Modification of Two Peptides of Bacteriorhodopsin with a Pentaamminecobalt(III) Complex†

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ABSTRACT: Bacteriorhodopsin (bR) was regenerated from the cation-depleted blue membrane with pentaammineaquocobalt(III) tetrafluoroborate ($[Co(NH_3)_5H_2O]^{3+}[BF_4^-]_3$). Illumination of the sample with orange light decreased the extinction at 568 nm concomitantly with a hypsochromic shift of the absorption maximum. The photocycle of this sample was inhibited, and the rate of proton pumping was reduced. Chymotryptic cleavage of the corresponding apomembrane into the two fragments C1 and C2 and their subsequent separation revealed that cobalt label is only attached to C1. The maximal incorporation of Co into this peptide was 0.3 Co/C1. After cleavage of C1 with cyanogen bromide and subsequent proteolysis with trypsin and chymotrypsin, this modification could be associated with peptides from cyanogen bromide fragments 6 and 9. The sequences were determined to be ^{101}Val-Asp-Ala-Asp-Gln and ^{228}Ala-Ile-Phe-Gly-Glu-Ala-Glu-Ala. These peptides contain the sequences Asp-Ala-Asp and Glu-Ala-Glu, respectively, which might be constituents of the same cation binding site. The observation that the incorporation of Co into bacteriorhodopsin is enhanced under illumination with orange light indicates that this site might be involved in the proton uptake.

Bacteriorhodopsin, the only protein of the purple membrane from *Halobacterium halobium*, absorbs maximally at 568 nm [for recent reviews on bacteriorhodopsin, see Ottolenghi (1980), Ovchinnikov et al. (1982), Hess et al. (1982), Stoeckenius and Bogomolni (1982), and Dencher (1983)]. On removal of cations by acidification or deionization of the purple membrane (Fischer & Oesterhelt, 1979; Kimura et al., 1984; Chang et al., 1985), the so-called blue membrane (bR^{blue})[1] is formed, which has an absorption maximum at 605 nm. Upon titration of bR^{blue} with cations the purple color is regained (Kimura et al., 1984; Chang et al., 1986). The distance of a negative counterion from the protonated Schiff base and a perturbation through charges close to the ionone ring (Nakanishi et al., 1980; Lugtenburg et al., 1986) is thought to modulate the color. In the case of bR^{blue} it was considered that the counterion is either protonated or removed from its original position by a conformational change of the protein (Fischer & Oesterhelt, 1979; Szundi & Stoeckenius, 1988). Recent experiments suggested that the purple to blue transition might not be directly dependent on cations but on protonation changes at the surface of the membrane which alter the microenvironment at the site of the chromophore (Szundi & Stoeckenius, 1987, 1988). Apparently, according to these data cations do not have to be bound to a specific site and are not essential for the stabilization of the purple chromophore.

However, specific cation binding sites were proposed by several authors to explain the data gained from the binding of cations to the blue membrane and the influence of different cations on functional properties of the purple membrane (Zubov et al., 1986; Dunach et al., 1986, 1987; Ariki et al., 1987; Corcoran et al., 1987). For example, evidence can be seen in results provided by Mercier and Dupuis (1988), who concluded from their fluorescence experiments on deionized and La^{3+}-modified bR that two different cation binding sites exist. Furthermore, Corcoran et al. (1987) and Dupuis et al. (1985) described the involvement of metal ions in the Schiff base and tyrosine deprotonation during the formation of the long-lived intermediate ^{412}M. Also, the retardation of the photocycle by La^{3+} (Drachev et al., 1984; Chang et al., 1985) or Fe^{3+} (Engelhard et al., unpublished results) might indicate a possible relevance of metal ions for the function of bR.

If distinct metal ion binding sites do indeed exist, it should be possible to identify them. Several attempts to characterize the cation binding sites and to distinguish between a lipid binding site and a protein binding site have already been reported. Diffraction studies (Katre et al., 1986) showed defined binding sites for Pb^{2+} which were located not only on the protein part but also on the lipid part of the density map. Ariki et al. (1987) concluded from the lanthanide luminescence of Eu^{3+}-modified bR that two high-affinity sites with six protein ligands were present. In a similar study, Corcoran et al. (1987) determined three different cation environments, two of which are surface sites and with six and three water molecules, respectively, as ligands. The cation in the third site is probably located in a hydrophobic region. Cladera et al. (1988) discuss in a thermal denaturation study a site that is linking carboxyl groups with the phospholipid head groups.

These results are an indication of the existence of at least one protein binding site. It could also be assumed that the carboxylate side chain functions of aspartic acids and/or glutamic acids are involved in cation binding. The modification of the purple membrane by EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide), a carboxyl-modifying agent, inhibits the formation of the blue membrane (Renthal & Wallace, 1980; Dunach et al., 1987). It is therefore suggestive that if a binding site exists, side-chain functions of Asp and/or Glu are involved.

Carboxyl groups can be modified by the pentaamminecobalt complex $[Co(NH_3)_5H_2O]^{3+}$ in which the H_2O is replaced by

† Dedicated to A. Trebst on the occasion of his 60th birthday.
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[1] Abbreviations: bR, bacteriorhodopsin; bR^{blue}, cation-depleted bacteriorhodopsin; CobR, cobalt-containing bacteriorhodopsin; $CobR^{hν}$, CobR modified by light; DBU, diazabicycloundecene; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

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a carboxylate (Isied et al., 1982). In this paper the modification of bacteriorhodopsin by this cationic Co complex is described, the labeled peptides are identified, and their sequences are determined.

Materials and Methods

All reagents used were of reagent grade. Cyanogen bromide was sublimated prior to use. The proteases were bought from Boehringer Mannheim, Mannheim, FRG; Worthington, Freehold, NJ; or Sigma Chemie, Steinheim, FRG, respectively. Pentaammineaquocobalt(III) tetrafluoroborate ($[Co(NH_3)_5H_2O]^{3+}[BF_4^-]_3$) was a gift from S. Isied, Rutgers University.

Bacteriorhodopsin was isolated from *Halobacterium halobium* (S 9) according to the method of Oesterhelt and Stoeckenius (1974). The blue cation-depleted membrane was prepared according to Kimura et al. (1984). It was deionized by dialysis against an ion-exchange resin (AG 50W-X8, Bio-Rad) suspended in double-distilled water. The apomembrane was obtained by illuminating purple membrane in the presence of hydroxylamine (Oesterhelt, 1982). The photocycle kinetics were measured and analyzed according to Maurer et al. (1987) and Müller et al. (unpublished results).

The blue membrane (40 mg in 40 mL of H_2O) was titrated with a 10-fold excess of the cobalt complex ($[Co(NH_3)_5H_2O]^{3+}[BF_4^-]_3$). The pH was adjusted to pH 6 by the addition of diazabicycloundecene (DBU), and subsequently, the sample was irradiated with orange light (filter OG 530, Schott, Mainz, FRG) from a projector lamp (Xenosol II, Zeiss, Oberkochen, FRG). The intensity of the orange light was approximately 3 kW/m^2. After appropriate times, samples were taken and washed three times with water. For the analysis of the Co incorporation the samples were also washed five times with 0.01 N HCl to remove not firmly bound Co.

The apomembrane was chymotryptically cleaved into the C1 and C2 fragments and separated on an LH-60 column (2.5 cm × 100 cm, Pharmacia, Freiburg, FRG) according to the method of Gerber and Khorana (1982). The flow rate was 10 mL/60 min (2.5 mL/fraction). Cyanogen bromide cleavage was carried out essentially as described by Gerber and Khorana (1982) except that $Mn[CH_3COO^-]_2$ was added as scavenger for cyanate (OCN^-). The C-terminal tail of bR was removed by papain according to Abdulaev et al. (1978).

The peptides from the cyanogen bromide cleavage of C1 and from the proteolytic digest of the cyanogen bromide fragments were dissolved in 88% HCOOH in H_2O and were subsequently separated by a Beckman Instruments (Palo Alto, CA) high-performance liquid chromatography (HPLC) system employing a reverse-phase C-18 analytical column (C_{18} μBondapak, Waters GmbH, Eschborn, FRG). The peptides were eluted by an acetonitrile (CH_3CN)–trifluoroacetate (TFA) gradient. The solvents can be used for monitoring at 230 nm.

Cobalt was determined by atomic absorption spectroscopy (observation wavelength 240.7 nm, Model AAS 300 from Perkin-Elmer, Überlingen, FRG). The 2.4-mL fractions from the LH-60 column were analyzed by taking 100-μL portions. The solvent was removed, and the samples were dissolved in 100 μL of formic acid and placed onto the automatic sample injector of the atomic absorption spectrophotometer. The fractions of two to three HPLC separations were collected, dried, and redissolved in 100 μL of formic acid before being analyzed for Co.

The cyanogen bromide fragments of CoC1 (0.3 μmol) were dissolved in 600 μL of 88% HCOOH in H_2O and separated in 50-μL portions on a reversed-phase column. The Co-containing fractions between the elution of CNBr-8 and CNBr-9 of each run were collected and freed of CH_3CN on a rotary evaporator. The last traces of solvent were removed by lyophilization (yield 80%). The peptide mixture was dissolved—though not completely—in 75 μL of buffer A (200 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.8, 8 M urea) and diluted with buffer B (buffer A without urea) to 2 M urea. Chymotrypsin (12 μg) and trypsin inhibitor (0.12 μg) in 10 μL of buffer B were added to this mixture, and the reaction was carried out for 2 h at 37 °C until an almost clear solution was obtained. The reaction was stopped by freezing the sample to −20 °C. Appropriate amounts were separated on a C_{18} reversed-phase column.

The tryptic digestion of CNBr-6 was done according to Walsh (1970). Peptides were sequenced manually according to the method of Tarr (1975, 1981). The PTH-amino acids were analyzed by HPLC (Tarr, 1982).

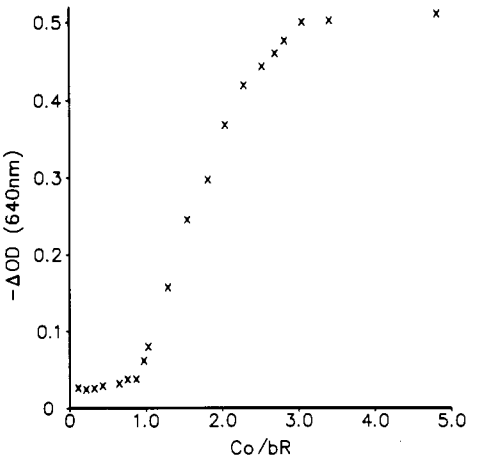

FIGURE 1: Titration of the blue membrane with $[Co(NH_3)_5H_2O]^{3+}[BF_4^-]_3$. For the determination of the bound Co the samples were centrifuged, and Co was determined in the supernatant by atomic absorption spectroscopy. These values were subtracted from the added amount of Co.

Table I: Photocycle Data of Native bR, Native bR Regenerated with $[Co(NH_3)_5H_2O]^{3+}$ (CobR), and the Corresponding Light-Activated Reaction Product ($CobR^{h\nu}$)

	L → M (μs)	M → bR (ms)
bR	70	3
CobR	70	5
$CobR^{h\nu}$	80	8 139

Results

Spectrophotometric Titration of Bacteriorhodopsin with Cobalt. The titration of bR^{blue} in distilled water at pH 5 with $[Co(NH_3)_5H_2O]^{3+}[BF_4^-]_3$ leads to a sigmoidal saturation curve with a dissociation constant of approximately 0.01 μM for the high-affinity site, which is characteristic for the binding of trivalent ions to bR (Ariki & Lanyi, 1985; Kohl et al., 1984) (Figure 1). Three equivalents of Co/bR is sufficient to turn the blue membrane into its purple state with an absorption maximum at 568 nm but with an extinction coefficient of ϵ = 59000. Raising the pH to 7 restores the extinction of native bR. The photocycle of this preparation is indistinguishable from other cation-regenerated (e.g., Mg^{2+}) purple membranes and similar to native purple membrane (Table I). It can also be shown that after its incorporation into vesicles the proton-pump efficiency is not inhibited and is comparable to that of a native preparation (data not shown).

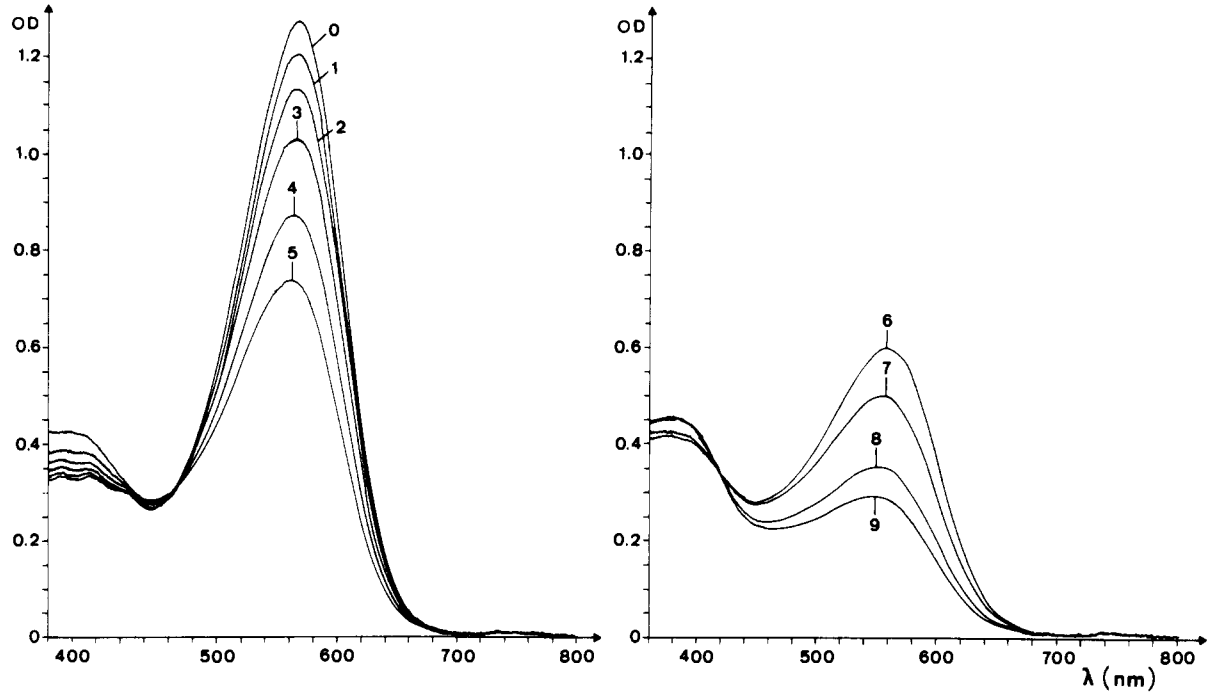

FIGURE 2: Illumination of CobR with orange light. Numbers indicate the time of irradiation in hours.

Modification of Bacteriorhodopsin with Cobalt. In order to convert the loose linkage of Co in CobR into a stable bond, a 10-fold excess of the Co reagent over bR was used, and the reaction was carried out at pH 6 at 40 °C either in the dark or under illumination with orange light. Whereas in the dark almost no change in the visible spectrum is observed, under irradiation with orange light the absorption maximum of 568 nm gradually decreases and shifts hypsochromically to about 540 nm (Figure 2). As the end of the reaction is approached, the absorbance at 380 nm is increased, indicative of the formation of free retinal. A clear isosbestic point is missing so that the decrease at 568 nm cannot be directly correlated with the increase at 380 nm, thus suggesting a complex transition. Taking the extinction coefficients of bR (63 000) and retinal (40 000) into account, only a fraction (20%) of free retinal is formed. The remaining absorbance with an absorption maximum of 540 nm could be attributed to bR modified with $[Co(NH_3)_5]$. This is further supported by the minute absorption shift of $CobR^{hv}$ upon acidification from 540 to 550 nm.

On analysis of the photocycle of this sample, a retardation by 1 order of magnitude is observed (Table I). Furthermore, a pronounced biphasic M-decay is observed. Upon incorporation of $CobR^{hv}$ into asolectin vesicles, the proton pump was found to be reduced (data not shown).

The extent of modification of bR by $[Co(NH_3)_5H_2O]^{3+}$ was determined by atomic absorption spectroscopy. After 8 h in the dark, only 1 Co/bR is incorporated. However, after irradiation with orange light under the same conditions about 2.5 Co/bR were obtained (Figure 3), indicating that the active photocycle enhances the formation of a Co–protein bond (see below). Apparently, the binding of the first Cobalt does not lead to a color shift, whereas the second Co correlates approximately with the shift of the absorption maximum.

Identification of the Co-Modified Chymotryptic Fragments. Chymotrypsin cleaves the apomembrane into the two fragments C1 and C2. The preparation of the apomembrane affords the irradiation of bR in the presence of hydroxylamine (Oesterhelt, 1982). This reaction, which was carried out at 20 °C, resulted in a loss of the label of about 80%. Apparently

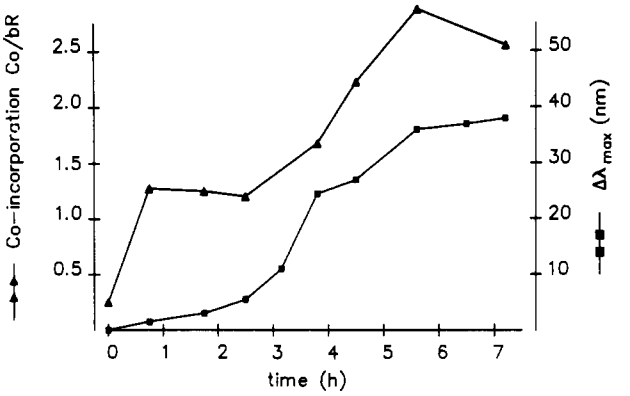

FIGURE 3: Extent of Co incorporation into bR in comparison to the shift of the absorption maximum. $\Delta\lambda_{max}$ is the difference between the absorption maximum of native bR at 570 nm and the corresponding maximum of the reaction product.

the hydroxylamine can split the Co–protein complex.

After proteolysis of the protein by chymotrypsin and separation of the two fragments (C1 and C2) by gel chromatography (LH 60), the incorporation of Co was measured. The chromatogram is shown in Figure 4. Co is found only in uncleaved opsin and in the C1 fragment which comprises the five C-terminal helices. About 10% of the original applied cobalt eluted together with free retinal between fractions 143 and 165. After 9 h of illumination a saturation is reached with 0.35 Co/C1.

When the sample is kept at 40 °C in the dark for 8 h, the value is 0.13 Co/C1; however, one has to consider that in order to obtain the apomembrane this sample had to be irradiated for 2 h.

Cyanogen Bromide Cleavage of C1. C1 was further cleaved with cyanogen bromide (CNBr), and the fragments were separated on a reverse-phase column (Figure 5). The recovery of peptides was generally between 70 and 80%. The Co label was found in CNBr-6 and on the left shoulder of CNBr-8 [the nomenclature follows Gerber and Khorana (1982)]. The maximum of the Co label does not coincide with the maximum of the optical density trace because—as described above—only

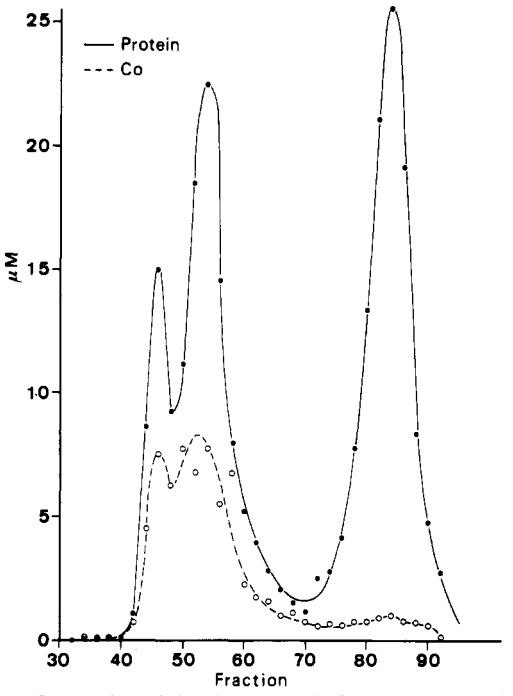

FIGURE 4: Separation of the chymotryptic fragments C1 and C2 on a LH-60 column. Uncleaved opsin is found in fractions 42–48 whereas C1 elutes in the second and C2 in the third position. Cobalt was determined by atomic absorption spectroscopy.

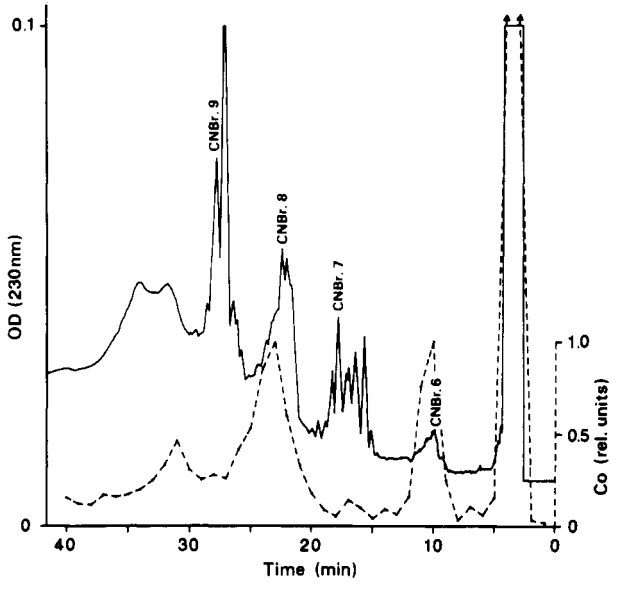

FIGURE 5: Separation of cyanogen bromide fragments of C1 on a C_{18} reverse-phase column. The buffers for the gradient were 10 mM TFA in H_2O (A) and 10 mM TFA in CH_3CN (B). The gradient started with 30% B and increased to 80% B in 30 min. For details of the experimental conditions, see Materials and Methods.

35% of the C1 was labeled with cobalt. In addition, a further loss was expected under the reaction conditions of the CNBr cleavage, which promote the destruction of the Co–protein bond. Particularly, OCN^-, which is formed as a side product during the reaction, might have replaced a ligand from the Co–protein complex. To reduce the side reactions, Mn-$[CH_3COO^-]_2$ was added as scavenger to the reaction mixture.

Chymotryptic Cleavage of CNBr-8/CNBr-9. The Co-containing shoulder of CNBr-8 was collected and subjected to chymotryptic cleavage. The peptide mixture was separated on a reverse-phase column (Figure 6). Three cobalt-containing peaks were identified. By manual Edman degradation of the peptide of peak I, which contained 10 times more Co

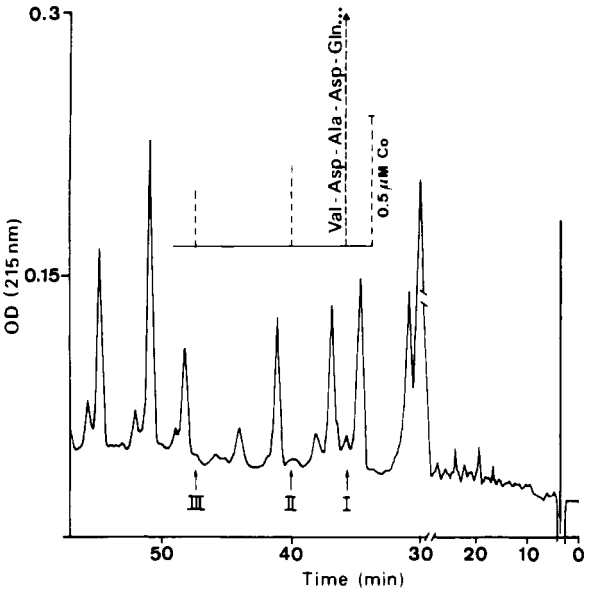

FIGURE 6: Separation of a chymotryptically cleaved mixture of CNBr-8 and CNBr-9 by HPLC (buffers as in Figure 5). The gradient was 0% B to 30% B in 60 min. The Co content of peaks I–III is shown in the insert.

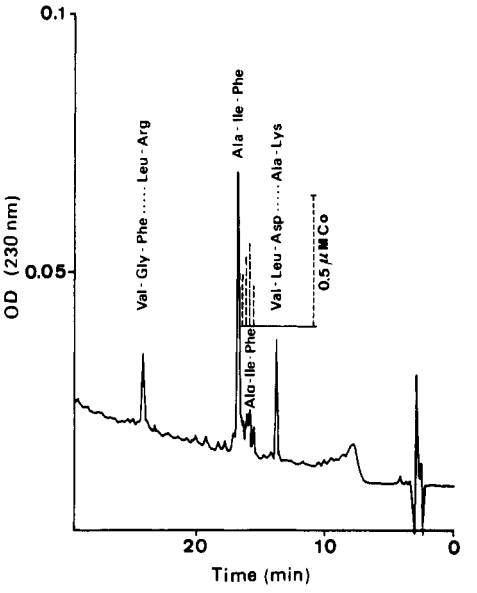

FIGURE 7: Separation of the tryptic digestion mixture of CNBr-6 by HPLC (buffers as in Figure 5). After injection, 100% A was continued for 5 min before B was started and gradually increased to 80% in 40 min.

than peaks II and III, the sequence ^{101}Val-Asp-Ala-Asp-Gln... was determined. This sequence is found in CNBr-9. Apparently, the hydrophilic Co label decreases the hydrophobicity of the CNB-9 fragment so that it elutes at much lower CH_3CN concentrations. The exact position of the Co label could not be determined because the Co–protein bond was found to be nonresistant to the Edman degradation procedure, the label thus being lost already during the first sequencing steps.

Tryptic Cleavage of CNBr-6. The HPLC of the tryptic digestion mixture of CNBr-6 is shown in Figure 7. The cobalt-containing fractions are located close to peak II and start with the sequence ^{228}Ala-Ile-Phe. To further characterize this heterogeneous mixture, it was subjected to a carboxypeptidase cleavage. A new peak also containing cobalt appeared at 28 min (Figure 8). The reaction was not complete as can be seen from the almost unchanged pattern of the original mixture of peptides. The first amino acids of this new

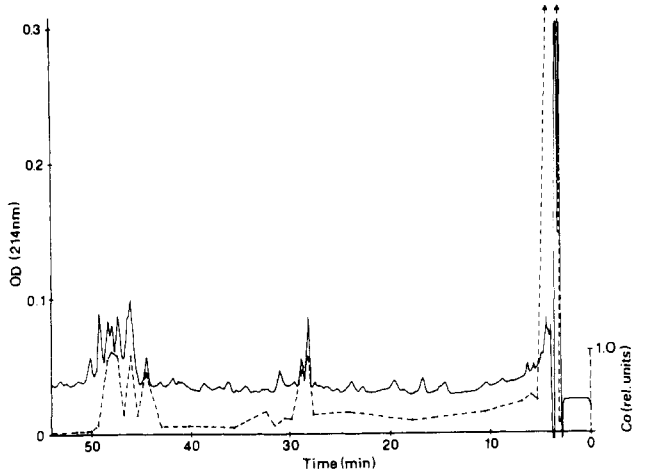

FIGURE 8: Separation of the Co-containing Ala-Ile-Phe... peptides (see Figure 7) that were cleaved by carboxypeptidase A by HPLC (buffers as in Figure 5). After injection, 100% A was continued for 5 min before B was started and increased to 30% in 60 min.

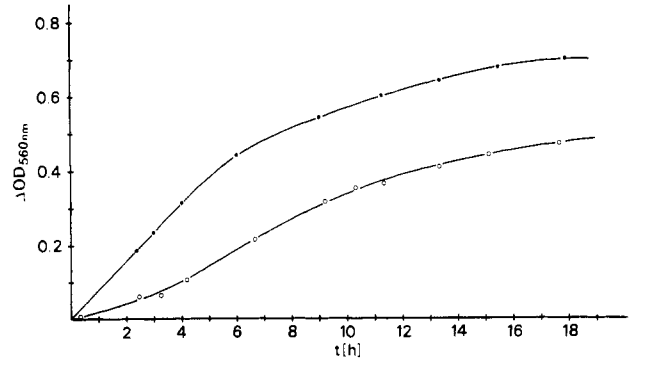

FIGURE 9: Time course of the optical density decrease (ΔOD) at 560 nm for the light-activated reaction of $[Co(NH_3)_5H_2O]^{3+}$ with purple membrane (closed circles) and papain-treated (open circles) purple membrane.

peptide were again Ala-Ile-Phe, similar to those of the peptide of peak II and to those of the original peptide mixture. Because the carboxypeptidase cannot cleave off Pro, the Co should be attached to the peptide ^{228}Ala-Ile-Phe-Gly-Glu-Ala-Glu-Ala-Pro-Glu-Pro.

To confirm this assignment, two different experimental approaches were undertaken. In a first set of experiments a papain-treated purple membrane which lacked the C-terminal tail was modified with the reagent $[Co(NH_3)_5H_2O]^{3+}$. The absorbance decrease was slower than that observed with native purple membrane (Figure 9). After bleaching with hydroxylamine, this sample was cleaved with chymotrypsin and separated on a LH-60 column. The Co incorporation into C1 occurred at a ratio of 1:0.17, half the amount of that obtained with native bR.

In a second experiment CobR was irradiated and then submitted to proteolysis by papain. The amino acid analysis of the corresponding, also cobalt-containing CNBr-6 fragment was only compatible with a cleavage at ^{235}Ala. Summarizing these results, the Co must be liganded to the peptide ^{228}Ala-Ile-Phe-Gly-Glu-Ala-Glu-Ala.

Modification of the Apomembrane. The experiments described above have shown that irradiation of CobR leads to an increased incorporation of the tightly bound cobalt label. This enhancement can be due either to an activation of the protein site during the photocycle or to the photochemistry of the cobalt complex, which has an absorption maximum at 540 nm. To discriminate between these possibilities, the apomembrane was deionized by dialysis against a cation-exchange resin in distilled water and subsequently treated with a 10-fold excess of $[Co(NH_3)_5H_2O]^{3+}$. The further reaction was carried out at 40 °C in the dark or under illumination. The Co content of the corresponding C1 fragments was 0.18 and 0.17 Co/C1, respectively. These data suggest that the photochemical reaction of the Co reagent does not enhance its reaction with the protein. After further fragmentation cobalt was again found in CNBr-6 and CNBr-9. Apparently, in bacterioopsin and bacteriorhodopsin the same sites of the protein are accessible to cations like $[Co(NH_3)_5H_2O]^{3+}$.

Discussion

The modification of carboxyl groups in proteins by cations and the subsequent identification of the labeled amino acids impose some difficulties regarding the choice of the label. Inorganic cations that are strongly bound to purple membrane in general do not form bonds that are stable under the conditions of the fragmentation of the protein, the isolation of the resulting peptides, and/or the sequence analysis of the peptides. Yet, a few compounds are applicable to modification of proteins [e.g., Grimes et al. (1974)] and were already successfully introduced as an α-carboxyl protecting group in peptide synthesis (Isied et al., 1982). In the latter case the cobalt complex $[Co(NH_3)_5H_2O]^{3+}$ was used as cationic reagent in which the water can be replaced by a carboxyl group. The resulting carboxyl complex is kinetically stable so that it can withstand the harsh conditions of the standard Merrifield synthesis (Barany & Merrifield, 1980). The steps comprise on the one hand a base treatment for neutralization and on the other hand an acid treatment to remove the amino protecting group. Therefore, this compound seemed to be suitable for the modification of carboxyl groups in bacteriorhodopsin because the Co–protein complex should be stable under the conditions of the protein fragmentation.

$[Co(NH_3)_5H_2O]^{3+}$ was found to convert the deionized blue membrane into the purple form, completely regenerating the photocycle properties. The subsequent modification by light reduced the photocycle turnover and the proton-pump efficiency. From these observations it can be concluded that the Co complex is a functional analogue to divalent ions such as Mg, Ca, or Mn.

The cobalt label was only found in two peptides located on the cytoplasmic surface of the purple membrane. The sequences of these peptides were ^{101}Val-Asp-Ala-Asp-Gln... and the tryptic peptide ^{228}Ala-Ile-Phe-Gly-Glu-Ala-Glu-Ala. Both fragments contain the carboxyl groups of Asp or Glu as potential ligands for Co. The exact position cannot be given because the label is already lost during the first steps of the sequence analysis. However, it is strongly suggested that the sequences Asp-Ala-Asp and Glu-Ala-Glu, which contain the only carboxyl side chain functions of the modified peptides, are involved in Co binding.

With respect to the topology of bR two possibilities should be considered. The two modified peptides might be part of two different binding sites. On the other hand the C-terminal peptide and the loop peptide might contribute to the same binding site. In the latter case the peptides must be spatially able to form this site. Structural models (Heyn et al., 1988) do not contradict this assumption. Especially, recent data from a molecular dynamic calculation (Jänig and Edholm, private communication) seem to support this notion. A schematic presentation of the results is shown in Figure 10 in which the circumference of bR and the position of retinal and the indicated amino acids are taken from the molecular dynamic calculation, which were kindly provided by F. Jänig and O.

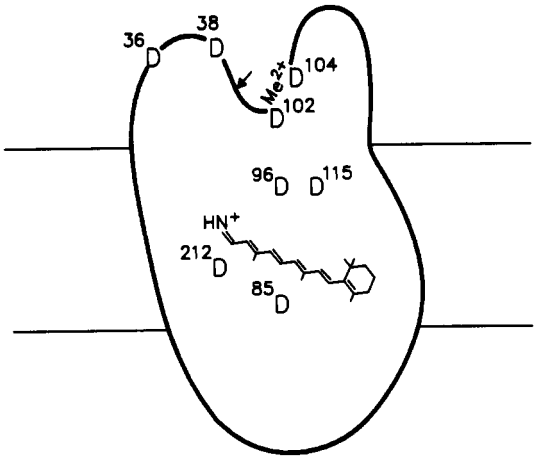

FIGURE 10: Two-dimensional scheme of bR with the relative positions of retinal and four internal and four external Asp from the loop between helices A and B and between helices C and D, respectively. The data were provided by F. Jänig and O. Edholm prior to publication. The amino acids are abbreviated with the one-letter code.

Edholm prior to publication. The C-terminal sequence was not included in this calculation. However, it would enter the bulk phase at the position of ^{227}Arg (marked by the arrow in Figure 10), which is quite close to the two aspartic acids (^{102}D and ^{104}D in Figure 10) of the loop between helices C and D.

This model is also supported by recent solid-state NMR experiments on [4-^{13}C]Asp-labeled bR. It could be shown that the cleavage of the C-terminus by papain transfers surface carboxyl groups of Asp residues from a salt-like environment to surroundings where they can form more hydrogen bonds (Engelhard et al., 1989). These data clearly favor only one binding site with at least four ligands probably constituted of the two Glu from the C-terminal peptide and the two Asp from the loop peptide. In this model the C-terminal tail folds over the membrane, thereby enveloping the cation and protecting it from the bulk phase.

Co(III) forms hexacoordinated octahedral complexes. With this stereochemistry Co(III) might be able to replace the divalent cations Mg and Ca in their binding sites, which were found as constituents of the purple membrane (Chang et al., 1985). A search for sequence homology in other proteins revealed at least two Ca binding proteins in which the sequence Asp-Ala-Asp was found. In thermolysin these amino acids are part of the Ca binding site (Holmes & Matthews, 1982). The second example, the Ca binding site in calmodulin, was already recently described by Helgerson and Dratz (1988). These data led the authors to propose a cation binding site in bR which also includes the sequence Asp-Ala-Asp. Apparently, the relatively large trivalent Co complex can occupy the same binding niche as the divalent cations. This might indicate either that the ligands are rather flexible in accommodating a large variety of cations or that the structural requirements of the site are minimal.

The question whether these modified peptides belong to the cation binding sites which were proposed by several authors (Zubov et al., 1986; Dunach et al., 1986, 1987; Ariki et al., 1987; Corcoran et al., 1987; Cladera et al., 1988) cannot yet be answered. However, the properties of the cation binding site described here might be similar to those of the hydrophobic site proposed by Corcoran et al. (1987).

The presence or absence of cations at specific sites certainly does not regulate the color of the chromophore (Szundi & Stoeckenius, 1987; Szundi & Stoeckenius, 1988). As Szundi and Stoeckenius (1988) pointed out, these findings do not contradict the existence of binding sites which might have functional properties. The results provided by the experiments presented above suggest that at least one specific protein binding site exists. The observation of the light-triggered Co incorporation is suggestive of a functional relevance of this site.

The modification of the two peptides influences not only the chromophore–protein interaction but also the photocycle kinetics, which become much slower (Table I). The peptide derived from CNBr-9 is a member of the connecting loop between helices C and D. Helix C comprises two Asp (^{85}Asp and ^{96}Asp) which were found to participate in the proton transfer (Mogi et al., 1988; Engelhard et al., 1985; Gerwert et al., private communication). It is noteworthy that Fe(III) [in a concentration range of 3–5 Fe(III)/bR] regenerates the purple color and also slows down the photocycle by 1 order of magnitude (Engelhard et al., unpublished results). Mössbauer experiments of these samples revealed through spin–spin coupling of the iron nuclei that the iron atoms are clustered in close spatial contact (Engelhard and Parak, unpublished results). Assuming that the trivalent iron binds to the same amino acid residues as $[Co(NH_3)_5H_2O]^{3+}$, a positively charged cluster might alter also the internal charge distribution and/or internal steric relations. ^{96}Asp might well be the target and mediator in these interactions.

In the dark, less binding of Co to the protein occurred. However, under illumination with wavelengths greater than 500 nm the optical density at 570 nm decreased (Figure 2), indicating a reaction of the reagent with the protein and thereby disturbing the protein–chromophore interaction. As already pointed out, two reasons might be responsible for the facilitation of the reaction by light. Since the Co reagent also has an absorption maximum above 500 nm, a photoproduct might be the reactive species (Balzani et al., 1967). However, this interpretation seems to be unlikely because $[Co(NH_3)_5H_2O]^{3+}$ is incorporated into the apomembrane in the dark as well as in the light with similar yields. The following second explanation is more plausible. Under constant illumination of bR a steady state is reached with ^{412}M as the major intermediate. One of these intermediates might be the species reactive toward the Co reagent. From a chemical point of view the reacting carboxyl group should be deprotonated, which is actually the case since it is a counterion to the cation. An explanation of the increase in reactivity in the light might be that this particular group comes into a structurally or stereochemically favorable position for the reaction. Structural changes were observed in the M-state by X-ray absorption spectroscopy (Engelhard et al., 1987). Irrespectively of the exact mechanism, apparently, this part of the membrane surface alters its structure during the transport of the proton and seems to be involved either functionally or spatially in the capture of the proton from the cytoplasma.

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Registry No. $[Co(NH_3)_5H_2O]^{3+}$, 14403-82-8; Co, 7440-48-4; H^+, 12408-02-5.

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