Histidine Tagging Both Allows Convenient Single-step Purification of Bovine Rhodopsin and Exerts Ionic Strength-dependent Effects on Its Photochemistry*

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Jacques J. M. Janssen†, Petra H. M. Bovee-Geurts, Maarten Merkx, and Willem J. DeGrip

From the Department of Biochemistry, Center of Eye Research, University of Nijmegen, 6500 HB Nijmegen, The Netherlands

For rapid single-step purification of recombinant rhodopsin, a baculovirus expression vector was constructed containing the bovine opsin coding sequence extended at the 3'-end by a short sequence encoding six histidine residues. Recombinant baculovirus-infected Spodoptera frugiperda cells produce bovine opsin carrying a C-terminal histidine tag (v-opshi6x). The presence of this tag was confirmed by immunoblot analysis. Incubation with 11-cis-retinal produced a photosensitive pigment (v-Rhohis6x) at a level of 15–20 pmol/10⁸ cells. The histidine tag was exploited to purify v-Rhohis6x via immobilized metal affinity chromatography. Optimized immobilized metal affinity chromatography yielded a binding capacity of ≥35 nmol of v-Rhohis6x per ml of resin and purification factors up to 500. Best samples were at least 85% pure, with an average purity of 70% (A₂₈₀ nm/A₅₀₀ nm = 2.5 ± 0.4, n = 7). Remaining contamination was largely removed upon reconstitution into lipids, yielding rhodopsin proteoliposomes with a purity over 95%.

Spectral analysis of v-Rhohis6x showed a small but significant red shift (501 ± 1 nm) compared to wild type rhodopsin (498 ± 1 nm). The pK₅ of the Meta I ↔ Meta II equilibrium in v-Rhohis6x is down-shifted from 7.3 to 6.4 resulting in a significant shift at pH 6.5 toward the Meta I photointermediate. Both effects are reversed upon incubation with 11-cis-retinal, producing a second photosensitive phase with a pK₅ of 6.6 ± 0.1. Remaining contamination was largely removed upon reconstitution into lipids, yielding rhodopsin proteoliposomes with a purity over 95%.

Rhodopsin is the major component of the outer segments of the vertebrate rod photoreceptor cell. This visual pigment consists of an integral membrane protein to which a chromophore, 11-cis-retinal, is covalently linked via a protonated Schiff base. Rhodopsin triggers the conversion of photon energy (light) into a graded membrane potential. The absorption of a photon leads to a number of discrete conformational changes in the protein moiety of the pigment (sequel of photointermediates → photocascade), finally resulting in the exposure of G-protein binding sites at the cytoplasmic surface of the protein. In the past decade, research has focused on analyzing the relationship between the structure of the receptor and its functional properties. Heterologous expression of the protein in combination with site-specific mutagenesis has become an attractive way to study this relationship. Several expression systems capable of producing recombinant bovine opsin in vitro have been described (1–5). Expression levels in these systems are usually quite low compared to total cell protein (<0.5%) and even to total membrane protein. For most analyses, recombinant rhodopsin therefore has to be extensively purified. Several methods have been described for the purification of recombinant rhodopsin (1, 4, 6). These methods often have the disadvantage that the obtained samples are still contaminated to a various extent with proteins derived from the cells, used for recombinant protein production (4, 6). Quite pure preparations can be obtained using immunoaffinity chromatography (1). However, this approach is expensive (monoclonal antibody, peptides for elution), laborious (antibody production, purification, and coupling), and fairly inefficient (low column capacity, recovery only in the order of 50%). Hence, this procedure is not very suitable for production of larger amounts (1–10 mg of purified protein) required for structural studies (crystallization, FT-IR spectroscopy,¹ NMR spectroscopy).

The recombinant baculovirus-based expression system is an excellent system for the production of larger amounts of recombinant bovine rhodopsin (6, 7). Thus far, we have used a combination of A-Sepharose affinity chromatography to purify rhodopsin produced in this system (6), which, combined with reconstitution into proteoliposomes, yields reasonably pure preparations (60–80%). However, it is quite inefficient. Because of contaminating viral glycoproteins, the column capacity for recombinant rhodopsin is small, and a laborious elution profile has to be applied. Here we report on an alternative approach, using a histidine tag engineered onto the C terminus of bovine opsin. Histidine tagging for purification of recombinant proteins by means of IMAC has been used for a number of proteins both in prokaryotic (8–10) and eukaryotic (11) expression systems, including recombinant baculovirus (12–14). None of these proteins, however, belonged to the superfamily of helical G-protein-coupled membrane receptors of which rhodopsin is a member. Membrane proteins have to be solubilized with the help of specific detergents which may impair affinity techniques. Hence, we have evaluated IMAC for purification of recombinant rhodopsin. Here we will demonstrate that an adapted IMAC allows relatively simple, highly efficient single-

¹ The abbreviations used are: FT-IR, Fourier transform infrared; IMAC, immobilized metal affinity chromatography; v-Rho, wild-type regenerated opsin produced in vitro by recombinant baculovirus; v-Rhohis6x, C-terminally histidine-tagged regenerated opsin produced in vitro by recombinant baculovirus; DoM, dodecyl-β-D-maltoside; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinpropanesulfonic acid; da, double-stranded; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis.

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RESULTS

Construction of the Histidine Tag Transfer Vector pAcJAC2 and Generation of Recombinant Virus—In order to introduce a tag consisting of six histidines at the C terminus of bovine opsin, the transfer vector pAcJAC2 was constructed (Fig. 1A). pAcJAC2 is derived from the baculovirus transfer vector pAcD21 (28). A short synthetic sequence encoding a histidine tag followed by a stop codon was introduced at the C terminus of v-Rhohis(\(\beta\)x) was collected from the 20% and/or 45% interface. Collected retinal lipid extract (100-fold molar excess). This mixture was layered on an Omega 30K filter (Filteron, Northborough, MA) and mixed with 1 volume of retina lipid extract (100-fold molar excess). This mixture was layered on top of a sucrose step gradient (10%, 20%, and 45% [w/v] in 3 × diluted buffer B) and spun overnight (100,000 × g, \(\sim\)16 h, 4 °C). Reconstituted v-Rhohis\(_{\beta}\) was collected from the 20% and/or 45% interface. Collected proteoliposomes were either diluted with the required buffer, and used for analysis, or with distilled water, pelleted (80,000 × g, 1 h, 4 °C), and stored at −80 °C until further use.

Fourier Transform Infrared (FT-IR) Difference Spectroscopy—FT-IR spectra of the rhodopsin → Meta II transition were recorded in principle near 3400 cm⁻¹ (OH stretching mode of water) and near 2900 cm⁻¹ (CH stretching mode of protein and lipids). Rhodopsin → Meta I difference spectra were recorded at 10 °C. Spectra were taken at 8 cm⁻¹ resolution in 5-min blocks before and after illumination at (1280 scans/spectrum). Each sample was illuminated for 30 s, 100 ng/ml leupeptin, pH 6.6). All subsequent manipulations were performed in the dark. The remaining rhodopsin was bleached away by a 5-min illumination. Sample hydration was monitored by the ratio between the peak absorbance near 3400 cm⁻¹ (OH stretching mode of water) and near 2900 cm⁻¹ (CH stretching mode of protein and lipids). Rhodopsin → Meta II difference spectra were recorded at 10 °C. Spectra were taken at 8 cm⁻¹ resolution in 5-min blocks before and after illumination at (1280 scans/spectrum). Each sample was illuminated for 30 s. The resulting films were hydrated with 5 μl of MES buffer (pH 5.5–6.0), or 20 μl HEPPS (pH 7.5–8.5) (24). The photocascade was triggered using a 5-min illumination. FT-IR spectra were recorded on a Mattson Cygnus 100 spectrometer (Madison, WI) equipped with a liquid nitrogen-cooled, narrow band MCT detector. Sample hydration was monitored by the ratio between the peak absorbance near 3400 cm⁻¹ (OH stretching mode of water) and near 2900 cm⁻¹ (CH stretching mode of protein and lipids). Rhodopsin → Meta II difference spectra were recorded at 10 °C. Spectra were taken at 8 cm⁻¹ resolution in 5-min blocks before and after illumination at (1280 scans/spectrum). Each sample was illuminated for 30 s.
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**A**

![Restriction Enzyme Cleavage Sites](image)

**B**

**Bovine opsin C-terminal sequence**

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GAC GAC GAG GCC TCC ACC ACC GTC TCC AAG ACA GAG ACC AGC CAA GTG GCG CCT GCC TAA GCCCCAGGGACTCCGTGGC
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Asp Asp Glu Ala Ser Thr Thr Val Ser Lys Thr Glu Thr Ser Gin Val Ala Pro Ala

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**Bovine opsin containing a histidine-tag at the C-terminus**

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GAC GAC GAG GCC TCC ACC ACC GTC TCC AAG ACA GAG ACC AGC CAA GTG GCG CCT GCC TAA GCCCCAGGGACTCCGTGGC
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Asp Asp Glu Ala Ser Thr Thr Val Ser Lys Thr Glu Thr Ser Gin Val Ala Pro Ala

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**Fig. 1. Cloning and C-terminal modification of v-ops**

**A.** A schematic diagram of the transfer vector pAcJAC2. This vector was used to produce recombinant baculovirus AcNPV/ops his expressing bovine opsin carrying 6 histidine residues at the C terminus. The plasmid contains the cDNA encoding bovine opsin (---). Opsi expression is controlled by the polyhedrin promoter (Ppolh). The *Drosophila* hsp70 promoter (P_hsp70) and the lacZ marker gene, used to identify recombinant virus, are identical with vector pAcDZ1 (28). Cleavage sites for several restriction enzymes are denoted. **B.** The amino acid and DNA sequences of bovine opsin and the histidine-tagged opsin (designated v-ops his6x) encoded by pAcJAC2, are displayed.

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In pAcJAC2, opsin biosynthesis is controlled by the polyhedrin promoter while the small heat shock promoter of hsp70, drives the biosynthesis of β-galactosidase, which functions as a reporter enzyme (17, 28).

**Expression of v-ops his6x using Recombinant Virus AcNPV/ops his---** Protein samples derived from recombinant virus-infected Sf9 cells were analyzed by immunoblot, using a polyclonal antiserum elicited against bovine opsin (Fig. 2A). Wild-type virus-infected Sf9 cells are used as a negative control, and native bovine opsin and wild-type v-ops as positive controls (Fig. 2A, lanes 1, 2, and 4). The presence of the histidine tag in v-ops his6x was confirmed by the following observations: 1) only v-ops his6x is recognized by the histidine tag antibody (see below), 2) the apparent molecular mass of v-ops his6x is larger (by about 2 kDa) than that of native opsin and v-ops (Fig. 2A, lane 1 versus lanes 2 and 3 versus lanes 2 and 4), 3) v-ops his8x does not react with the monoclonal antibody 1D4 (Fig. 2B, lane 1 versus 2), since the C-terminal histidine tag extension eliminates the epitope for this antibody (29). Hence, recombinant virus AcNPV/ops his directs expression of a fully intact histidine-tagged v-ops. The expression level varies between 20 and 30 pmol/10⁶ cells, which is comparable to wild-type v-ops (6).

**Regeneration and Purification of v-Rhohis6x---** To convert expressed v-ops his6x into v-Rhohis6x, we incubated total membranes, harvested at 3 days post-infection from infected Sf9 cells, with 11-cis-retinal. Spectral analysis of regenerated samples showed that this resulted in 18 ± 2 pmol of v-Rhohis6x per 10⁶ cells (n = 7). For purification by IMAC, regenerated samples were solubilized using DoM as a detergent and loaded onto
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A Ni²⁺-NTA agarose column. This column appeared to bind v-Rhohis₆x with high capacity. Proteins, which have natural affinity for metal ions are bound as well. A variety of washing and elution conditions have been tested to remove this contamination as much as possible without sacrificing binding capacity of the column and recovery of v-Rhohis₆x. Eluted fractions were analyzed by SDS-PAGE (protein pattern), immunoblotting (anti-rhodopsin CERNJS858 antiserum), and by UV/Vis spectroscopy (A₅₉₀ to detect and quantify v-Rhohis₆x, ratio A₂₈₀/ A₅₉₀ as a purity indicator). Typical results are presented in Fig. 3 and Table I. Lane 1 in Fig. 3, A, B, and C, presents the total extract of Sf9 membranes after regeneration. Only by immunoblot analysis can glycosylated v-opshis₆x be identified (arrow) together with a minor amount of unglycosylated species (arrowhead). The nonbound, flow-through fraction shows a very similar protein pattern (lane 2), except that most v-Rhohis₆x has bound, since only minimal amounts of the glycosylated form are detected in this fraction. Additional washings with extraction buffer (buffer C) elute a complex protein population (Fig. 3, lanes 3 and 4), probably representing specifically or very weakly bound proteins. The nonglycosylated v-opshis₆x is already strongly present in these fractions (Fig. 3, B and C, lanes 2-4, arrowhead) and apparently is not very well retained by the column. Protein contamination, weakly interacting with the column, could be eluted by raising the imidazole concentration to 25 mM (Fig. 3, lane 5). Under these conditions, a minor amount of v-Rhohis₆x was eluted. Most of the specifically bound v-Rhohis₆x, however, was eluted upon raising the imidazole concentration to 100 mM (Fig. 3, lane 6). This fraction also contains a minor amount of nonglycosylated species and is still contaminated by several other minor bands (Fig. 3A, lane 6, open arrowheads).

The amount of nonglycosylated v-opshis₆x in the DoM extract and column fractions varied between different experiments, but it was always present at immunodetectable levels (data not shown). In addition, immunoblots showed the presence of a third opsin species (Fig. 3B, lanes 1–4) which on SDS-PAGE gels migrated between the glycosylated and nonglycosylated v-opshis₆x. This species did not react with the anti-histidine tag antibody (Fig. 3C, lanes 1–4). We were unable to detect this band in the final purified v-Rhohis₆x fraction (Fig. 3B, lane 6).

An overview of IMAC results obtained under two conditions, as determined by spectroscopic analysis, is given in Table I. Nearly complete binding of v-Rhohis₆x to the Ni²⁺-NTA agarose was attained. At pH 8.0 (buffer C), a pH at which IMAC is usually performed, an average recovery of 84% was achieved with a purification factor of at least 450. An attempt to reduce contamination by weakly binding proteins, by applying the membrane extract in the presence of a higher imidazole concentration (10 mM; buffer D), resulted in a much higher loss of v-Rhohis₆x and in fact lowered the purification factor (not shown). Finally, the performance of the procedure we developed at pH 8.0, was evaluated at pH 6.5, since rhodopsin and most of its mutants are thermally much more stable at the
TABLE I

General characteristics of v-Rhohis6x purification by IMAC as determined by UV/Vis spectroscopy

Values given are mean ± S.D. of three experiments. The percentage solubilization is related to the amount solubilized under standard conditions (6), which was taken as the 100% value.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Buffer E</th>
<th>Buffer C</th>
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<tbody>
<tr>
<td>Solubilization (%)</td>
<td>100 ± 20</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Nonbound (%)</td>
<td>8 ± 5</td>
<td>5 ± 6</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>84 ± 4</td>
<td>80 ± 5</td>
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<tr>
<td>A280/A500</td>
<td>2.7 ± 0.4</td>
<td>2.4 ± 0.4</td>
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FT-IR Spectroscopy

—FT-IR difference spectroscopy provides adduced behavior (Table II). Addition of KCl up to 1 M increased the ionic strength by produced by the histidine tag. Increasing the ionic strength by solubilization is related to the amount solubilized under standard conditions (6), which was taken as the 100% value.

The efficiency of this IMAC procedure is spectrally illustrated in Fig. 4A. Curve 1 represents the total membrane extract applied to the column, while curve 2 presents the spectrum of the combined purified v-Rhohis6x fractions.

The contamination in the v-Rhohis6x fraction obtained after IMAC resides in several minor bands. These are largely removed upon subsequent reconstitution of v-Rhohis6x into retinal lipid proteoliposomes, which represent a more native-like environment, we routinely use to analyze functional properties of recombinant rhodopsin (6). To simplify reconstitution, the detergent used during regeneration (Dox) is exchanged during IMAC for nonylglucoside, which is more easily exchanged for phospholipids (30). The resulting v-Rhohis6x proteoliposomes contain at least 95% rhodopsin on a protein base and are suitable for all functional analyses (Fig. 3A, lane 8).

Spectral Properties and Photocascade of v-Rhohis6x—Curve 2 in Fig. 4A is a typical absorbance spectrum of the combined v-Rhohis6x fractions obtained after IMAC purification. Illumination of v-Rhohis6x “bleaches” the main absorbance band at 500 nm, and we used difference spectra to calculate the Amax more accurately. Unexpectedly, the absorbance band of v-Rhohis6x turns out to be slightly red-shifted (Amax = 601 ± 1 nm, n = 7) relative to wild-type (Amax = 498 ± 1 nm) (Fig. 4B). This slight but significant red shift has been observed in all samples produced so far, both before and after IMAC purification. The shift is independent of the presence of 10 mM Ni2+ ions (complexed histidine tag) or 10 mM EDTA (free histidine tag).

Analysis of the later part of the photocascade of reconstituted v-Rhohis6x presents another subtle effect of histidine tagging: at pH 6.5, the Meta I ↔ Meta II equilibrium is clearly shifted toward Meta I (Fig. 5B) in comparison to wild-type rhodopsin (Fig. 5A). At pH 5.5, v-Rhohis6x again behaves similar to wild-type (Fig. 5C: little Meta I formed, normal formation of Meta III). Again, this effect is not influenced by excess Ni2+ ions or complexing agent (EDTA). Analysis over a larger pH range, in fact, demonstrate that the pKa of the Meta ↔ Meta II equilibrium is down-shifted about 1 pH unit from 7.3 in wild-type to 6.4 (Fig. 6).

Hence, we reasoned that these subtle changes in v-Rhohis properties might be due to the additional surface charge introduced by the histidine tag. Increasing the ionic strength by addition of KCl up to 1 M concentration indeed reverted Amax and photocascade of v-Rhohis6x to wild-type behavior (Table II).

Structural Analysis of the Rho → Meta II Transition by FT-IR Spectroscopy—FT-IR difference spectroscopy provides detailed structural information on the transitions in the rhodopsin photocascade (e.g. Refs. 25–27, 31, and 32). We have used this approach to investigate whether histidine tagging would also exert influence on the conformational changes accompanying Meta II formation.

Comparison of the FT-IR difference spectra for the rhodopsin to Meta II transition in native rhodopsin and v-Rhohis6x shows that these spectra are highly similar (Fig. 7). All major bands characteristic of the formation of Meta II (numbers in cm−1) are present in v-Rhohis6x as well and within experimental error (±2 cm−1) at the same frequency as wild-type. Hence, the histidine tag does not detectably influence the conformational changes accompanying photococxitation of rhodopsin.

DISCUSSION

Purification of Recombinant Membrane Receptors—G-protein-coupled membrane receptor proteins have been expressed in all common in vitro expression systems (mammalian cell lines, insect cell lines, yeast, Escherichia coli), but the levels of expression are always low (<0.5% of cellular protein). Hence, for more elaborate functional studies as well as for structural studies, a very efficient purification procedure is needed. The most selective and frequently used approach is to exploit receptor affinity for an (ant)agonist or a monoclonal antibody. For instance, the most popular way to purify recombinant rhodopsins is by immunoaffinity chromatography over immobilized 1D4 (1, 33).

However, this approach has several disadvantages: it suffers from rather low recoveries (≤50% in our hands), low column capacity (1–2 nmol/ml bed volume), and expensive exploitation (monoclonal antibodies, peptide for elution). It is therefore not very suitable to purify larger amounts of recombinant protein. As an alternative, we adapted lectin affinity chromatography over concanavalin A-Sepharose to the recombinant baculovirus system (6, 17). This technique yields good recoveries (≥80%) and is fairly inexpensive, but due to the large contamination with viral glycoproteins, a complex elution profile has to be applied and the column capacity for rhodopsin binding is low (approximately 1 nmol/ml bed volume). As a consequence, it only affords rather low purification factors (50–100) and is quite laborious in the case of larger batches. We therefore searched for other alternatives and IMAC in combination with histidine tagging looked like a good candidate (8–13).

IMAC Purification of Histidine-tagged Rhodopsin—We selected the C-terminal of rhodopsin as a suitable site for appending a 6x histidine tag since 1) this site allows immunoaffinity purification and therefore should be well accessible and 2) C-terminal extension does not significantly influence functional properties of the protein (33, 34).

Recombinant histidine-tagged bovine opsin could be successfully, functionally expressed in the recombinant baculovirus/insect cell expression system. Regeneration with 11-cis-retinal resulted in a pigment (v-Rhohis6x) that could be purified almost to homogeneity in a single step using IMAC under optimized conditions. This purification procedure is rapid, fairly inexpensive, yields good recoveries (≥80%) with an excellent purification factor (≥500), and can handle, thanks to the high column capacity (≥35 nmol/ml bed volume), relatively large batches of rhodopsin. Interestingly, the histidine tag at the C terminus was found to have a subtle influence on the spectral and photolytic properties of this visual pigment, but these effects could be reversed by increasing the ionic strength.

The presence of the histidine tag at the C terminus of bovine opsin was first confirmed by immunoblot analysis. The modified C terminus was no longer recognized by the monoclonal antibody Rho-1D4 (29), and v-opshis6x was clearly identified by a polyclonal antibody elicited against a hexahistidine peptide.

2 G. L. J. De Caluwé and W. J. De Grip, unpublished data.
In addition, the apparent molecular mass of v-opshisBx on the immunoblots has increased relative to v-ops. Our histidine tag should increase the molecular mass by approximately 0.7 kDa. The observed shift of the apparent molecular mass, seen on the immunoblots, is at least 2 kDa, however. This might reflect a direct or indirect (SDS binding) effect due to the additional charge introduced by the histidine residues, which would change the migration behavior on the gel. Such a large effect has not been reported before but it might be more pronounced in the case of membrane proteins, which by themselves usually show aberrant behavior in SDS-PAGE.

Ultimate proof for the presence of the histidine tag in v-opshisBx of course lies in the successful application of IMAC using Ni²⁺ chelation. With this technique we could combine a high column capacity with high purification factors and good recoveries. The A₃₀₀/A₁₀₀₀ ratio indicates that, under optimized conditions, the combined purified fractions on average contain at least 70% v-Rhohis₆ₓ. However, peak column fractions have been obtained in which this was as high as 80–85%. Immuno­blot analysis shows that the purified v-Rhohis₆ₓ samples contain some nonglycosylated v-opshisBx. Lack of glycosylation was reported to reflect impaired protein folding and loss of regeneration capacity (17, 35). Therefore, one of the contaminants present in the v-Rhohis₆ₓ samples is v-opshisBx. Protein staining of PAGE gels reveals in addition to nonglycosylated v-opshisBx two minor bands at ca 25–30 kDa. Hence, these three proteins are the major contaminants after IMAC. Upon subsequent reconstitution into proteoliposomes, this remaining contamination is also largely removed. In the case of opsins this is due to its relative low stability in detergents and tendency to aggregate, which impairs correct reconstitution into a lipid matrix. Indeed, absolute FT-IR absorption spectra, where the amide I (1620–1690 cm⁻¹) and amide II (1530–1560 cm⁻¹) bands are extremely sensitive to protein secondary structure (36), do not suggest the presence of any (partially) misfolded opsin,⁹ while this was clearly observed for the mutants E134D and E134R (31).

In addition to glycosylated and nonglycosylated (rhod)opsin, a third anti-opsin immunoreactive band was detected, primarily in the IMAC wash fractions. The latter band migrates between the first two bands and is not recognized by the anti-histidine tag antibodies. This opsin species is only formed in minor amounts (<5% of total), and, presently, it is not clear where it derives from. It could be due to chemical cleavage of the histidine tag on the column, e.g. in the Ni²⁺-complexed from, or derive from endogenous carboxypeptidase activity present in the Sf9 cells. Both reactions might remove up to the entire tag, which would explain why this species has no or only low affinity for the Ni²⁺-NTA agarose and is not recognized by the anti-histidine tag antibodies. Thus far, no one has described a similar observation or any carboxypeptidase activity in Sf9 cells although a C-terminal histidine-tagged protein has been purified from this expression system before (13). However, we only did observe this (partial) removal of the histidine tag from v-Rhohis₆ₓ because of the relative large difference in apparent molecular mass between v-Rho and v-Rhohis₆ₓ and the availability of the anti-histidine tag antiserum. In the earlier reports, no large difference in molecular mass between the native and histidine-tagged proteins was observed, and no antiserum against the histidine tag was available. On the other hand, carboxypeptidase activity has been previously put to good use to remove a polyhistidine peptide fused to dihydrofolate reductase after purification from expression in E. coli (9). Hence, if cleavage of the histidine tag does become a serious problem for certain proteins, it is recommendable either to include carboxypeptidase inhibitors during extraction and purification or to protect the histidine tag with a C-terminal proline residue or to insert/append it elsewhere into the protein.

Functional Properties of v-Rhohis₆ₓ—The expressed v-opshis₆ₓ smoothly recombines with the chromophore, 11-cis-retinal, into a functional photopigment. However, unexpectedly, the absorbance spectrum of v-Rhohis₆ₓ shows a small but significant red shift (A₃₀₀ = 501 nm) and the pKₐ of the Meta I ↔ Meta II equilibrium is shifted down. These effects are not due to the presence of a Ni²⁺-histidine tag complex, since they do not depend on the presence of Ni²⁺ ions and are not reversed by addition of an excess of the complexing agent EDTA. However, both shifts are fully reversed by the addition of 1 M KCl. Hence, presently we think that these subtle functional effects result from the additional surface charge introduced by the histidine tag at the cytoplasmic surface of v-Rhohis₆ₓ. This could lead to an alkaline shift in the surface pHi, which could explain the apparent downshift of the pKₐ of the Meta I ↔ Meta

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Fig. 5. Spectral analysis of late photointermediates (Meta I, Meta II, and Meta III). v-Rho (A) and v-Rhohis6x (B and C) were reconstituted in retina lipid proteoliposomes. Spectra shown in A and B were recorded at pH 6.5. The spectrum shown in C was obtained at pH 5.5. Upon illumination, the dark spectrum (spectrum 1) is converted into an equilibrium mixture of Meta II (380 nm) and Meta I (480 nm) (spectrum 2), which slowly decays under formation of Meta III (level after 30 min; spectrum 3). Addition of hydroxylamine then converts all photo­
products into opsin, retinaloxime, and remaining rhodopsin (spectrum 4). A final illumination bleaches away remaining rhodopsin (spectrum 5).

II equilibrium. In addition, electrostatic effects (either local or more general like an increase in the protein dipole) could be involved. Both effects would indeed be suppressed by an increase in ionic strength. It would be quite exciting if electro­
static effects could at least partially be responsible for the observed phenomena, since to our knowledge such "long range influence" has not been documented yet. We are presently investigating this in more detail by mapping bulk-pH dependence, measuring surface pH, establishing reversal by removal of the histidine tag, and insertion of the histidine tag in other positions.

In order to establish whether the effect of the histidine tag would also penetrate at the structural level, FT-IR analysis was performed. The band shape of the amide I band indicates a very similar secondary structure composition for rhodopsin and v-Rhohis6x and the FT-IR difference spectra corresponding to the rhodopsin to Meta II transition are also highly similar. All characteristic bands of this transition (at 970, 1562, 1643, 1686, 1712, 1728, 1750, and 1768 cm⁻¹; the latter two of which have recently been assigned to the C=O stretching mode of aspartic acid 83 (31, 32)) are also present in the difference spectrum obtained with v-Rhohis6x. Hence, according to anal­
ysis by FT-IR, no significant differences exist in the structural alterations accompanying receptor activation in rhodopsin or v-Rhohis6x.

Conclusion—The use of IMAC in combination with histidine tagging has considerably improved and simplified the purifica­
tion of recombinant rhodopsin. This will now permit us to further scale up the cell culture volume and recombinant protein production, which thereby will yield sufficient protein for more detailed structural studies, requiring modified protein (FT-IR, NMR, crystallization).

The adaptations we have introduced in the IMAC (use and exchange of detergents, lower pH, stepwise imidazole elution) should make it applicable for membrane proteins in general. It cannot be foreseen whether the subtle influence of the histidine
tag on functional properties, observed for rhodopsin, would also always emerge in other membrane receptors. However, the histidine tag apparently need not influence the conformational changes accompanying receptor activation, and any functional effects can easily be suppressed by an increase in ionic strength or, if properly placed, be abolished by its proteolytic removal.

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