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STRUCTURAL STUDIES
ON
ALPHA-CRYSTALLIN
AND ON
RACEMIZATION
OF
ASPARTIC ACID RESIDUES

PIET
VAN
DEN
OETELAAR
STRUCTURAL STUDIES ON ALPHA-CRYSTALLIN AND
ON RACEMIZATION OF ASPARTIC ACID RESIDUES
STRUCTURAL STUDIES ON ALPHA-CRYSTALLIN AND
ON RACEMIZATION OF ASPARTIC ACID RESIDUES

een wetenschappelijke proeve op het gebied van de
WISKUNDE EN NATUURWETENSCHAPPEN

Proefschrift
ter verkrijging van de graad van doctor
aan de Katholieke Universiteit te Nijmegen,
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Petrus Johannes Maria van den Oetelaar

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Voor Désirée
"Ik heb sedert eenigen tijd herwaarts weder verscheidde observatien ontrend de oogen van Ossen, en koeijen gedaan, om dat ik veel tijts (sedert mijn eerste observatien) in gedagten hadde, dat ik het regte maaksel van het cristalijn lichaam, soo als ik het behoorde te sien, nog niet gesien hadde."

Antoni van Leeuwenhoek, 1684.
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INTRODUCTION
The ultimate goal of studies on the structure of lens proteins, like those described in this thesis, is to obtain an answer to the following questions: why is the lens transparent and what happens in cataract? Therefore, for a full appreciation of structural investigations on lens proteins, at least a short introduction on lens transparency and cataract is inevitable.

LENS TRANSPARENCY

The lens is transparent because it does not absorb in the visual region of the spectrum and scattering is reduced to a minimum. The former is easily explained by the virtual absence of chromophores. The latter is more difficult to comprehend. Large scattering cellular bodies like cell nuclei and mitochondria are only present in the peripheral cells. Upon the differentiation to lens fiber cells these organelles are lost. A second reason for the minimum scattering was already realized in 1674 by Antoni van Leeuwenhoek, while observing a cow lens through one of his fine microscopes. Ten years later he reported to the London Royal Society as follows:

"(...) als wij met aan dagt aan schouwen het (...) cristaline lichaam, soo als het suiijver uijt het oog genomen is soo bevinden wij dat der geen glas so het selvige in doorschijnenthedt overtreft, hoe wel het selvige uijt soo veel duijsenden van draatgens is te samen gevoegt, dat wij wel mogen seggen, het is een wonder in ons oog; te meer als wij gedenken hoe nauw en digt dese draatgens aan malkanderen moeten sijn vereenigt, op dat het ligt lijn-regt daar door soude konnen gaan, want soo sulks anders was, soo soude het cristaline lighaam niet doorschijnend maar wit in onse oogen sijn vertoonen."

Van Leeuwenhoek was fully correct, since it is indeed the close packing and high order of organization of the lens fiber cells that minimizes scattering from the cell membranes (2).

The morphology of the lens has also been described by Van Leeuwenhoek with remarkable accuracy:
"Het Crystaline lichaam dat men de cristaline vogt noemt, en in hardigheid bij na over een komt met een geconfite note Muskaat, heb ik met een scheer-mes onstukken gesneden, ende het selvige alsoo in deelen observerende, heb ik het selvige bevonden te bestaan uit kringen gewijz schibbige deelen, op malkanderen liggende, (...) (en) dat ieder schibbe weder uit kringen gewijz deelen te samen was gesteld, (...)" (1)

"The Crystallin Humor, which in hardness almost resembles a Nutmeg preserv'd, I have with a rasor cut asunder, and observing it in parcels, I found it to be made up of orbicular Scaly parts, lying upon one another, (...) (and) that every scale was composed again of circular parts (...)"

A more recent description of the morphology of the lens can be found in Ref. 2.

The third group of scattering particles are the lens proteins or crystallins as they are called. If all individual proteins would act as independent scatterers, only about 30% transmission would be expected (3). Since this is not the case, a certain degree of spatial organization of the proteins must be present. Benedek (4) indicated that the proteins need not be in a paracrystalline state, but that a high density of packing is sufficient to reduce scattering. In a most elegant study, Delaye and Tardieu (5) showed that, with increasing concentration of crystallins, the optical density of the solution rises until a concentration of about 100 mg/ml while rapidly decreasing at higher concentrations (the protein concentration in the human lens is about 300 mg/ml). A short-range, glass-like spatial order of the crystallins was found to be the cause of this phenomenon. These observations make clear that knowledge of the structure of the lens proteins and the nature of their interactions will greatly improve our understanding of the molecular basis of lens transparency.

CATARACT

The major pathological change of the eye lens concerns the development of opacities (cataract). This may either have an age-related, a metabolic or a traumatic origin. A USA Department of Health, Education and Welfare publication from 1977 (cited in Ref. 6) reports that the ca. 300,000 cataract surgeries performed in 1972 account for one half of all the operations for eye disorders in the United States. Only five other surgical operations are performed more frequently (7). One and a half million Americans, which is 1% of the total population, suffers from significant visual impairment because of
cataract (6). Impressive as these figures may be, they turn insignificant when one realizes how serious the cataract problem can be in third-world countries. A study in the plains of the Punjab, for example, showed that 22.5% of the population from 50 to 59 years old either had a cataract or had already had a cataract removed, and this figure rose to 31.4% among people aged 60 or more (7).

Although cataract can have many causes, it is often characterized by changes at the molecular level of the crystallins. The processes that take place include oxidation, crosslinking, degradation, aggregation, glycation and subsequent Maillard reactions, deamidation, and racemization. These alterations give rise to changes in the solubility of the crystallins, thus increasing the number of scattering particles and eventually causing cataract. Many of the processes are to a lesser extent also observed in the aging lens (for reviews see Refs. 8-10). It has been suggested that one can envision senile cataract as the result of a combination of risk factors superimposed on the normal aging processes (7,8,11).

**OUTLINE OF THIS THESIS**

The protein content of the mammalian lens is extremely high: 30-40% of its wet weight. Based on parameters like molecular weight and isoelectric point, the crystallins (constituting about 95% of the protein content of the lens) can be subdivided into three classes, namely the α-, β- and γ-crystallins (12). Elucidation of primary structures and crystallographic analyses have resulted in detailed knowledge of the structural organization of calf γ-crystallin. Because of the high sequence similarity of the β- and γ-crystallins, predictions for the bovine β-crystallin structure could be made. Furthermore, the aggregation states of the β-crystallins have been well studied (13).

Although in the early days of lens research studies on the individual crystallins started with bovine α-crystallin, nowadays our knowledge of its structure shows less detail than for the β- and γ-crystallins. Attempts to grow α-crystallin crystals have been unsuccesful until now, thus excluding the possibility of X-ray analysis. The main cause for this lack of success is believed to be the varying number and ratio in which the four subunits αA₁, αA₂, αB₁ and αB₂ are present in the α-crystallin aggregate, resulting in a
protein which is heterogeneous both in size and charge (14). However, with regard to its quaternary structure, previous work in this laboratory has resulted in a model of bovine \( \alpha \)-crystallin in which about 40 subunits build up an aggregate of about 800,000 Da (15). Studies of Thomson and Augusteyn (16,17) who performed isolation at 37°C instead of 4°C raised serious doubts about the molecular weight of \( \alpha \)-crystallin and consequently about the validity of the model of its quaternary structure. Based on these studies a micellar structure was recently proposed (18).

Chapter 2 deals with the quaternary structure of bovine \( \alpha \)-crystallin and in 2.1 it is reported to what extent the observed molecular weight of \( \alpha \)-crystallin depends on isolation conditions like, for instance, temperature. The dynamic character of the quaternary structure of \( \alpha \)-crystallin is dealt with in chapter 2.2 in which the intermolecular exchange of subunits by \( \alpha \)-crystallin and homopolymeric aggregates of \( \alpha \)-crystallin subunits is described.

In chapter 3 we step down to the next level of protein organization, i.e. the tertiary structure of the \( \alpha \)-crystallin subunits. The two principal techniques used in this study, urea-gradient isoelectric focusing and two-dimensional isoelectric focusing, are presented along with results obtained for several proteins in chapter 3.1. The observations made for \( \alpha \)-crystallin have implications for the organization of its subunits, which is discussed in chapter 3.2.

Racemization of amino acid residues, i.e. the conversion of the L into the D enantiomer, can be regarded as a change in the primary protein structure. Although recognized as a notorious problem in peptide synthesis for many years, racemization of aspartic acid residues \( \text{in vivo} \) is encountered in the literature for only little more than a decade. It has been shown to be one of the many post-translational modifications of proteins in the aging human lens. However, with regard to the relevance of this process in cataractogenesis, contradicting results have been published (19-21). One of the aims of this thesis is to investigate the role of aspartic acid racemization in cataract. In that respect it was essential to use analytical methods of higher precision than that used in the studies mentioned above. Various enantio-selective methods have been developed in the recent years. Two of them which were found to be useful for this study are reported in chapters 4.1 and 4.2. Using these methods, protein fractions from a large number of normal and cataractous human lenses were analyzed. The kind of relationships that were found between racemization and age or cataract are described in
chapter 4.3. The kinetics of the racemization of amino acid residues has, up till now, been described as a reversible first-order reaction showing identical rate constants for the optical inversion of both the L and the D antipode. However, since amino acid residues are present in a chiral protein environment, this view is incorrect and a more complete description of the kinetics of aspartic acid racemization in human lens proteins is given in chapter 4.4. In that same chapter, evolving theories on the mechanism of aspartic acid racemization are described.

The non-stoichiometry of the methyl esterification of carboxyl groups, catalyzed by the eukaryotic enzyme protein carboxyl methyltransferase and its nonexisting substrate specificity, seemed a logic quality of this enzyme when the group of Clarke suggested that it specifically recognizes the γ-carboxyl group of D-aspartic acid residues. They further postulated that methylation and subsequent demethylation of these residues could result in the re-inversion of the D into the L enantiomer (22). Our study, testing whether this racemization-repair hypothesis is valid for human eye lens proteins, is reported in chapter 4.5.

REFERENCES


THE QUATERNARY STRUCTURE OF BOVINE
ALPHA-CRYSTALLIN
THE INFLUENCE OF ISOLATION CONDITIONS ON THE MOLECULAR WEIGHT OF BOVINE ALPHA-CRYSTALLIN

Piet J. M. van den Oetelaar, Julius Clauwaert, Marc van Laethem and Herman J. Hoenders

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The Influence of Isolation Conditions on the Molecular Weight of Bovine α-Crystallin*

(Piet J. M. Van den Oetelaar, Julius Claauwaert, Marc Van Laethem, and Herman J. Hoenders)

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The molecular weight of bovine α-crystallin, isolated at 37°C, was studied and found to be about 800,000. This contrasts with the results of Thomson and Augusteyn (Thomson, J. A., and Augusteyn, R. C. (1983) Exp. Eye Res. 37, 367-377) who isolated a species of about half this molecular weight. We show here that this form of α-crystallin can only be isolated under unphysiological conditions with regard to buffer pH and ionic strength.

The vertebrate eye lens contains about 35% by weight of structural proteins, which have been subdivided into the water-soluble crystallins and a water-insoluble fraction. The crystallins form the bulk of lens proteins and are classified as α-, β-, and γ-crystallins. The former two have a quaternary crystalline form that makes up the bulk of lens proteins and are classified as α- and β-crystallins. This form of α-crystallin can only be isolated at 37°C, whereas α-crystallin that is isolated at the normally used temperature of 4°C has a much higher molecular weight. In the experiments of Thomson and Augusteyn this so-called α-crystallin had a minimum molecular weight of 630,000. The fact that α-crystallin can only be isolated at 37°C led them to the assumption that this is the native form of α-crystallin. This two forms differ in their sedimentation coefficient (12 S and 19 S, respectively) but not in other parameters such as amino acid composition, subunit distribution, near- and far-UV circular dichroism spectra, and immunochromatographic properties. In a subsequent article of the same authors, the reversibility of urea-induced dissociation of α-crystallin is assumed to be another indication for the native state of this species (14). In the light of these findings, Thomson and Augusteyn (13, 14) reject the three-layered model. They believe that α-crystallin has a more simple structure, in which the subunits occupy equivalent sites.

In this paper, we present evidence that not only the temperature is critical for the isolation of α-crystallin, but also ionic strength and pH. The conditions used by Thomson and Augusteyn (13, 14) to isolate α-crystallin (low ionic strength and alkaline pH) favor partial dissociation of α-crystallin (15). Since we were able to isolate α-crystallin only if using these circumstances, we assume that this form of α-crystallin arises by the rather unphysiological isolation conditions.

MATERIALS AND METHODS

Lens Handling—Calf (6 months) and cow (3 years) lenses were obtained from the local slaughterhouse. Immediately after killing of the animal the eyes were removed and transferred into small plastic bags. These were placed in a Dewar vessel, filled with water at 37°C, and transported to the laboratory where they arrived within 15 min. In an empty beaker, placed in a 37°C waterbath the lenses were removed and decapsulated. Cortices were scraped off and homogenized on ice or at 37°C with 4 volumes of buffer.

The extracts were centrifuged for 30 s in a Beckman microfuge B. After this step the temperature had dropped 0.5°C maximally. Within 20 min after the death of the animal the water-soluble cortex extracts could be applied on the gel filtration column or used for ultracentrifugus experiments.

Four buffers were used. The temperature dependency of the pH was taken into account. Buffer 1 was a high strength phosphate buffer, 20 mM Na phosphate, 100 mM NaCl, 2 mM EDTA, 0.2 mM dithioerythritol, pH 6.9, ionic strength, 533 mM. Buffer 2 was a low-strength Tris buffer (according to Ref 13) 50 mM Tris/HCl, 2 mM

1 In this article we adopt the nomenclature introduced by Thomson and Augusteyn (13). They called the cold-isolated form of α-crystallin αm and the warm-isolated form αw.
The Molecular Weight of \( \alpha \)-Crystallin

**FIG 1.** Sedimentation velocity analysis of 37 °C extracted calf cortex. Run at a rotor temperature of 37 °C in high-strength phosphate buffer, pH 6.9, ionic strength, 333 mM. Total protein concentration 20 mg/ml. Sedimentation is from right to left for 24 min at 56,000 rpm. Schlieren angle 70°.

EDTA, 0.2 mM dithioerythritol, pH 8.0, ionic strength, 36 mM. Buffer 3 was a low-strength HEPES buffer: 10 mM HEPES, 0.02% (w/v) NaNO\(_3\), pH 7.0, ionic strength, 44 mM at 37 °C, 2 mM at 25 °C. Buffer 4 was a high-strength HEPES buffer: 10 mM HEPES, 25 mM NaCl, 120 mM KCl, 0.02% (w/v) NaNO\(_3\), pH 7.0 or 8.0, ionic strength, 149 or 154 mM at 37 °C, 147 or 152 mM at 25 °C.

**Gel Chromatography**—Water-soluble extracts (0.5 ml) were layered on agarose Bio-Gel A-5m (Bio-Rad) (column dimensions 1.6 X 170 cm). Elution was performed with the buffer used in the extraction procedure at a flow rate of 7.2 ml/h at room temperature (25 °C). Top fractions of 5.5 ml of the \( \alpha \)-crystallin peak were pooled and, after concentration on an Amicon filter, used to measure sedimentation coefficients.

**Analytical Ultracentrifugation**—Sedimentation coefficients of total extracts were determined with schlieren optics in a Beckman Spinco model E analytical ultracentrifuge, equipped with electronic speed control, from the rate of the movement of the maximum of the schlieren peak, and corrected for the density and viscosity of the buffer relative to water at 20 °C (\( s^0 \)). Correction to zero concentration was performed according to the formula \( s^0 = s^0 + k \cdot c \), with \( k = 0.011 \) for \( \alpha \)-crystallin and \( c \) is the protein concentration in mg/ml, as presented by Siezen and Berger (16). Sedimentation was performed at 56,000 rpm at 20 or about 37 °C. For the 37 °C experiments the rotor was heated overnight at 37 °C. During these runs the temperature dropped from 36.8 °C to 35.9 °C. Sedimentation experiments with isolated \( \alpha \)-crystallin were carried out with UV optics at a protein concentration of 1 mg/ml, so that sedimentation coefficients did not need a correction.

**Quasi-elastic Light Scattering**—The diffusion coefficient of the \( \alpha \)-crystallins has been determined from their quasi-elastic light scattering. The single-chopped photon-count autocorrelation function of the light scattered by a dilute mono-disperse solution of small Brownian particles, decays exponentially with a decay time proportional to the searched diffusion coefficient \( D \),

\[
G(t) = A + B \exp(-2DK^2t)
\]

where \( t \) is the channel number, \( T \) is the chosen sample time, and \( K \) is the known modulus of the scattering vector, and \( A \) and \( B \) are constants. Our set-up, experimental procedures, and methods of data analysis have been described previously (17). The concentration of \( \alpha \)-crystallins ranged from 0.5 to 1.5 mg/ml and no concentration effects on the diffusion coefficient were observed in this concentration range. Since our set-up did not allow measurements at 4 °C, we decided to carry out experiments at 25 °C and 37 °C in order to investigate the influence of low isolation temperature on the diffusion coefficient.

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**RESULTS**

**Analytical Ultracentrifugation of Total Extracts**—Calf cortices, extracted with high-strength and low-strength buffer, were analyzed by analytical ultracentrifugation without further purification to make a rapid estimation of the sedimentation coefficient possible. Sedimentation runs were carried out at rotor temperatures of 20 °C and 37 °C. Fig. 1 shows the sedimentation profile of a sample, run at 37 °C, immediately after extraction at 37 °C with high-strength buffer, pH 6.9. A fast sedimenting peak, representing \( \alpha \)-crystallin, is observed. Due to the uncommon temperature, we were not able to correct its sedimentation coefficient to obtain the \( s^0 \) value.

The changes in the sedimentation profiles of samples, extracted and incubated at 37 °C in low-strength Tris buffer, pH 8.0, were followed for several hours. Fig. 2A shows a pattern, obtained immediately after extraction, consisting of three peaks. The fastest sedimenting peak had a \( s^0 \) of 18 S. Upon incubation the fast sedimenting peak disappeared gradually. Fig. 2B shows the situation after 2 h. Prolonged incubation of a high-strength phosphate buffer-extracted sample at 37 °C did not result in a change of the sedimentation profile (not shown). To examine whether the

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2 The abbreviation used is HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
The Molecular Weight of α-Crystallin

TABLE I

Physicochemical data of α-crystallin as a function of isolation conditions

<table>
<thead>
<tr>
<th>pH</th>
<th>Ionic strength</th>
<th>Isolation temperature</th>
<th>Analysis temperature</th>
<th>Sedimentation constant</th>
<th>Diffusion constant</th>
<th>Friction ratio</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>149</td>
<td>37</td>
<td>20</td>
<td>19.7 ± 0.5</td>
<td>2.33 ± 0.03</td>
<td>1.46 ± 0.05</td>
<td>850,000</td>
</tr>
<tr>
<td>7.0</td>
<td>147</td>
<td>25</td>
<td>25</td>
<td>14.9 ± 0.5</td>
<td>2.33 ± 0.03</td>
<td>1.46 ± 0.05</td>
<td>592,000</td>
</tr>
<tr>
<td>8.0</td>
<td>154</td>
<td>37</td>
<td>20</td>
<td>19.6 ± 0.5</td>
<td>2.51 ± 0.09</td>
<td>1.49 ± 0.05</td>
<td>815,000</td>
</tr>
<tr>
<td>8.0</td>
<td>152</td>
<td>25</td>
<td>20</td>
<td>12.1 ± 0.5</td>
<td>2.40 ± 0.03</td>
<td>1.41 ± 0.05</td>
<td>442,000</td>
</tr>
<tr>
<td>7.0</td>
<td>4</td>
<td>37</td>
<td>25</td>
<td>17.7 ± 0.5</td>
<td>2.73 ± 0.06</td>
<td>1.51 ± 0.05</td>
<td>732,000</td>
</tr>
</tbody>
</table>

Fig. 3 Elution profile of cortex extracts of 6-month-old calf on agarose A-5m. Extraction and elution was done with the low-strength Tris buffer, pH 8.0, at 37 °C (—) or 4 °C (— — —)

Fig. 4 Elution profile of cortex extracts of 3-year-old cow on agarose A-5m. Extraction and elution was done with the high-strength phosphate buffer, pH 8.0, at 37 °C (—) or 4 °C (— — —)

Table 1 shows the sedimentation coefficients of α-crystallin were constant for 24 h, when incubation took place in a 50 mM Tris buffer of pH 6.9 and ionic strength of 333 mM. However, incubation of α-crystallin in a 20 mM phosphate buffer of pH 8.0 without other salts added, also resulted in a rapid transfer to a 14 S (s20) particle.

Gel Chromatography—Gel chromatography on agarose A-5m was performed in either low- or high-strength buffer. Fig. 3 shows the elution profiles in a low-strength buffer of samples, extracted at 4 and 37 °C. The α-crystallin peak of the latter was retarded by 1 h, compared to that of the 4 °C extract. The retention times of the other crystallins were not affected. The difference in elution times of α-crystallin was reflected in the sedimentation coefficients of the top fractions. Values of 16 S and 13 S (s20w) for the top fractions of α- and γ-crystallin, respectively, were found at a protein concentration of 11 mg/ml. After correction to zero concentration, the values 18 S and 15 S (s20w) were obtained.

When elution took place in a high-strength buffer, the different extraction temperatures did not influence the retention time of α-crystallin (Fig. 4). The sedimentation coefficients of the top fractions were 16 S and 17 S (s20w) for the 4 and 37 °C extracts, respectively. Correction to zero concentration yielded the s20w values 18 S and 19 S for α-crystallin, Isolated under Various Conditions—α-Crystallin was isolated from calf lenses by gel chromatography on agarose A-5m with low- or high-strength HEPES buffer of pH 7.0 or 8.0 at 37 °C.

Sedimentation constants of about 19.7 ± 0.5 S were found for calf α-crystallin, isolated by gel chromatography in the high-strength buffer at 37 °C. Only when isolated at 37 °C, pH 8.0, a lower value of 14.9 ± 0.5 S was found. When the low-strength buffer was used, even at pH 7.0, the isolation at 37 °C resulted in a 4 S (s20w) species. However, isolation at 25 °C still yielded a 17.7 ± 0.5 S (s20w) particle. Using bovine α-crystallin, similar results were obtained, although slightly higher sedimentation coefficients were found (data not shown).

Diffusion coefficients were measured of α-crystallin, isolated at the same combinations of conditions. When the high-strength buffer was used, only the combination of high pH (8.0) and high temperature (37 °C) resulted in an increase of...
the diffusion coefficient from a mean value of 2.35 ± 0.04 to 2.51 ± 0.09 (Table I) Low ionic strength and high extraction temperature resulted already at pH 7.0 in an increase from 2.41 ± 0.05 to 2.73 ± 0.05. The diffusion coefficients of α-crystallin, isolated from bovine lenses, essentially showed the same behavior (data not shown), be it that they were always slightly less than those of calf α-crystallin

**DISCUSSION**

Confusion was raised about the molecular weight of α-crystallin, when Thomson and Augusteyn (13, 14) claimed the existence of an α-crystallin species, possessing half the molecular weight of the generally accepted value. These investigators had reason to believe that this species, which they called αα-crystallin, in contrast to the classical αα-crystallin, was the native form of the protein. They stated that it was very critical to maintain a temperature of 37°C during isolation of αα-crystallin. Our results prove that pH and ionic strength of the isolation buffer also have great influence on the molecular weight of α-crystallin.

We tried to investigate the in vivo state of α-crystallin by analytical ultracentrifugal analysis of water-soluble lens extracts. In this manner results were obtained within 40 min after the death of the animal. Figs 1 and 2A indicate that, when a sample is analyzed immediately after extraction at 37°C in a low- or high-strength buffer, the existence of αα-crystallin can be demonstrated. With these results in mind, Fig 3 of Ref 13 puzzles us, because that sedimentation profile of a 37°C low-strength buffer extract shows a complete lack of αα-crystallin, although the conditions used to prepare that sample were highly comparable to those used to obtain Fig 2A. The destabilizing effects of low ionic strength and high pH were demonstrated by the rapid dissociation in a low-strength buffer of pH 8.0 (Fig 2B). Irrespective of the type of buffer, the sedimentation coefficient of α-crystallin was unchanged for more than 24 h in high-strength buffers of pH 6.9.

Using exactly the same conditions as Thomson and Augusteyn (13), we were able to isolate by gel filtration αα-crystallin with a sedimentation constant of 15 S (Fig 3). It could also be reproduced that only αα-crystallin with a sedimentation constant of 18 S is isolated when extraction took place at 4°C. The buffer used in these procedures was a low-strength buffer of pH 8.0. If, however, a high-strength buffer with pH 6.9 was used, we only found αα-crystallin, even if extraction was performed at 37°C (Fig 4). These findings suggest that, besides temperature, pH and ionic strength play a more important role during isolation of α-crystallin than Thomson and Augusteyn supposed (13). It should be noted that, although only the extraction was performed at 37°C, whereas the fractionation was carried out at room temperature, we were able to isolate αα-crystallin, which is consistent with the results of Thomson and Augusteyn (13). The diffusion constant varied only within 0.01 × 10^{-7} cm^{2}/s if the analysis temperature of αα-crystallin, isolated at 37°C in high-strength buffer of pH 6.9 or 8, was repeatedly changed from 37°C to 25°C and back.

It cannot be excluded that the influences of pH, ionic strength, and temperature work synergistically. In fact, we believe they do, since the ultracentrifugal experiments with high-strength buffer only at the combination of 37°C and pH 8.0 showed a decrease of the sedimentation coefficient and an increase of the diffusion coefficient. Using a low-strength buffer, already at pH 7.0 a smaller particle (442,000) was isolated at 37°C, showing the cooperative effects of pH, ionic strength, and temperature.

The conditions pH 8.0, low ionic strength, and 37°C, used by Thomson and Augusteyn (13, 14) in the isolation of αα-crystallin, are rather unphysiological. First, the pH differs more than 1 unit from the intralenticular pH, which by means of 31P NMR of intact lenses, was shown to be 6.9 (18). Second, the ionic strength of the cytosol of the normal bovine lens is about 190 mM (19). Taking into account the partial dissociation of Trs at pH 8.0, an ionic strength of 39 mM can be calculated for their buffer, which is only one-fifth of the physiological ionic strength. In order to avoid ionic interactions, gel filtration has to be performed at relatively high ionic strength. We therefore used a 333 mM buffer. This is nearly 1.8 times the physiological level. However, previous studies (15) have shown that ionic strengths >150 mM do not influence the sedimentation coefficient of α-crystallin, whereas lower ionic strengths have a dramatic effect. Finally, the lenticular temperature, presumed to be 37°C (13, 14), may be doubted.

Extensive literature search could not give us any data about the bovine lens temperature. Schwartz and Feller (20) showed that the rabbit anterior lens surface and the mid lens region have a temperature of 33.6° and 35.4°C, respectively, being 4.2° and 2.4° less than the body temperature. Nakao (21) found comparable figures 34.8°C in the anterior lens region (−3.8°) and 36.5°C in the posterior lens region (−1°). We assume that the bovine lens, more often exposed to lower environmental temperatures than that of the laboratory rabbit, also shows a temperature gradient. This is a reasonable assumption since loss of heat caused by evaporation at the surface of the cornea has been shown to be used by birds and some mammals such as ox to cool the blood entering the brain (22-24). Since the body temperature of cattle is about 38.5°C (25, 26), the mean lenticular temperature will be some degrees below 37°C. Since the conditions of pH and ionic strength, necessary for the isolation of αα-crystallin both favor dissociation, we wondered whether αα-crystallin might be formed by dissociation out of αα-crystallin. If so, this should be reflected in the retention time of αα-crystallin on the gel chromatography column, because gel chromatography (Figs 3, 4, and Fig 1 of Ref 13) and dissociation (Fig 2) take place at a comparable time scale. Therefore, we made a calibration curve of the A22 column used by Thomson and Augusteyn (13) by calculating the retention times of the peaks of the chromatogram and plotting them against the logarithm of the molecular weight (Fig 5). The elution time of αα-corresponds to a molecular weight of 560,000. Applying the same approach to our own chromatographic data, a molecular weight of 541,000 was calculated. These molecular weights do not correspond to the sedimentation coefficients of about 12 S (Table I and Ref 13) and the molecular weight of 442,000 (Table I) based on αααα and D. It is obvious that during a certain period the elution rate of αα-crystallin has been faster than can be expected on the basis of its size. This can only be explained if one assumes that αα-crystallin first migrates as αα and, upon exposure to the denaturing eluent, dissociates to form αα. This would be in accordance with the sedimentation profiles obtained immediately after extraction at 37°C (Figs 1 and 2A), which clearly show an αα peak. Rechromatography strengthens the notion that αα-crystallin is applied to the column as αα. Upon rechromatography, as performed by Thomson and Augusteyn (13), αα-crystallin emerged at exactly the same elution volume (115 ml). However, as can be expected, the elution volume of αα-crystallin then increased from 127 to 133 ml, indicating a molecular weight of 495,000, which is in better agreement with the expectations for a 12 S particle.

The results presented in this paper demonstrate that the molecular weight of αα-crystallin not only depends on temper-
The Molecular Weight of α-Crystallin

nature, but also on the pH and the ionic strength of the solvent. Under physiological conditions α-crystallin is relatively stable and has a molecular weight of about 800,000, depending on the age of the animal and the lens region from which it is extracted. We therefore conclude that αn is the native form of α-crystallin. This conclusion is supported by thermodynamic considerations. Hydrophobic interactions play an important role in the aggregation of α-crystallin (27-31). Because of the positive sign of the entropy, the Gibbs energy decreases with increasing temperature if ionic strength and pH are kept constant. Therefore, it is more likely that isolation at 37°C results in larger aggregates. If smaller particles are isolated, this can only be caused by the ionic strength and pH of the solution which affect the strength of the ionic interactions and hydrogen bridges.

The nature of αn-crystallin still remains an unresolved matter. The monomer/dimer relationship between αm and αn, as suggested by Thomson and Augusteyn (14), has been postulated previously (9, 32-36). However, in all these papers, the presence of a 12 S particle can be related to alkaline pH, low ionic strength, or prolonged incubation at higher temperatures. Although this particle is not the native α-crystallin, it must be a thermodynamically favorable organ-

See text for details.
THE DYNAMIC QUATERNARY STRUCTURE OF BOVINE ALPHA-CRYSTALLIN

INTERMOLECULAR EXCHANGE OF SUBUNITS BY HOMOPOLYMERIC AGGREGATES AND ALPHA-CRYSTALLIN

Piet J. M. van den Oetelaar, Petri F. H. M. van Someren, John A. Thomson, Roland J. Siezen and Herman J. Hoenders

ABSTRACT

The structural bovine eye lens protein α-crystallin was dissociated in 6 M urea and its four subunits A₁, A₂, B₁ and B₂ were separated by means of ion-exchange chromatography. Homopolymeric reaggregates of these subunits were prepared by removal of the denaturant via dialysis. It was found that upon incubation of mixtures of two homopolymers under native conditions, subunits were exchanged. A new species was formed within 24 h as demonstrated by isoelectric focusing. Moreover, it was found that native α-crystallin molecules also exchanged subunits when incubated with homopolymeric aggregates of A₂ or B₂ subunits. Subunit exchange between native α-crystallin molecules is postulated and this "dynamic quaternary structure" is presented as a way of the polydisperse protein to adapt itself to changes in cytoplasmic conditions upon aging of the lens tissue, by producing a new, again thermodynamically stable population.

INTRODUCTION

Calf lens α-crystallin is a multi-subunit protein composed of A and B type 20,000 Da subunits that occur in an average ratio of about 3:1 (Siezen, Bindeis and Hoenders, 1978). The A₁ and B₁ subunits are formed out of the primary gene products A₂ and B₂, respectively, by a phosphorylation step (Voorter, Mulders, Bloemendal and De Jong, 1986; Chiesa, Gawinowicz-Kolks and Spector, 1987; Chiesa, Gawinowicz-Kolks, Kleiman and Spector, 1987a,b). Many studies have dealt with the quaternary assembly of α-crystallin. A model for the architecture of polydisperse molecules has been proposed with 14±2, 13±3 and 15±2 subunits in three concentric layers (Bindeis, Siezen and Hoenders, 1979; Siezen, Bindeis and Hoenders, 1980). However, in the past few years there has been some discussion about the molecular weight of native α-crystallin. Thomson and Augusteyn (1983, 1984) described an α-crystallin of 320,000 Da, isolated at 37°C. Hydrodynamic studies and symmetry considerations led to dodecameric models of α-crystallin, either as a protein with an intermediate tetrahedral shell organization of its subunits or as a micelle-type aggregate (Thomson, 1985; Augusteyn and Koretz, 1987). On the other hand, others are still in favor of a molecular weight of about 800,000 Da (Van den Oetelaar, Clauwaert, Van Laethem and Hoenders, 1985; Tardieu, Laporte, Licinio, Krop and Delaye, 1986). The latter authors presented an extended version of the three layer model, characterized by tetrahedral symmetry and 12, 24 and 24 sites occupied in the first, second and third layer, respectively.
The subunits of \( \alpha \)-crystallin are very prone to form aggregates. In fact, single subunits only exist in concentrated solutions of denaturing agents and they aggregate upon dilution of these agents or their removal by dialysis. In order to form reaggregates, the ratio of the subunits is not critical. It was found that not only dissociated native \( \alpha \)-crystallin could be reassociated (Bloemendal, Bont, Jongkind and Wisse, 1962; Li and Spector, 1973; Siezen and Bindels, 1982; De Block, Dom and Clauwaert, 1986; Tardieu, Laporte, Licinio, Krop and Delaye, 1986) but also mixtures of isolated A and B subunits in various ratios (Van Kamp, Van Kleef and Hoenders, 1974; Bindels, 1982). Moreover, it turned out to be possible to form homopolymers by association of purified subunits (Li and Spector, 1972, 1973, 1974; Bindels, 1982; Thomson, 1985). In general, reaggregated \( \alpha \)-crystallin and these homopolymers are smaller than the native \( \alpha \)-crystallin and generally do not exceed about 20-22 subunits (Bloemendal, Zweers, Benedetti and Walters, 1975; Siezen, Bindels and Hoenders, 1978; Siezen and Berger, 1978).

In this paper we show that subunits are exchanged between homopolymeric molecules in the absence of denaturing agents. Moreover, subunit exchange is also demonstrated between homopolymers and native \( \alpha \)-crystallin molecules.

**MATERIALS AND METHODS**

*Isolation of alpha-crystallin*

Lenses of 6-months-old calves were gently stirred at 5°C for 15 min in a buffer containing 20 mM sodium phosphate, 100 mM sodium sulphate and 1 mM EDTA, pH 6.9. In this way only the outer cortex dissolved. Insoluble material was pelleted by centrifugation at 10,000 g for 30 min. The water-soluble proteins were reconcentrated by dialysis in tubing with a cutoff of 3,000 Da (Spectrapor) against a solution of 40% (w/v) polyethylene glycol 20,000 (Fluka) in the same buffer. Subsequently, the water-soluble proteins were fractionated by gel filtration at 4°C on Bio-Gel A-5m (LKB, column dimensions: 27 mm i.d., 90 cm length) and the low molecular weight \( \alpha \)-crystallin fraction was concentrated by centrifugation at 44,000 g for 18 h (Siezen and Berger, 1978).

*Isolation of alpha-crystallin subunits*

Subunits were purified by anion exchange chromatography in the presence of 7 M urea. A sample of 10 mg \( \alpha \)-crystallin was dialyzed against the starting buffer (5 mM Tris-HCl, 7 M urea, 0.02% dithioerythritol, pH 8.0) and subsequently layered on top of a DEAE-cellulose column (DE-52, Whatman, dimensions: 16 mm i.d., 47 cm length), equilibrated with the same buffer. After the non-bonded material was eluted from the column, the subunits were fractionated by gradient elution with an increasing concentration of Tris-HCl from 5 to 100 mM. The purity of the isolated subunits was checked by isoelectric focusing in the presence of 7 M urea.
Preparation of homopolymeric aggregates

The isolated subunits now present in a solution of 7 M urea at a concentration of 0.5 mg/ml, were dialyzed against 5 mM Tris-HCl, pH 8.0 for 24 h, with seven renewals of the dialysis buffer. The homopolymeric reaggregates obtained in this manner were concentrated to 10 mg/ml by dialysis against a solution of 40% polyethylene glycol 20,000 in 5 mM Tris-HCl, pH 8.0 and stored at -70°C.

Iodination of A2-aggregates

The inside wall of a glass reaction vial was coated with the catalyst Iodogen (Pierce) by evaporation of a solution in trichloromethane under nitrogen. A solution of αA2-aggregates (1 mg/ml) was incubated with 125I2 (477 μCi, Amersham) for 15 min. The non-bound iodine was complexed with potassium iodide and the protein fraction was isolated by gel filtration on a short Sephadex G-25 column (Pharmacia). A protein fraction with a specific activity of 52 μCi/mg was isolated.

Subunit exchange experiments

Usually two types of homopolymers were filtered separately through 0.2 μm Millex filters (Millipore) and mixed in a 1:1 ratio at a final protein concentration of 5 mg/ml. Incubations were performed both at 37°C and 5°C. Samples were taken at zero-time and after 2, 4, 6, and 24 h and stored at -70°C until further analysis. In every experiment, pure homopolymers were incubated as a control. Buffer change for experiments in buffers other than 5 mM Tris-HCl, pH 8.0, was effected by dialysis against the appropriate buffer.

Isoelectric focusing

Homopolymeric aggregates and hybrid aggregates resulting from subunit exchange experiments were analyzed on 3% T, 3% C acrylamide gels containing 13% (v/v) glycerol and an ampholyte mixture of the following composition: 1.81% (v/v) Ampholines 6-8 and 2.26% (v/v) Ampholines 3.5-10 (LKB) and 1.81% (v/v) Pharmalytes 2.5-5 (Pharmacia). Subunit composition of samples was analyzed on 5% T, 3% C acrylamide gels containing 7 M urea and the same ampholyte mixture. Gels of 10 x 20 cm with a thickness of 0.3 mm were cast between a glassplate treated with γ-methacryloxypropyltrimethoxysilane (Aldrich) and another one treated with dimethyldichlorosilane, as described by Tegelstrom and Wyoni (1986). In this way the gel was covalently bound to a glass support. Flat-bed electrophoresis was carried out in a Desaphor electrophoresis unit (Desaga), using 1 M phosphoric acid and 1 M sodium hydroxide as electrode solutions and an LKB 2297 Macrodrive 5 as power supply. Limit settings were 2500 V and 70 mA. Prefocusing was carried out for 45 min at 15 W constant power. Subsequently the samples were focused for 30 min at 8 W and 105 min at 15 W constant power. Tap water cooling was used to ensure a temperature of about 15°C. Occasionally, the pH gradient was determined by cutting the gel in pieces of 0.5 cm and extracting the ampholytes with 2 ml of water for 48 h. The pH of the resulting solution was measured.

After fixing for 1 h at 60°C in an aqueous solution of 30% (v/v) ethanol and 11.5% (v/v) trichloroacetic acid, and rinsing with 2% (v/v) acetic acid, the gels were stained for 45 min at 60°C in a solution of 1.15% (w/v) PAGE Blue 83 (BDH Chemicals) in 25% (v/v) ethanol and 8% (v/v) acetic acid. Gels were destained in a solution of 25% (v/v) ethanol and 8% (v/v) acetic acid until a clear background was obtained and subsequently dried under a stream of hot air. Autoradiographs were made by exposing Sakura X-ray films to the gel at -70°C. An LKB UltroScan gel scanner was used for Coomassie-stained gels as well as for autoradiographs.

Analytical ultracentrifugation

Sedimentation velocity analyses were carried out in a Beckman Spinco model E analytical ultracentrifuge at 56,000 rpm near 20°C using UV-optics.
Figure 1. Fractionation of α-crystallin subunits. Subunits of 200 mg α-crystallin were isolated by anion-exchange chromatography on DEAE-cellulose in Tris buffer, pH 8.0, containing 7 M urea. In the part of the chromatogram shown in this figure, the concentration of Tris linearly increases from 30 to 100 mM. Fractions of 10 ml were collected and top fractions of the four main peaks were pooled.

Figure 2 (left). Isoelectric focusing of purified subunits. Homogeneity of the isolated subunits was analyzed by isoelectric focusing in the presence of 7 M urea. Lane 1, A1; lane 2, A2; lane 3, B1; lane 4, B2; lane 5, α-crystallin. The pH-gradient, non-linear because of the mixture of ampholytes used, is indicated.

Figure 3 (right). Isoelectric focusing of homopolymers. Aggregates of purified subunits were analyzed by isoelectric focusing in the absence of denaturants. Lane 1-4: αr(A1); αr(A2); αr(B1) and αr(B2), respectively.
RESULTS

PREPARATION AND CHARACTERIZATION OF HOMOPOLYMERS

Separation and isolation of the α-crystallin subunits A₁, A₂, B₁ and B₂ was carried out by anion-exchange chromatography on DEAE-cellulose (Fig. 1). The purity of the subunits was analyzed by isoelectric focusing in the presence of 7 M urea and is shown in Fig 2. As judged by gel scanning, purity was >95% for A₁, A₂, and B₂ and 85% for B₁. The purified subunits formed reaggregates upon removal of urea through dialysis against 5 mM Tris, pH 8.0. They will be referred to as α_r(A₁), α_r(A₂), α_r(B₁) and α_r(B₂). Sedimentation and diffusion constants obtained by analytical ultracentrifugal analysis indicated molecular weights of 248,000 for α_r(A₂) and 483,000 for α_r(B₂) (Thomson, 1985). Isoelectric focusing in the absence of urea reveals that the homopolymers show charge heterogeneity (Fig. 3). Although the reaggregates focus in a broader range than the unfolded subunits, several sharp bands are present for all reaggregates. The pI-ranges found were: α_r(A₁), 4.5-5.0; α_r(A₂), 4.8-5.2; α_r(B₁), 6.1-6.5; α_r(B₂), 6.5-6.9. It is remarkable that all of these pI-ranges are lower than the pI's of the respective subunits (Van den Oetelaar, Bezemer and Hoenders, 1987): 0.6-1.1 units for α_r(A₁) and α_r(A₂) and 0.3-0.7 units for α_r(B₁) and α_r(B₂). Apparently, positively charged groups are masked either by folding or by aggregation of the subunits. The broad pI ranges of the homopolymeric aggregates indicate a charge heterogeneity which may be caused by a heterogeneity in structure of these aggregates with respect to number, folding and packing of subunits.

SUBUNIT EXCHANGE BETWEEN HOMOPOLYMERS

The differences in pI range of the four homopolymeric aggregates were used as a tool to study subunit exchange in mixtures of two types of homopolymers in the absence of denaturant. In the first experiment, α_r(B₂) was incubated with iodinated α_r(A₂) in a 1:1 ratio. Isoelectric focusing (Fig. 4A) shows that already after 2 h of incubation the bands focusing in the α_r(A₁) region become more alkaline, whereas those in the α_r(B₂) region shift towards a more acidic pI. The new hybrid aggregates focusing in the alkaline region of the gel have incorporated a few labeled A₂ subunits, as is indicated by the autoradiograph of this gel (Fig. 4B).
Figure 4: Subunit exchange between $^{125}$I-$\alpha_c(\alpha_2)$ and $\alpha_p(B_2)$. The pure homopolymers and mixtures thereof were analyzed by isoelectric focusing. The Coomassie-stained gel is shown in panel A. Panel B depicts the autoradiograph. Lane 1, $\alpha_c(\alpha_2)$; lane 7, $\alpha_p(B_2)$. Lanes 2-6 show the focusing pattern of the 1:1 mixture of these two homopolymers after incubation at 37°C for 0, 2, 4, 6 and 24 h. In lane 1, 2 and 4 some labeled protein has precipitated at the application site in the lower part of the gel. Relative proportions and compositions of the bands and population of bands indicated with a number are given in Table I.
As the incubation proceeds, more and more bands are observed with intermediate pI's and the label is more uniformly distributed over these bands. Within 24 h a steady-state situation is reached and no changes in the focusing pattern were observed when incubation was extended to 7 days. The newly formed hybrid aggregates have isoelectric points in the pI range from 5.7-6.4. However, the major part (87%) of the Coomassie-staining is observed in the narrow pI 5.9-6.0 region which falls within the average calculated from the pI regions of $\alpha_r(A_2)$ and $\alpha_r(B_2)$, namely 5.7-6.1. It is interesting to note here that when we mixed these homopolymers in other ratios (3:1 and 1:3) the resulting hybrid population again showed a narrow pI range around that of the number average of the pI's of the homopolymers.

By comparing the relative intensities of the bands in the Coomassie-stained gel and the autoradiograph, it is possible to calculate the relative amount of $A_2$ subunits in several bands. These results are summarized in Table I. Obviously, the isoelectric points of the bands are a reflection of the relative amounts of the acidic $A_2$ and the more basic $B_2$ chains. In the 24 h steady-state situation the subunit composition of the major band shows an equal amount of $A_2$ and $B_2$ chains. Since the $\alpha_r(A_2)$ and $\alpha_r(B_2)$ homopolymers were mixed in an equimolar ratio, the 1:1 subunit composition of the final protein population indicates a complete redistribution of the subunits over the hybrid aggregates.

The ability to form hybrids is not just limited to a mixture of $\alpha_r(A_2)$ and $\alpha_r(B_2)$. We also tested the following combinations of two homopolymers: $\alpha_r(A_1)/\alpha_r(B_1)$, $\alpha_r(A_2)/\alpha_r(B_1)$ and $\alpha_r(A_1)/\alpha_r(B_2)$. In each case steady-state situations were obtained within 24 h, which did not change if incubation was extended up to 7 days. In the combinations $\alpha_r(A_1)/\alpha_r(B_1)$ and $\alpha_r(A_2)/\alpha_r(B_1)$ a broad band was observed after 24 h, focusing at the intermediate pI, very similar to what was observed for $\alpha_r(A_2)/\alpha_r(B_2)$ in Fig. 4. Subunit exchange was incomplete in the combination $\alpha_r(A_1)/\alpha_r(B_2)$. Fig. 5 shows that at 24 h the homopolymers have exchanged some subunits since the clusters of both protein populations are shifted towards each other. However, absence of Coomassie-staining in the intermediate pI region indicates that subunit exchange was incomplete. This did not change upon prolonged incubation.

Exchange of subunits between homopolymers did not seem to be critical towards pH or ionic strength since identical results were obtained when the pH of the buffer was changed to 8.0 or when the buffer concentration was increased to 50 mM. Subunit exchange between $\alpha_r(A_1)$ and $\alpha_r(B_1)$ was studied at protein concentrations of 1, 5 and 10 mg/ml and no differences were observed.
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Band numbers refer to those shown in Figs. 4A and 4B. Bands with a faint appearance in either of these two figures gave unreliable subunit compositions and are not reported.

The proportion of each band is expressed in percent as obtained by gel scanning of the Coomassie-stained gel shown in Fig. 4A. Note that since not all bands are reported the sum of the percentages in this row will not amount to 100%. The subunit composition of the bands is expressed as the amount of A<sub>2</sub> chains relative to the total amount of A<sub>2</sub> and B<sub>2</sub> chains and is calculated from the gel scans of the coomassie-stained gel shown in Fig. 4A and the autoradiograph thereof, depicted in Fig. 4B.
in hybrid formation. However, temperature plays a crucial role. Subunit exchange, reaching a steady-state within 24 h at 37°C, was undetectable upon incubation at 5°C even after 7 days for all mixtures tested.

**SUBUNIT EXCHANGE BETWEEN NATIVE ALPHA-CRYSTALLIN AND HOMOPOLYMERS**

In order to study whether subunit exchange also occurred with native α-cry stallin molecules, we incubated α-cry stallin with two types of homopolymers at 37°C, in each case in a 1:1 ratio. The progression of the reaction of α-cry stallin with αr(B₂) was analyzed using isoelectric focusing and is depicted in Fig. 6. Already after 2 h numerous new bands have appeared, covering the whole pI region between the most acidic α-cry stallin and the αr(B₂) species. All of the original αr(B₂) aggregates and nearly all of the α-cry stallins have hybridized at this stage. After 24 h the pI range of the hybrids has narrowed down and most Coomassie-staining material is now concentrated in a small region at intermediate pI of 5.6-6.0. The reaction of α-cry stallin with αr(A₂) was performed at 37°C and 1:1 mixtures were analyzed at zero-time and after 24 h in the analytical ultracentrifuge (Fig. 7) since the isoelectric points of the two populations are too similar to analyze by isoelectric focusing. At the start of the incubation two sedimenting peaks are observed, representing α-cry stallin (17.0 S) and the αr(A₂) homopolymer (8.2 S). After 24 h only a single new peak is present with intermediate sedimentation velocity (9.3 S). These two experiments demonstrate that subunit exchange also occurs between native α-cry stallin and both αr(A₂) and αr(B₂) aggregates.

**DISCUSSION**

We used homopolymers of the four subunits A₁, A₂, B₁ and B₂ as model molecules to study the interaction of α-cry stallin aggregates. There are few techniques available to study the mutual interactions of individual α-cry stallin molecules directly. The homopolymers offer the advantage that they differ in pI and any change in their subunit composition results in a change of their pI, which is detectable by isoelectric focusing.
**Figure 5** (left): *Subunit exchange between $\alpha_r(A_1)$ and $\alpha_r(B_2)$.* Isoelectric focusing gel of the pure homopolymers of $A_1$ and $B_2$ and mixtures thereof. Lanes 1 and 6 show $\alpha_r(A_1)$ and $\alpha_r(B_2)$, respectively. Lanes 2-5 show the focusing pattern of their 1:1 mixture after incubation at 37°C for 0, 4, 6, and 24 h.

**Figure 6** (right): *Subunit exchange between $\alpha$-crystallin and $\alpha_r(B_2)$.* Isoelectric focusing gel of $\alpha$-crystallin and $\alpha_r(B_2)$ and mixtures thereof. Lane 1 and 2: $\alpha$-crystallin before and after 24 h of incubation at 37°C. Lane 8 and 9: $\alpha_r(B_2)$ after and before incubation. Lane 3-7: 1:1 mixture after incubation at 37°C for 0, 2, 4, 6 and 24 h.

**Figure 7:** *Sedimentation analysis of a 1:1 mixture of $\alpha$-crystallin and $\alpha_r(A_2)$.* Directly after mixing, $\alpha$-crystallin and $\alpha_r(A_2)$ sediment as two separate peaks (panel A), whereas after 24 h of incubation at 37°C only one single peak is detected (panel B). UV-scans were taken after 20 min of centrifugation at 56,000 rpm. Sedimentation is from left to right.
The experiment in which iodinated α (A.) was incubated with α (В ) shows that the changes in pI of the bands are accompanied by changes in the I-label content of the molecules. This is a first indication that homopolymeric molecules exchange their subunits and that this reaction continues until, within 24 h, a completely new population of hybrid molecules is formed which is relatively homogeneous with regard to its subunit composition. The second indication for exchange of subunits is found in the observation that the narrow pI range of the hybrid population is the number average of the pI's of the original two homopolymer populations. Subunit exchange is not limited to the combination of α (A.) and α (В .) but was observed for several combinations of all four homopolymer aggregates.

We carried out similar experiments with α-crystallin to assess whether the native population undergoes subunit exchange. Upon its incubation with α (A.) or α (В ) we monitored a rapid redistribution of all subunits as a new species appeared with intermediate pI or sedimentation velocity, simultaneously with the disappearance of the original α-crystallin and homopolymer population.

These experiments show that α-crystallin is capable of exchanging its subunits with homopolymers in the absence of denaturing agents. There seems to be no reason to assume that the subunit exchange observed between α-crystallin and α (A.) or α (В ) would not also happen between two α-crystallin molecules. To our knowledge, this kind of interaction is without precedent in the literature. Nevertheless, it might be a more universal characteristic of protein aggregates that has not been noticed until now and with a yet unknown function. As far as α-crystallin is concerned, this "dynamic quaternary structure" might provide us with an explanation for several unusual and unstoichiometric properties of this protein. First, charge microheterogeneity of both native and reassociated α-crystallin is the result of a random distribution of A- and B-type subunits in the aggregates; it was previously hypothesized that this distribution could arise by exchange of subunits (Siezen, Bindels and Hoenders, 1978). Second, upon aging, the size distribution of α-crystallin gradually shifts towards a higher average molecular weight (Siezen, Bindels and Hoenders, 1979; Bindels, De Man and Hoenders, 1982; Bessems, Hoenders and Wollensak, 1983). An $M_r$ of 780,000 was observed in the younger calf cortex and of 1,100,000 in the older nucleus. Since this growth takes place in the absence of protein synthesis, it cannot be explained by incorporation of newly synthesized subunits. Moreover, a simple oligomerization of α-crystallin molecules can be excluded because of
the unstoichiometric, gradual increase of the molecular weight. Third, rechromatography of five molecular weight subpopulations, isolated by means of size exclusion chromatography on Bio-Gel A-5m, revealed a slow re-equilibration of these subpopulations towards the initial size distribution (Siezen and Owen, 1983). Fourth, another type of unstoichiometric reaction is encountered in the study of the dissociation of α-crystallin by denaturing agents such as urea or guanidine hydrochloride. It has been shown that with increasing concentration of denaturant, the size of α-crystallin gradually decreases without the appearance of single subunits (Wisse, Zweers, Jongkind, Bont and Bloemendal, 1969; Siezen and Bindels, 1982; Thomson and Augusteyn, 1984; Bindels, Van den Oetelaar and Hoenders, 1986). It is obvious that these results cannot be explained by simple stoichiometric dissociation and hence other processes have to be involved. Subunit exchange may contribute to all these phenomena.

This exchange phenomenon may also explain the observation by Manski and Malinowski (1980, 1983) that the addition of reaggregated B chains to native α-crystallin caused complete loss of the antigenic determinants which are depending on the quaternary structure of α-crystallin. Remarkably, these authors found that upon addition of reaggregated A chains these antigenic determinants were conserved, suggesting that subunit exchange did not occur, whereas we observed subunit exchange between α-crystallin and α₂. Since Manski and Malinowski did not state the temperature at which their experiment was performed, the subunit exchange may have been prevented by a low temperature. On the other hand, if their experiment was performed at a high enough temperature the subunit exchange would not be detected if the A chains determine the antigenicity of α-crystallin.

Upon aging, several changes take place in the bovine lens that might affect the stability of α-crystallin. It was observed that the electrolyte composition of the cytoplasm of the fiber cells changes with age (Rink and Twenhoven, 1985; Bloemendal, Hockwin, Hoenders, Ohrloff and Rink, 1985), and it has been pointed out that the quaternary structure of α-crystallin is dictated by parameters such as the ionic strength and pH of its medium (Siezen, Bindels and Hoenders, 1980; Van den Oetelaar, Clauwaert, Van Lathem and Hoenders, 1985; Tardieu, Laporte, Licinio, Krop and Delaye, 1986; De Block, Dom and Clauwaert, 1986). During aging, the α-crystallin subunits undergo several post-translational modifications like C-terminal clipping, resulting in an increase in negative charge (Van Kleef, De Jong and Hoenders, 1975; Van Kleef, Willems-Thijssen and Hoenders, 1976). It was postulated that
these degraded chains are involved in the super-aggregation of $\alpha$-crystallin molecules (Siezen, Bindels and Hoenders, 1979). Furthermore, due to dehydration of the bovine lens nucleus, the cellular protein concentration increases with age (Rink, 1977; Rink, Muennighoff and Hockwin, 1977; Hockwin, Rast, Muennighoff and Twenhoven, 1978). We suggest that these changes disturb the thermodynamic equilibrium of the existing $\alpha$-crystallin population. By means of intermolecular exchange of subunits, this lens protein is capable of adequately responding to destabilizing changes in physiological conditions and producing a new, again thermodynamically stable population. Future experiments may be directed towards simulating age-related increase in size of $\alpha$-crystallin through subunit exchange/uptake of postsynthetically shortened A and B chains.

The molecular mechanism of the reaction is still unclear. Either subunits are transferred from one molecule to another during collision or subunits may detach from one molecule and adhere to another after having existed as a free subunit for a short period of time. From the experiments described here, no conclusions can be drawn as to the validity of one of these models.

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REFERENCES


THE TERTIARY STRUCTURE OF BOVINE ALPHA-CRYSTALLIN SUBUNITS
PROTEIN FOLDING AND AGGREGATION STUDIED BY
ISOELECTRIC FOCUSING ACROSS A UREA-GRADIENT AND
ISOELECTRIC FOCUSING IN TWO DIMENSIONS

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Netherlands.
SUMMARY

Isoelectric focusing across a concentration gradient of urea is used to study the folding/unfolding and association/dissociation processes of proteins. Myoglobin, albumin, RNase, papain, β- and α-crystallin were analyzed with this technique and examples are given of visualized dissociation steps and of equilibrium-unfolding intermediates. Furthermore, a two-dimensional isoelectric focusing technique is presented that is useful to deduce whether a transition of a protein aggregate observed upon urea-gradient isoelectric focusing must be attributed to a change in the protein's tertiary or quaternary structure.

INTRODUCTION

A large number of studies in the field of biochemistry and biophysics have been devoted to the phenomenon of protein folding. Much progress has been made in the last few decades, resulting in several models to describe the process (1-4). However, none of these has yet been generally accepted, not in the last place because of the fact that the experimental conditions to a certain degree determine which model may be applicable for the protein under investigation. The current interest in the process of protein folding and the factors that govern it is no longer purely academic. The new biotechnological production methods of proteins by recombinant DNA techniques and our ability to adapt proteins to our needs by means of directed mutations, once again have made us aware of our limited knowledge and that for a rational and successful use of these new techniques more insight is needed into the principles that determine the native protein structure and into the processes by which this structure is formed.

Denaturation of the native protein structure by agents like urea or guanidine hydrochloride, or the renaturation from concentrated solutions thereof, is a frequently used method to study protein structure and the

Abbreviations used: UGIF, urea-gradient isoelectric focusing; 2DIF, two-dimensional isoelectric focusing. IEF, isoelectric focusing; pI, isoelectric point; HSA, human serum albumin; T, total concentration of acrylamide and bis-acrylamide in percents; C, fraction in percents of bis-acrylamide in T; TEMED, N,N,N',N'-tetramethylene diamine;
process of folding and association. Changes in protein structure are often detected as changes in absorbance, fluorescence, circular dichroism or optical rotatory dispersion. Other detection techniques used include enzyme activity, sedimentation velocity, light scattering, scanning microcalorimetry or nuclear magnetic resonance (2-5). The introduction of rigid porous silica gels have added high-performance gel permeation chromatography to this list (see e.g. Refs. 6 and 7).

Electrophoresis across a urea-gradient in plain polyacrylamide gels as a technique to study protein folding was introduced by Creighton (for reviews on this topic the reader is referred to Refs. 8 and 9). In this technique, due to molecular-sieving by the pores in the polyacrylamide gel, urea-induced conformational changes that are accompanied by alterations in size are detected as changes in the electrophoretic mobility.

Changes in the net charge of the protein have seldomly been used to monitor changes in protein structure. In this paper we report the use of the high resolving power of isoelectric focusing in the study of urea-mediated de- and renaturation. We show that electrofocusing perpendicular to a concentration gradient of urea visualizes these processes. Several kinds of proteins were subjected to analysis by urea-gradient isoelectric focusing. The complexity of their structure varied from that of a small single-domain protein like RNase A to that of a multishubunit aggregate like the bovine eye-lens protein α-crystallin. Using this latter protein as a model, it was shown that isoelectric focusing in an intermediate concentration of urea followed by isoelectric focusing in 8 M urea in a second dimension, helps to deduce whether transitions of oligomeric proteins observed in UGIF gels are related to the dissociation/association of aggregates or to the un-/refolding process of isolated single subunits.
MATERIALS AND METHODS

Chemicals
Acrylamide, bis-acrylamide, TEMED and ammonium persulphate were all of electrophoresis purity and purchased from Bio-Rad. γ-Methacryloxypropyltrimethoxysilane came from Aldrich. Agarose-indubiose A37 was obtained from IBfraee. Urea and sucrose were purchased from Janssen Chimica. triethanolamine, phosphoric acid, sulphuric acid and sodium hydroxide were obtained from Merck. Pharmalytes were from Pharmacia. PAGE Blue 83 was from BDH Chemicals. All other chemicals were of analytical grade and came from local suppliers.

Proteins
Human serum albumin was obtained from Behringwerke, Warburg, F.R.G. Papain (type IV), RNase A (type XII A) and horse heart myoglobin (type III, iron in the ferric-state) were all from Sigma. Bovine α- and β-crystallin were isolated from calf eye-lenses by gel permeation chromatography as described (10). The monoclonal human gamma globulin spectrotype was a gift from dr. W. Flapper and isolated from a human gammapathic serum by high-performance size exclusion chromatography as previously reported (11).

Urea-gradient isoelectric focusing
Two glass plates (10x20 cm) were treated with γ-methacryloxypropyltrimethoxysilane and dimethyldichlorosilane, respectively. In this way the gel was covalently bound to a glass support (12). The mould was prepared by sandwiching three 0.6 mm spacers, greased with white vaseline, between the two glass plates, clamped together by large bulldog clips. The mould was filled from the bottom through the open shorter side, by a peristaltic pump that was fed by a gradient-mixer. A linear concentration gradient of urea was formed and stabilized by the increasing density of the urea. The light solution contained acrylamide and bis-acrylamide (3.5% T, 3% C; as defined by 13), 4.8% (v/v) Pharmalytes, the mixture of Pharmalytes depending on the protein to be analyzed, 0.07% TEMED and 0.05% ammonium persulphate. The heavy solution additionally contained 7 M urea. A few milliliters of the light solution were pumped into the mould before the gradient was started. The filling was finished with a few milliliters of the heavy solution. The gel was allowed to polymerize for about 2 h and used immediately. The linearity of the urea-gradient was checked occasionally by staining of the heavy solution with Coomassie and subsequent scanning of the polymerized gel.

Flat-bed electrophoresis was carried out in a Desaphor electrophoresis unit (Desaga), using 1 M phosphoric acid and 1 M sodium hydroxide as electrode solutions and an LKB 2297 Macrodrive 5 as power supply. One hour of prefocusing and 4 h of focusing were performed with a constant power of 5 W and a maximum voltage of 1000 V. After prefocusing, the protein solution was applied across the gel in a well cut from a strip of silicon rubber. Proteins were applied either in their native state as an aqueous solution, or in a denatured form dissolved in a solution of 7 M urea. Disulphide bridges were never reduced.

Cooling with tap water ensured a temperature of about 15°C. After focusing, the pattern of the osmotic iso-pH lines was checked to verify a straight pH gradient throughout the gel.

Two dimensional isoelectric focusing
First dimension: isoelectric focusing in various concentrations of urea. Polyacrylamide gels (4.5% T, 2.6% C) with a pH gradient of 4 to 9 were made in tubes of 13 cm length and 2.7 mm inner diameter. A gel stock-solution was
prepared of 2.4 ml of an acrylamide/bis-acrylamide solution (40%/1.07%, w/v), 14 μl TEMED, 0.25 ml Pharmalyte 4-6.5 and 0.25 ml Pharmalyte 6.5-9 in a final volume of 7.0 ml. Gel solutions with the appropriate urea concentrations were made of 1.0 ml gel stock-solution, 180 mg urea per M final concentration and water to a volume of 2.6 ml. After deaeration, 0.4 ml of an also deaerated 0.5% ammonium persulphate solution (w/v) was added and the resulting solution was cast into the tubes. An overlayer of iso-butanol ensured a horizontal meniscus. An ampholyte overlayer stock-solution was prepared of 0.125 ml Pharmalyte 4-6.5, 0.125 ml Pharmalyte 6.5-9, 0.3 g sucrose and water to a final volume of 5.0 ml. Ampholyte overlayer solutions with the appropriate concentration of urea were prepared from 0.5 ml of the ampholyte overlayer stock-solution and 60 mg of urea per M final concentration. Water was added to a final volume of 1.0 ml. The iso-butanol was discarded and the polymerized gels were layered with 100 μl of the corresponding ampholyte overlayer solution. The tubes were mounted in a home made container. A solution of 4 g triethanolamine / l was used as catholyte in the lower compartment. The upper compartment was filled with 2 g sulphuric acid / l as anolyte. An ISCO model 493 power-supply was used to prefocus for 1 h at 360 V, constant voltage. After prefocusmg, 100 μg of α-crystallin was applied to the gels through the ampholyte overlayer. Electrophoresis was carried out overnight at 360 V - constant voltage. Immediately after the gels were removed from the tubes, they were frozen at -20°C.

Second dimension: isoelectric focusing in 8 M urea. IEF in the second dimension was always carried out in 8 M urea with 4.5% T, 2.6% C polyacrylamide slab gels. For two slabs (150 x 170 x 0.75 mm) the following solution was prepared: 5.0 ml acrylamide/bis-acrylamide (40%/1.07%, w/v), 27 μl TEMED, 1.35 ml Pharmalyte 4-6.5, 1.35 ml Pharmalyte 6.5-9, 21.5 g urea and distilled water to a final volume of 42.0 ml. After deaerating 2.7 ml of a 1% ammonium persulphate solution (w/v) was added. The gels were cast between 2D glass plates and clamped in a Bio-Rad Protean II Slab Cell. A solution of 4 g triethanolamine / l was used as the lower buffer (catholyte), the upper buffer (anolyte) contained 2 g sulphuric acid / l. An 8 M urea ampholyte overlayer of about 5 ml, containing 0.5 ml Pharmalyte 4-6.5, 0.5 ml Pharmalyte 6.5-9, 2 g sucrose and 19.2 g urea in a final volume of 40 ml, was layered through the upper buffer onto the gel. Prefocusing was performed at 1000 V, constant voltage, for 1 h. The upper electrode solution and ampholyte overlayer were discarded and the remaining solution on the top of the gel was removed with a paper tissue. A small layer of 10% agarose (w/v) in ampholyte overlayer solution was cast on top of the gel and whirled a bit in order to prevent the remaining of a thin layer of upper buffer. The first-dimension gel rod was pressed into this agarose solution and covered with it. Reference samples were prepared by polymerizing the protein in tubes, using the mentioned gel solution, containing 8 M urea. Slices of 3 mm length, containing 100 μg of protein, were placed into the agarose layer. After the agarose had stiffened the upper electrode solution was poured onto the gel and a new ampholyte overlayer was applied. Electrophoresis was carried out overnight at 1000 V - constant voltage.

Fixing and staining
Fixing of the gels was performed in a solution of 12% trichloroacetic acid and 30% ethanol in water. The gels were Coomassie stained with 1.15% PAGE Blue 83 in destaining solution consisting of 25% ethanol and 8% acetic acid. The UGIF gels were dried on their glass support by a stream of hot air. The 2DIF gels were dried on a sheet of paper on a sintered glass filter, covered with plastic wrap. The water was evaporated from the gel under vacuum.
RESULTS

UREA-GRADIENT ISOELECTRIC FOCUSING

The denaturation profile of horse heart ferric myoglobin as detected upon UGIF is shown in Fig. 1. The upper part of the figure shows the unstained gel. Native myoglobin is visible because of the brown-red color given to it by its prosthetic group. At high concentrations of urea the intensity of the band gradually decreases to zero. This process starts at about 5 M urea and is complete at about 7 M. The Coomassie-stain of this gel in the lower part of Fig. 1 shows more detail. Multiple bands are now visible because the gel had to be overloaded with respect to the Coomassie sensitivity in order to visualize unstained myoglobin (upper part of Fig. 1). In the low urea region of the gel, two additional species are now revealed with more acidic pI's. These two bands merge at a concentration of about 5 M urea. Although the intensity of these bands is too low to be visible on the photograph of the unstained gel, they were detectable as faint red bands in the fresh gel and, therefore, very likely represent ferrous myoglobin (14). The Coomassie-stained gel now also reveals a new band of higher pI whose appearance coincides with the disappearance of the above-mentioned bands. Since this protein band is colorless, it obviously represents the apo-form of the molecule. Ragone et al. (15) have shown that the lack of ability to bind the heme in concentrated solutions of guanidine hydrochloride is caused by concomitant conformational changes in both structural units of the protein. Because of the higher sensitivity of the Coomassie stain, the lower part of Fig. 1 makes clear that this process starts at a concentration of about 3.5 M and that even at the highest concentration of 7 M urea a small portion of the native metmyoglobin still remains. Our results are in good agreement with those of Puett (16) who reported an increasing loss of the Soret absorption with increasing concentration of urea, showing a midpoint at 5.4 M urea. Interesting are the bands that are only present at intermediate concentrations of urea. These bands possibly represent intermediates in the unfolding process.

The urea-induced unfolding of human serum albumin is visualized by the UGIF gel shown in Fig. 2. The broad focusing area at low concentrations of urea reflects the microheterogeneity of the protein and has been mentioned before (17-21). At about 3 M urea there is a large and sudden change in pI
Figure 1: UGIF of horse heart metmyoglobin. The protein (10 mg) was applied across the gel. In the upper part of this figure the unstained gel is shown and myoglobin is visible by the red-brown color of its heme group. The lower part of this figure shows the same gel after staining with Coomassie. The concentration gradient of urea was enclosed by strips of the two limiting concentrations. The pH gradient was non-linear and was estimated from the Pharmalyte composition (3.3% 6-8 and 1.4% 8-10.5). The pH values in the figure are only given as an indication of the pH gradient and are by no means intended to be exact. A pI of about 7.5 was reported for the main band of metmyoglobin (17,38).

Figure 2: UGIF of human serum albumin. The protein (0.4 mg) was applied across a UGIF gel with a Pharmalyte composition of 1.0% 2.5-5; 2.9% 4-6.5 and 1.0% 6-8. Reported values for the pI are 4.9 for native albumin and 6 in concentrated urea (17,20).
and at about 4 M urea all of the albumin has undergone this transition. Nearly all of the protein is now focused in this new sharp band and only a minor part in a slightly more acidic one. An interesting phenomenon is observed between 4 and 6 M urea: the major band splits into two bands at about 4 M, while the minor band shows the same behavior at about 5 M. The two middle bands of the four now present merge at a concentration of about 5.5 M urea, resulting finally at 7 M urea in two major and one minor band.

Changes in pI upon increase of the concentration of urea were not observed for each protein studied. In the case of RNase A a single straight band running across the gel was found (UGIF gel not shown). Upon UGIF of papain, an enzyme known to be unusually stable to high concentrations of denaturant, three isozymic forms were revealed, all focusing as straight bands (Fig. 3). The purified monoclonal component of the gamma globulin fraction from the serum of a patient suffering from monoclonal gammapathy, was found to exist as a spectrotype of nine species. As shown by the UGIF of this protein (Fig. 4), increasing concentrations of urea have no effect on the pI's. Apparently, the two kappa and two gamma chains, joined by four disulphide bridges, are only minimally affected by urea in the absence of reducing agents, which is in good agreement with observations of Tanford and co-workers (22,23).

As examples of oligomeric proteins we chose the low molecular weight fraction of the β-crystallins from the bovine eye lens. These so-called βL-crystallins are composed of two or three non-covalently associated 22,000 to 26,000 Da subunits (10). UGIF of this complex protein population (Fig. 5) reveals that the first equilibrium transitions occur at concentrations of urea as low as 1.5 M where bands vanish and appear. Similar gradual and abrupt transitions are observed at the higher concentrations of 2, 3.5 and 5.5 M urea. Again, as was the case for myoglobin, it is interesting to note that certain bands, originating at lower concentrations of urea, exist only at intermediate concentrations of denaturant and disappear at the higher concentrations. Although it is likely that the transitions seen at low concentrations of urea are related to dissociation of the oligomers, and that those observed at higher concentrations are related to unfolding of the single subunits, it is impossible to unequivocally distinguish between these possibilities solely on the basis of the UGIF experiment.

The technique of 2DIF that we used to overcome this problem will be discussed later in the context of the multimeric bovine eye-lens protein α-crystallin. This protein is composed of four types of 20,000 Da subunits
Figure 3 UGIF of papain. An amount of 0.5 mg of protein was applied to the UGIF gel. Although the pI of papain is 8.75 (39), best results were obtained using Pharmalytes 9-11 (4.8%).

Figure 4 UGIF of a monoclonal gamma globulin spectrotype. An amount of 1.2 mg was applied. The Pharmalyte composition was 2.4% 4-6.5 and 2.4% 6.5-9.

Figure 5 UGIF of β<sub>L</sub>-crystallin. An amount of 2 mg of β<sub>L</sub>-crystallins isolated from calf lenses was applied onto a UGIF gel with a Pharmalyte composition of 1.0% 2.5-5, 3.5% 4-6.5 and 1.9% 6.5-9. The pI of the members of this population of di- and trimers ranges from 5.9 to 7 (10).
called $A_1$, $A_2$, $B_1$ and $B_2$. The $A_2$ and $B_2$ chains are the two primary gene products showing 57% sequence homology (10) and they are transformed into the $A_1$ and $B_1$ chain by a single phosphorylation step (24). No disulphide bridges are present in the molecules. Because of the variation in the relative subunit composition and their absolute number, $\alpha$-crystallin is in fact a population of proteins with respect to size and charge (25). Calf-lens $\alpha$-crystallin that was used in these experiments is composed of about 40 subunits and has a molecular weight around 800,000 (26). The acidic $A$ and basic $B$ chains are present in a ratio of about 3:1 (25). Despite the fact that models have been proposed for the architecture of this protein (27,28,29), a detailed description of the quaternary structure of $\alpha$-crystallin and the tertiary structure of its subunits is still lacking.

Only a limited portion of the large native aggregates entered the UGIF gel due to the sieving effect (Fig. 6). Protein precipitated at the application site is visible as a very thin line in the low urea region of the gel. Due to both the charge-population character of the protein and the molecular sieving of the gel pores no sharp focusing lines can be expected. Between 1.5 and 2 $M$ urea faint blurred bands appear at a more alkaline pI than that of the native protein. The two most alkaline bands sharpen between 3 and 4 $M$ urea and then run as two thin parallel bands towards the 7 $M$ urea region, where they undoubtedly represent the unfolded $B_1$ and $B_2$ subunits. The two dips in the $B_1$ and the single dip in the $B_2$ band at about 3.5 $M$ urea are caused by irregularities of the pH gradient, as judged from the osmotic iso-pH lines seen on the fresh gel. The pattern in the acidic region of the gel is more complex. At 3 $M$ urea a couple of diffuse double bands is present. Upon increase of the concentration of urea, the lower of both double bands splits, resulting in two sets of three bands each. At about 5.5 $M$ urea the triple bands both have merged to single bands that run parallel towards the 7 $M$ urea region, where they represent the unfolded single $A_1$ and $A_2$ chains. The less dense bands visible in this region are age-related degradation products of the four main bands. An identical focusing pattern was obtained when denatured $\alpha$-crystallin was applied in a 7 $M$ urea solution to the UGIF gel (not shown).

As argued in the case of $\beta_L$-crystallin, no definite conclusions can be drawn as to the nature of the triple bands of $A_1$ and $A_2$. They might represent either equilibrium-unfolding intermediates of the two $A$ subunits, or small aggregates formed as intermediates in the dissociation process of the native multimeric protein. These kinds of small aggregates have been detected in previous equilibrium dissociation experiments of $\alpha$-crystallin (6,30).
Figure 6: UGIF of α-crystallin. An amount of 0.08 mg of α-crystallin isolated from calf lenses was applied onto a UGIF gel with a Pharmalyte composition of 1.0% 2.5-5, 1.5% 4-6.5 and 1.9% 6.5-9. The pI's of the subunits in 7 M urea are reported to be 5.6 (A1), 5.9 (A2), 6.8 (A3) and 7.2 (B2) (40). Large aggregates, precipitated at the application site, form a thin straight band at the low-urea side of the gel. Molecular sieving and the charge heterogeneity of native α-crystallin results in a broad and diffuse band between 0 and 1 M urea.

Figure 7: 2DIF of α-crystallin. Focusing of 0.1 mg of α-crystallin in the first dimension was performed in gel rods containing 2.3% Pharmalytes 4-9 and urea in a concentration of 8 M Taur, top left, 4.3 M Taur, left, or 2.7 M Taur, right. The second dimension was carried out in a slab gel containing the same amount of Pharmalytes and 8 M urea. A reference sample of α-crystallin was also run in the second dimension slab gel and is indicated with 'α'.
In order to elucidate whether the triple bands of α-crystallin A chains represent unfolding or dissociation intermediates, we designed a two-dimensional isoelectric focusing technique. The usual O'Farrell method (31) is not applicable in this situation since, once treated with urea, the four subunits have identical mobility upon sodium dodecylsulphate polyacrylamide gel electrophoresis. Therefore, as separation method in the second dimension, we performed slab-gel isoelectric focusing in the presence of 8 M urea.

In the first dimension, α-crystallin was focused in the presence of 8 M urea (Fig. 7A), 4.3 M urea (Fig. 7B), and 2.7 M urea (Fig. 7C). The functioning of the system is proven by the diagonal formed by the spots if both the first and second dimension were carried out in 8 M urea (Fig. 7A). The first dimension of the 2DIF gel shown in Fig. 7B was carried out in 4.3 M urea, a concentration at which the triple bands of A1 and A2 are present (Fig. 6). The 2DIF is essentially identical to that of Fig. 7A, although the spots of A1 and A2 are somewhat broader due to the fact that the triple bands occupy a longer part of the first-dimension gel rod than do the single A1 and A2 bands in the 8 M first-dimension gel rod. The fact that no other bands focus in a vertical line above the A1 and A2 spots indicates that the triplets do not represent hetero-oligomers, i.e. aggregates of different subunits. Another possible explanation, assuming residual quaternary structure for the triplets, is that we are dealing with homo-oligomers of A1 and A2, i.e. (A1)n and (A2)n respectively, the simplest possibility being mono-, di- and trimers. However, since high-performance size exclusion chromatography in the presence of 4.2 M urea indicated that no trimers and probably no dimers are present (6), we conclude that the two triple bands seen in the UGIF gel represent three forms of the single A1 and A2 subunit, respectively, that differ in certain secondary or tertiary structural aspects.

Fig. 7C shows what is found if the concentration of denaturant in the first dimension of a UGIF experiment allows aggregates to be present. In this case α-crystallin was focused in the presence of 2.7 M urea. At the top of the gel rod in which the first dimension was performed, large aggregates that were unable to penetrate have precipitated. Complete dissociation of these aggregates by 8 M urea in the second dimension gives rise to a vertical lane containing all the subunits. Two groups of smaller aggregates were able to penetrate the first-dimension gel rod. In the second dimension, the
aggregates from these two groups have dissociated into their constituting subunits. This results for the group with more acidic pi in four horizontal bands representing all four subunits. The relative intensities of these bands show that the low pi aggregates are enriched in A chains. The aggregates of the group with more alkaline pi are composed of only B chains as is indicated by the fact that upon dissociation in the second dimension only a $B_1$ and a $B_2$ band are obtained.

**DISCUSSION**

UGIF electrophoresis of a protein takes place in an IEF slab gel in which a concentration gradient of urea perpendicular to the pH gradient is present. If the urea-induced changes in the conformation of the protein are reflected in changes in net charge, UGIF offers a unique continuous visualization of the dissociation and unfolding processes. Alternatively, if the protein is applied in a denatured state, visualization of the renaturation process is obtained. In this technique it is indeed essential that conformational changes are accompanied by changes in pi. Our study of RNase A, papain and monoclonal gamma globulin indicates that this is not always the case. However, if changes in charge do occur, this may lead to very interesting observations. The various patterns that are possible have been mentioned in a brief paper on isoelectric focusing across a discontinuous urea-gradient (32). Many of these patterns and combinations thereof can be observed in the gels shown here.

One way to study the process of protein (un)folding is to analyze the kinetics of the process. Because of the rapidity of the reaction, intermediates are seldomly detected and hence (un)folding is often described as a two-state process (2,3). In equilibrium studies on the other hand, intermediates may be populated under certain favorable conditions like certain concentrations of urea (2,3,9). UGIF visualizes equilibrium situations across the complete range of urea concentrations generally used for (un)folding studies. As Kim and Baldwin have pointed out, folding probably proceeds along a pathway determined by the most stable intermediates (2) and, therefore, UGIF has the potency of providing us with information of the folding pathway by revealing stable and metastable intermediates, and their relation with each other and the folded and unfolded protein. The relevance of the study of
equilibrium intermediates for the understanding of the folding process is becoming evident since recently equilibrium intermediates have been reported that are identical to transient folding intermediates observed in kinetical folding studies (15,33-35).

In the case of HSA we discerned two transition regions. Since this is a monomeric protein, interpretation of the UGIF pattern should not be hampered by the possibility of dissociation steps. Therefore, the large shift in pI at about 3 M of urea at first glance reflects a large change in tertiary structure and might be related to the unfolding of one or more domains. Indeed, the large change in pI of albumin upon denaturation by urea was attributed to the exposure of positively charged groups (18). According to others this change in pI is caused by dissociation of negatively charged ligands like fatty acids and bilirubin (20,22). The HSA used in our experiments typically contained 0.3% fatty acids (as stated by the manufacturer), which, when calculated as palmitic acid, equals a ratio of 0.8 moles of palmitic acid to one mole of albumin. However, this concept of ligand dissociation cannot explain a previous observation that albumin, deprived of its ligands by exhaustive dialysis in 8 M urea and subsequently renatured, again focuses at its original acidic pI (18). In our hands, replication of this experiment resulted in focusing of both the dissociated and refolded albumin at the alkaline pI (gel not shown) and, therefore, we favor the view of ligand dissociation to explain the pI shift at 3 M urea. Although the transitions observed between 4 and 5.5 M urea involve much smaller changes in pI, they might still reflect large changes in domain structures. It is not likely that these transitions represent the minimal structural elements of the folding process or kernels as they have been defined (2,36) since these are believed not to be stable enough to be directly detected as intermediates (3). Furthermore, it has been shown that some secondary structure is present in HSA at these concentrations of urea (7). The disulphide interchange, catalyzed by the free thiol group and suggested as an explanation for the microheterogeneity of albumin at high concentrations of urea (37), might be the molecular basis of the transition between the conformers observed between 4 and 5.5 M urea upon UGIF.

UGIF of the multimeric bovine eye-lens protein α-crystallin between 4 and 5.5 M urea results in similar band-splitting patterns as for albumin. By means of 2DIF it could be ruled out that these patterns arose from the dissociation process and, therefore, one can conclude that at intermediate concentrations of urea three stable or metastable conformers of both the αA1
and $\alpha\beta_2$ subunit exist. It is important to note that the UGIF gel of the renaturation process of $\alpha$-crystallin, that was obtained by applying the protein in a solution of 7 M urea (gel not shown) was identical to the denaturation gel (Fig. 6), indicating that the equilibrium-folding and -unfolding processes give rise to the same intermediates. The implications of these findings for the folding model and the tertiary structure of the $\alpha$-crystallin subunits will be discussed in the following chapter.

In conclusion we feel that UGIF is an elegant equilibrium method to produce a continuous image of the folding or unfolding and association or dissociation of proteins. Because of the high resolving power which is characteristic of IEF techniques, transitions of closely similar structures can be made visible. In the case of proteins with quaternary structure it is necessary to deduce whether transitions are related to association/dissociation or folding/unfolding. If the subunits differ in $M_r$, the O'Farrell two-dimensional method can be used in which the first dimension is carried out in the concentration of urea at which the intermediate is observed. However, if the subunits are of the same $M_r$ and only differ in $pI$, as is the case for most isozymes, the presence of heterogeneous aggregates can be detected by 2DIF in which the first dimension is performed in the desired concentration of urea and the second dimension IEF in the presence of 8 M urea.

REFERENCES

FOLDING/UNFOLDING AND AGGREGATION/DISSOCIATION OF BOVINE ALPHA-CRYSTALLIN SUBUNITS

EVIDENCE FOR UNFOLDING INTERMEDIATES OF THE ALPHA-A SUBUNITS

Piet J. M. van den Oetelaar and Herman J. Hoenders

Preliminary results were reported at the 13th International Congress of Biochemistry, August 25-30, 1985, Amsterdam, The Netherlands.
The aggregation and dissociation behavior of bovine α-crystallin as well as the folding and unfolding of its subunits were investigated by equilibrium studies using tryptophan fluorescence measurements and two isoelectric focusing techniques, viz. isoelectric focusing across a urea gradient and isoelectric focusing in two dimensions with different concentrations of urea. It was found that the αB chains lose their ability to aggregate and start unfolding at a lower concentration of urea than the αA chains. Equilibrium intermediates were found upon unfolding or refolding of αA subunits, which can be explained by a two-domain organization of these molecules.

INTRODUCTION

One of the most intriguing characteristics of the eye lens is that this organ is optically clear despite its extremely high protein content. This, of course, is a necessity for its proper functioning and can only be achieved by a high degree of organization of the fiber cells (Kobayashi and Suzuki, 1975; Van Heyningen, 1975; Duncan and Jacob, 1984). A further requirement is a high density of packing of the structural lens proteins (crystallins), but no paracrystalline state is required (Benedek, 1971). Indeed, short-range, glass-like protein interactions have been established in intact lenses by means of small-angle X-ray scattering (Délaye and Tardieu, 1984). Therefore, an insight into the structure of the crystallins will not only be helpful in the understanding of the in situ organization of these proteins but also in the understanding of the factors and processes that disrupt this organization and eventually cause cataract.

Bovine α-crystallin has received much attention in studies concerning the structure of the lenticular proteins. The protein is composed of 20,000 Da subunits and the amino acid sequences of the two primary gene products, αA2 and αB2, have been resolved (Van der Ouderaa, De Jong and Bloemendal, 1973; Van der Ouderaa, De Jong, Hilderink and Bloemendal, 1974). Two other α-crystallin subunits, αA1 and αB1, arise from the former ones by a single phosphorylation step (Voorter, Mulders, Bloemendal and De Jong, 1986; Chiesa, Gawinowicz-Kolks and Spector, 1987). Conflicting models have been presented.

Abbreviations used: UGIF, urea-gradient isoelectric focusing; ZDIF, two-dimensional isoelectric focusing.
on the quaternary structure of \( \alpha \)-crystallin, describing either a 800,000 Da three-layer aggregate (Bindels, Siezen and Hoenders, 1979; Van den Oetelaar, Clauwaert, Van Laethem and Hoenders, 1985; Tardieu, Laporte, Licinio, Krop and Delaye, 1986) or a 320,000 Da aggregate possibly of micellar structure (Thomson and Augusteyn, 1983, 1984; Augusteyn and Koretz, 1987). Little is known about the secondary structure of the subunits. By means of circular dichroism analyses it was found that \( \alpha \)-crystallin contains 50% \( \beta \)-structure and virtually no \( \alpha \)-helix (Liang and Chakrabarti, 1982).

Concerning the tertiary structure of the subunits no direct information is available. However, based on hydropathy profiles, secondary structure predictions, circular dichroism and considerations on structural similarities with \( \beta \)- and \( \gamma \)-crystallins, a 4-motif folding pattern containing \( \beta \)-structure was proposed for all three crystallin classes (Siezen and Argos, 1983). Based on the gene structure of bovine \( \alpha A \) and the sequence similarity between \( \alpha A \) and \( \alpha B \), Wistow (1985) suggested that the tertiary structure of these subunits consists of a globular N-terminal domain of two symmetry-related motifs and a somewhat larger C-terminal domain (also of two motifs) with an exposed C-terminal arm. No resemblance was suggested between these motifs and the 'Greek key' motifs of the \( \beta \)- and \( \gamma \)-crystallins beyond common \( \beta \)-structure. Structural similarity was found between the globular C-terminal domain and the small heat shock proteins of \textit{Drosophila} and \textit{Caenorhabditis elegans}.

The most informative technique for the conformational study of proteins with known primary structure is X-ray analysis. However, up till now attempts to grow crystals from \( \alpha \)-crystallin solutions have been unsuccessful and it is believed that this has to be ascribed to the size and charge heterogeneity of the protein, caused by the variety of subunit number and composition amongst the individual molecules (Siezen, Bindels and Hoenders, 1978). If \( \alpha \)-crystallin has the dynamic quaternary structure that we postulated (chapter 2.2), this will certainly contribute to making crystallization of this protein a difficult if not impossible task.

Therefore, in the mean-time other methods have to be used for further conformational studies of \( \alpha \)-crystallin. In chapter 3.1 we discussed UGIF and 2DIF as methods to study the urea-induced equilibrium folding and unfolding as well as aggregation and dissociation of proteins; moreover we showed the first results obtained with \( \alpha \)-crystallin using these techniques. Here we report new results, together with fluorescence analyses, which indicate that intermediates are involved in the (un)folding pathway of the bovine \( \alpha A \) and \( \alpha B \) chains. The consequences of these findings for the tertiary structure of the
subunits and the quaternary structure of the \( \alpha \)-crystallin molecule are discussed.

**MATERIALS AND METHODS**

**PROTEIN PREPARATIONS**

Bovine \( \alpha \)-crystallin was isolated from extracts of cortical parts of calf lenses by gel filtration and preparative ultracentrifugation as described in chapter 2.2. The same procedures were followed for the isolation of human \( \alpha \)-crystallin from a pool of normal human lenses and of rat \( \alpha \)-crystallin. The isolation of bovine \( \alpha \)-crystallin subunits by ion-exchange chromatography in 6 M urea and their subsequent renaturation by removal of urea via dialysis has also been described in chapter 2.2.

**METHODS**

Urea-gradient isoelectric focusing, carried out as reported in chapter 3.1, was used to study both the denaturation and renaturation process of \( \alpha \)-crystallin, depending on whether the applied protein was dissolved in the absence or presence of 7 M urea. The technique of 2DIF has also been described (chapter 3.1).

Equilibrium fluorescence measurements were performed on samples containing 10 \( \mu \)g/ml \( \alpha \)-crystallin in 50 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris), pH 6.0. The concentration of urea in the preparations ranged from 0 to 7 M. After 4 h of incubation at room temperature, fluorescence emission spectra were recorded with an Aminco-Bowman spectrophotofluorometer (American Instruments, Inc., Silver Spring, Md.) using an excitation wavelength of 278 nm and an excitation- and emission-bandwidth of 5.5 nm.

Polyacrylamide gel electrophoresis in the presence of urea was carried out in 13% gels of 0.7 mm thickness using a BioRad Protean II slab gel electrophoresis system. The technique was performed as described by Bloemendal, Zweers, Benedetti and Walters (1975) using an electrode buffer containing 4.3 M urea, adjusted to pH 7.5.

**RESULTS**

In chapter 3.1 we presented UGIF as a powerful tool for the visualization of urea-mediated denaturation and renaturation processes of proteins. In Fig. 6 of that chapter we showed the denaturing UGIF gel of calf \( \alpha \)-crystallin. The renaturing UGIF gel of the same protein is now presented here (Fig. 1). Superimposing these two gels learns that they are fully identical. At the 7 M urea side of the UGIF gel in Fig. 1, the four main \( \alpha \)-crystallin chains, \( \text{A}_1 \)-
Figure 1 (above): Renaturing UGIF gel of bovine α-crystallin. Dissociated and unfolded α-crystallin, applied as a 7 M urea solution, was focused across a traverse urea-gradient. The main subunits, A₁, A₂, B₁, and B₂, are indicated. The other bands represent age-related degradation products.

Figure 2 (left): Polyacrylamide gel electrophoresis of bovine α-crystallin and its subunits in the presence of urea. Electrophoresis was carried out with a 4.3 M urea electrode buffer of pH 7.5. Lane 1, αB₂; 2, αB₁; 3, αA₂; 4, αA₁; 5, α-crystallin.

Figure 3 (below): 2DIV of bovine α-crystallin. Isoelectric focusing in the first dimension was performed in 3.3 M urea. The slab gel isoelectric focusing in the second dimension was carried out in the presence of 8 M urea.
A₂, B₁ and B₂, are present as single subunits. The other, less dense bands represent post-translationally modified subunits. Going from high to low urea concentration, the B₁ band runs straight until about 2 M. The B₂ band behaves similar, except that it undergoes a gradual acidic shift between 3.5 and 3 M urea. Below 2 M both bands have disappeared. Both the A₁ and A₂ band split twice at about 5.5 and 5 M urea. The two new bands curve towards the lower pH region and merge at about 3.5-3 M, at the same time becoming diffuse. The original band runs straight until about 3.5 M urea and then also curves slightly towards more acidic pH. At about 2.5-2 M all of these bands gradually disappear.

In order to conclude whether the triple aA bands, present between 3.8 and 4.6 M urea, arise from oligomeric aggregates or represent different unfolding states of the subunits, further analysis was necessary. In chapter 3.1 it was demonstrated by means of 2DIF that these triple bands are composed of only A₁ or A₂ chains and it was concluded that they do not represent small aggregates composed of various types of subunits. High-performance gel permeation chromatographic analyses indicated that it was unlikely that the triple bands represent small oligomers of one type of subunits. Further evidence for the monomeric character of the triple bands is obtained by polyacrylamide gel electrophoresis at pH 7.5 in the presence of 4.3 M urea. On this gel A₁ and A₂ both run as a single band (Fig. 2). Because of the molecular sieving of the proteins in this type of gel, the separation is not only based on the charge of the proteins but also on their size. Therefore, since both A₁ and A₂ run as a single band, the possibility of differences in quaternary structure as the cause of the triple aA bands on UGIF gels must be rejected.

The states of the subunits at lower concentrations of urea (aggregates or single chains) was analyzed by means of 2DIF (Fig. 3). The first dimension was performed in 3.3 M urea. At this concentration aggregates are still present as is indicated by the fact that a band focusing at the acidic side of the first dimension gel rod yields a vertical lane of various spots upon electrofocusing in 8 M urea in the second dimension. It is striking that these aggregates are virtually devoid of B chains. The latter have been focused at their own pI in the first dimension as can be concluded from the fact that they focus on the diagonal in the second dimension. The reason why A₂ focuses as two bands in the second dimension is not known. This was not observed in nearly identical 2DIF gels in which the first dimension was carried out in the presence of 3 or 3.6 M urea.
Figure 4: Emission maxima of the tryptophan fluorescence emission spectra of α-crystallin (○), αA₂ (□) and αB₂ (▲) at various concentrations of urea.

Figure 5: Denaturing UGIF gel of human α-crystallin. The main subunits, αB₂, αA₂ and αAₓ, are indicated. The non-marked bands represent age-related degradation products.
Since the tryptophan fluorescence emission spectrum shows a red-shift with increasing solvent exposure of the tryptophan residues, the emission maximum wavelength can be used to monitor structural changes of proteins (Burstein, Vedenkina and Ivkova, 1973). A continuous red-shift of $\lambda_{\text{max}}$ is observed for $\alpha$-crystallin upon increasing concentrations of urea (Fig. 4). In order to investigate whether the relatively different behavior of the $\alpha A$ and $\alpha B$ chains found upon UGIF is confirmed by their tryptophan fluorescence, we recorded the emission spectra of isolated $A_2$ and $B_2$ subunits at various concentrations of urea. As is shown in Fig. 4, the change in $\lambda_{\text{max}}$ is biphasic for both $A_2$ and $B_2$. However, the $A_2$ chains seem to be more resistant towards denaturation by urea than the $B_2$ chains. At 1.7 M urea $\lambda_{\text{max}}$ of $B_2$ reaches a plateau, whereas $A_2$ shows no change up to approximately this concentration of denaturant. The plateau region of $B_2$ stretches from 1.7 to 3 M urea and that of $A_2$ from 3.5 to 4.2 M. At these plateaus, the shift in $\lambda_{\text{max}}$ is about 85% of the ultimate shift observed at 7 M urea. At the same urea concentrations where the $\lambda_{\text{max}}$ plateau regions are seen, the band of the $B_2$ chains on the UGIF gels is somewhat diffuse and the $A$ chains show their characteristic triple bands (Fig. 1). The values for $\lambda_{\text{max}}$ at the highest concentrations of urea, 350 nm for $A_2$ and 352 nm for $B_2$, indicate complete water exposure of the tryptophan residues (Burstein, Vedenkina and Ivkova, 1973). The pattern for $\alpha$-crystallin is more or less the average of that of $A_2$ and $B_2$, but the subunit plateaus are not resolved. Siezen and Bindels (1982) found a slight plateau at 1.5-2 M in a similar experiment with $\alpha$-crystallin. Although the shape of the rest of the curve was identical, their $\lambda_{\text{max}}$ values are consistently 4 nm lower than ours.

It would be interesting to know whether the unfolding behavior reported above is limited to the bovine $\alpha$-crystallin subunits or whether it is of a more universal character and also exhibited by the subunits of $\alpha$-crystallin of other species. The denaturing UGIF pattern of human $\alpha$-crystallin is given in Fig. 5. The $B$ chains focus as sharp bands, starting at about 2 M urea. A small shift in $pI$, as observed for the bovine $B_2$, is not present. At about 3 M the $A_2$ chain becomes visible as two bands that merge at about 5.5 M urea. The third, most acidic band that was observed for the bovine $\alpha A$ chains upon UGIF, is absent in human $\alpha A_2$. The human $\alpha A_1$ chain focuses from about 4 M urea as a single band. The focusing pattern of rat $\alpha$-crystallin is much the same as that of man (Fig. 6). Again, the $B$ chains start focusing at 2 M urea. The rat $B_2$ chain shows the same gradual shift in $pI$ between 3.5 and 4.5 M as does the bovine $B_2$ between 3 and 3.5 M. The rat $A$ chains start focusing at 3 M urea, the $A^{\text{ins}}$ and degraded $A_2$ chains as single bands, the $A_2$ as a double band.
Figure 6: Denaturing UGIF gel of rat α-cry stallin. The main subunits αB₂, αAₓNB and αA₂ are indicated. The large number of other bands visible on this somewhat overloaded gel represent degradation products.

Figure 7: Denaturing UGIF of rat α₂ subunit.

Figure 8: Schematic representation of the UGIF pattern of bovine α₁ chains. The focusing pattern is composed of the native (N) and unfolded (U) form and three intermediates (I₁, I₂, I₃) of the αA subunit.
merging at about 5 M urea. The latter is more clearly visible in the UGIF gel that was made of isolated rat A2 subunit (Fig. 7). Isolated rat A ins showed only the dissociation-derived shift at about 3 M and then runs as a straight band (UGIF gel not shown).

DISCUSSION

The two types of bovine α-crystallin subunits, A and B, differ markedly in their stability towards urea. First, a red-shift of the fluorescence emission spectrum is observed for B2 in the presence of very low concentrations of urea, whereas below 1.5 M the spectrum of A2 remains unaffected (Fig. 4). Second, the λ max plateau region in the biphasic λ max -graph is reached for the B2 chain at substantially lower concentrations of urea than for A2. Third, 2DIF showed that the B chains lose their ability for aggregation at lower concentrations than the A chains (Fig. 3).

The biphasic red-shift of λ max is evidence for the existence of one or more equilibrium unfolding intermediates (Cantor and Schimmel, 1980; Kim and Baldwin, 1982; Pace, 1986). These intermediates give rise to some peculiar focusing patterns in the parts of both the denaturing and renaturing UGIF gel that more or less correspond to the urea concentrations of the λ max plateau regions. At the point at which there is a small shift in the pI of the bovine αB2 subunit in the UGIF gel (3-3.5 M), the band is somewhat diffuse. Presumably a rapid transition exists between the intermediates which prevents them from focusing as sharp bands. Remarkably this phenomenon is not observed for the B1 chain that differs only in a single phosphorylation from the B2 chain (Voorter, Mulders, Bloemendal and De Jong, 1986; Chiesa, Gawanowics-Kolks, Kleiman and Spector, 1987). Both bovine αA1 and αA2 focus as triple bands at about 4 M urea in the UGIF gels. This indicates that there exist equilibrium unfolding intermediates. Since no Coomassie-staining material is present between the triple bands, it is concluded that the conditions in the gel, of which the low temperature is probably the most crucial (Creighton, 1986), are such that transitions between the intermediates are slow compared to the time scale of the focusing process, if these transitions are present at all. As for the A chains, the phosphorylation responsible for the formation of αA1 from αA2 (Voorter, Mulders, Bloemendal
and De Jong, 1986; Chiesa, Gawnowics-Kolks, Kleiman and Spector, 1987) apparently does not affect the (un)folding behavior.

The fact that the renaturing and denaturing UGIF gels of bovine α-crystallin can be fully superimposed indicates that both, the equilibrium folding and unfolding processes, give rise to the same intermediates. Even though the equilibrium intermediates need not be populated during the kinetical (un)folding process (Creighton, 1986), their existence indicates that (un)folding does not follow a two-state mechanism (Pace, 1986) and generally indicates that the protein has more than one domain (Pace, 1986).

More insight into the domain structure of αA subunits, therefore, may be gained from their UGIF pattern. In our opinion this focusing pattern of the A chains can be explained as follows. As indicated by 2DIF (Fig. 3), at low concentrations of urea the A subunits are present as aggregates. Since these aggregates are microheterogeneous as shown in chapter 2.2, they will focus as diffuse bands. Therefore, the blurred band in the A1 and A2 region of the UGIF gels likely represents aggregates composed of native (N) subunits (Fig. 8). At higher concentrations of urea the aggregates are dissociated, resulting in sharper focusing patterns, and two of the three sharp A bands undergo a relatively large change in pI. Since only single subunits are present at these concentrations of urea as we demonstrated by means of alkaline urea gel electrophoresis (Fig. 2), these pI changes must be attributed to changes in tertiary structure. Therefore, these two bands reflect (un)folding intermediates (I1 and I2). The single band that is observed at the high-urea side of the UGIF gels represents the unfolded A chain (U). The extension of this band into the low-urea side of the gels (I3) focuses sharply at urea concentrations where also aggregates are present. The pI of this band is not much affected by the concentration of urea. It is very unlikely that this band represents the unfolded form (U) of the A subunits at these low concentrations of urea. Therefore, we assume that it represents a third intermediate (I3), the pI of which is very similar to that of U.

The relative intensities of all three bands (I1, I2 and I3) do not change with the concentration of urea. In our opinion this means that no transitions between the three states exist. Once a molecule has been trapped in one of these states, it can follow only one route of (un)folding. This means that the concept of sequential or hierarchic folding (Kim and Baldwin, 1982; Ghélis and Yon, 1982; Pain, 1987) is not applicable to the αA subunits, since in that case the bands should show changes in intensities upon changes of urea concentration. On the other hand, the unchanging intensities of the three
bands is very well compatible with a recent model suggesting that the 
(un)folding process is composed of a multiplicity of pathways (Lesk and Rose, 
Pain, 1987). The model implies that in the case of protein folding one part 
of the molecule has acquired its structure while other parts may still be 
folding. Keeping this in mind, the αA focusing pattern can be represented by 
the following scheme for the (un)folding:

As stated before, judged from the unchanging intensities of the bands, 
transitions between the intermediates are unlikely to occur. Evidence is 
accumulating that critical (un)folding steps and (un)folding intermediates of 
large proteins are related to protein domains (Creighton, 1986) which in turn 
are believed to correspond to exons (Gilbert, 1978; Blake, 1978; Ghélys and 
Yon, 1982). The intermediates $I_1$ and $I_2$ could both have one domain largely 
unfolded and another grossly intact, the folded and the deteriorated domain 
being different in both intermediates. If we approach the problem as a folding 
process by interpreting the UGIF gel from high to low urea concentration, the 
folded domain serves as a template for the folding of the second domain. The 
formation of interdomain contacts causes the shift in $pI$. For some part of the 
population of αA molecules ($I_3$) the folding processes do not result in the 
creation of either domain although partial secondary structures will be 
present. When the urea concentration is low enough, these molecules will be 
transposed directly to the native structure by a consensus way of folding. 
Obviously, this view implies a two-domain structure for the αA chains. This 
is in accordance with the hypothesis of Wistow (1985) that the exon structure 
of the bovine αA gene predicts a two-domain protein in which each globular 
domain is composed of two β-structured motifs. Based on the 57% sequence 
similarity, he predicted a similar structure for the αB chains. Since the β 
chains reveal too little detail on the UGIF gels, it is impossible at this 
stage to draw a corollary on their tertiary structure from the results 
presented here.

In the model described above for the folding of the αA subunits all three 
pathways result in the same native structure. Another possibility is that the 
three pathways lead to three differently folded chains. The three-layer model
of the architecture of α-crystallin built up from only four subunits (Bindels, Siezen and Hoenders, 1979; Tardieu, Laporte, Licinio, Krop and Delaye, 1986) implies that they have to occupy non-equivalent positions. Apparently this is the main objection to this model (Augusteyn and Koretz, 1987). However, if the subunits are present in several native conformations this could explain their non-equivalent positions.

With the observed difference in stability of αA and αB, it is now possible to explain results that were obtained earlier by high-performance gel permeation of α-crystallin in urea-containing solutions (Bindels, Van den Oetelaar and Hoenders, 1986). At concentrations of urea between 3 M and 3.8 M the monomers eluted as two not completely resolved peaks. Upon analysis of the subunit composition of these peaks by means of isoelectric focusing in the presence of 6 M urea, it was found that the fast moving peak was enriched in B chains whereas the slower one was enriched in A chains. Only at higher concentrations of urea the A chains were also fully unfolded and coeluted with the B chains at a smaller elution volume. Therefore, our observation that the B chains unfold at lower concentrations of urea than the A chains is clearly reflected in the hydrodynamic volume of the α-crystallin subunits at intermediate concentrations of urea. As can be calculated from the elution volumes and the calibration plot of the gel permeation column used, the hydrodynamic radius of the αA subunit increases by a factor 1.4 upon unfolding.

When compared with the bovine αA subunits, the rat and human αA chains behave quite differently upon UGIF. First it is striking that the rat and human A2 subunit both focus as two bands at intermediate concentrations of urea, whereas the rat AINS and the human A1 do not. Second, the third - most acidic - band observed for the bovine A1 and A2 is absent in the A2 of the other two species. This could mean that the folding process of these subunits is quite different from that of the bovine αA chains, implying that the domain structure is different. However, this is not to be expected. Apart from the AINS which differs by a peptide fragment of 22 residues (Cohen, Westerhuis, De Jong and Bloemendal, 1978), the αA chains within one species likely differ only by phosphorylation (Voorter, Mulders, Bloemendal and De Jong, 1986). Furthermore, the primary structure of αA and αB is highly conservative (De Jong, 1981), which makes it very likely that similar domains are present in all of these molecules. Therefore, the most plausible explanation for a different UGIF behavior of these αA subunits is that the intermediates are only stable at very defined conditions, which for these specific proteins might not be met during UGIF.
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DETECTION OF ASPARTIC ACID ENANTIOMERS BY CHIRAL CAPILLARY GAS CHROMATOGRAPHY

DETERMINATION OF IN VIVO RACEMIZATION AND REDUCTION OF METAL-INDUCED BACKGROUND

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DETERMINATION OF IN VIVO RACEMIZATION AND REDUCTION OF METAL-INDUCED BACKGROUND

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SUMMARY

Racemization of aspartyl residues in proteins is a post-translational process, related to ageing. A method is presented for the detection of aspartic acid enantiomers in protein hydrolysates, based on chiral capillary gas chromatography. It is fast, easy and preferable to the usual diastereomeric dipeptide technique. We present evidence that traces of metals that are extracted from the glassware during acidic hydrolysis are the main cause for high background racemization, which often troubles accurate measurements. Effective ways to reduce this background and its standard deviation to acceptable levels are discussed, and a mathematical approach to correct for background racemization is given. Hydrolysates of aged human eye lens proteins were used to demonstrate the enantiomeric separation.

INTRODUCTION

The biochemistry of living organisms exhibits a strong enantioselectivity1. Protein synthesis is highly discriminative to amino acid enantiomers and uses only L-amino acids. Therefore, the finding of D-aspartyl residues in tooth enamel proteins from living humans was very surprising2. However, especially because of the work of Bada and co-workers3–5, aspartic acid racemization is now accepted as a fundamental post-translational process. Because of its low rate constant, in vivo aspartic acid racemization can only be detected in long-living tissues, such as the eye lens6–8, tooth enamel and dentine2,9 and white brain matter10,11. These studies have shown that racemization is a spontaneous, slow but continuous process with a rate constant of ca. 0.14% year−1 (ref. 12). In proteins from nuclear parts of old normal human lenses, the D-enantiomer of aspartic acid can comprise up to 15% of the total aspartate content13. There is a strong belief that racemization plays an important role in ageing processes3,5,8,14.

Until now, aspartic acid racemization in proteins has mostly been detected by
the method of Manning and Moore. Diastereomeric dipeptides are prepared by coupling the enantiomeric amino acids with L-leucine-N-carboxyanhydride, and then separated by ion-exchange chromatography on a regular amino acid analyser. Since the peaks of L-Leu-D-Asp and L-Leu-L-Asp overlap with other dipeptides, a pre-purification of aspartic acid by ion-exchange chromatography is essential and makes this method very laborious and time-consuming.

Two decades ago, chiral gas chromatographic (GC) liquid phases, capable of separating amino acid enantiomers, were reported by Gil-Av et al. and Chang et al. The low thermal stability of these chiral peptide phases, however, generally made them unsuitable for less volatile amino acids. (For a review on enantioselective GC phases, see ref. 18).

Chiral diamide phases, derived from L-valine bound to cross-linked polysiloxane, exhibit greater efficiency, higher enantiomeric separation factors, shorter retention times and better thermal stability. Three such phases are now available in fused-silica capillary columns. These are Chirasil-Val (Chrompack and Alltech) developed in the group of Bayer and Frank, RSL-007 (Alltech) from Saeed et al. and XE-60-S-valine-S-α-phenylethylamide (Chrompack) from the group of Koenig. The third of these exhibits the best enantiomeric separation factors for most amino acids, including aspartic acid. Because of their good thermal stability, all common amino acids can be resolved by analysis of a total protein hydrolysate in a temperature-programmed run. Aspartic acid enantiomers can be detected without pre-purification. These columns meet the demand that the elution time of D-amino acids is a little shorter than that of their optical antipodes. Only then is a good quantification of small amounts of a D-amino acid in the presence of an excess of the L-form possible.

Catalysis of racemization by metal ions is known as a notorious problem in peptide synthesis, but has been given little attention in the literature on aspartic acid racemization. Nevertheless, great effort should be given to the reduction of the concentrations of metals, since especially during hydrolysis they can cause a very high background and so obscure results.

In this paper we describe the detection of aspartic acid enantiomers in protein samples by a chiral capillary GC method. Furthermore, advice is given on how to reduce hydrolysis-induced background racemization and how to correct data mathematically for this background.

EXPERIMENTAL

Preparation of protein samples

The isolation of proteins from human lens fractions by extraction and gel chromatography has been described. Bovine serum albumin (Sigma, A-8002) was used to determine the background racemization. In order to remove traces of metal ions, the protein solutions were dialysed for 24 h against 10 mM EDTA in water of Milli-Q quality and subsequently against Milli-Q water for 76 h. The Milli-Q system is from Millipore. The dialysis solutions were changed three times a day. The samples were lyophilized prior to acidic hydrolysis. L-Asp and D-Asp used in the reproducibility tests were purchased from Sigma.
Acidic hydrolysis

Single-use Duran 50 borosilicate hydrolysis tubes (Schott) were pre-extracted for 48 h with 6 M hydrochloric acid (Merck, p.a.) at 100°C to remove acid-extractable metal ions. Hydrolysis was performed for 6 h at 110°C under vacuum in 0.5 ml of 6 M hydrochloric acid (Merck, Suprapur) in tubes that were sealed in a flame. The hydrolysates were lyophilized prior to derivatization.

Preparation of amino acid derivatives

Dry protein hydrolysates were esterified using 0.6 ml of 4 M hydrochloric acid in 2-propanol in closed reaction vials at 110°C for 50 min. After evaporation under nitrogen at 40°C, acylation was performed with a mixture of 0.5 ml of trifluoroacetic acid anhydride and 0.5 ml of dichloromethane for 1 h at room temperature. The redundant reagents were evaporated at 30°C under nitrogen. The derivatized hydrolysates were dissolved in dichloromethane.

Gas chromatographic analysis of aspartic acid enantiomers

Samples were injected onto a fused-silica capillary column (20 m × 0.22 mm I.D.) coated with the chiral polysiloxane phase XE-60-S-valine-S-α-phenylethylamide (Chrompack, Middelburg, The Netherlands). The Hewlett-Packard 5710A gas chromatograph was used in the split mode, with helium as carrier gas and a flame ionization detector. The oven temperature was kept at 135°C until both aspartic acid enantiomers had eluted from the column (8 min), after which the less volatile compounds were removed by heating the column to 180°C for 4 min. The injector and detector temperatures were 250°C, the carrier gas pressure was 1.7 bar, the vent 80 ml min⁻¹ and the gas flow-rate in the column was 1% of the vent. After every hundred analyses, a few centimetres were broken off from both ends of the column and the septum was changed. Peak heights were automatically recorded by a Hewlett-Packard 3390A integrator. The percentage racemization is expressed as the \( \frac{D \times 100}{D + L} \) ratio. The reproducibility of the determination of this ratio was tested by ten-fold analysis of standard samples containing both aspartic acid antipodes in a 35%, 5% and 0.6% ratio.

Determination of metal ion concentrations

In order to quantify the extraction of metal ions from the hydrolysis tubes during the acidic hydrolysis step, 0.5 ml of 6 M hydrochloric acid (Merck, Suprapur) was heated at 110°C for 6 h in six pre-extracted and six non-treated tubes. These twelve samples and three fresh Suprapur hydrochloric acid samples were analysed with a Jarrell-Ash Atom Comp Model 975 inductively coupled argon plasma multi-channel atomic emission spectrometer system equipped with a scanning monochromator. Specifications of the main characteristics of the system have been given elsewhere²⁷.

Amino acid analysis

Samples were analysed on a LKB 4151 Alpha Plus amino analyzer, with post-column ninhydrin derivatization.

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RESULTS

Gas chromatographic separation of D- and L-aspartic acid

The separation of the aspartic acid enantiomers of a human eye lens protein hydrolysate with a chiral capillary column is shown in Fig. 1. D-Asp elutes before L-Asp. The resolution was calculated to be 1.42 at retention times of ca. 7.5 min.

Fig. 1 Enantiomeric separation on a chiral capillary column. The applied sample is a hydrolysate of the cortical urea-soluble protein fraction of a 55-year-old human eye lens. The analysis revealed an aspartic acid racemization of 10.5% Peaks S = solvent, 1 = L-Ala, 2 = L-Val, 3 = L-Thr, 4 = L-Ile; 5 = L-Leu; 6 = L-Pro, D = D-Asp, L = L-Asp

The reproducibility of the ratio determination is shown in Table I. The coefficient of variation (C.V.) is ca. 8%. Because of large relative errors in the case of small amounts of D-Asp, the coefficient is much higher for small percentages of D-Asp (0.6%). However, one should realize that owing to the acidic hydrolysis, samples

TABLE I

REPRODUCIBILITY OF THE $\frac{D \times 100}{(D + L)}$ ESTIMATION BY CHIRAL GC ANALYSIS OF ASPARTIC ACID ENANTIOMERS

Mixtures of D- and L-Asp were derivatized and chromatographed as mentioned in Experimental. Results of each mixture are based on ten analyses.

<table>
<thead>
<tr>
<th>Mean $\frac{D \times 100}{(D + L)}$ ratio</th>
<th>S D</th>
<th>C V (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34.82</td>
<td>2.91</td>
<td>8.36</td>
</tr>
<tr>
<td>49.93</td>
<td>0.41</td>
<td>8.32</td>
</tr>
<tr>
<td>60.62</td>
<td>0.13</td>
<td>20.97</td>
</tr>
</tbody>
</table>
contain at least 2% racemized aspartic acid. We wish to emphasize that we investigated the reproducibility of the determination of the $D \times 100/(D + L)$ ratio and not that of the GC analysis, which of course has a CV much less than 8%.

The detection limit, defined as a signal-to-noise ratio of 2, is ca. 250 pmol at a splitting ratio of 1%, in which case only 25 pmol are actually applied to the column.

The lifetime of the column is extremely good. Only a slight decrease in retention time due to bleeding of the liquid phase, but no detectable loss of resolution were noticeable after 6 months of continuous operation.

Racemization during acidic hydrolysis

Since the racemization rate is enhanced in strong acid$^{28-31}$, the hydrolysis time should be kept as short as possible. We therefore investigated the time needed for total liberation of aspartic acid. Acidic hydrolysis of bovine serum albumin was carried out as described for varied periods of time. The amounts of free Asp, as determined by amino acid analysis, are depicted in Fig. 2. Obviously, 6 h are sufficient to release all aspartate residues.

The metal ions that catalyse racemization in the hydrolysis medium can originate from the sample and from the glass of the hydrolysis tubes. The remedy for the first source is exhaustive dialysis against an EDTA solution, followed by ultra-pure water. Leaching of the glass during the hydrolysis step can greatly be reduced by a 48-h pre-extraction of the tubes with 6 M hydrochloric acid at 100°C. Concentrations of thirteen metal ions, present in the hydrochloric acid after a dummy hydrolysis in treated and non-treated tubes, are shown in Table II. Obviously, the pre-treatment reduces the total amount of acid-extractable metals dramatically. Furthermore, the standard deviation (SD) of their concentration is also greatly decreased.

![Graph](image)

Fig. 2 Release of aspartic acid as a function of duration of hydrolysis. The amount of free aspartic acid was measured on an amino acid analyser after hydrolysis in 6 M hydrochloric acid at 110°C for the times indicated.
TABLE II
METAL CONCENTRATIONS IN HYDROCHLORIC ACID AFTER HYDROLYSIS IN PRE-EXTRACTED AND NON-EXTRACTED TUBES

Concentrations are presented as parts per billion (10^9). The upper row shows the normal background concentrations in the Suprapur hydrochloric acid used in this experiment. Standard deviations are given in parentheses. The composition of the Duran 50 glass is 80.5% SiO₂, 12.9% B₂O₃, 3.8% Na₂O, 2.2% Al₂O₃, and 0.4% K₂O.

<table>
<thead>
<tr>
<th></th>
<th>Zinc</th>
<th>Lead</th>
<th>Cadmium</th>
<th>Aluminium</th>
<th>Iron</th>
<th>Manganese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrochloric acid, fresh (n = 3)</td>
<td>&lt;4</td>
<td>53 (13)</td>
<td>4 (1)</td>
<td>&lt;25</td>
<td>47 (6)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>Hydrochloric acid after hydrolysis in pre-extracted Duran 50 tubes (n = 6)</td>
<td>17 (5)</td>
<td>74 (9)</td>
<td>4 (2)</td>
<td>190 (30)</td>
<td>320 (300)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Hydrochloric acid after hydrolysis in non-treated Duran 50 tubes (n = 6)</td>
<td>22 (10)</td>
<td>83 (13)</td>
<td>5 (2)</td>
<td>1600 (200)</td>
<td>165 (75)</td>
<td>5 (1)</td>
</tr>
</tbody>
</table>

The effect of reduction of metal concentration on the background racemization is shown in Fig. 3. Clearly, the removal of acid-extractable metals from the tubes is very effective in reducing the background and its S.D. Sample dialysis contributes only slightly to the reduction of the racemization background in the case of commercial bovine serum albumin (Fig. 3). For protein extracts the effect may be expected to be higher. Derivatization-induced racemization, measured with optically pure, only derivatized L-Asp, was undetectable. Furthermore, it turned out that the amount of protein played no role in the racemization.

DISCUSSION

Chiral capillary GC is a suitable technique for the analysis of aspartic acid racemization in vivo. It offers various advantages over the classical diastereomeric dipeptide method, originally introduced by Manning and Moore and used in most studies in this field. Because of the high resolution of the individual amino acids and the moderate enantioselectivity offered by the GC technique, the aspartic acid enantiomers can be detected in a sample as complex as a protein hydrolysate (Fig. 1). This eliminates the need to isolate the aspartic acid from the other amino acids. Furthermore, the analysis time is well reduced. Elution times in the diastereomeric method exceed 1 h, whereas on a 20-m capillary GC column retention times are as short as 7.5 min. The 250-pmol detection limit is about the same for both methods. $\chi_100/(D+L)$ ratios can be determined with a C.V. of 8% (Table I). The reproducibility of the GC analysis as such is of course much better. Therefore, this method is more accurate than the dipeptide analysis, for which Bada mentioned a “reproducibility of $\pm 5-10\%$”.

Background racemization caused by the hydrolysis step has been given little attention in the literature. The problem is seldom acknowledged, and quantitations are scarce and vary considerably. We have convincingly shown that metal ions, originating from the sample and especially from the glassware, play a major role in the racemization during the hydrolysis step (Fig. 3). Although well-documented reports on that role are not available, there is some information on the catalytic properties
of metals and metal complexes on the racemization rate of amino acids. Metals that have been positively identified as catalysts include: cobalt$^{32-36}$, copper$^{32,35-42}$, iron and aluminium$^{39}$, magnesium$^{35,38}$, zinc$^{35,36,41}$ and nickel$^{35,36,43}$. The concentrations of all those metals, except iron, were all increased during hydrolysis in non-treated tubes (Table II). If precautionary steps such as dialysis of the samples and pre-extraction of the tubes are undertaken, the background racemization and its S.D. are reduced to acceptable levels (Fig. 3).

Fig. 3. Effect of tube-treatment and sample-dialysis on the background racemization of aspartic acid. Bovine serum albumin, dialysed and non-dialysed, was hydrolysed in extracted and non-extracted tubes. Racemization and standard deviation were calculated from five individual hydrolyses.
For samples treated in this way, the S.D. of the background is of the same order of magnitude as that of the GC analysis. Therefore, if extreme accuracy is required, this cannot be achieved by multiple GC analysis only. The sample should also be hydrolysed in duplicate or triplicate. We have achieved relative standard deviations (R.S.D.) of 1% by hydrolysing eye lens protein fractions in triplicate and triplicate analysis of each hydrolysate. We have some preliminary results, indicating that this accuracy can also be achieved by the HPLC separation of fluorescent diastereomeric amino acid derivatives, as described by Aswad.

At this point we should comment on the question of how measured Dχ100/(D+L) ratios may be corrected mathematically for background racemization. Simply subtracting this background, as is often done, is not correct since it neglects the facts that not only L-Asp but also D-Asp racemizes during hydrolysis and that the equimolar enantiomeric ratio of a racemic mixture does not change on hydrolysis. Therefore, after subtracting the background from the measured ratio, the resulting value has to be multiplied by a factor of 50/(50-background). Let us consider a hypothetical experiment, in which the background amounts to 2% racemization. In that case, a realistic measured amount of racemization of, e.g., 15.0%, would be corrected to 13.5%, in contrast to 13.0% if the background were simply subtracted.

We have described here a method for the analysis of aspartic acid enantiomers, based on chiral capillary GC. The simplicity of the method and the short analysis time might give a new impulse to the investigation of in vivo racemization of aspartic acid, its implications for protein structures and its role in ageing processes.

ACKNOWLEDGEMENTS

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ANALYSIS OF ASPARTIC ACID RACEMIZATION

EVALUATION OF A CHIRAL CAPILLARY GAS CHROMATOGRAPHIC AND A DIASTEREOMERIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

Piet J.M. van den Oetelaar, José van Beckhoven and Herman J. Hoenders

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SUMMARY

A recently developed chiral gas chromatographic method and a diastereomeric high-performance liquid chromatographic method for the analysis of aspartic acid enantiomers in protein hydrolysates have been evaluated. Although both techniques are fast and convenient, the latter is preferred because of its higher reproducibility and shorter analysis time. Furthermore, this method offers the possibility of on-line derivatization and analysis.

INTRODUCTION

Aspartic acid racemization is of interest for two reasons. First, it has been recognized as a geochronological tool for dating fossils. Secondly, it has been identified as a post-translational protein modification. In vivo racemization of aspartic acid in proteins is a rather slow non-enzymatic process with a rate constant of about 13.10⁻³ year⁻¹. High D/L ratios can therefore be found only in long-living tissues in which the protein turnover is low or absent, such as the lens of the eye, tooth enamel and dentine, and white brain matter. The effects of the optical isomerization of aspartic acid residues upon protein structure and its rôle in aging have been the subject of much speculation, but have still to be elucidated. It has been suggested that aspartic acid racemization is involved in cataract formation, but this is not accepted by other workers. Recently it has also been shown that D-Asp as well as L-IsoAsp residues are substrates for the ubiquitous enzyme protein carboxylmethylase (EC 2.1.1.24). It was postulated that this enzyme might function in a racemization- or isomerization-repair pathway.

For studies of the in vivo racemization of Asp, most investigators have used ion-exchange separation of diastereomeric dipeptides as introduced by Manning and Moore. Since in this procedure the isolation of aspartic acid is a necessity and elution times exceed 1 h, it is very laborious and time-consuming. In a previous
However, high-performance liquid chromatography (HPLC) often offers even better reproducibility and shorter analysis times. Furthermore, HPLC is becoming a routine technique, and is very well suited to automation. Many enantioselective HPLC techniques have recently been developed. The separation is based either on a chiral mobile or stationary phase or a pre-column derivatization with a chiral agent transforming the enantiomers into diastereomers. Chiral stationary phases have recently been reviewed by Däppen et al. Ligand-exchange phases for amino acids have been extensively studied and were reviewed by Davankov. Most methods are characterized by rather high enantioselectivity which is adequate for samples with few components, but causes peak overlap in such complicated mixtures as a protein hydrolyzate. Recently, less enantioselective HPLC methods for amino acid enantiomers have been described, all using the successful pre-column derivatization with o-phthalaldehyde (OPA) in which the traditional coupling agent β-mercaptoethanol has been replaced by chiral thiols such as N-acetyl-L-cysteine (NAC) or tert.-butyloxycarbonyl-L-cysteine. In this paper we evaluate a pre-column derivatization type HPLC method and a chiral GC technique for the detection of D- and L-Asp in protein hydrolyzates.

MATERIALS AND METHODS

Sample preparation and acidic hydrolysis

The isolation of human eye lens proteins was performed as described. Bovine serum albumin (Sigma, A-8002) was used to determine background racemization. As pointed out before, it is essential to remove metal traces from samples and hydrolysis tubes in order to minimize background racemization during acidic hydrolysis. Therefore, protein samples were dialyzed against an EDTA solution and ultra-pure water. Hydrolysis tubes were pre-boiled with 6 M hydrochloric acid. Acidic hydrolysis was performed for 6 h at 110°C under vacuum. In reproducibility tests, mixtures of D- and L-Asp (Sigma) were used.

Gas chromatography

Lyophilized hydrolyzates were derivatized with acidic isopropanol and trifluoroacetic acid anhydride as before. Analyses were performed with a fused-silica capillary column (20 m x 0.22 mm I.D.) coated with the chiral polysiloxane XE-60-S-α-phenylethylamide (Chrompack, Middelburg, The Netherlands). An Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector was operated in split mode with helium as the carrier gas. Aspartic acid enantiomers were chromatographed isothermally at 135°C. Less volatile derivatives were removed by increasing the oven temperature to 180°C for 4 min. The injector and detector temperatures were 250°C, the column pressure was 1.7 bar, vent 80 ml/min and the splitting was set at 1%. Automatic data handling was performed by an Hewlett-Packard 3390A integrator.
CHROMATOGRAPHIC ANALYSIS OF RACEMIZED ASPARTIC ACID

High-performance liquid chromatography

The derivatization and analysis were adapted from the description given by Aswad\textsuperscript{23} To 4 mg OPA, dissolved in 300 μl methanol, were added 250 μl of 0.4 M Na\textsubscript{2}BO\textsubscript{3}, pH 9.4, 390 μl water and 60 μl of 1 M NAC. The NAC could be dissolved in water by the addition of small amounts of sodium hydroxide. The final pH of the OPA NAC solution should be between 9 and 10.

The derivatization and injection were by means of a Gilson autosampler Model 231 equipped with a Gilson dilutor Model 401. A 22-μl volume of the OPA NAC reagent was mixed with 3 μl of an aqueous hydrolyzate containing about 10–100 nmol Asp. After 2.5 min the reaction was stopped by lowering the pH through the addition of 200 μl of 50 mM sodium acetate, pH 5.2. Of the resulting solution, 100 μl were placed on a combined 10-cm C\textsubscript{18} column and 1-cm guard column (Hyper-sil-ODS, ChromSep cartridge system, Chrompack). Gradient elution with 50 mM sodium acetate, pH 5.7 (eluent A) and a mixture of eluent A with methanol (20:80, eluent B) was performed with two Beckman Model 100A pumps controlled by a Beckman Model 420 programmer. After 3.5 min of isocratic elution with 10% eluent B, the percentage of eluent B was increased to 100% in 0.5 min and after 3 min reduced to 10% in 0.5 min. The flow-rate was 0.8 ml/min. A Perkin-Elmer Model 204-A fluorimeter was used to monitor the fluorescence at 445 nm. Excitation was at 340 nm, and spectral bandwidths were 10 nm. Peak areas were integrated automatically by an Hewlett-Packard 3353 LAB-DATA system equipped with a 18652A A/D-converter.

RESULTS AND DISCUSSION

We have evaluated two chromatographic methods for the analysis of D- and L-Asp in protein hydrolyzates. In the GC procedure a capillary column was used, coated with a chiral liquid phase, as developed by Konig \textit{et al}.\textsuperscript{27,28} The chiral phase enables direct separation of the aspartic acid enantiomers. For the HPLC analysis, the amino acid enantiomers have to be converted into diastereomers. For this purpose they were derivatized with OPA and NAC as described by Aswad\textsuperscript{23} and Nimura and Kinoshita\textsuperscript{24} to form highly fluorescent diastereomeric isoindols\textsuperscript{24,29} that can be separated on a regular reversed-phase column.

The enantiomeric separations of hydrolyzates of human lens protein fractions by these GC and HPLC methods are shown in Figs. 1 and 2. In both methods, the D-Asp derivative is eluted before that of L-Asp, thus enabling the detection of small amounts of D-Asp in the presence of an excess of its optical antipode. Although both techniques are capable of resolving D- and L-aspartic acid, the enantioselectivity of the GC method is a little higher, having a resolution factor of 1.4 vs. 1.0 for the HPLC method (Table I). However, the HPLC technique is inferior to the GC method only in respect of the resolution. In all other aspects it is superior. Table I shows that the total analysis, including derivatization and column regeneration, is about three times faster. The reproducibility of the determination of the racemization percentage, defined as \(\frac{D}{D + L} \times 100\), is much higher for the HPLC method. The detection limit differs by two orders of magnitude (Table I).

We also investigated the optimum conditions for the HPLC separation. Its performance is only satisfactory within sharply defined limits of pH, methanol con-
Fig 1 Chiral GC of a protein hydrolyzate. The hydrolyzed urea-insoluble protein fraction of the cortex of a 60-year-old human lens was analyzed on a 20-m chiral capillary column, revealing a racemization percentage, D 100(L + 1), of 13.7%, not corrected for background racemization. Identified peaks: 1 = dichloromethane, 2 = L-Ala, 3 = L-Val, 4 = L-Thr, 5 = L-Ile, 6 = L-Leu, 7 = L-Pro, 8 = D-Asp, 9 = L-Asp.

Fig 2 Diastereomeric HPLC separation of a protein hydrolyzate. The hydrolyzed urea-soluble protein fraction of the nucleus of an 86-year-old human lens was derivatized for 2.5 min with the OPA-NAC reagent. After the diastereomeric derivatives of D-Asp (1) and L-Asp (2) had eluted, the percentage of eluent B was increased to 100% in order to elute all other amino acids. The uncorrected D 100(L + 1) ratio was 18.5%. The gradient profile is indicated by the dashed line.

centration and eluent flow-rate. The effects of these parameters on the resolution are shown in Fig. 3. Especially the pH of the eluent is very critical (Fig. 3A). The sharpness of the curve in Fig. 3A is explained by the fact that the elution behaviour as a function of pH is nearly identical for both diastereomeric aspartic acid derivatives.
TABLE I
CHARACTERISTICS OF THE HPLC AND GC SEPARATIONS OF D- AND L-ASPARTIC ACID

<table>
<thead>
<tr>
<th></th>
<th>HPLC</th>
<th>GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>3.1 (D) 3.3 (L)</td>
<td>7.2 (D), 7.5 (L)</td>
</tr>
<tr>
<td>Column regeneration (min)</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Sample derivatization (min)</td>
<td>2.5</td>
<td>15*</td>
</tr>
<tr>
<td>Total analysis time (min)**</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Resolution***</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Coefficient of variation (%)§</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Detection limit (pmol)</td>
<td>1</td>
<td>250**</td>
</tr>
</tbody>
</table>

* The batchwise derivatization of twelve samples takes 180 min
** This includes sample derivatization, L-Asp elution time and column regeneration
*** Calculated as \( \frac{(t_L - t_D)}{(W_L 0.625 + W_D 0.625)} \) where \( W_L 0.625 \) is the peak width of L-Asp at 0.625 height
§ Only the racemization percentage, \( \frac{D}{100 + L} \), was verified
** At a splitting ratio of 1%

Fig 3 Optimum conditions for the HPLC separation of D- and L-Asp. Racemic mixtures of aspartic acid were chromatographed under the following conditions: (A) flow-rate 0.8 ml/min, 10% eluent B, (B) flow-rate 0.8 ml/min, pH 5.7, (C) pH 5.7, 10% eluent B.
and only differs for a very narrow pH region. The optimum pH of 5.7 in our system differs from that of 5.9 as found by Aswad\textsuperscript{21}. This may be due to the different characteristics of the reversed-phase matrix in the respective set-ups. Nimura and Kinoshita\textsuperscript{24} developed a similar method but did not mention the pH of the elution buffer. For an optimum resolution, the HPLC separation was performed with a flow-rate of 0.8 ml/min and 10% eluent B, pH 5.7.

CONCLUSION

In comparison to the diastereomeric dipeptide technique\textsuperscript{17} which is generally used to study aspartic acid racemization \textit{in vivo}\textsuperscript{3,8}, both the GC and HPLC methods described here offer much shorter analysis times and higher reproducibilities. In spite of a lower resolution, the HPLC technique is preferable because of its short total analysis time and low coefficient of variation. Furthermore, since the HPLC derivatization can be carried out at room temperature in aqueous solutions, this method can be fully automated by means of an “intelligent” autoinjector.

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RACEMIZATION OF ASPARTIC ACID RESIDUES IN PROTEINS FROM NORMAL AND CATARACTOUS HUMAN LENSES

AN AGING PROCESS WITHOUT INVOLVEMENT IN CATARACT FORMATION

Piet J. M. van den Oetelaar and Herman J. Hoenders
ABSTRACT

A highly sensitive method was used to determine D- and L-aspartyl residues in protein fractions from normal and cataractous human lenses. A linear relationship with age was found in all fractions from normal lenses. However, no correlation with cataract could be established. It is concluded that racemization of aspartyl residues is a continuous process in eye-lens proteins but plays no role in cataract formation.

INTRODUCTION

Due to their long lifetime, the human eye-lens proteins are subject to many post-translational modifications (Harding, 1985). About one decade ago racemization of aspartic acid residues was recognized as one of these processes when Masters, Bada and Zigler (1977) reported the age-related increase of the enantiomeric D/L ratio of aspartic acid in the total nuclear protein fraction from normal human lenses. This was confirmed by Garner and Spector (1978) who reported a similar increase in the total WI protein fraction from normal human lenses. No significant increase was found in either the total WS fraction (Garner and Spector, 1978) or the total cortical protein fraction (Masters, Bada and Zigler, 1978) from normal lenses.

With respect to the racemization in proteins from cataractous lenses, the different approaches of the two groups of investigators make it difficult to directly compare their results. In their first paper on racemization in eye-lens proteins, Masters, Bada and Zigler (1977) reported that the racemization rate of the total nuclear protein fraction from class I and II cataracts could not be distinguished from that of normal lenses. However, the nuclear protein fraction from five class IV cataracts of 57 yr and older showed a higher D/L ratio than that of normal lenses of the same age, whereas in one class III cataract this fraction showed a much lower ratio. In their second paper (Masters, Bada and Zigler, 1978) these authors report regression lines for CoWS, CoWI, NuWS and NuWI fractions that are based on the combined data from a small number of normal and cataractous lenses, suggesting that in

Abbreviations used: Co, cortex; Nu, nucleus; WS, water-soluble; WI, water-insoluble; US, urea-soluble; UI, urea-insoluble; HMW, high molecular weight.
their view no significant differences exist between these two types of lenses with regard to Asp racemization.

Both groups also studied the extent of racemization of individual proteins, isolated from pools of cataractous lenses. Masters, Bada and Zigler (1978) observed the highest D/L values in HMW and WI proteins and based on the assumption that HMW proteins are precursors of the WI fraction they suggested that racemization precedes WI formation in cataracts. Garner and Spector (1978) reach a quite different conclusion. They stated that the low D/L ratio which they observed for the 20 and 43 kDa WI proteins from cataractous lenses indicated an enhanced insolubilization of these proteins, causing the low overall D/L ratio for the total WI fraction for cataractous lenses. Their conclusion was that the racemization rate is the same for all proteins and that the D/L ratio of a certain protein fraction depends on the age of the composing proteins.

In chapter 4.4 of this thesis we will deal with the kinetics and mechanism of aspartic acid racemization in proteins (a concise report of that study has been published, Van den Oetelaar and Hoenders, 1987). It will be shown that the overall rate is strongly affected by the conformational state of the protein. In proteins that still possess their native structure, the rate of the D-L inversion can be significantly higher than that of the L-D inversion. With respect to the mechanism of this reaction, increasing evidence is found that racemization proceeds via a succinimide intermediate formed by cyclization of Asn and Asp residues (Clarke, 1987; Van den Oetelaar and Hoenders, 1987; chapter 4.4 of this thesis). Hydrolysis of the succinimide results for about 25% in the restoration of the normal peptide bond and for about 75% in the formation of an isopeptide bond. Both D-Asp and L-isoAsp have been found to be substrates for the enzyme protein carboxyl methyltransferase (Aswad, 1987; Clarke, 1985). It was suggested that this enzyme might be involved in a racemization repair pathway (McFadden and Clarke, 1982). However, repair was not found to take place at a significant level in erythrocyte proteins in vivo and in vitro (Brunauer and Clarke, 1986) as well as in human eye-lens proteins (chapter 4.5). Therefore, the repair function of the enzyme remains to be demonstrated.

In recent years, new techniques have greatly improved the reproducibility of racemization measurements and also background racemization has been significantly reduced (Van den Oetelaar, Van Beijsterveldt, Van Beckhoven and Hoenders, 1986; Van den Oetelaar, Van Beckhoven and Hoenders, 1987). These developments stimulated us to reexamine racemization in eye-lens proteins. A
large number of cataractous lenses was included in this investigation in order to study the role of racemization in cataract formation.

**MATERIAL AND METHODS**

*Fractionation of lens proteins*

Ten normal human lenses (28- to 86-yrs-old) were obtained within 20 h after death and stored at -70°C. Twenty-five cataractous lenses were obtained within 24 h after surgery and refrigerated at -70°C. The lenses were dissected into cortex and nucleus (70% and 30% by weight, respectively) and subsequently homogenized in a buffered solution containing 20 mM sodium phosphate, 100 mM sodium sulphate and 1 mM EDTA, pH 6.9. WS and WI protein fractions were prepared by the general extraction procedure as described by Bloemendal (1981). The US and UI protein fractions were prepared by extraction of the WI fraction with the phosphate buffer containing 7 M urea.

*Analysis of aspartic acid enantiomers*

Protein samples were dialyzed against an EDTA solution and ultra pure water (Milli Q, Millipore) to remove metal traces. This step was essential in order to minimize background racemization during acidic hydrolysis and improve reproducibility (Van den Oetelaar, Van Beijsterveldt, Van Beckhoven and Hoenders, 1986). For the same purpose, hydrolysis tubes were pre-boiled with 6 M hydrochloric acid for 48 h. Acidic hydrolysis was performed in 6 M Suprapur hydrochloric acid (Merck) for 6 h at 110°C under vacuum. Six hours were sufficient to quantitatively release Asp (Liardon & Jost, 1981; Brunauer & Clarke, 1986; Van den Oetelaar, Van Beijsterveldt, Van Beckhoven and Hoenders, 1986). Bovine serum albumin (Sigma, A-8002) and gamma globulin (Sigma, G-5009) were used to determine background racemization, which did not exceed 1.5%. Fluorescent diastereomeric amino acid derivatives were prepared by treatment of the hydrolyzate with N-acetyl-L-cysteine and o-phthalaldehyde as described by Aswad (1984), and were subsequently analyzed by reversed-phase HPLC. An evaluation of this method has been published (Van den Oetelaar, Van Beckhoven and Hoenders, 1987).

**RESULTS AND DISCUSSION**

We measured the enantiomeric ratio of Asp in protein fractions from both normal and cataractous human lenses. A high correlation with age was found for the D/L ratios of all six protein fractions from normal lenses, viz. CoWS, CoUS, CoUI, NuWS, NuUS and NuUI (Table I). For the sake of comparison with the older literature we here evaluate the D/L values of different ages by linear regression analysis, although it was shown that this approach is based
on an incomplete picture of the racemization in proteins (Van den Oetelaar and Hoenders, 1987; chapter 4.4). Due to its high reproducibility, substantially better correlation coefficients were obtained with the diastereomeric HPLC method than with the diastereomeric dipeptide liquid chromatographic technique (Masters, Bada and Zigler, 1977). Whereas the D/L ratio for the WS proteins was found to be constant by these authors, we observe a definite increase with age for this protein fraction in both cortex and nucleus. Furthermore, the rate constants that we observe for the US and UI fractions (Table I) are rather high compared with those reported earlier for WI from normal total lenses, i.e. $1.7 \times 10^{-3}$ yr$^{-1}$ (Garner and Spector, 1978) and $1.67 \times 10^{-3}$ yr$^{-1}$ for CoWI and NuWI from normal and cataractous lenses (Masters, Bada and Zigler, 1978). Standard deviations were only reported by the latter authors. Presumably due to the limited number of samples and the modest reproducibility of the method, a standard deviation of $0.30 \times 10^{-3}$ was obtained. Because of this high value, the results of their study and those reported here are within two standard deviations. The discrepancy for the WS proteins remains unsolved.

## Table I

**Overview of Racemization Rate Constants of Protein Fractions from Normal Human Lenses**

<table>
<thead>
<tr>
<th></th>
<th>$k$ [$10^{-3}$ yr$^{-1}$]</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoWS</td>
<td>1.43 ± 0.11</td>
<td>0.973</td>
</tr>
<tr>
<td>CoUS</td>
<td>2.30 ± 0.16</td>
<td>0.981</td>
</tr>
<tr>
<td>CoUI</td>
<td>2.36 ± 0.17</td>
<td>0.979</td>
</tr>
<tr>
<td>NuWS</td>
<td>1.43 ± 0.09</td>
<td>0.982</td>
</tr>
<tr>
<td>NuUS</td>
<td>2.31 ± 0.14</td>
<td>0.987</td>
</tr>
<tr>
<td>NuUI</td>
<td>2.59 ± 0.24</td>
<td>0.967</td>
</tr>
</tbody>
</table>

D- and L-Asp of protein fractions from normal human eye-lenses, aged 28 - 86 years, were measured as fluorescent diastereomeric derivatives by reversed-phase HPLC. The racemization data were corrected for background racemization by the method given by Van den Oetelaar, Van Beekhoven and Hoenders (1986) prior to statistical analysis. Data were plotted as $\ln[(1+D/L)/(1-D/L)]$ vs. age. The slope of the least-squares regression line equals $k_{\text{observed}} = k + k'$. It is generally assumed that the enantiomerization of L-Asp tends towards a racemic mixture ($D/L = 1$) by reversible first-order kinetics, implying $k = k'$. This will certainly be true for the free amino acid, however we will demonstrate (Van den Oetelaar and Hoenders, 1987; chapter 4.4) that in the chiral protein environment the D/L ratio at equilibrium can be well below unity due to the diastereomeric situations in proteins created by the enantiomerization and because of the structural constraints in the native proteins. The lowest equilibrium D/L ratio, corrected for background, was 0.14 for the CoWS fraction. The non-unity ratios in protein fractions containing rather native proteins was attributed to an increased inversion rate of the D-Asp residues. Rate constants up to $19 \times 10^{-3}$ yr$^{-1}$ (for CoWS) were found for D-Asp, whereas a uniform rate of $2.5 \times 10^{-3}$ yr$^{-1}$ for L-Asp was found for all protein fractions of normal human lenses.
Figure 1 D/L ratios of protein fractions from cataractous lenses. The lines represent the regression lines obtained for normal lenses.
The role of racemization in the process of cataract formation was studied at twenty-five cataractous lenses, aged 47 to 86 yr. This group consisted of 7 sclerotic, 4 brown, 8 dark brown and 6 nigra cataracts, 6 of which were associated with diabetes. For none of the protein fractions a correlation could be found between the extent of racemization and cataract or any type of cataract. As can be seen in Fig. 1, the D/L ratios obtained for the cataract lenses scatter around the regression lines obtained for the normal lenses. By studying this large number of cataractous lenses it has now become evident that the approach of Masters, Bada and Zigler (1978) in which they do not distinguish between normal and cataractous lenses was too simple. Remarkably, Garner and Spector (1978) who did consider cataract lenses as a separate group found that the D/L ratios of total WI of a group of 14 cataract lenses were consistently lower than those of normal lenses of the same age.

Our finding that cataract does not correlate with the extent of racemization is not unlogical since so many causes and processes are involved in cataract formation (Harding and Crabbe, 1984) that it would be surprising when racemization would have been found to be a driving force. We will state elsewhere (Van den Oetelaar, 1987; chapter 4.4) that the racemization rate is enhanced if the native protein conformation is lost. This explains the high D/L values found in some instances for proteins from cataractous lenses. However, other processes may be responsible for the rapid insolubilization and thus decrease the D/L ratio by the supply of relatively young proteins in other instances as suggested by Garner and Spector (1978). In neither case can racemization be seen as a primary cause for cataract.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the technical assistance of Chris Vleugels, José van Beckhoven, Ludy van Beijsterveldt and Frans van Nieuwenhoven. The communication of unpublished results by Dr. Steven Clarke was greatly appreciated. This paper was published within the framework of EURAGE, the European Community concerted action on cellular aging and disease.
REFERENCES

KINETICAL AND MECHANISTIC CONSIDERATIONS ON THE RACEMIZATION OF ASPARAGINE AND ASPARTIC ACID IN PROTEINS

P. J. M. van den Oetelaar, G. I. Tesser and H. J. Hoenders

We have studied the process of racemization of asparagine and aspartic acid residues in proteins. Measurement of D- and L-aspartic acid in hydrolyzates of protein fractions from human eye lenses, differing in degree of perturbation of their structure, revealed that the D-L inversion reaction proceeds with a rate constant of $2.5 \times 10^{-3}$ yr$^{-1}$, irrespective of the structural state of the protein. The reverse D-L reaction proceeds about eight times faster, leading to racemization values of 12% D at equilibrium. However, as the protein structure is altered by various post-translational processes during the long life-span of the lens, the rate constant of the D-L inversion drops to that of the L-D reaction. Analysis of $\beta$-substituted alanine derivatives and computer-graphics molecular modelling of the tripeptide L-Ala-(D,L)-Asp-L-Ala and the Asp$^{114}$ region in the bovine eye lens protein $\gamma^{II}$-crystallin, revealed that five- and six-membered cyclic compounds formed by intramolecular reactions of the Asx side chain are capable of stabilizing the carbanion intermediate and may explain the relatively rapid racemization of Asx residues in proteins. The formation of the five-membered cyclic imide was found to be highly dependent on the local secondary structure. Since an imide at position 114 in $\gamma^{II}$-crystallin perturbed the protein structure over a sequence of ten residues whereas a D-Asp residue in that position was compatible with the native backbone, it has to be concluded that it is not primarily the racemized status of an amino acid residue but the process of its formation that is responsible for the distortion of the protein structure.

INTRODUCTION.

The fact that aspartic acid can exist in two optical forms was discovered by Pasteur (1852), when he showed that chemical synthesis of Asp yielded an optically inactive mixture, whereas biological synthesis produced an optically active molecule. In nature solely L-amino acids are used for protein synthesis. This situation, however, is unstable since chiral molecules tend to racemize towards a racemic mixture in which the D and L form are present in equimolar amounts. However, the high activation energies and the rapid turnover of proteins prevent D-amino acids to accumulate in proteins to detectable amounts. Aspartic acid is the only exception to this rule.

Racemization of protein-bound Asp in vivo was discovered in tooth enamel and dentine (Masters-Helfman & Bada, 1975, 1976) and later also in eye lens proteins (Masters et al., 1977; Garner & Spector, 1978) and brain (Man et al., 1983; Fisher et al., 1986). Speculations have been made about the denaturing impact of Asp racemization on the protein structure, but no direct relation has been described (Poplin & DeLong, 1978; McKerrow, 1979).
Recently, Asp racemization in proteins has gained renewed attention when it was found that D-Asp, together with L-isoAsp, forms a substrate for the enzyme protein carboxyl methyltransferase. It has been postulated that protein methylation by this enzyme plays a role in the repair of these altered Asp residues (Clarke, 1985).

The human eye lens is an attractive object for aging studies, since it does not loose its cells throughout its entire life-span. Furthermore, protein-synthesis and -turnover ceases soon after cell differentiation. Therefore, racemization values reflect the age of the tissue and are not affected by a rapid metabolism. The characteristic growth-pattern of the lens, by which new cells, differentiating from the anterior epithelium, are layered on the organ's surface, makes it possible to isolate concentric portions of specific age periods. A common procedure is the dissection into cortex and nucleus which constitute the younger and older parts, respectively (Bloemendal, 1977, 1981; Hoenders & Bloemendal, 1983). Upon aging, lens proteins are subject to various post-translational modifications that include oxidation, crosslinking, degradation, deamidation, non-enzymatic glycation and subsequent Maillard reactions, phosphorylation and racemization (Kramps et al., 1978; Hoenders & Bloemendal, 1981, 1983; Harding, 1985; Voorter et al., 1986; Chiesa et al., 1987). In the embryonic lens, at least 95% of the protein is water-soluble (Bours & Foedisch, 1986). However, upon aging, insults to the native structure accumulate in these proteins. This results in a decreasing solubility and the "nativeness" of the cortical and nuclear proteins is decreased in the order water-soluble (WS) > 7M-urea-soluble (US) > 7M-urea-insoluble (UI) protein fraction. We measured the D-Asp content in these three protein fractions from both the cortices and nuclei from human eye lenses in order to gain insight into the influence of the conformational state of a protein on the kinetics of the racemization process.

The racemization of amino acids proceeds via a carbanion intermediate (Neuberger, 1948). Their incorporation into a protein results in a two- to four-fold increase of the racemization rate constant compared to that of the free amino acid (Manning, 1970; Bada & Schroeder, 1975; Williams & Smith, 1980).

(1) Abbreviations: Co, cortex; Nu, nucleus; WS, water-soluble; WI, water-insoluble; US, 7M-urea-soluble; UI, 7M-urea-insoluble; PCM, protein carboxyl methyltransferase (EC 2.1.1.77, previously EC 2.1.1.24); dabco, 1,4-diazabicyclo-[2,2,2]-octane; Z, benzyloxy carbonyl; Np, p-nitrophenyl; Bu\textsuperscript{t}, tert.-butyl.
However, Asp, when included in a protein structure shows an enormous increase of the racemization rate, much larger than that for other amino acids. Increases of up to $10^5$-fold have been reported (Masters & Friedman, 1980).

Earlier investigations into the phenomenon of amino acid racemization revealed that racemization in activated acylamino acids is more rapid than in unactivated acylamino acids (Jones et al., 1967). Not only activation as such plays a role, the effect of temperature and predominantly that of hydrogen ion concentration are of importance (Young, 1967). Even in neutral medium, racemization of free amino acids was observed (Bada et al., 1970; Bada, 1972, 1974).

We explored the mechanism of racemization in order to find an explanation for the extremely high rates found for racemization of Asp residues in proteins. Firstly, we studied the racemization of \( \beta \)-substituted alanine derivatives. Secondly, computer-assisted molecular modelling was used to study the formation of carbanion-stabilizing intermediates in the racemization of amino acid residues in protein environments. Further, modelling experiments gave insight into the effect of the racemization process on the protein structure.

**EXPERIMENTAL PROCEDURES.**

*Fractionation of lens proteins.*

Ten normal human lenses (28 to 86 yrs-old) were obtained within 20 h after death and stored at -70°C. The lenses were dissected into cortex and nucleus and subsequently homogenized in a buffer solution containing 20 mM sodium phosphate, 100 mM sodium sulphate and 1 mM EDTA, pH 6.9. WS and WI protein fractions were prepared by the general extraction procedure as described by Bloemendal (1981). The US and UI protein fractions were prepared by extraction of the WI fraction with the phosphate buffer containing 7 M urea.

*Analysis of aspartic acid enantiomers.*

Protein samples were dialyzed against an EDTA solution and ultra pure water (Milli Q, Millipore) to remove metal traces. This step was essential in order to minimize background racemization during acidic hydrolysis (Van den Oetelaar et al., 1986). For the same purpose, hydrolysis tubes were pre-boiled with 6 M hydrochloric acid. Acidic hydrolysis was performed in 6 M Suprapur hydrochloric acid (Merck) for 6 h at 110°C under vacuum. Six hours were sufficient to quantitatively release Asp (Liardon & Jost, 1981; Brunauer & Clarke, 1986; Van den Oetelaar et al., 1986). Bovine serum albumin (Sigma, A-8002) was used to determine background racemization, which did not exceed 1.5%. Fluorescent diastereomeric amino acid derivatives were prepared by treatment of the hydrolyzate with N-acetyl-L-cysteine and o-phthalaldialdehyde and were subsequently analyzed by reversed-phase high-pressure liquid chromatography (HPLC). An evaluation of this method was published (Van den Oetelaar et al., 1987).
**Synthesis of beta-substituted alanine derivatives**

Z-Asn-OH (15 g, 60 mmoles) was dissolved in pyridine (75 ml) and treated with dicyclohexylcarbodiimide (12 g, 90 mmoles) in 38 ml pyridine at 20°C. The resulting suspension was filtered after 2 h and the filtrate was concentrated *in vacuo*, cooled at 4°C and diluted with hydrochloric acid (60 ml, 6 M), filtered and the filtrate was concentrated *in vacuo*. The solid residue was recrystallized from 1,2-dichloroethane and gave a crude product containing still some starting material. A sample (2 g) was subjected to counter current distribution using n-butanol / acetic acid / water (4:1:5, by vol.) as the solvent system. The purified Z-Ala(CN)-OH (1 g; m.p. 129-131°C; \([\alpha]_D^{20} -44.8°, c 1.4 \text{ in } \text{N-N-dimethylformamide}\) was used as such in the synthesis of the p-nitrophenyl ester.

The p-nitrophenyl esters were synthesized from the corresponding hydroxy compounds by the method outlined by Bodanszky and Du Vigneaud (1959, 1962).

- **Z-Ala-ONp**: m.p. 79.5-80.5°C; \([\alpha]_D^{20} -38.1° (c 1.4 \text{ in ethylacetate})\).
- **Z-Asp(OBu^t)-ONp**: m.p. 84-85°C; \([\alpha]_D^{20} -35.5° (c 1.28 \text{ in methanol})\).
- **Z-Ala(CN)-ONp**: m.p. 137-138°C, \([\alpha]_D^{20} -81.8° (c 2 \text{ in aceton})\). Elemental analysis found for Z-Ala(CN)-ONp: C 58.43, H 3.96, N 11.26%. Calculated: C 58.54, H 4.09, N 11.38%.

**Analysis of the beta-substituted alanine derivatives.**

Melting points were determined with a Tottoli apparatus and are uncorrected. Optical rotations of solutions containing 1% of the derivative and 0.5% dabco in dried acetone were determined with a Perkin-Elmer 241 polarimeter. Prior to elemental analysis, samples were dried *in vacuo* over phosphorus pentoxide at 40-60°C for 20 h. Amino acid analyses were performed with a modified Jeol JLC-6 analyzer with samples hydrolyzed in sealed evacuated ampoules at 110-115°C for 24 h in 5.7 M hydrochloric acid (Merck, suprapur). 1H-NMR spectra of solutions containing 1% of the alanine derivative and 0.2% dabco in dried deuteroacetone were recorded with a Bruker WH-90 spectrometer using tetramethylsilane as the internal standard.

**Computer-assisted molecular modelling**

The computer-assisted molecular modelling of L-Ala-(D,L)-Asp-L-Ala was carried out at the Laboratory of Molecular Biology, Department of Crystallography, Birkbeck College, University of London, with the kind assistance of Dr. H. Driessen, using the program FRODO (Jones, 1978) for interactive computer color graphics on an Evans and Sutherland Picture System 2, as modified by I.J. Tickle. The 1.6 Å coordinates of bovine γ- crystallin (Summers et al., 1984) were used for a model-study of the racemization process in a protein environment. Three dimensional projections of all models were visualized by the program MIDAS (Tickle, 1982) using the Evans and Sutherland Picture System 2.
RESULTS.

KINETICS.

We determined the content of D-Asp in protein hydrolyzates as a measure for the \textit{in vivo} racemization of asparagine and aspartic acid residues in eye lens proteins. As pointed out in the introduction, the dissection of the lenses into cortices and nuclei and the subsequent extraction procedure roughly divided the lens proteins into six classes of age and of degree of structural perturbation. The kinetics of the racemization were studied using the D-Asp/L-Asp ratios measured in these six protein fractions of ten lenses aged 28 to 86 yr. As will be shown later, Asn is likely to be also a source of D-Asp in protein hydrolyzates. However, in this section we find it convenient to discuss the kinetics in terms of racemization of L-Asp residues. Irrespective of the origin of D-Asp in protein hydrolyzates, this approach makes no difference to the overall kinetics.

It is commonly thought that the racemization of protein-bound amino acids obeys first-order kinetics identical with that found for free amino acids (Petit, 1974; Dungworth, 1976; Masters & Friedman, 1980; Smith & Evans, 1980; Engel \textit{et al.}, 1981; Frank \textit{et al.}, 1981; Liardon & Jost, 1981; Bada, 1984, 1985). For aspartic acid this means:

\[ L-\text{Asp} \xrightarrow{k_{1}} D-\text{Asp} \quad [1] \]

The mathematics of these kinetics have been described repeatedly by the group of Bada (Bada & Schroeder, 1972, 1975; Masters & Friedman, 1980; Bada, 1984, 1985) and others (Dungworth, 1976; Williams & Smith, 1977). The kinetic equation most commonly used for amino acids is written as (Bada, 1984, 1985):

\[ \ln \left( \frac{1 + \frac{D}{L}}{1 - K' \cdot \frac{D}{L}} \right) - \ln \left( \frac{1 + \frac{D}{L}}{1 - K' \cdot \frac{D}{L}} \right)_{t=0} = (1 + K') \cdot k \cdot t \quad [2] \]

where \((D/L)\) is the enantiomeric ratio measured at a certain time \(t\) and corrected for background racemization as described by Van den Oetelaar \textit{et al.}
The reciprocal of the equilibrium constant, $K'$, equals $1/K = k'/k$. Except for amino acids with more asymmetry centers than only the $\alpha$-carbon (e.g. isoleucine and threonine), this term is 1.0 for free amino acids. The $t=0$ part of equation [2] accounts for the initial racemization in the sample or that introduced during processing, e.g. by protein hydrolysis (Van den Oetelaar et al., 1986). Analogous to the racemization of free amino acids, the rate constants $k$ and $k'$ of the forward and reverse inversion reaction are thought to be equal also for protein-bound residues. If we adopt this approach, the racemization rate constant can be calculated from the least square fit of an $\ln[(1+D/L)/(1-D/L)]$ vs. age plot. The thus determined rate constants for the six protein fractions and the correlation coefficients of the lines are shown in Table I. These results are in reasonable accordance with those reported earlier for eye lens proteins and calculated in the same manner (Masters et al., 1977; Garner & Spector, 1978).

However, the validity of describing protein-bound racemization in this way can be questioned. Unlike amino acids in dilute solutions, a racemizing amino acid in a polypeptide chain is covalently joined to other amino acids. This is likely to affect the racemization process, since racemization takes place in a chiral environment of all-L amino acids. Inversion of one amino acid in this L-matrix, gives rise to diastereomeric situations, as is shown in [3].

\[
\begin{array}{c}
\text{k} \\
\text{...L-L-L-L-L-L-L...} \\
\text{k'}
\end{array}
\begin{array}{c}
\text{k'} \\
\text{...L-L-D-L-L-L-L...} \\
\text{k}
\end{array}
\]

The kinetics of the system will probably still appear first order; however, equilibrium no longer needs to be at 50% D (racemic mixture). The kinetic equation describing this situation can be written as (Moore & Pearson, 1981):

\[
\ln(D_{eq} - D) - \ln(D_{eq}) = -(k+k') \times t
\]

where $D$ is the percentage D-Asp relative to the total Asp measured at time $t$ and corrected for background racemization. $D_{eq}$ is the percentage D-Asp at infinite time (equilibrium).
TABLE I

Racemization rate constants based on reversible first order equation, supposing equilibrium at 50% D.

<table>
<thead>
<tr>
<th>protein fraction</th>
<th>$k$ (10^{-3} yr^{-1})</th>
<th>correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoWS</td>
<td>1.43 ± 0.11</td>
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</tr>
<tr>
<td>CoUS</td>
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<td>NuUI</td>
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<td>0.967</td>
</tr>
</tbody>
</table>

D- and L-Asp of protein fractions from normal human eye lenses, aged 28 - 86 years, were measured by reversed-phase HPLC as fluorescent diastereomeric derivatives. Data were plotted as $\ln[(1+D/L)/(1-D/L)]$ vs. age. The slope of the regression line of the least-squares fit equals $k_{\text{observed}} = k + k'$. The assumption that equilibrium is at 50% D, infers $k = k'$. Identical results were obtained from a plot of $\ln(D_{eq} - D)$ vs. age, with $D_{eq} = 50\%$. The racemization data were corrected for background racemization by the method given by Van den Oetelaar et al. (1986) prior to statistical analysis.

TABLE II

$D_{eq}$ and correlation coefficients of the best regression lines.

<table>
<thead>
<tr>
<th>protein fraction</th>
<th>$D_{eq}$</th>
<th>correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoWS</td>
<td>12%</td>
<td>0.988</td>
</tr>
<tr>
<td>CoUS</td>
<td>31%</td>
<td>0.982</td>
</tr>
<tr>
<td>CoUI</td>
<td>50%</td>
<td>0.979</td>
</tr>
<tr>
<td>NuWS</td>
<td>16%</td>
<td>0.989</td>
</tr>
<tr>
<td>NuUS</td>
<td>50%</td>
<td>0.987</td>
</tr>
<tr>
<td>NuUI</td>
<td>50%</td>
<td>0.967</td>
</tr>
</tbody>
</table>

The racemization in protein fractions of human eye lenses, aged 28 - 86 years, was measured by diastereomeric reversed-phase HPLC and plotted as $\ln(D_{eq} - D)$ vs. age. The value for $D_{eq}$ that rendered the highest correlation coefficient by least-square linear regression fitting of the data, represents the percentage D-Asp ($= [100\times D]/[D+L]$) at equilibrium. The values measured for D were corrected for background racemization (Van den Oetelaar et al., 1986) prior to statistical analysis.
The forward and reverse racemization rate constants $k$ and $k'$ were calculated from the \( \ln(D_{eq} - D) \) vs. age plot, where \( D_{eq} \) is the percentage D-Asp (\( = \frac{[\text{D}]}{[\text{D} + \text{L}]} \)) at equilibrium, given in Table II. Rate constants were calculated from the slope, \( -(k+k') \), and the equilibrium constant $K$ of the reaction, \( ie \ K = \frac{D_{eq}}{L_{eq}} = \frac{k}{k'} \).

Our assumption that $D_{eq}$ does not equal 50% and, therefore, that $k \neq k'$ proved correct when we analyzed the same racemization data of the eye lens protein fractions mentioned above by plotting \( \ln(D_{eq} - D) \) vs. age. Iteratively the value was found for $D_{eq}$, yielding the best fit. As shown in Table II, for some protein fractions the $D_{eq}$ value deviates substantially from 50%. The more native a protein fraction is, the more the equilibrium is shifted towards a lower percentage D. This implies that $k$ and $k'$ indeed are not equal for these fractions. They can be calculated from the slope of the \( \ln(D_{eq} - D) \) vs. age plot, which equals \( -(k+k') \), and the equilibrium constant $K = \frac{D_{eq}}{L_{eq}} = \frac{k}{k'}$. These values are given in Table III. For all fractions, the rate constant $k$ of the L→D inversion is about the same: \( 2.5 \times 10^{-3} \ \text{yr}^{-1} \). The rate constant $k'$ of the D→L inversion has this same value for the perturbed protein fractions CoUI, NuUS and NuUI. However, in the other, more native fractions, the reverse reaction proceeds much faster, showing rate constants up to \( 19.3 \times 10^{-3} \ \text{yr}^{-1} \) for the most native CoWS proteins, which is almost eight times as fast as the general rate constant for L→D conversion.

### TABLE III

<table>
<thead>
<tr>
<th>protein fraction</th>
<th>$k \ (10^{-3} \ \text{yr}^{-1})$</th>
<th>$k' \ (10^{-3} \ \text{yr}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoWS</td>
<td>2.64 ± 0.15</td>
<td>19.34 ± 1.06</td>
</tr>
<tr>
<td>CoUS</td>
<td>2.82 ± 0.19</td>
<td>6.27 ± 0.43</td>
</tr>
<tr>
<td>CoUI</td>
<td>2.36 ± 0.17</td>
<td>2.36 ± 0.17</td>
</tr>
<tr>
<td>NuWS</td>
<td>2.14 ± 0.12</td>
<td>11.24 ± 0.61</td>
</tr>
<tr>
<td>NuUS</td>
<td>2.31 ± 0.14</td>
<td>2.31 ± 0.14</td>
</tr>
<tr>
<td>NuUI</td>
<td>2.59 ± 0.24</td>
<td>2.59 ± 0.24</td>
</tr>
</tbody>
</table>
The mechanism of racemization was studied in two ways. Firstly, we measured proton exchange and changes in optical rotation of β-substituted alanine derivatives. Secondly, we explored by molecular modeling techniques the possibilities for carbanion stabilizing intermediates to be formed in proteins.

In the first study we compared the chiral stability of three amino acid derivatives that had different side chains and were activated in order to accelerate the process of racemization. Benzylloxycarbonyl / p-nitrophenyl esters of alanine, O4-tert.-butyl aspartic acid and of 3-cyanoalanine (Ressler & Ratskin, 1961) were used. The benzylloxycarbonyl functions provided the amino acids with a peptide bond on the amino side. Therefore, racemization of these compounds might partly be comparable with racemization of protein-bound residues. The alanine derivatives were treated with bases in hexadeuteroacetone, dried over a molecular sieve. The bases used were triethylamine and 1,4-diazabicyclo-[2,2,2]-octane at a concentration of 0.5% (2).

In 1H-NMR spectra the β-CH₂ group, unlike the proton in α-position, gives a signal whose position depends strongly on the side chain substituent (Table IV). However, in contradistinction with the α-protons (Table V), the β-methylene protons do not exchange perceptibly if the side chain bears a more electronegative group. On the other hand, a profound effect of the substituent in the side chain was observed with respect to racemization, measured as change in optical rotation (Fig. 1).

As will be discussed later, these results can be explained by a mechanism containing a 6-membered ring as intermediate. Besides this ring, another cyclic Asx-derivative can be a candidate as intermediate in the racemization reaction. It is well documented that Asn can form a 5-membered cyclic imide

(2) Unexpectedly, dabco exchanged all of its protons rapidly against deuterons following dissolution in deuteroacetone. The use of the deuterated base (obtained by dissolution of dabco in hexadeuteroacetone and leaving the solution overnight) simplified the 1H-NMR measurements, since its deuterons now could not obscure the 1H-NMR signals of the investigated compounds. Apart from this, a possible exchange of protons will then not be counteracted by the base providing protons.
Figure 1. Relative change in optical rotation of Z-Ala-ONp (n), Z-Asp(OBu$^\text{t}$)-ONp (O) and Z-Ala(CN)-ONp (Δ), expressed in percents. Measurements were performed on solutions containing 1% of the Ala derivative and 0.5% dabco in dried acetone.

TABLE IV

Chemical shift (ppm) of Z-Ala(R)-ONp compounds, dissolved in hexadeuteroacetone.

<table>
<thead>
<tr>
<th>R-substituent</th>
<th>H</th>
<th>COOBU$^\text{t}$</th>
<th>CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>aH</td>
<td>4 52</td>
<td>4 89</td>
<td>4.90</td>
</tr>
<tr>
<td>CH3</td>
<td>1 59</td>
<td>3.00</td>
<td>3.25</td>
</tr>
<tr>
<td>benzyl-$^\text{H}_2$</td>
<td>5 12</td>
<td>5.14</td>
<td>5.20</td>
</tr>
</tbody>
</table>

TABLE V

H/D exchange on the alpha-carbon of Z-Ala(R)-ONp derivatives, dissolved in hexadeuteroacetone.

<table>
<thead>
<tr>
<th>R-substituent</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>30min</th>
<th>60min</th>
<th>120min</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COOBU$^\text{t}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>10</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>CN</td>
<td>17</td>
<td>25</td>
<td>33</td>
<td>41</td>
<td>56</td>
<td>72</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Expressed in percent. A dash indicates a not measured point. Measurements were performed on solutions containing 1% Z-Ala(R)-ONp and 0.2% dabco in dried hexadeuteroacetone.

Although this reaction is studied in polypeptides only for Asn, it can be argued that Asp can undergo the same cyclization when in a hydrophobic environment like that of the interior of a globular protein (Bernhard, 1983) or when the β-carboxyl group is protonated by specific interactions with other residues (Blow et al., 1969; Koeppe & Stroud, 1976; Phillips, 1976).

The resonance structures of the succinimide ring involves charge transfer between nitrogen and both the α- and β-carbonyl function:

Structure III and IV involve the peptide bond and can be drawn for any protein-bound amino acid residue. Resonance with the β-carbonyl (V), however, is limited to the succinimide. This latter structure allows the peptide carbonyl to intensify resonance with the α-carbanion, formed by proton abstraction as an intermediate in the racemization process:
L-Ala\(^\text{L}\)–L-Asp–L-Ala in \(\alpha\)-helix configuration

L-Ala–L-Asp–L-Ala in \(\beta\)-sheet configuration

L-Ala–D-Asp–L-Ala in \(\alpha\)-helix configuration

L-Ala–D-Asp–L-Ala in \(\beta\)-sheet configuration

The limited approach of \(N^\text{C} - C^\gamma\) and obstruction by the carbonyl moiety prevent cyclic imide formation in situations b and c.

**TABLE VI**

**Favorability of cyclic imide formation, as judged by molecular modelling of L-Ala-(D,L)-Asp-L-Ala**

<table>
<thead>
<tr>
<th>Asp configuration</th>
<th>(\phi)</th>
<th>(\psi)</th>
<th>(\omega)</th>
<th>secondary structure</th>
<th>closest distance (N-C^\gamma) ((\AA))</th>
<th>formation of cyclic imide</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>-60</td>
<td>-60</td>
<td>180</td>
<td>(\alpha)-helix</td>
<td>2.45</td>
<td>favorable</td>
</tr>
<tr>
<td>L</td>
<td>-81</td>
<td>69</td>
<td>180</td>
<td>(\beta)-sheet</td>
<td>3.93</td>
<td>unfavorable, obstructed by carbonyl</td>
</tr>
<tr>
<td>D</td>
<td>-60</td>
<td>-60</td>
<td>180</td>
<td>(\alpha)-helix</td>
<td>3.68</td>
<td>unfavorable, obstructed by carbonyl</td>
</tr>
<tr>
<td>D</td>
<td>-80</td>
<td>70</td>
<td>180</td>
<td>(\beta)-sheet</td>
<td>2.17</td>
<td>favorable</td>
</tr>
</tbody>
</table>

Asp, either in the L or D configuration, was given the ideal \(\alpha\)-helical and \(\beta\)-sheet dihedral angles (\(\phi\), \(\psi\) and \(\omega\)). By means of computer-graphics molecular modelling, the closest distance attainable between the Asp \(\gamma\)-carbon and the nitrogen of the succeeding peptide bond were found by rotation around the \(C^\alpha-C^\gamma\) bond. Projections of these structures are shown in Fig. 2.
We questioned whether the imide formation would have a preference for certain secondary structures and studied this by computer-assisted modelling of the tripeptide L-Ala-(D,L)-Asp-L-Ala. Alanine was chosen for the residues adjacent to Asp in order to introduce a chiral environment with a minimal possibility of steric hindrance. Torsion angles were chosen to be those for ideal α-helix or β-sheet secondary structure. By rotating the aspartyl side chain around the C–C$_\alpha$ axis, we searched for the closest distance of N-Ala and C$_\gamma$-Asp (Table VI and Fig 2). With Asp in the L configuration the closest distance was 2.45 Å in the α-helix equivalent structure. This situation might be in favor of a cyclization. In the equivalent of the β-sheet structure, ring closure is impossible because of obstruction by the peptide-carbonyl and the fact that the approach of N to C$_\gamma$ is limited to 3.93 Å. The same experiment was repeated with a racemized Asp in the tripeptide. The short N-C$_\gamma$ distance of 2.17 Å now favors imide formation in a β-sheet type of situation. With D-Asp in an α-helix like environment, the 3.68 Å N-C$_\gamma$ distance and steric hindrance by the peptide carbonyl would prevent cyclization (Table VI and Fig 2).

We next used computer-graphics molecular modelling to study the process of racemization and its impact on protein structure. The modelling was performed on Asp residue 114 of bovine βII-crystallin. The primary structure of this 20,000 Da lens protein has been elucidated (Croft, 1972; Bhat & Spector, 1984) and X-ray crystallography at 1.6 Å resolution (Wistow et al., 1983; Summers et al., 1984) revealed a structure composed of four 'Greek key' motifs. The reason why we chose this specific residue is that its dihedral angles (φ = -74°, ψ = -44°, right handed helix generally distorted from α-helix, Wistow et al., 1983) are not unfavorable to the formation of a cyclic imide and because it is located in a loop at the surface of the molecule. The primary structure in this part of the protein is highly variable among the various β-crystallins of different species (Summers et al., 1986). The side chain is directed towards the solvent region and not involved in hydrogen bonds with other residues or nearby water molecules. These features warrant that the effect on the protein structure seen during computer-graphics simulation of ring closure are not caused by breaking of hydrogen bonds or displacement of the bulky side chain. Although it may be questionable whether this specific Asp residue might thermodynamically be capable of ring closure, we feel it can be used as a model for this intramolecular reaction of Asn and possibly Asp in a realistic protein environment.

It was possible to racemize L-Asp$^{114}$ by simply interchanging the
Figure 3: Overlay representation of the Asp$_{114}$ region in bovine $\tau^{II}$-crystallin with Asp in the L and the D configuration. The latter structure is represented by the dashed line. The primary structure of the fragment shown (residues 110 to 118) is: Pro-Ser-Leu-Gln-Asp-Arg-Phe-His-Leu.

Figure 4: Overlay representation of the normal Asp$_{114}$ region in bovine $\tau^{II}$-crystallin (thin line) and the cyclic imide derivative after computational refinement (bold line). Distortion of the structure stretches out from residue 109 to 118.
α-hydrogen and the side chain. The side chain could be so orientated that the original backbone structure was conserved (Fig. 3). Furthermore, the same bond distances and torsion angles present in L-Asp$^{114}$ could be given to its optical antipode. This indicated that the D-Asp$^{114}$ residue is filled in the main chain structure without problems.

However, formation of a flat cyclic imide, as a possible intermediate in the Asx-racemization of residue 114, distorted the local structure to a great extent. Computational refinements of the protein backbone, necessary to achieve bond distances and torsion angles that are acceptable for a peptide structure, was needed for residues 109 to 118 (Fig. 4).

**DISCUSSION.**

The kinetics of the racemization of protein-bound amino acids are thought to be comparable with the reversible first order kinetics of the racemization of free amino acids (Petit, 1974; Dungworth, 1976; Masters & Friedman, 1980; Smith & Evans, 1980; Engel et al., 1981; Frank et al., 1981; Liardon & Jost, 1981; Bada, 1984, 1985). This may be questioned since in dilute solutions, prepared with achiral solvents, an amino acid will not experience any chiral interactions with other molecules, whereas in a protein, an amino acid finds itself in a matrix of all-L residues. This creates diastereomeric situations as represented by equation [3] and, therefore, differences in energy are expected. Indeed, steric interactions for L and D residues in proteins have been estimated to differ ~1 kcal/mole (Petit, 1974). In the case of protein-bound aspartic acid, this is several percent of the activation energy which was calculated to be about 20.8 kcal (Friedman & Masters, 1982). These differences in activation energy might very well result in large differences in the racemization rate constants of the forward and reverse reaction.

We have now shown that there exists indeed a difference between $k$ and $k'$ for aspartic acid racemization in some fractions of eye lens proteins (Table III). The fractionation that we carried out is based on differences in solubility and results in a crude subdivision of the lenticular proteins into various classes of perturbation of the protein structure. In the more denatured fractions (CoUI, NuUS and NuUI) the racemization equilibrium was at 50% and the forward and reverse racemization rate therefore are equal.
However in the more native protein fractions (CoWS, CoUS and NuWS) equilibrium was at a lower percentage of D-Asp. The rate constant $k$ for the L→D inversion in the native protein fractions was still identical to that of the perturbed proteins. However, that of the D→L inversion, $k'$, on the other hand was much larger. This can be understood as follows. Post-translational modifications as seen in eye lens proteins will disturb the tertiary structure but still leave much of the secondary structure intact. Therefore, the constraints on the formation of the carbanion and other intermediates, which greatly perturb the local secondary structure (as will be discussed later), will be comparable in native and denatured protein fractions. This explains why the L→D and D→L rate constants for the denatured proteins and the L→D rate constant for the native proteins are identical. In order to explain the high D→L rate constant for the native fractions, we suggest that racemization in native proteins causes a reversible perturbation of the local structure. The D→L inversion will result in the complete recovery of the protein's native structure thereby decreasing the energy of the molecule. This results in an increase of the rate of the D→L inversion which we found to be almost eight-fold for the most native protein fraction (CoWS), relative to the denatured fractions.

The racemization rates reported in the literature show a distinct difference between the free and protein-bound aspartic acid. The racemization rate constant of free aspartic acid in solution is surpassed by some other amino acids (Smith & Evans, 1980; Bada, 1971, 1984). Incorporation of aspartic acid into a protein structure, however, turns it into the most rapid racemizing residue. In contrast with the two- to four-fold increase observed for other amino acids (Manning, 1970; Bada & Schroeder, 1975, Williams & Smith, 1977, Smith & Evans, 1980; Bada, 1985), dramatic increases up to even $10^5$-fold have been reported for Asp (Smith & Evans, 1980; Masters & Friedman, 1980; Friedman & Masters, 1982; Bada, 1982; Liardon & Hurrell, 1983). Although not explicitly stated by the authors, this can also be concluded from time-course experiments of the racemization during acidic hydrolysis (Masters & Friedman, 1979, 1980; Friedman & Masters, 1982; Smith & Evans, 1980) in which the initial rate of racemization when Asp is still protein-bound is much higher than in a later stage, when Asp is hydrolyzed and the racemization of the free amino acid is measured.

An explanation for this exceptional behavior of Asp must be contained in the mechanism of its racemization and since the racemization enhancement is seen in proteins, the mechanism must involve adjacent amino acid residues.
Alteady four decades ago, Neuberger (1948) postulated that amino acid racemization proceeds via a carbanion intermediate, formed by abstraction of the α-carbon hydrogen. This hypothesis has since been proven correct by NMR studies showing the parallelism between racemization and deuterium or tritium exchange on the α-carbon (Buckingham et al., 1967; Matsuo et al., 1967, 1970; Manning, 1970; Pasini & Casella, 1974; Smith & Evans, 1983). This parallelism is also present in the activated β-substituted alanine derivatives that we studied (cf. Table V and Fig. 1). Differences in the racemization rate, therefore, must be explained by different capabilities amongst the amino acids to stabilize the incipient carbanion. Neuberger (1948) predicted that electron withdrawing substituents on α-carbon would stabilize the carbanion intermediate. Our finding that the rates of racemization and the exchange of the α-protons occur in the order CN > COOBu t >> H support this theory. Curiously, the observed chemical shifts present no indications for the acidity of the protons in this type of compounds, since the exchange of protons is accelerated in the order α-proton >> β-proton. Bada (1984, 1985) felt that the differences in the racemization rates observed for free amino acids could be fully explained by differences in the electronegativity of the R-substituent, indexed by the Taft constant σ*. However, this can only partly be true. For instance, judged by the σ* value of the serine substituent, the racemization rate of this amino acid in proteins should be higher than that of aspartic acid, just as it is in free solution (Bada, 1984, 1985). However, this has repeatedly found not to be the case (Smith et al., 1978; Smith & Evans, 1980; Smith & Sivakua, 1983; Engel et al., 1981; Bada, 1984). Detailed kinetic and thermodynamical investigations of the racemization of amino acids (Smith et al., 1978) and substituted arylglycines (Smith & Sivakua, 1983) led these authors to the conclusion that the correlation between σ* and log k is poor and that the stabilization of the carbanion is a complex combination of inductive, resonance, steric, proximity and solvent effects.

Our results also indicate that the exceptionally high racemization rate constant in proteins seen for Asp is not only caused by the high σ* value of its substituent. Its capability of stabilizing the carbanion is also increased by intramolecular reactions. For instance, the mechanism of racemization of Z-Asp(OBu t)-ONp may tentatively be explained by a 6-membered cyclic intermediate formed through the reaction of the β-carbonyl group with the carbonyl function of the preceding peptide bond [8]. This mechanism explains the fast racemization of protein-bound Asx relative to other amino acids since, firstly, it involves the β-carbonyl function of the Asp-derivatives which
indicates that this reaction is unique to Asx and, secondly, the reaction with the carbonyl function of the preceding peptide bond shows the involvement of adjacent amino acid residues. Furthermore, the \( \beta \)-methylene protons do not take part in the reaction, as indicated by the absence of deuterium exchange.

\[
\begin{align*}
\text{(IX)} & \quad \text{(X)} & \quad \text{(XI)} & \quad \text{(XII)} \\
R \quad R' \quad \text{represent adjacent parts of the protein and } R'' \quad \text{is an alcohol moiety in the case of an esterified } \beta-\text{carboxyl or NH}_2 \quad \text{in the case of asparagine. Racemization can occur in structures XI and XII, in which the carbanion is stabilized by resonance.}
\end{align*}
\]

Another possible intermediate, capable of stabilizing the carbanion is that of a 5-membered cyclic imide [5]. This could not be formed in the \( \beta \)-substituted alanines since they lacked a peptide bond on their carboxyl-side due to the \( p \)-nitrophenyl deriva
tization. However, the formation of a cyclic imide by nucleophilic attack on the \( \beta \)-carbonamide of Asn by the amino function of the succeeding peptide bond (see [5]) is well documented (Battersby & Robinson, 1955; Bernhard et al., 1962; Bernhard, 1983; Bornstein, 1970; Bornstein & Balian, 1977; Barber & Clarke, 1985; Johnson & Aswad, 1985; Yamada et al., 1985· Wold, 1985; Didonato et al., 1986; Meinwald, 1986). It should be remembered that D-Asp found in protein hydrolyzates can originate not only from L-Asp but also from L-Asn that is rapidly deamidated under acidic hydrolysis conditions. Aspartic acid residues with esterified \( \beta \)-carboxyl moieties have also been reported to form succinimides (Bernhard et al., 1962; Bernhard, 1983; McFadden & Clarke, 1986). Even Asp can undergo this cyclization when the dissociability of the \( \beta \)-carboxyl group is reduced in hydrophobic environments (Bernhard, 1983). Increases of the \( pK_a \) have actually been reported for Asp\(^{102} \) in chymotrypsin (Blow et al., 1969) and bovine trypsin (Koeppe & Stroud, 1976) and for glutamate\(^{35} \) in lysozyme (Phillips, 1976).

The cyclic imide pathway is capable of explaining several features of the racemization of Asx residues in proteins. The stability of the incipient carbanion is increased since the peptide carbonyl is more available for
resonance in the imide intermediates (see [6] and [7]). The inductive effect responsible for the higher racemization rate of free Asn relative to that of Asp (Masters & Friedman, 1979; Friedman & Masters, 1982; Bada, 1984, 1985) will also be a reason for the increase of the racemization in the succinimide intermediate because the structure of this cyclic carbonamide derivative is comparable to that of the Asn side chain. Furthermore, the formation of the imide involves the succeeding amino acid and therefore explains why the rate enhancement is seen in proteins. The wide variation in racemization rates observed among proteins (Masters & Friedman, 1979, 1980) might be explained by the fact that the imide formation is highly defined by the microenvironment. Our modelling experiments with the tripeptide showed a dependence on the secondary structure, but other factors as the local hydrophobicity or the nature of the adjacent residues will be important. Especially Asn-Ser (Bernhard, 1983) and Asn-Gly (Bornstein & Ballan, 1977) sequences were shown to be prone to cyclization. Finally, since formation of a glutimide is less likely (Bernhard et al., 1962), this mechanism is also consistent with the low racemization rate of glutamine and glutamic acid residues in proteins.

A scheme summarizing the reaction pathways leading to D-Asp is shown in Fig. 5. Included are the methylation of D-Asp and L-isoAsp. These residues have been described as the substrates for the ubiquitous enzyme PCM and their methylation by this enzyme was postulated to play a role in the repair of proteins damaged by racemization or transpeptidation of Asp (Clarke, 1985). The cyclic imide is unstable. Upon hydrolysis the normal Asp-peptide and the isoAsp-peptide are formed in a ratio 25:75 (Aswad, 1984; Murray & Clarke, 1984). D-Asp and L-isoAsp will be readily recognized and methylated by PCM. Since esterified Asp residues are more prone to cyclization (Bernhard et al., 1962; Bernhard, 1983), they will rapidly return to their imide state and reenter the hydrolysis - methylation - cyclization cycle. This might be the molecular basis for the suggested repair function of PCM for proteins with altered Asp residues (Clarke, 1985). Murray and Clarke (1984) showed that the enzyme has no affinity for L-Asp and D-isoAsp residue. In the long run, therefore, L-Asp and D-isoAsp are the predominant species in the imide part of the scheme in Fig. 5. In this view, D-Asp found in protein hydrolyzates mainly originates from D-isoAsp.

Computer-graphics model building indicated that the local backbone structure of the peptide is highly decisive on whether a cyclic imide can be formed. Ideal torsion angles for an α-helix configuration were found to be cyclization-permissive to L-Asp and restrictive to D-Asp. For the ideal
Figure 5 Tentative scheme of the reaction pathways of L-Asp and L-Asn residues in proteins. Besides a direct inversion of L-Asp and L-Asn into their optical antipodes, these residues may either form a five-membered cyclic imide through nucleophilic attack on the β-carbonyl carbon by the α-nitrogen of the succeeding amino acid in the polypeptide chain or form a six-membered cyclic ester by intramolecular reaction of the β-carbonyl group with the carboxyl function of the preceding peptide bond (an ortho ester in the case of D-Asp(OBu’)-ONp). Hydrolysis of the imide breaks the bond between nitrogen and either the β-carbonyl or the α-carbonyl carbon. The resulting normal and isopeptide bond respectively are usually formed in a ratio 25:75 (Aswad, 1984; Murray & Clarke, 1984). In living tissues the free carboxyl groups of D-Asp and L-isoAsp are subject to methylation by the ubiquitous enzyme PCM and it was suggested that PCM plays a role in the repair of altered Asp residues (Clarke, 1985). The high rate of cyclization of esterified Asp-derivatives (Bernhard et al., 1962; Bernhard, 1983) might provide the molecular basis for this hypothesis. Due to the continuous methylation of L-isoAsp and D-Asp, the equilibrium state will be characterized by a domination of L-Asp and D-isoAsp in the imide-part of this scheme. A last pathway proposed by Barber and Clarke (1985) and not included in this scheme involves the aspartic acid anhydride intermediate in which the β-carbonyl carbon is bound to the oxygen of the α-carbonyl function.

torsion angles of a β-sheet configuration the situation was the reverse (Table VI, Fig. 2). However, although racemization of Asx is possible through a cyclic imide in some situations, this does not mean that it is harmless to the protein structure. Assuming absolute rigidity of all the other bonds and angles, a situation certainly not the case in reality, the protein backbone had to be perturbed over a distance of not less than ten residues when trying to form an imide in a concrete situation like that of the Asp residue of β crystallin. Thus, despite the absence of structural constraints, cyclization of Asx residues can be considered to be a great insult to the protein structure. On the other hand, the inclusion of a D-Asp residue in a polypeptide does not necessarily have to upset the protein structure. We have shown that in a not sterically hindered situation the D configuration can be fully compatible with the protein backbone (Fig. 3). We therefore conclude that, in contrast to what might be intuitively believed, it is not the
racemized status of an amino acid residue, but the racemization process leading to it, that is responsible for the perturbation of the protein structure.

ADDENDUM.

When this manuscript was in a final stage, we became aware of a publication by T. Geiger and S. Clarke (J. Biol. Chem. 262, 1987, 785-794) in which they report on deamidation, isomerization and racemization of Asn and Asp residues in the hexapeptide L-Val-L-Tyr-L-Pro-(D,L)-(Asn,Asp)-Gly-L-Ala. They provide evidence by means of HPLC analyses that the racemization of Asp and Asn proceeds mainly via the succimide intermediate. Their kinetical studies predict that at equilibrium only 28% of the peptides will be in the epimerized D-form, which supports our findings that in native proteins equilibrium is at 12-31% D-Asp. The overall rate constant for the formation of D-Asp in the L-Asp hexapeptide (1% in 14 days) is about 100 times as fast as the rate constants for eye lens proteins reported in this paper. This clearly demonstrates the restrictions laid on the cyclic imide formation by the structural constraints in proteins.

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DOES PROTEIN CARBOXYL METHYLTRANSFERASE REPAIR RACEMIZED ASPARTYL RESIDUES IN HUMAN EYE LENS PROTEINS?

Piet J. M. van den Oetelaar and Herman J. Hoenders
Eukaryotic protein carboxyl-O-methyltransferase catalyzes the S-adenosyl-L-methionine-dependent methyl-esterification of altered aspartyl residues both in natural proteins and synthetic peptides. These methylation sites include β-carboxyl groups of aspartyl residues in the D-configuration and the α-carboxyl groups of L-isoaspartyl residues. It has recently been proposed that the methyl-esterification of racemized D-Aspartyl residues could be part of a repair mechanism restoring the original L-configuration. According to this hypothesis, it is conceivable that incubation of racemized proteins in the presence of protein carboxyl methyltransferase and the methyl donor will result in a decrease in D-aspartate content. However, our results, obtained by using highly racemized eye-lens proteins, fail to show any significant variation in the D-aspartate content mediated by the methyl-esterification reaction.

**INTRODUCTION.**

The enzyme PCMT catalyzes the transfer of methyl groups from AdoMet to the carboxyl groups of various methyl accepting protein substrates. The enzymatically formed protein methyl esters are unstable and undergo spontaneous and/or enzymatic hydrolysis (1). This posttranslational modification, which implies the reversible neutralization of negative charges, has been widely regarded as an on/off mechanism regulating a variety of basic cellular events, both in prokaryotes and in eukaryotes (2). In addition, it has recently been proposed that in mammalian cells PCMT recognizes proteins with D-aspartyl (3) and L-isoaspartyl residues (4). The racemization of L-Asp residues in proteins, leading to the enantiomeric D-Asp residues, occurs spontaneously during protein ageing (5-6, and chapter 4.4 in this thesis). It is important to note, in this respect, that in human erythrocytes, where D-Asp residues have been identified as methyl accepting sites (3), the extent of the membrane protein methylsterification increases during cell aging (7,8).

In the light of these considerations, it has been proposed that the protein methyl-esterification could be envisioned as part of a repair pathway of racemized aspartyl residues (3). According to this hypothesis the spontaneously formed D-aspartyl residues are selectively methylated by the cytosolic PCMT and the subsequent enzymatic and/or spontaneous demethylation could restore the original L-configuration (Fig. 1).

Because of its characteristic growth-pattern, the human eye-lens offers a unique possibility to test the validity of this hypothesis. Since this organ never sheds its cells and protein synthesis rapidly ceases after cell differentiation (9), lens proteins can reach exceptionally high ages. Moreover, it is well established that lens proteins accumulate D-aspartate at a high rate (10-12, and chapter 4.3 in this thesis). Therefore, the eye-lens proteins represent a suitable model system to elucidate the physiological role of the selective methyl-esterification of aged proteins. Furthermore, preliminary work by Clarke's group showed the presence of PCMT and protein substrates in bovine and human lenses (14,15).

In this study highly racemized eye-lens proteins have been used in order to obtain direct evidence for the existence of a repair pathway. Our results suggest that, although lens proteins are very good substrates for PCMT, it is unlikely that this enzyme is involved in a repair mechanism of racemized aspartic acid residues.

MATERIALS AND METHODS.

Materials
S-adenosyl-L-[methyl-14C]methionine (specific activity 50-60 Ci/mol) was purchased from The Radiochemical Centre (Amersham, Bucks, U.K.). S-adenosyl-L-homocysteine was supplied by Sigma (St. Louis, U.S.A.). All other chemicals were from local sources and of the best grade available. A citrate-phosphate buffer (pH 6.0) was prepared as described (15).

Purification of protein carboxyl methyltransferase
The enzyme was purified from calf brain according to the procedure of Kim et al. (16).

Enzymatic assay for protein carboxyl methyltransferase
PCMT assay was carried out as previously reported (17). The standard assay mixture contained 15 µl of citrate-phosphate buffer (pH 6.0), 2 nmoles of S-adenosyl-L-[methyl-14C]methionine (140 dpm/pmoles), 40 µg of eye-lens proteins and 1000 units of purified PCMT in a final volume of 125 µl. Incubation was performed at 37°C for different periods of time and the [14C]methyl incorporation into the substrates was determined by extraction.
with isoamyl alcohol of the $^{14}$C-methanol derived from base labile protein methyl esters.

**Preparation of lens fractions and lens proteins**
Cortical and nuclear parts of human lenses were pooled and homogenized in buffer containing 20 mM sodium phosphate, 100 mM sodium sulphate and 1 mM EDTA, pH 6.9. WS and WI protein fractions were prepared by the general procedure outlined by Bloemendal (18). The US and UI protein fractions were prepared by extraction of the WI fraction with phosphate buffer containing 7 M urea. Purified proteins were isolated from the WS and US fractions of individual lenses by high-performance gel permeation chromatography on TSK G3000SW and G4000SW columns (Toyo-Soda) in the appropriate buffer, as described before (19,20).

**Analysis of aspartic acid enantiomers in proteins**
Chiral capillary gas chromatography was used for the detection of D- and L-aspartic acid in protein hydrolyzates, as previously described (21,22). Aspartic acid racemization is expressed as the amount of D-Asp relative to that of total Asp. Low standard errors of the mean were achieved by performing the hydrolysis in triplicate and subsequent triple gas chromatographic analysis of each hydrolyzate, resulting in nine analysis per sample.

**Amino acid analysis**
Samples were analyzed on an LKB 4151 Alpha Plus Amino Analyzer, using post-column ninhydrin derivatization.
An essential tool to directly test the involvement of PCMT in the repair of racemized proteins is the availability of a sensitive and reproducible method to measure the amount of D-aspartyl residues in proteins. In this respect, the use of a chiral capillary gas chromatographic method has been recently discussed (21,22). Using this procedure, the D-Asp content of different proteins from old human lenses was determined (Table I). The results obtained are in reasonable agreement with previous reports on the extent of racemization in lens proteins (10-12).

The WS and WI proteins, prepared from a pool of human lenses, were assayed as substrates for PCMT. These proteins are extensively methylated by the enzyme, the WI proteins being somewhat better substrates than the WS ones (Table II, last column). The levels of methyl-esterification that we found are the highest so far reported for naturally occurring proteins. It is worthwhile mentioning here that the extent of the methyl incorporation appears to be of the same order of magnitude as the D-Asp content in both fractions (Table II), indirectly suggesting the nature of the methyl accepting site in these proteins.

Therefore, lens proteins seem to be a useful tool to demonstrate the existence of a repair pathway of D-Asp residues. Highly racemized human WI lens proteins were enzymatically methyl-esterified for 24 h at 37°C. This prolonged incubation time was elected in order to allow the proteins to

### TABLE I

<table>
<thead>
<tr>
<th>Protein fraction</th>
<th>D/(D+L)-Aspartate [%]</th>
<th>Cortex</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HM</td>
<td>4.5 ± 0.8</td>
<td>12.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>s</td>
<td>5.5 ± 0.9</td>
<td>6.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>e&lt;sup&gt;1&lt;/sup&gt; + e&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.2 ± 0.6</td>
<td>3.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>e&lt;sup&gt;1&lt;/sup&gt; - e&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5.0 ± 0.6</td>
<td>5.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>US</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>9.3 ± 0.4</td>
<td>9.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>12.5 ± 0.4</td>
<td>8.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>14.9 ± 0.4</td>
<td>13.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>UI</td>
<td>17.9 ± 0.4</td>
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<sup>g</sup> Nomenclature refers to Ref 19
undergo several methylation and demethylation cycles. At the end of the incubation the proteins were hydrolyzed and the D-Asp content was determined. The hypothesis being tested here, predicts that the incubation of the racemized proteins in the presence of PCMT will result in a decrease of D-Asp content. However, the results reported in Table III fail to show a significant drop in D-Asp content of lens proteins upon incubation with AdoMet and purified PCMT.

However, on the basis of this result, the requirement in the postulated repair mechanism of other cellular factors, such as racemase/esterase enzyme(s) and/or small molecules and cofactors, cannot be ruled out. A first

<table>
<thead>
<tr>
<th>incubation mixture</th>
<th>D/(D+L)-Asp content [%]</th>
<th>D-Asp content [nmoles/mg]</th>
<th>$^{13}$C-methyl incorporation [nmoles/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI + AdoMet</td>
<td>4.3</td>
<td>15.61 ± 0.87</td>
<td></td>
</tr>
<tr>
<td>WI + AdoMet + PCMT</td>
<td>16.06 ± 0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WI + AdoMet + AdoHcy + calf lens homogenate</td>
<td>8.76 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WI + AdoMet + PCMT + calf lens homogenate</td>
<td>8.89 ± 0.13</td>
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</table>

WI proteins from pooled human lenses were incubated for 24 h at 37°C. AdoHcy was used in one incubation to suppress the endogenous PCMT activity present in the calf lens homogenate. At the end of the incubation, the proteins were hydrolyzed and the relative amount of D-Asp was determined. The extent of the methyl incorporation was evaluated in a parallel incubation carried out with $S$-adenosyl-$L$-[methyl-$^{13}$C]-methionine.
approach to mimic the \textit{in vivo} condition was carried out by adding to the methyl-esterification mixture an aliquot of the homogenate of the cortical fraction of calf lens. It is conceivable that if the mentioned factors are operative in the lens \textit{in vivo} they should be more active in the youngest cells. However, even under these latter experimental conditions, no decrease in the D-Asp content could be detected (table III).

\textbf{DISCUSSION}

The data reported in this paper indicate that proteins from human lenses are the best substrates for the enzyme PCMT described up till now. Furthermore, it has been shown that the extent of the methyl incorporation is of the same order of magnitude as the D-Asp content, suggesting that D-Asp residues indeed are the methylation sites in these proteins. However, our attempts to demonstrate a possible involvement of PCMT in the repair of racemized aspartyl residues by successive methylation/demethylation steps were unsuccessful.

The groups of Clarke and Aswad also failed to substantiate the repair-role of PCMT for D-Asp residues (24,25). However, in their attempts to verify their hypothesis they struck on another repair function of this enzyme. First they found that the L-isoAsp residues in small synthetic peptides are stoichiometrically methylated by PCMT (26,27). No methylation of D-Asp residues was found in these peptides. Next, it was demonstrated for a hexapeptide that L-isoAsp residues are formed through the succinimide pathway (24) as was suggested in the previous chapter of this thesis. Finally, it was found that in a 27 h incubation of the L-isoAsp form of tetragastin (Trp-Met-Asp-Phe-NH$_2$) in the presence of PCMT and AdoMet, repeated cycles of enzymatic methylation and spontaneous demethylation of the L-isoAsp resulted in a transformation of 50% of these residues into the normal L-Asp residues (24,25). Because of the racemization in the transient succinimide intermediate, D-Asp and D-isoAsp peptides (totally ca. 20% after 27 h) were formed as side products.

It has still to be elucidated why no methylation of D-Asp and D-isoAsp in small peptides is observed, while on the other hand methylated D-Asp residues could be isolated from proteolytic digests of methylated erythrocytes proteins (26,28), indicating that D-Asp residues in proteins are substrates for PCMT.
Another question not yet answered is whether L-isoAsp residues which have been shown to be substrates in small peptides are also methylated in proteins. And if so, might the methylation of eye-lens proteins reported here not be directed towards L-isoAsp residues as well as D-Asp residues? The methylation of L-isoAsp in proteins is currently being investigated by us using spectrotypes of monoclonal gammaglobulins that very likely are formed by deamidation of aspartic acid residues and, therefore, might contain isoAsp residues which are formed in a 3:1 ratio together with Asp residues by hydrolysis of the succinimid intermediate (4,28).

REFERENCES.


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SUMMARY

STRUCTURAL STUDIES ON α-CRYSTALLIN AND ON RACEMIZATION OF ASPARTIC ACID RESIDUES

The function of the structural lens proteins, called crystallins, is to maintain the transparency of the lens which they achieve by short-range interactions. The lens fiber cells as well as the crystallins remain present during the entire life-span. Upon aging, the crystallins are subject to several post-translational modifications which may destabilize their structure. Since this structure is so essential to lens transparency, these changes can, eventually, lead to cataract. This thesis deals with the native structure of bovine α-crystallin (chapters 2 and 3) and the racemization of aspartyl residues in human lens proteins (chapter 4), which is one of the many post-translational modifications observed in these proteins.

Of the bovine eye lens proteins, both the α- and β-crystallins possess a quaternary structure, i.e. they are composed of subunits. In the case of α-crystallin, these are essentially αA_1, αA_2, αB_1 and αB_2. The apparent number of subunits composing an α-crystallin molecule strongly depends on the isolation conditions. At 4°C a molecule of about 800,000 Da (ca. 40 subunits) is isolated from the lens cortex. However, isolation at 37°C in combination with high pH or low ionic strength, induces dissociation and a much smaller molecule is obtained (chapter 2.1). This qualifies the α_m-crystallin, which is isolated at such conditions (Thomson and Augusteyn, Exp. Eye Res. 3, 1984, 367-377), as an isolation artifact. Our results were confirmed by Tardieu et al. (J. Mol. Biol. 192, 1986, 711-724) who also refined the three layer model of bovine α-crystallin, originally proposed by our laboratory (Bindels et al., Ophthalmic Res. 11, 1979, 441-452).

Upon aging the size distribution of α-crystallin shifts towards a higher average molecular weight. Until now, the molecular mechanism of this process was puzzling since it concerns a non-stoichiometric, gradual increase that cannot be explained by a simple oligomerization of α-crystallin molecules. Attachment of newly synthesized subunits is also impossible since the process takes place in the absence of protein synthesis. Other non-stoichiometric changes in molecular weight were previously observed upon rechromatography or in experiments which showed a gradual decrease of molecular size with increasing concentration of urea or guanidine hydrochloride without the
appearance of single subunits. These observations can now be explained by our conception of the 'dynamic quaternary structure' of \( \alpha \)-crystallin. We have shown that both \( \alpha \)-crystallin molecules and homopolymeric aggregates of its subunits are in a rapid equilibrium with each other by intermolecular exchange of their subunits under native conditions. The time-scale of this process is such that a complete redistribution of all subunits has taken place within 24 hours (chapter 2.2)

With regard to the tertiary structure of the \( \alpha \)-crystallin subunits, no direct information is available. However, some predictions, based on primary structure as well as gene structure, have been made in the past. Using, amongst others, urea-gradient isoelectric focusing, which is explained in chapter 3.1, a remarkable difference in stability towards urea was found for the \( \alpha A \) and the \( \alpha B \) subunits with regard to their aggregation/dissociation and folding/unfolding. We observed that the urea-mediated un- and refolding of the \( \alpha A \) subunits is characterized by the existence of three intermediates. If folding through these intermediates results in one native structure, this implies a two-domain organization of the \( \alpha A \) chains. On the other hand, it cannot be excluded that the intermediates are indicative of differently folded native structures and this possibility might well explain the non-equivalent positions occupied by the subunits in the three layer model of \( \alpha \)-crystallin (chapter 3.2).

Racemization of aspartic acid is a modification at the level of the primary structure with possible implications for higher-level structures. In order to study this process in human lens proteins, precise enantioselective methods are essential. Both a chiral capillary gas chromatographic and a diastereomeric high-performance liquid chromatographic technique were found to meet our requirements (chapters 4.1 and 4.2). The latter method offered the possibility of on-line derivatization and analysis. Furthermore, we found that extraction of metal ions from the glassware during the acidic hydrolysis of the proteins causes high and varying amounts of background racemization. Precautionary actions are necessary to circumvent this problem (chapter 4.1).

A linear relationship with age was found for the extent of aspartic acid racemization in all six protein fractions of normal human lenses. The observed order of the overall racemization rate constants of these fractions was: cortex WS = nucleus WS < cortex US = nucleus US < cortex UI < nucleus UI (WS = water-soluble protein fraction, US = urea-soluble, UI = urea-insoluble). However, no correlation could be found with cataract and it is concluded that racemization of aspartyl residues is an aging process which plays no role in
proteins from normal lenses revealed that, in contrast to the general assumption, the rate constants of the forward (L→D) and the reverse (D→L) reaction are not equal. This is attributed to the chiral environment that is perceived by aspartyl residues in proteins. It was found that the rate constant of the forward reaction was identical for all protein fractions. However, the rate constant of the reverse reaction was higher for those fractions composed of grossly native proteins and was reduced for fraction containing post-translationally modified proteins. The lack of ability of these proteins to regain their native structure by a simple reinversion of the configuration of aspartic acid is thought to explain this observation (chapter 4.4). Two reaction mechanisms are proposed explaining why aspartic acid is exceptionally successful in stabilizing the carbanion intermediate at which racemization occurs. For one of these mechanisms (the succinamide-intermediate route) the structural requirements were explored by computer-assisted molecular modelling (chapter 4.4).

The isolation of D-aspartic acid, methylated at its β-carboxyl moiety from enzymatically hydrolyzed erythrocyte proteins, resulted in the suggestion that the enzyme protein carboxyl methyltransferase (E.C. 2.1.1.24) could function in a racemization-repair mechanism by methylating racemized aspartic acid residues which upon demethylation would return to their original configuration (McFadden and Clarke, Proc. Natl. Acad. Sci. U.S.A. 79, 1982 2460-2464). We tested the validity of this hypothesis for human eye lens proteins and could not find a decrease of the D/L ratio in these proteins upon incubation with protein carboxyl methyltransferase. On the other hand, we did observe that the lens proteins are the best substrates reported thus far for this enzyme (chapter 4.5). This leaves open the possibility that, in fact, L-isoaspartyl residues are recognized and methylated as has been previously observed for small peptides containing this unusual residue.
SAMENVATTING

STRUCTUURONDERZOEK AAN α-CRYSTALLINE EN AAN
RACEMISATIE VAN ASPARAGINEZUURRESIDUEN

De helderheid van de lens wordt onder andere bereikt door interacties over korte afstand van de structurele ooglenseiwitten, de crystallines. Evenals de lensvezelcellen worden de crystallines nadat ze eenmaal gevormd zijn nooit vervangen en blijven aanwezig gedurende het hele leven van het individu. Tijdens het verouderen ondergaan de crystallines diverse posttranslationele veranderingen die een destabilisering van hun structuur tot gevolg kunnen hebben. Omdat de structuur van de crystallines van essentieel belang is voor het behoud van de helderheid van de lens, kunnen deze veranderingen uiteindelijk leiden tot cataract (staar). In dit proefschrift wordt de natieve structuur van runder-α-crystalline behandeld (hoofdstukken 2 en 3), alsmede een van de vele posttranslationele modificaties van menselijke ooglenseiwitten, namelijk racemisatie van asparagine-zuur-residuen (hoofdstuk 4).

Van de rundercrystallines bezitten zowel de α- als de β-crystallines een quaternaire structuur, dat wil zeggen zij zijn opgebouwd uit subeenheden. In het geval van α-crystalline zijn dat hoofdzakelijk α₁, α₂, β₁ en β₂. De isolatie-omstandigheden zijn zeer van invloed op het schijnbare aantal subeenheden waaruit α-crystalline is opgebouwd. Zo wordt bij 4°C een molecuul van ongeveer 800.000 Da (±40 subeenheden) geïsoleerd uit de lens cortex, terwijl isolatie bij 37°C in combinatie met hoge pH of laag ionsterkte dissociatie induceert en resulteert in een veel kleiner molecuul (hoofdstuk 2.1). Deze bevindingen betekenen dat het α₁-crystalline, geïsoleerd onder laatstgenoemde condities (Thomson en Augusteyn, Exp. Eye Res. 37, 1984, 367-377), als een isolatie-artefact moet worden beschouwd. Onze resultaten zijn bevestigd door Tardieu et al (J. Mol. Biol. 192, 1986, 711-724). Deze auteurs hebben tevens het drie-lagen-model verfijnd dat oorspronkelijk door ons laboratorium was voorgesteld voor de quaternaire structuur van runder-α-crystalline (Bindels et al., Ophthalmic Res. 11, 1979, 441-452).

Tijdens het verouderen van de lens verschuift de grootteverdeling van α-crystalline naar een groter gemiddeld molecuulgewicht. Het moleculaire mechanisme van dit proces was tot heden niet duidelijk; het betreft een niet-stechiometrische, geleidelijke toename die niet eenvoudig verklaard kan
worden door oligomerisatie van α-crystalline moleculen. Bovendien vindt dit proces plaats in afwezigheid van eiwitsynthese, zodat aanhechting van nieuwgesynthetiseerde subeenheden ook niet tot de mogelijkheden behoort. Daarnaast zijn niet-stechiometrische veranderingen van het molecuulgewicht bij rechromatografie van geïsoleerde moleculelgewichtsfracties beschreven en bij experimenten waarin een geleidelijke groottevermindering bij toenemende concentratie ureum of guanidine HCl werd gevonden zonder dat daarbij losse subeenheden verschenen. Deze waarnemingen kunnen nu verklaard worden met wat we de "dynamische quaternaire structuur" van α-crystalline genoemd hebben. We hebben aangetoond dat zowel α-crystalline als homopolymere aggregaten van zijn subeenheden in evenwicht met elkaar verkeren door snelle uitwisseling van hun subeenheden onder natieve omstandigheden. De tijdbasis waarop dit proces zich afspeelt is zodanig dat volledige herverdeling van alle subeenheden binnen 24 uur plaatsvindt (hoofdstuk 2.2).

Er is geen directe informatie beschikbaar met betrekking tot de tertiaire structuur van de runder-α-crystalline subeenheden. Enkele voorspellingen gebaseerd op aminozuurvolgorde en de genstructuur zijn echter in het verleden wel gedaan. Door onder andere gebruik te maken van ureumgradient isoelctrische focusering (verklaard in hoofdstuk 3.1) is een opmerkelijk verschil in stabiliteit ten opzichte van ureum aangetoond voor de αA en de αB subeenheden voor wat betreft hun aggregatie/dissociatie en vouwing/ontvouwing. We vonden dat de ureum-gemedieerde ont- en hervouwing van αA subeenheden wordt gekarakteriseerd door het bestaan van drie intermediairen. Indien vouwing via deze intermediairen resulteert in een natieve structuur, betekent dit dat de αA subeenheden een twee-domein-organisatie bezitten. Anderzijds kan niet worden uitgesloten dat de drie intermediairen duiden op het voorkomen van verschillend gevouwen natieve conformaties. Dit zou een verklaring kunnen zijn voor de niet-equivalente posities, die door de subeenheden in het drie-lagen-model van α-crystalline worden ingenomen (hoofdstuk 3.2).

Racemisatie van asparaginezuurresiduen is een verandering op het niveau van de primaire structuur met mogelijke implicaties voor de structuur op hogere niveaus. Teneinde dit posttranslationele proces aan menselijke lensiwitten te kunnen onderzoeken, was het essentieel te beschikken over nauwkeurige enantioselectieve methoden. De chirale capillaire gaschromatografische en de diastereomere hoge-druk-vloeistofchromatografische techniek, die aan onze eisen voldeden, staan beschreven in de hoofdstukken 4.1 en 4.2. Deze laatste methode bood de mogelijkheid om de derivatisering en de analyse geïntegreerd te automatiseren. Verder werd gevonden dat, indien geen
voorzorgsmaatregelen worden getroffen, de extractie van metaalionen uit het glaswerk tijdens de zure hydrolyse een hoge achtergrond racemisatie met een grote spreiding tot gevolg heeft (hoofdstuk 4.1).

Voor elk van de zes eiwitfracties van normale menselijke lenzen werd een lineaire relatie gevonden tussen de mate van racemisatie en de leeftijd. De volgorde van de racemisatiesnelheidskonstante, zoals door ons vastgesteld, luidt: cortex WS = nucleus WS < cortex US = nucleus US < cortex UI < nucleus UI (waarbij WS = water oplosbare, US = ureum oplosbare en UI = ureum onoplosbare eiwitfractie). Er kon geen correlatie worden aangetoond met de mate van cataract en wij concluderen dan ook, dat de racemisatie van asparaginезuurresiduen een verouderingsproces is, maar geen rol speelt bij de vorming van cataract (hoofdstuk 4.3). Door nauwkeurige analyse van de racemisatie in eiwitten van normale lenzen kon worden vastgesteld dat, in tegenstelling tot wat algemeen gedacht wordt, de snelheidsconstanten van de voorwaartse (L→D) en de omgekeerde (D→L) reactie niet gelijk zijn. Wij schrijven dit toe aan de chiraliteit van de omgeving, zoals die door asparaginezuurresiduen wordt ervaren. De reactiesnelheidskonstante van de voorwaartse reactie was iets hoger voor de fracties die voornamelijk uit natieve eiwitten bestaan en was gelijk aan die van de voorwaartse reactie voor die fracties, waarin posttranslationele modificatie eiwitten voorkomen. Wij verklaren deze waarneming met de onmogelijkheid voor laatstgenoemde eiwitten om door middel van de inverse van het betreffende D-asparaginезuurresidue terug te keren tot hun natieve conformatie (hoofdstuk 4.4). Twee reactiemechanismen worden voorgesteld om te verklaren waarom juist asparaginезuurresiduen er zo uitzonderlijk goed in slagen om het carbamoion te stabiliseren dat als intermediair tijdens de racemisatie van het γ-koolstof atoom van aminozuren wordt gevormd. Voor één van deze mechanismen (die van de succinimide-route) is met behulp van computersimulatie onderzocht welke eisen dit mechanisme stelt aan de ruimtelijke structuur rond het asparagine- of asparaginезuurresidue (hoofdstuk 4.4). De isolatie van β-carboxyl gemethyleerd D-asparaginезuur uit enzymatisch gehydrolyseerde erythrocyteiwitten resulteerde in het voorstel dat het enzym protiene-carboxyl-methyltransferase (E.C. 2.1.1.24) deel zou kunnen uitmaken van een racemisatiereparatiemechanisme. Dit zou geschieden door katalyse van de methylverestering van garacemiseerde asparaginезuurresiduen, die vervolgens door verzeping van de methylester terug zouden keren tot hun oorspronkelijke L configuratie (McFadden en Clarke, Proc. Natl. Acad. Sci. U.S.A. 79, 1982, 2460-2464). Wij hebben deze hypothese getoetst voor menselijke lenseiwitten en
geen vermindering van de D/L verhouding na incubatie met het methylerende enzym gevonden. Anderzijds bleken de lenseiwitten te fungeren als de beste tot nu toe beschreven substraten voor het enzym (hoofdstuk 4.5). Dit laat de mogelijkheid open dat het niet D-asparaginezuurresiduen maar L-isoasparaginezuurresiduen zijn die worden herkend en gemethyleerd, zoals is aangetoond voor kleine peptiden die dit ongebruikelijke aminozuur bevatten.


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bedankt/grazie/thanks

Piet
PUBLICATIONS


Submitted:
The following chapters of this thesis have been or will be submitted for publication:
- P.J.M. van den Oetelaar, P.F.H.M. van Someren, J.A. Thomson, R.J. Siezen and H.J. Hoenders: The dynamic quaternary structure of bovine \( \alpha \)-crystallin. Intermolecular exchange of subunits by homopolymeric aggregates and \( \alpha \)-crystallin.

- P.J.M. van den Oetelaar, B.M. de Man and H.J. Hoenders. Protein folding and aggregation studied by isoelectric focusing across a urea-gradient and isoelectric focusing in two dimensions.

- P.J.M. van den Oetelaar and H.J. Hoenders: Folding/unfolding and aggregation/dissociation of bovine \( \alpha \)-crystallin subunits. Evidence for unfolding intermediates of the \( \alpha \)A subunits.


- P.J.M. van den Oetelaar, G.I. Tesser and H.J. Hoenders: Kinetical and mechanistic considerations on the racemization of asparagine and aspartic acid in proteins.

- C. Manna, P. Galletti, P.J.M. van den Oetelaar and H.J. Hoenders: Does protein carboxyl methyltransferase repair racemized aspartyl residues in human eye lens proteins?


Hij is getrouwd met Desirée de Rooij en vader van Pepijn.
STELLINGEN

I

De racemisatie van asparagine(zuur)residuen speelt geen aanwijsbare rol bij de cataractogenese.
Dit proefschrift.

II

De quaternaire structuur van runder α-crystalline heeft een dynamisch karakter.
Dit proefschrift.

III

Met de door Liardon en Jost gebruikte methode is het onmogelijk om de racemisatiesnelheid van eiwitgebonden aminozuren te bepalen.

IV

De conclusie van Beswick en Harding, dat glucose-6-fosfaat de subunits van α-crystalline niet "crosslinkt", is onwaarschijnlijk.

V

Het additionele gebruik van een UV-detector naast een kleine hoek laserlichtverstrooier en een brekingsindexdetector draagt niet bij tot een verbeterde bepaling van de molecuulmassa van chromatografisch gescheiden eiwitten.

VI

In een tijd waarin veel van het laboratoriumglaswerk door kunststofproducten is vervangen, doet de term "in vitro" enigzins archaïsch aan.
De bewering van Yuasa en medewerkers, dat de enantiomerenscheiding van aminozuren met behulp van een natieve cellulosekolom superieur is aan de voornaamste andere technieken, wordt niet door hun resultaten gerechtvaardigd.

Houdbaarheidsstermijnen van 70 jaar en langer, zoals zij voor crystallines in de humane ooglens gelden, zullen voor farmaceutische eiwitpreparaten voorlopig nog niet bereikt worden.

Zonder een controle van de gebruikte substraatpeptiden op de aanwezigheid van gemodificeerde asparaginezuurresiduen, is de conclusie, dat proteine-carboxyl-methyltransferase specifiek L-Asp methyleert, onacceptabel.

Veel gestuntel bij eenzijdig openende klapdeuren kan voorkomen worden door de deurklink aan de duwzijde te verwijderen.

De huidige propaganda voor condoomgebruik als afdoende beschermingsmaatregel tegen AIDS-besmetting is moeilijk te rijmen met het onbetrouwbare karakter dat dit rubberproduct heeft verworven op het gebied van geboorteregeling.

Een eiwitrijk orgaan als de runderooglens zou niet als slachtafval maar als een voedzaam onderdeel van ons menu moeten worden beschouwd.

Nijmegen, 21 januari 1988. P. J. M. van den Oetelaar.