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Reaction of 1-Fluoro-2,4-dinitrobenzene with the Free $\alpha$ Chains of Human Hemoglobin

EVALUATION OF THE pK OF THE TERMINAL AMINO GROUP*

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SUMMARY

The reaction of 1-fluoro-2,4-dinitrobenzene with isolated $\alpha$ chains in their mercaptide form with $\beta$-mercuribenzoate (PMB) was studied. From kinetic measurements with the change in absorbance at 353 nm upon substitution of the terminal valine, the pK of this group was calculated to be 7.30. The pH-independent reaction constant was estimated to be $0.282 \text{ M}^{-1} \text{sec}^{-1}$. Comparing the proton-binding behavior of untreated $\alpha$ chains with that of the chains substituted in the terminal valine ($\alpha^\text{PMBDNP}$), the pK of this group was calculated to be 7.4. Histidines did not appear to be dinitrophenylated. However, from the rate of liberation of protons upon the reaction of the chains with 1-fluoro-2,4-dinitrobenzene we concluded that they do interact with the reagent without forming a stable derivative. A pK of 6.85 and a pH-independent reaction constant of $0.023 \text{ M}^{-1} \text{sec}^{-1}$ per individual group was calculated for these residues. From oxygen equilibration studies on a mixture in equal amount of $\alpha^\text{PMBDNP}$ and $\beta$ chains in their mercaptide form with $\beta$-mercuribenzoate we calculated a value of 1.35 for the Hill parameter n. The $s_{10,\nu}$ of this mixture was 4.95 while it is near 2.5 when the $\alpha$ chains are not dinitrophenylated (Antonini et al., J. Mol. Biol., 17, 29 (1966)), indicating that the dinitrophenylation strongly favors the formation of tetramers. This high sedimentation value is consistent with a partial specific volume $\bar{\nu} = 0.731$, as compared to $\bar{\nu} = 0.749$ commonly used for hemoglobin (Svedberg and Pedersen, The ultracentrifuge, Oxford University Press, New York, 1940).

A determination of this pK by the specific method as done by Hill and Davis (1) for human carboxyhemoglobin has not yet been carried out for the free $\alpha$ chains. In proton-binding studies of these chains, the pK of the terminal amino group was assumed to be near 8 (4, 5). However, Janssen, de Bruin, and van Os (5) pointed out that the large rotational freedom present for this group in the crystals of carboxyhemoglobin (6) might suggest that its pK is also low in the free $\alpha$ chains, where its rotational freedom should be at least as great.

A study of the pK of the NH$_2$-terminal group in the free chains is of particular importance because this group is considered to be one of the alkaline Bohr groups (1, 2, 6, 7). The pK shift upon oxygenation is thought to result from a change in interaction between this group and the carboxyl terminal of its partner $\alpha$ chain (6).

This paper presents a study of the reaction between the free chains and FDNB.$^1$ The rate of reaction was measured by following the change in absorbance (8) at 353 nm or by measuring the release of protons with time upon reaction.

The proton-binding behavior of both unsubstituted and fully substituted $\alpha$ chains was consistent with the kinetic measurements, indicating that the pK of the amino group is indeed fairly low (near 7.4).

EXPERIMENTAL PROCEDURE

Materials

Adult hemoglobin was prepared by the toluene procedure of Drabkin (9). The isolated $\alpha$ chains were prepared by the method of Bucci and Fronticelli (10). Because of their higher stability, the chains were used without removal of the $\beta$-mercuribenzoate from the $-\text{SH}$ groups and will be designated as $\alpha^\text{PMB}$. The concentration of the $\alpha^\text{PMB}$ solutions was measured with the molar extinction coefficient $e_{540} = 14,000 \text{ M}^{-1} \text{cm}^{-1}$ at 540 nm for the CO derivative. FDNB was obtained from Sigma and was used without further purification. For all experiments a freshly prepared saturated FDNB solution (approximately 10

The pK of the $\alpha$-amino group of the $\alpha$ chain of human carboxyhemoglobin is estimated as close to 6.7 (1, 2). A similar pK value for this group was found in bovine carboxyhemoglobin (3).

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$^1$ The abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; DNP-, 2,4-dinitrophenyl-; PMB $\beta$-mercuribenzoate; $\alpha^\text{PMBDNP}$, $\alpha^\text{PMB}$ chain dinitrophenylated in the NH$_2$-terminal; PMB system, a mixture in equal amount of $\alpha$ and $\beta$ chains in their mercaptide form with $\beta$-mercuribenzoate.
mm) in 0.1 m NaCl was used. The concentration was checked with the dinitrophenylbenzylamine method of Hill and Davis (1). Carbon dioxide-free solutions of NaOH were prepared from a saturated stock solution. All other chemicals were analytical grade.

The preparation of DNP-substituted αPMB chains was carried out in the following way. Solutions of αPMB \((10^{-4} \text{ m in heme})\) were reacted with FDNB \((5 \times 10^{-4} \text{ m})\) in 0.2 m phosphate buffer at pH 7 for 1 hour at room temperature. The unreacted FDNB was removed by passage through Sephadex G-50. The reaction mixture was separated by chromatography on carboxymethylcellulose with a linear pH and ionic strength gradient made with two cylindrical bottles containing 1 liter of 0.05 m phosphate buffer at pH 6.2 and 0.1 m phosphate buffer at pH 7.0, respectively. The first bottle was connected to the column (2.5 x 20 cm), which had been equilibrated with 0.05 m phosphate buffer at pH 6.0. The protein solution was adjusted to pH 6.0, then dialyzed against the pH 6.0 buffer before adsorption on the column. A typical elution pattern is shown in Fig. 1. The leading peak contained the DNP-substituted material. (see “Results”).

Methods

**Measurement of Rate of Reaction Following Change in Optical Density at 353 nm**—Of a deionized αPMB solution \((2 \times 10^{-4} \text{ m})\), 10 ml were mixed with 5 ml of 0.2 m phosphate buffer at the desired pH and 5 ml of 0.02 m FDNB in a jacketed vessel thermostated at 25°. At regular time intervals, 2-ml samples were pipetted into test tubes containing 6 ml of 0.2 m phosphate buffer at pH 5.4 at 0° kept in an ice bucket. In this way the reaction rate was lowered about 2000 times. The excess FDNB was removed by dialyzing the samples at 4° against three changes of 2 liters of 0.02 m phosphate buffer at pH 5.4. Both FDNB and 2,4-dinitrophenol dialyzed very rapidly out of the dialysis bags. Control FDNB solutions dialyzed in this way showed a residual absorbance near 0.002, at 353 nm and were used as blanks in the spectrophotometric measurements. A Cary 14 spectrophotometer was used to measure the absorbance of the solutions.

**Measurement of Rate of Reaction Following pH Changes**—For these measurements the same equipment was used as described for the hydrogen ion titrations. The procedure was as follows. Of a deionized αPMB solution \((4 \times 10^{-4} \text{ m})\), 3 ml were put into the titration vessel and adjusted with KCl to a μm of 0.1. The desired pH was obtained by adding known amounts of 0.1 m HCl. Of a 0.2 m FDNB solution roughly adjusted with CO2-free NaOH to about the same pH, 3 ml were put into a separate vessel at the same temperature (25°). This was done to avoid pH changes upon mixing caused by differences in temperature. At zero time the FDNB solution was transferred into the titration vessel through a plastic tube by nitrogen pressure.

**Hydrogen Ion Titration**—The hydrogen ion titration curves were measured with roughly the same titration equipment as described by de Bruin, Janssen, and van Os (3). Instead of a digital voltmeter a Sargent-Welch recorder model SRG was used; full scale deflection (10 inches) corresponded to 0.1 or 0.2 pH unit. All protein solutions were carefully deionized by passing them through a mixed bed ion exchange column. The solution was brought to an ionic strength of 0.1 with KCl and the titration was carried out at 25° under a stream of nitrogen.

**Peptide Maps**—These were obtained following the method of Baglioni (11). High voltage electrophoresis was carried out with a model D Gilson electrophorator.

**Quantitative Amino Acid Analyses**—Quantitative amino acid analyses were performed with a Beckman model 120C amino acid analyzer and the standard 4-hour run.

**Oxygen Binding**—Oxygen-binding curves were measured spectrophotometrically according to the method of Rossi-Fanelli and Antonini (12).

**Sedimentation Velocity**—Sedimentation velocity measurements were carried out with a Beckman model E ultracentrifuge with the schlieren optics.

**Treatment of Kinetic Data**

**Spectrophotometric Kinetics**—As given by Hill and Davis (1) the rate of reaction of FDNB with the unprotonated groups of Class i can be described by

\[
\frac{d[X]}{dt} = \sum_i k_r^i [N_i] [P]
\]

in which \([X]\) represents the product, the \([N_i]\) represents the concentrations of the unprotonated groups of Class i, \(k_r^i\), the corresponding reaction constant, and \([P]\) the concentration of FDNB. As discussed later only the reaction of the terminal valine with FDNB is detected spectrophotometrically, so we are dealing with only one term in Equation 1. The concentration of the unprotonated α-NH2 group is related to the total concentration, \([P]\), of the unreacted αPMB chains by

\[
[N] = [P] = \frac{K}{K + (H^+)} [P] \quad \text{(2)}
\]

in which \(α\) is the degree of ionization of the NH2-terminal amino group, \(K\) its ionization constant, and \((H^+)\) the proton activity. Substituting Equation 2 into Equation 1
Reaction of FDNB with Free $\alpha$ Chain

Vol. 246, No. 17

Intercept of this line with the $(H^+)$ axis is $K$. When $K_{H^{OD}}$ is measured at several pH values a plot of $1/K_{H^{OD}}$ versus $(H^+)$, see Equation 4, will result in a straight line. The intercept of this line with the $1/K_H$ axis equals $1/k^a$ and the intercept at the $(H^+)$ axis is $K$.

Rate of Proton Release—The quantity $d[X]$ in Equation 1 is equal to the number of protons released by the chains upon reaction with FDNB. So in this kind of measurements Equation 1 can be written as

$$\frac{d(H^+)}{dt} = [P] k^a [N]$$

If only the initial stage of the reaction is considered, where $[P] = [P_o]$ the concentrations of all possible reactive sites are related to $[P_o]$ by a constant namely the number of groups per chain. Including this constant in $k^a$ an equation similar to Equation 3 is obtained

$$\frac{d(H^+)}{dt} = [P_o][F] k^a = [P_o][F] K_{H^{PH}}$$

As $dH^+/d[P_o] = dZ_H$, the change in proton charge, we can write

$$\frac{dZ_H}{d[H^+]} = [F] K_{H^{PH}}$$

This equation shows that measurement of $dpH/dt$ at zero time combined with measurements of the buffer capacity at that pH gives $K_{H^{PH}}$. It is clear that, if only the $\alpha$-amino group react with FDNB, $K_{H^{OD}} = K_{H^{PH}}$.

RESULTS

Characterization of DNP-substituted $\alpha$-PMB Chains

Optical Density Measurements—In Fig. 1 also the ratio $R = A_{353}/A_{450}$ of the various fractions is plotted. $R$ is 1.75 in untreated $\alpha$-PMB chains and has a maximum value $R_{max} = 2.88$ in the NH$_2$-terminal substituted chains as calculated from the molar extinction coefficient $\varepsilon_{353} = 16,200$ of the DNP-valerylleucine peptide (8). Fig. 1 shows that $R$ reaches a plateau value of about 2.87 in the leading peak consistent with a complete substitution of the terminal valine, and drops to a value of 1.88 in the trailing peak, indicating the presence of unsubstituted chains. The value of 1.88 is somewhat higher than the expected value (1.75) probably because the leading peak tails into this peak. It is noteworthy that only a very small amount of material had a value for $R$ of 3.3, which indicates a minor reaction of other groups with FDNB. The difference absorption spectrum of the CO derivatives of the $\alpha$-PMB-DNP and $\alpha$-PMB chains were obtained by adjusting the path length of a variable path length cuvette (type X DC 14 Precision Cell) until the optical density of the solutions to be compared was the same at 540 nm. From the difference spectrum, which corresponded to the spectrum of the DNP-substituted terminal peptide with a maximum at 353 nm a molar extinction coefficient for this peptide was calculated equal to 16,000 $\text{m}^{-1}\text{cm}^{-1}$.

Peptide Analysis—Peptide maps of the material eluted with the first peak in Fig. 1 showed the absence of Peptide $\alpha$T1 and the presence of only one additional yellow spot with a lower electrophoretic mobility and a larger $K_F$ value in chromatography than the $\alpha$T1 peptide. The amino acid composition of the eluted yellow peptide was that expected for the $\alpha$T1 peptide. The amino acid analysis of the terminal peptide DNP-valyleucine, extracted after partial hydrolysis as described by Hill and Davis (1), showed the presence of leucine with a recovery of about 80%.

pH Measurements. These showed that the isoeionic pH of the material of the leading and second peak was in all preparations pH 7.38 and 7.55, respectively. The latter pH is equal to the isoeionic point of untreated $\alpha$-PMB chains.

Determination of pH of $\alpha$-Amino Group

Hydrogen Ion Titrations—In Fig. 2 the differential titration curves for unsubstituted and fully substituted $\alpha$-PMB chains are shown. The analysis of such curves is described by de Bruin...
Fig. 3. Difference in protons bound by $\alpha^{PMB}$ and DNP-substituted $\alpha^{PMB}$ chains. The drawn line was calculated on the basis of $pK = 7.4$ and $\omega = 0.06$.

et al. (3) de Bruin and van Os (13). It represents the slope $\Delta pH/\Delta Z_H$ of the normal pH against $Z_H$ curve, in which $Z_H$ is the mean proton charge. This slope is equal to the reciprocal of the buffer capacity. Without going into the details of the analysis of such curves it is enough to mention that the distance between the two peaks shown is ionic strength independent and equal to the number of groups titrated in the neutral region. In the curve for the $\alpha^{PMB}$ chains this distance is 8.1, which means that in $\alpha^{PMB}$ 7 histidines and the $\alpha$-amino group are titratable. This result is in full agreement with earlier reports (4, 5). The peak to peak distance in DNP-substituted $\alpha^{PMB}$ chains is 7.2 eq. So there is one group less titratable than in the $\alpha^{PMB}$ chains. Considering the results obtained in the previous section the group missing in the substituted $\alpha^{PMB}$ chains has to be the DNP-substituted NH$_2$ terminal. Substitution of lysines can be ruled out because this should have produced a shift of the right peak in the curve for $\alpha^{PMB}$ DNP relative to the same peak in the curve for $\alpha^{PMB}$ in Fig. 2. It is also evident that the height of the right peak is higher in the substituted chains, meaning a diminished buffer capacity of the protein in this region of about 10%. This would indicate that the $pK$ value of the amino group is considerably lower than 8, because the disappearance of a group with a $pK$ near 8 would have changed the buffer capacity about 20% (3). In Fig. 3 the difference in protons bound by $\alpha^{PMB}$ DNP and $\alpha^{PMB}$ is shown. The curve shows clearly that the $pK$ of the amino terminal is near 7.4. The value for $\omega$, the electrostatic interaction factor obtained from a plot of pH ~ log $\alpha/1-\alpha$ against $Z_H$ (14), where $\alpha$ is the degree of ionization, was 0.060. This value is as usual (2) lower than that calculated on the basis of the radius of the macromolecule, $\omega = 0.078$ for the $\alpha^{PMB}$ chain at $\mu_m = 0.1$.

Spectrophotometric Kinetic Measurements—Fig. 4 shows two examples of experiments done at pH 6.645 and pH 7.660, respectively. As is seen straight lines are obtained even to a degree of substitution of more than 70% indicating a homogeneous reaction. In Fig. 5 the kinetic data are plotted as $1/K_H p^{H+}$ against $p^{H+}$ ($\Delta$). From the two intercepts we calculated $k^0 = 0.282 \pm 0.01$ m$^{-1}$ sec$^{-1}$ and $pK 7.3$. The $pK$ value found is slightly lower than the $pK$ calculated from Fig. 3, but the agreement is nevertheless satisfying. The $k^0$ value for this group in carboxyhemoglobin as measured by Hill and Davis (1) is somewhat lower, viz. $k^0 = 0.239 \pm 0.009$ m$^{-1}$ sec$^{-1}$.

pH-Kinetic Measurements—Fig. 6 shows an example of the change in pH upon reaction of $\alpha^{PMB}$ chains with FDNB. Thirty seconds after addition of FDNB a straight line is obtained. The slope of this line was taken as the slope at zero time without any correction, as the spontaneous hydrolysis of FDNB was very slow in the pH region investigated. The initial change in pH was very probably caused by small differences in temperature and pH of the $\alpha^{PMB}$ and FDNB solution before mixing. $K_H p^{H+}$ values were calculated with the buffer capacity data available from the acid-base titrations. In spite of the fact that all data so far presented are consistent with the hypothesis that only the NH$_2$-terminal valine reacted with FDNB under our conditions, the $K_H p^{H+}$ values did not correspond to the...
Reaction of FDNB with Free α Chain

Vol. 246, No. 17

Fig. 7. Oxygen binding equilibrium of a mixture in equal amounts of αPMB-DNP and αPMB chains. Y is the degree of oxygenation and P02 is the partial pressure of oxygen in millimeters of Hg. The measurements were done in 0.01 m phosphate buffer at pH 6.5 (Δ) and 7.5 (○).

Fig. 8. Concentration dependence of the sedimentation rate of: □, mixture in equal amounts of αPMB-DNP and αPMB chains; ○, αPMB-DNP chains. Both compounds were in their CO derivatives. The measurements were carried out in 0.05 m phosphate buffer at pH 6.9.

*K* values; indicating that additional groups reacted with FDNB, which were not detected spectrophotometrically. However, the plot in Fig. 5 of 1/K* against (H+) (○) is still linear suggesting that the various classes of reacting groups had similar pK and k* values. Subtracting the values of K* from the value of K* + ΔK* values were obtained which represented the rate of reaction of the additional groups in the pH measurements. A plot of 1/ΔK* against (H+) (○) gave values of pK 6.85 and k* 0.17 m⁻¹ sec⁻¹. The pK is consistent with the pK of the ionizable histidines in the α chains (4, 5).

Functional Properties of Substituted Chains—Attempts to measure the oxygen affinity in the αPMB-DNP chains failed because the material completely denatured during the deoxygenation procedure. However, a few measurements performed on the substituted chains after removal of the PMB seemed to indicate that the oxygen affinity was increased or at least equal in the substituted chains as compared to the normal chains (15). Fig. 7 shows the Hill plot of the oxygen-binding data of the 1:1 mixture of the αPMB-DNP and βPMB chains, designated as the DNP-PMB system. The value p1 (1.6 mm of Hg) also proves that upon DNP substitution the oxygen affinity increases, for the p1 of the PMB system is about 3 mm of Hg (16). The Bohr effect was, however, totally absent; the two curves measured at pH 7.65 and 6.5, respectively, coincided completely.

All the results concerning the degree of substitution of the material of the leading peak in Fig. 1 indicated that the NH2 terminus of the αPMB chains was fully substituted. Since titration studies of the same material showed that only one group was no longer titratable, we conclude that this peak contained only αPMB chains homogeneously substituted with DNP in their terminal value. The second peak in the same preparation appeared to be unreacted αPMB chains, as judged from the value of R and of the isoionic point. Also, in the chromatography it appears that the amount of material showing a value of R higher than 3, (expected if some lysines were substituted) is very small. In other words the chromatographic analysis indicates that, at neutral pH, essentially only the terminal valine is substituted by FDNB. Substitution of histidines was in no way detectable, in agreement with the findings of Neer and Konigsberg (8) for carboxyhemoglobin.

However the pH kinetics measurements showed that the release of protons by the αPMB chains upon the reaction with FDNB cannot be accounted for by the substitution of the NH2 terminus alone. The rate of release of protons can only be explained assuming the presence of another “reactive” site with a pK 6.5 which does not produce a stable DNP derivative. The only way out of this apparent contradiction is to hypothesize that some groups are able to catalyze the hydrolysis of FDNB, or that they form unstable derivatives which do not survive mild treatment like the dialysis and chromatography carried out in our experiments. In view of the fact that the pK of these groups is 6.85 they are most likely histidyl residues.

DISCUSSION

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hydrolysis. For the remaining difference it is possible that the pK 6.4 chosen by Hill and Davis (1) for the histidines is somewhat low. Acid base titrations would assign a pK near 7 to those groups in hemoglobin (4, 5). This value introduced in their calculations would increase the kₚ per histidyl residue. Also, it is possible that while 10 histidines can be protonated in hemoglobin per αβ dimer, less than these react with FDNB. Of course some modification of the structure of the chains upon substitution with FDNB might produce an additional liberation of protons detected in the pH measurements. However, this seems improbable to us in view of the behavior of the kinetics of the reaction and of the physicochemical properties of the substituted chains, especially their proton-binding characteristics.

The remote position of the cysteine 104 from the terminal valine in the α chains suggests that the reactivity of the α chains with FDNB was not influenced by the presence of PMB substituting that residue.

The average pK value of 7.35 for the terminal valine is remarkably higher than the value of 6.72 reported by Hill and Davis (1) for the same group in carboxyhemoglobin. This supports their hypothesis that this low pK is produced in hemoglobin with FDNB might produce an additional liberation of protons detected in the pH measurements. However, this seems improbable to us in view of the behavior of the kinetics of the reaction and of the physicochemical properties of the substituted chains, especially their proton-binding characteristics.

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Note Added in Proof—In a recent letter to one of us (S. H. B.) Dr. Perutz said that in collaboration with Dr. Ten Eyck he was able to locate, in human oxyhemoglobin, "the position where the guanidinium group of the Arginine 141α spends part of its time. It is in the internal cavity immersed in water without being bonded to any other group of the protein... . Its distance from the α-amino group of the Valine 1 of the other α chain is near 6 Å."

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REFERENCES