Topographic Studies of Spin-labeled Bacteriorhodopsin

EVIDENCE FOR BURIED CARBOXYL RESIDUES AND IMMOBILIZATION OF THE COOH-TERMINAL TAIL*

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Chemical modification and electron spin resonance techniques were used to study the topography of carboxyl residues in purple membranes. The results showed that buried carboxyl groups are located in hydrophobic protein domains at least 16 Å from the membrane surface and that the carboxyl-terminal tail is partially immobilized.

Carboxyl groups on bacteriorhodopsin in purple membranes were covalently spin-labeled with 4-amino-2,6,6-tetramethylpiperidine-N-oxyl using N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline as a highly specific coupling agent. Spin-labeled bacteriorhodopsin preparations containing an average of 2.1 ± 0.5 spins/molecule retained photocycling and proton-pumping functions. Accessibility to the paramagnetic broadening agents, Fe(CN)$_6^{3-}$ and Ni$^{2+}$, revealed a highly mobile surface group quenched at low concentrations of these agents, and a buried, immobilized group whose ESR signal remained at high quencher concentration. Treatment with denaturing agents greatly increased the mobility and quenching of these buried residues. A series of stearic acid spin labels bound to purple membranes was used to define the depth of paramagnetic interactions. Fe(CN)$_6^{3-}$ interactions were limited to surfaces whereas Ni$^{2+}$ and Cu$^{2+}$ effects extended into hydrophobic domains. A double modification procedure, which first blocked surface groups, selectively spin-labeled only buried carboxyl group(s) having a strongly immobilized signal. ESR analysis of the isolated carboxyl-terminal tail after trypsin treatment showed it had increased mobility, indicating that it is moderately immobilized in the native structure. These data provide evidence consistent with several models of bacteriorhodopsin tertiary structure which place carboxyls within hydrophobic domains of the protein.

The purple membrane found in Halobacterium halobium has been shown to contain only a single protein, bacteriorhodopsin, which functions as a light-driven proton pump (1). The static structure of the protein has been characterized by x-ray diffraction, electron diffraction, and circular dichroism techniques (2-4). It is thought to consist primarily of seven α-helical transmembrane rods and a "soluble tail" that comprises approximately the last 15-20 amino acid residues of the carboxyl terminus (3, 5). The primary sequence of bacteriorhodopsin has been determined and contains a large fraction of hydrophobic residues as expected for an integral membrane protein (6). Despite recent advances in resolution of electron diffraction techniques applied to purple membranes, the precise secondary and tertiary structure of bacteriorhodopsin remains unknown (7-9). Nevertheless, several detailed structural models have been postulated based on x-ray and electron diffraction data, proteolytic cleavage points, chemical modification data, and general thermodynamic considerations (8-11).

An additional method that would make it possible to evaluate some of these models of the arrangement of the bacteriorhodopsin polypeptide in the purple membrane is the chemical modification of selected functional groups by the introduction of spin label reporter groups (12). Although lack of site specificity can be an inherent limitation in functional group modification (13), useful structural information can still be obtained if the complex ESR spectrum can be deconvoluted by biophysical and biochemical methods (14). This approach allows the study of the local topography and mobility of different domains of the membrane protein in the vicinity of the covalently bound labels.

We have applied the spin label procedure to study the location of carboxyl residues in bacteriorhodopsin. Previous chemical modification studies have indicated carboxyl structural involvement in the photocycle (15, 16). Recently, Fourier transform infrared spectroscopy studies have suggested that buried carboxyl residues may be involved in the proton translocation mechanism (17, 18). In addition, the position of charged residues, including carboxyls, within hydrophobic membrane protein domains is a distinguishing feature in the selection of possible bacteriorhodopsin structural models (9). Thus, the localization of carboxyl residues is of interest from both a structural and functional viewpoint.

MATERIALS AND METHODS

Halobacterium halobium strain S-9 was grown in 15-liter batches and purple membranes were isolated and purified according to Ref. 10. Protein was determined by using an extinction coefficient of 63,000 M$^{-1}$ cm$^{-1}$ in light-adapted samples. Bacteriorhodopsin was denatured by addition of 8 M urea, 1% SDS, 10 mM HEPES, pH 7.0, and subsequently

1 The abbreviations and trivial names used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EEDQ, N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; Tempamine, 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl; TA-BR, tempamine spin-labeled bacteriorhodopsin; AES, 2-aminoethanesulfonic acid; SDS, sodium dodecyl sulfate; 5NS, 2-(3-carboxypropyl)-4-dimethyl-3-oxazolidinyl oxyl; 10NS, 2-(4-carboxyethyl)-2-oxyl-4,4-dimethyl-3-oxazolidinyl oxyl; 16NS, 2-(14-carboxyhexadecyl)-2-ethylo
boiled for 15 min. 16% polyacrylamide running gels with 0.1% SDS were prepared with a 5% acrylamide stacking gel according to Laemmli (20).

**Spin-labeling of Bacteriorhodopsin—**Bacteriorhodopsin was covalently spin-labeled by reacting Tempamine with protein carboxyl amino acid residues using EEDQ as the coupling agent (Fig. 1). EEDQ is a hydrophobic, highly specific reagent for the activation and subsequent modification of carboxyl residues (21) and its reaction with bacteriorhodopsin has been characterized (16). Purple membranes (2.5 mg/ml bacteriorhodopsin) suspended in 0.10 M MES, pH 6.0, were incubated with Tempamines for 10 min prior to addition of EEDQ. Tempamine (200 mM) was present in large excess relative to the concentrations of the carboxyl-activating reagent and carboxyl residues on the protein in order to drive the reaction to the amide structure. The molar ratios of the reagents during the 200 mM Tempamine + 10 mM EEDQ reaction were 115 Tempamine/5.8 EEDQ/1 protein carboxyl. EEDQ (in 100% methanol) was added to a rapidly vortexing sample and incubated for 24 h in a shaking water bath at 37°C. Appropriate controls were run with methanol alone. The reaction was terminated and samples washed repeatedly by dilution in ice-cold 0.10 M NaCl, 0.01 M HEPES, pH 7.0, and centrifugation at 100,000 x g for 30 min. The ESR signal of the supernatant at very high gain was used to monitor removal of unreacted Tempamine. In final washed samples, the unreacted Tempamine concentration did not exceed 2% of the protein-bound signal. Tempamine was obtained from Aldrich Chemical Co.; EEDQ was from Sigma Chemical Co.

A sequential double chemical modification of bacteriorhodopsin carboxyl groups was developed to separate the spin label only buried reagent. Tempamine was first modified by reaction of 250 mM AES and 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at pH 4.5 according to a previously described procedure (16). After extensive washing, the second spin label modification by 200 mM Tempamine and 10 mM EEDQ was carried out as described above.

**Stearic Acid Spin Labels—**The stearic acid spin labels 5NS, 10NS, 16NS were obtained from Syva, Palo Alto, CA (5NS and 16NS) and Molecular Probes, Junction City, OR (10NS). Purple membrane suspensions (10 mg/ml) in 0.10 M NaCl, 0.01 M HEPES, pH 7.0, and centrifugation at 100,000 x g for 30 min. The ESR signal of the supernatant at very high gain was used to monitor removal of unreacted Tempamine. In final washed samples, the unreacted Tempamine concentration did not exceed 2% of the protein-bound signal. Tempamine was obtained from Aldrich Chemical Co.; EEDQ was from Sigma Chemical Co.

**Correlation times, τs, in seconds, were calculated from ESR spectral data in certain cases using the equation defined in Ref. 22:**

\[
\tau_s = 6.5 \times 10^{-12} \Delta H_0 (h_0/h_1)^{-1} - 1
\]

where \(\Delta H_0\) is the width of the midline in gauss and \(h_0\) and \(h_1\) are the heights of the mid- and high-field lines, respectively. This equation is valid in the fast tumbling range (20 ± 2°C).

ESR—ESR spectra were recorded on a Varian E-109E spectrometer interfaced to a PDP-11/34 computer. Samples of TA-BR were routinely placed in 50-μl precalibrated capillaries with an inside diameter of 0.9 mm. ESR spectra were recorded at a frequency of 9.14 GHz (X-band) and at a power setting of 10 milliwatts. A modulation amplitude of 1.25 G was used for 2.1 spin-labeled bacteriorhodopsin and 1.6 G for the spin-labeled stearic acids. The value of the gain is indicated in each figure. The spin content of labeled bacteriorhodopsin was calculated from the second integral of the first derivative of the 15.5 mol/mol of bacteriorhodopsin. Spectra were recorded at ambient room temperature (20 ± 2°C).

Results

**Stoichiometry of the Spin-labeling Reaction—**The amount of spin label covalently coupled to bacteriorhodopsin was found to depend on the concentration of the carboxyl-activating agent, EEDQ, present during the reaction. The concentration dependence of EEDQ on the spin-labeling reaction was studied using a large excess of Tempamine nucleophile (200 mM). EEDQ concentrations ≤1.0 mM produced insignificant "bleeding", while higher concentrations (10–30 mM) yielded progressively increased levels of nucleophile coupling (1.9–4.25 TA/bacteriorhodopsin). Only a few of the 19 carboxyl residues found in bacteriorhodopsin were modified by this procedure. ESR spectra clearly revealed an increase in the immobilized spin content of samples that was correlated with a distance, d, was obtained by measurement of a model and the membrane-perpendicular projection of that segment of the chain was calculated as \(d \cos \gamma\). For example, the distance between the 10 and 16 carbons (\(d = 7.5\) Å) was used with \(\gamma = 45^\circ\), obtained from \(S_{\text{sw}'}\) for 16NS to calculate a perpendicular projection of 3.3 Å for that segment. Since the angle \(\gamma\) at the 16 carbon is greater than that for the 10 to 16 carbons, this estimate of the projection is a lower limit.

**Trypsin Treatment—**Trypsin treatment of spin-labeled bacteriorhodopsin was carried out essentially according to Gerber et al. (5). Spin-labeled bacteriorhodopsin (10 mg/ml) was treated with a 1:100 weight ratio of trypsin/bacteriorhodopsin in 80 mM NaCl, 10 mM CaCl2, 40 mM Tris, pH 7.0 at 37°C, and samples were periodically withdrawn. In the case of continuous ESR kinetic experiments, samples were incubated in the EPR cavity at 37°C using the Varian E4540 variable temperature controller. The trypsin used in experiments had been recrystallized twice, dialyzed, and lyophilized (Sigma Chemical Co.).

**Reconstitution of Bacteriorhodopsin into liposomes—**Bacteriorhodopsin liposomes were prepared using partially purified asolectin through a modification of the sonication procedure of Racker (25). Phospholipids (40 mg) were dried under a stream of nitrogen in the dark. To this dried preparation, 1.0 ml of 150 mM KCl, 10 mM phosphate buffer, pH 7.0, was added and this suspension was sonicated in a bath-type sonicator for 10 min. Immediately after sonication, 2 mg of spin-labeled bacteriorhodopsin were added to the phospholipid suspension, and the mixture was sonicated for an additional 10 min under nitrogen.

**Light-induced Proton Pumping and Volume Assays—**The internal volume of the vesicles was calculated on the basis of the unquenched internal signal from 1 mM of the spin label Tempone in the presence of 60 mM NaFe(CN)6 as described in Ref. 26. The activity of the reconstituted TA-BR liposomes was tested by measuring pH gradients established by light-induced proton pumping in terms of spin-labeled amine uptake (26). ESR spectra of TA-BR liposomes in the presence of 0.5 mM N,N'-dimethyl-Tempamine and 57 mM Na3Fe(CN)6 show the internal unquenched N,N'-dimethyl-Tempamine signal as clearly resolved narrow lines superimposed on a broad spectrum consisting of ferricyanide-quenched spins in bulk water and the spin signal of the labeled protein. The magnitude of the background did not interfere with measurement of changes in height of the A1 resonance line which were used to calculate the transmembrane pH gradient.

**Results**

**Fig. 1. Summary of spin-labeling reaction for carboxyl residues of bacteriorhodopsin.**

![Diagram of spin-labeling reaction](http://www.jbc.org/)

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**4,4-dimethyl-3-oxazolidinylxoyl; MES, 4-morpholinethanesulfonic acid; Tempone, 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl.**
with higher stoichiometries of spin-labeling (data not shown). Higher stoichiometries of labeling resulted in a broadening of the central line width ($\Delta H_0$), growth of the low field shoulder of the $h_+\拿来$ peak, and the appearance of two new extrema with a maximum splitting of 68.4 G characteristic of a strongly immobilized signal. In order to quantify spin content, computer double integration of the first derivative ESR spectrum was carried out for both "native" and SDS-urea-heat-denatured samples to preclude underestimation due to spin-spin interaction. Although the ESR spectra of denatured bacteriorhodopsin showed much greater mobility than the native sample, no significant differences in spin content for the 10 mM EEDQ sample were observed.

Higher stoichiometries of labeling also resulted in bleaching of the 570 nm retinal-protein chromophore. The 4.25 TA-BR sample was completely bleached, exhibiting a 370 nm spectral peak characteristic of free retinal. To avoid changes in protein structure that might accompany extensive modification, and to work with spin-labeled bacteriorhodopsin that retained its functional properties, all further work was carried out with 200 nM TA + 10 mM EEDQ-modified bacteriorhodopsin which contained an average of 2.1 spins/bacteriorhodopsin molecule (2.1 TA-BR).

Properties of 2.1 TA-BR—Spin-labeled 2.1 TA-BR was found to have slightly altered visible spectral characteristics. A shift in the $\alpha$-band 570 nm absorbance peak to 555 nm occurred as well as a reduction in the molar extinction coefficient, from 63,000 to 43,100 M$^{-1}$ cm$^{-1}$. Accompanying this change in the $\alpha$-band was a general increase in absorbance in the $\beta$-band between 300 and 500 nm. The modified membranes appeared slightly redder than control purple membranes. Despite these differences, many other spectral responses and functions were retained. TA-BR exhibited light-dark adaptation. Control purple membranes kept in the dark showed an absorption maximum at 558 nm and illumination caused a 10 nm red shift in this absorption maximum and a 14% increase in absorbance. The TA-BR dark-adapted protein had an absorption maximum at 548 nm which shifted to 555 nm upon illumination with an absorbance increase of 4%. Acid titration resulted in a red-shifted chromophore in TA-BR as in the case of control bacteriorhodopsin.

The TA-BR sample retained photocycling activity and exhibited slightly slower kinetics for decay of the $M_{415}$ nm intermediate. Chemical bleaching of TA-BR by hydroxylamine and light produced a retinaloxime species absorbing at 365 nm, as in the case of control bacteriorhodopsin, indicating no modification of retinal had occurred. The bleached membrane was reconstituted by addition of all-trans-retinal and yielded a 555 nm retinal-protein chromophore. These characteristics suggested that the structure and function of bacteriorhodopsin were not substantially affected by the 2.1 Tempamine spin label chemical modification.

Effects of Denaturing Agents—To demonstrate the sensitivity of TA-BR to structural changes, we tested the effects of denaturation on the ESR spectra. Previous studies with other spin-labeled proteins have demonstrated a transition from a moderately immobilized to a sharp, highly mobile ESR spectrum that correlated with loss of $\alpha$-helical structure measured by circular dichroism (27). The latter technique also demonstrated a large loss in $\alpha$-helical structure when bacteriorhodopsin was solubilized in SDS in the absence of urea or heating (28).

In the absence of any chemical denaturing agents, heating resulted in loss of the 555 nm chromophore, and a significant change in mobility of protein-bound spin labels. The heat-denatured sample showed an increase in all resonance line heights and a narrowing of the central line width, but the shoulder of the $h_+$ peak remained (Fig. 2A). Thus, the overall ESR spectrum of the spin labels showed more mobility than the control, but a large immobilized component remained. The change in mobility of the protein-bound spin labels as a function of SDS-urea concentration is shown in Fig. 2B. As the concentration of SDS-urea was increased, there were substantial increases in the resonance line heights, a decrease in $\Delta H_0$, and loss of the $h_+$ shoulder. The $h_+$ line height increased to a maximum of 175% of the control value when the concentration reached 4 M urea and 0.5% SDS. The ESR spectrum of TA-BR at high concentrations of SDS-urea appeared as a homogeneous population of spins possessing high mobility ($\tau_c = 7.61 \times 10^{-10}$ s), indicative of the release of previously immobilized labels.

Accessibility of Spin-labeled Bacteriorhodopsin to Paramagnetic Broadening Agents—As seen in Fig. 3, the control (0 mM Fe(CN)$_6^{3-}$) spectrum appears to be composed of at least two components of different mobility as evidenced by the broad low field shoulder adjoining the sharp $h_+$ peak. In order to distinguish spectral components located at the protein surface from other labeled sites, the spin-exchange broadening between protein-bound spin labels and paramagnetic ions in solution (presumed to require direct contact between colliding paramagnetic species (29)) was used to deconvolute the complex ESR spectrum. Sodium ferricyanide (Na$_3$Fe(CN)$_6$) and nickel chloride (NiCl$_2$) were employed as paramagnetic broadening agents since they possess similar relaxation rate constants for interactions with a nitroxide radical in water but differ in charge characteristics (14). These compounds are extremely water soluble and chemically inert with respect to functional groups of bacteriorhodopsin, and do not contribute an ESR signal of their own.

Titration of spin-labeled native bacteriorhodopsin with Na$_3$Fe(CN)$_6$ resulted in the progressive broadening of the spin signal until only a highly immobilized signal remained at high (200–273 mM) Fe(CN)$_6^{3-}$ concentrations (Fig. 3). Low concentrations of Fe(CN)$_6^{3-}$ selectively quenched the more mobile components of the spectrum, while the immobilized components were not affected. ESR spectra showed a significant loss of the $h_+$ peak that occurred in the 0–200 mM Fe(CN)$_6^{3-}$ concentration range while the underlying $h_+$ shoulder was not affected. In addition, computer subtraction of Fe(CN)$_6^{3-}$-quenched spectra from control TA-BR spectra revealed difference spectra demonstrating high mobility (e.g. bottom of...
Fig. 3. Paramagnetic broadening of 2.1 TA-BR by increasing concentrations of Fe(CN)$_{6}^{3-}$. Difference spectrum at bottom obtained by computer subtraction of 200 mM Fe(CN)$_{6}^{3-}$ from 0 mM spectrum. 2.1 TA-BR (10 mg/ml) was suspended in 150 mM KCl, 10 mM phosphate, pH 7.0. All ESR spectra recorded at 5 x 10$^3$ gain.

The presence of a large immobilized ESR signal that remained at high Fe(CN)$_{6}^{3-}$ concentrations suggested the existence of buried spin label residues in the native membrane protein structure. Denaturation is expected to open the bacteriorhodopsin structure and increase the accessibility of Fe(CN)$_{6}^{3-}$ to previously buried protein domains. The ESR spectra in Fig. 4 show that Fe(CN)$_{6}^{3-}$ was substantially more effective in quenching the SDS-urea-treated signal at equivalent concentrations of probe. At 20 mM Fe(CN)$_{6}^{3-}$, the decrease in the $h_{\perp}$ line height for the SDS-urea-treated sample was 61% compared to 25% for the native sample. At 200 mM Fe(CN)$_{6}^{3-}$, the SDS-urea TA-BR signal was completely broadened while a strongly immobilized spectrum remained in the native TA-BR sample.

Paramagnetic broadening by Ni$^{2+}$ was also found to remove the mobile components of the complex TA-BR spectrum. Fig. 4 also shows that a large immobilized signal remained at high concentrations of Ni$^{2+}$ which appeared more strongly immobilized than the corresponding Fe(CN)$_{6}^{3-}$-broadened spectrum. This may be due to the fact that Ni$^{2+}$ showed a much greater line-broadening efficacy than Fe(CN)$_{6}^{3-}$ at equivalent concentrations. Substantial quenching of native TA-BR occurred at 20 mM Ni$^{2+}$ while effects of 20 mM Fe(CN)$_{6}^{3-}$ were minimal. Moreover, 40 mM Ni$^{2+}$ and 245 mM Fe(CN)$_{6}^{3-}$ produced nearly identical line shapes with slightly different intensities.

The substantial differences in the concentration dependence between positively charged Ni$^{2+}$ and negatively charged Fe(CN)$_{6}^{3-}$ suggested the important influence of the negatively charged surface potential of purple membrane sheets. To corroborate this interpretation, the effect of ionic strength on quenching was investigated by increasing the concentration of NaCl while keeping Fe(CN)$_{6}^{3-}$ constant at 40 mM. Increased concentrations of NaCl resulted in a decreased $h_{\perp}$ intensity (>50% at 2.0 M), increased $\Delta H_0$ (100%), and saturation of the changes at high NaCl concentration. The 2.0 M NaCl spectrum in the presence of 40 mM Fe(CN)$_{6}^{3-}$ appeared similar to 120-160 mM Fe(CN)$_{6}^{3-}$ in the absence of salt.

The effects of Fe(CN)$_{6}^{3-}$, Ni$^{2+}$, and Cu$^{2+}$ on the spectra of 5-, 10-, and 16-doxylstearic acid spin labels were compared. As seen in Fig. 5, the ESR spectra of 5NS bound to purple membranes exhibited a strongly immobilized spectrum plus a small contribution from an aqueous mobile component. The 5NS signal was found to have a maximum hyperfine splitting ($2A_0$) of 63.1 G, as previously described by Chignell and Chignell (31). The apparent order parameter, $S_{app}$, was calculated to be 0.91 and the half-amplitude of motion, $\gamma$, was 21°. The nitroxide moiety was calculated to be 5.9 Å from the membrane surface. The amplitude and shape of the spectra...
were unaffected by the presence of 10–100 mM Fe(CN)$^\text{3-}$ except for the loss of the small, aqueous mobile component. However, Ni$^{2+}$ decreased the amplitude of the 5NS spectra without altering the line shape (data not shown). Only 51% of the central line ($h_0$) height remained in the presence of 20–200 mM Ni$^{2+}$. The 10NS label was also found to have a large hyperfine splitting ($2A_1 = 61.0$ G) and its spectrum closely resembled that of the 5NS label (data not shown). Calculations gave $S^{\text{max}} = 0.84$, $\gamma = 31^\circ$, and a distance of the nitroxide moiety of 11.3 Å from the surface.

In contrast to the strongly immobilized 5NS and 10NS spectra, the 16NS spectrum is only moderately immobilized (2 $A_1 = 56.8$ G, $S^{\text{max}} = 0.59$, $\gamma = 45^\circ$). This orientation gives a calculated distance of 16.6 Å for the 16NS nitroxide from the surface. The presence of a larger aqueous mobile signal superimposed on the membrane signal is due to increased partitioning of 16NS into the aqueous phase. As in the case of 5NS, high concentrations of Fe(CN)$_3^-$ had no effect on the 16NS ESR spectra other than to broaden the aqueous mobile component. In the presence of 100 mM Ni$^{2+}$, the amplitude of the 16NS $h_0$ line was essentially unchanged (92% of the control).

The paramagnetic broadening of 5NS, 10NS, and 16NS by Cu$^{2+}$ exhibited even greater differences when compared to the effects of Fe(CN)$_3^-$ (Fig. 5). The 5NS signal was almost completely broadened by 10 mM Cu$^{2+}$, with only 16% of the $h_0$ line height remaining. No additional broadening was observed at higher Cu$^{2+}$ concentrations (<200 mM). The more deeply buried 10NS and 16NS nitroxides were also broadened, but to a lesser extent. At 10 mM Cu$^{2+}$, 55% of the 10NS $h_0$ line height remained and 62% remained for 16NS. Stearic acid spin label spectra that were reduced in amplitude retained the same line shape as the control sample and did not show an increase in the central line width ($\Delta h_0$). At low concentrations of Cu$^{2+}$, the low field Cu$^{2+}$ ESR signal did not significantly contribute to the ESR signal in the nitroxide region. A small sloping baseline was observed in the presence of 100–200 mM Cu$^{2+}$, but did not interfere with quantitation of nitroxide ESR spectral parameters.

To determine if divalent cations without paramagnetic broadening properties affected the mobility of the 5NS and 16NS labels, ESR spectra were recorded in the presence of equivalent concentrations of Ca$^{2+}$. For both the 5NS and 16NS stearic acid spin labels, Ca$^{2+}$ had no effect on spin mobility, but caused a small increase in the partitioning of the label into the membrane. At 10 mM Ca$^{2+}$, the extent of this change was so small that no increase in the immobilized $h_0$ line height was measurable.

These results indicate that Fe(CN)$_3^-$ only broadens spin labels by collisional interactions and that it does not penetrate the purple membrane to the depth of the 5NS nitroxide. In contrast, the paramagnetic broadening effects of Ni$^{2+}$ and Cu$^{2+}$ extend considerably beyond the membrane surface.

**Selective Labeling of Buried Carboxyls by Prior Blocking of Surface Groups**—As a biochemical approach to further corroborate the results obtained with paramagnetic quenching agents, a sequential double modification procedure was developed to selectively prereact and thereby block surface carboxyl residues so that spin-labeling of only buried residues occurred. In order to block surface residues, the first carboxyl modification used a premanently charged non-spin label nucleophile, AES. After extensive washing, the second modification using EEDQ and Tempamine was carried out under conditions identical with the single step modification (200 mM TA + 10 mM EEDQ). The stoichiometry of the doubly modified samples prepared by this procedure contained an average of 0.4 ± 0.1 spin/bacteriorhodopsin molecule. ESR spectra of the AES/TA doubly modified BR showed only a strongly immobilized spin signal with a central line width of 7.6 G and a maximum hyperfine splitting of 68.7 G (Fig. 6). The ESR signal was not broadened by high concentrations of Fe(CN)$_3^-$.

Paramagnetic interactions of the AES/TA-BR spin labels with Cu$^{2+}$ were also examined in an attempt to estimate their depth from the membrane surface. The interaction of 10 mM Cu$^{2+}$ and AES/TA-BR resulted in only a moderate decrease in amplitude (75% of control remained) with no apparent broadening. Higher Cu$^{2+}$ concentrations caused no further decreases in the amplitude and line shape of the AES/TA spin label spectra.

**Trypsin Treatment**—To further resolve which surface groups had been labeled, 2.1 TA-BR was treated with trypsin. Trypsin treatment of bacteriorhodopsin has been shown to cleave at a single site and release the COOH-terminal tail from the membrane (5). The tail is composed of 20 predominantly polar amino acid residues and contains 5 carboxyl groups (5). Trypsin treatment of 2.1 TA-BR resulted in the progressive increase in the mobile components of the total spectrum. The kinetics of this change was monitored by either recording sequential ESR spectra or by following the increase in magnitude of $h_{11}$ (Fig. 7A). The 200% increase in line height of the $h_{11}$ resonance line was almost complete in 5 h. SDS-polyacrylamide gel electrophoresis showed only the loss of a $M_r = 1,500$ fragment from the $M_r = 26,000$ spin-labeled protein and yielded a single new band of the expected molecular weight. The trypsin-treated TA-BR membranes were centrifuged (100,000 x g, 30 min) to separate the membrane protein from the soluble peptides. As seen in Fig. 7B, ESR spectra of the clear supernatant fraction showed only a highly mobile spin population which was calculated to have a $\tau_m = 1.65 \times 10^{-10}$ s. The ESR signal of this fraction was completely quenched by a low concentration of Fe(CN)$_3^-$ (20 mM). The trypsin-treated TA-BR membrane pellet was washed and...
resuspended at the same concentration as before trypsin treatment. ESR spectra showed the loss of most of the more mobile components compared to the untreated TA-BR spectrum. However, a significant mobile feature remained after trypsin treatment. A large immobilized signal remained in the presence of high concentrations of Fe(CN)$_6^{3-}$.

Proton-pumping Activity of Spin-labeled BR Studied in Reconstituted Liposomes—To obtain information on the sidedness of the surface-labeled sites, 2.1 TA-BR was incorporated into liposomes by a procedure shown to produce unilamellar liposomes. It had been previously shown through proteolytic digestion experiments that essentially all (95%) bacteriorhodopsin incorporated into unilamellar liposomes is oriented with the COOH-terminal tail (cytoplasmic face) facing outward (33). Such “inside-out” orientation results in light-induced proton pumping to the liposome interior and internal acidification. The incorporation and functional integrity of TA-BR liposomes were verified by measuring internal lipid volume and light-dependent proton pumping.

An ESR volume measurement with Tempone gave a volume of 0.75 μg/ml lipid. Light-induced proton pumping, assayed by quantitating the distribution of N,N’-dimethyl-Tempamine, resulted in internal acidification and a pH gradient of 0.6 unit. Hence, the gross orientation and function of bacteriorhodopsin were preserved after spin-labeling. To permit comparison with digestion of TA-BR in purple membrane sheets, liposomes containing TA-BR were also trypsin treated. At room temperature, a substantial increase in the low field peak height occurred over a period of 8 h with kinetics similar to that obtained in TA-BR purple membrane sheets. An ESR spectrum of the supernatant after centrifugation showed the presence of highly mobile spins, as in the case of trypsin treatment of TA-BR purple membrane sheets.

Quenching of TA-BR in purple membrane sheets and in liposomes was compared under conditions of identical ionic strength and buffer composition. Incorporation of TA-BR into liposomes resulted in a small increase in the overall mobility of the ESR spectrum. Quenching in liposomes by Fe(CN)$_6^{3-}$ resulted in the loss of most of the mobile spectral components whereas all of the mobile components were quenched in the TA-BR purple membrane sheets. The sharp inflection in the $h_0$ line and small peak in the $h_z$ line that remained in the quenched liposome spectra were absent in the 200 mM Fe(CN)$_6^{3-}$ purple membrane sheet spectra (Fig. 8). The quenched components can be assigned to spins on the cytoplasmic purple membrane surface facing the liposome exterior. The mobile component that remains in the Fe(CN)$_6^{3-}$-quenched liposomes may be due to a small number of spins on the extracellular surface of purple membranes, although fractional misorientation cannot be excluded.

**FIG. 8.** ESR spectra of Fe(CN)$_6^{3-}$-quenched 2.1 TA-BR in purple membrane sheets and in reconstituted liposomes. Samples were suspended in 150 mM KCl, 10 mM PO$_4^-$, pH 7.0. Note the sharp inflections in the Fe(CN)$_6^{3-}$-quenched liposome spectra that are absent from the corresponding quenched purple membrane sheets.

**FIG. 6.** Interactions of paramagnetic broadening agents and AES/TA doubly modified bacteriorhodopsin. AES/TA-BR (8 mg/ml) was suspended in 0.1 M NaCl, 0.01 M HEPES, and Fe(CN)$_6^{3-}$ or Cu$^{2+}$ was added to give the indicated concentrations. A h spectra recorded at $2 \times 10^4$ gain. Notice that the Cu$^2+$ spectra retains the same line shape as the Fe(CN)$_6^{3-}$ sample and is only slightly decreased in intensity.

**FIG. 7.** Changes in ESR spectra due to cleavage of COOH-terminal tail by trypsin treatment. Trypsin treatment was in 40 mM Tris, pH 8.0, 80 mM NaCl, 10 mM CaCl$_2$ at a trypsin/TA-BR weight ratio of 1:100. A, kinetics followed by ESR recording of $h_1$ resonance line using 2.5× higher gain than in B at 37 °C. B, ESR spectra of TA-BR (5 mg/ml) before trypsin addition and after 4 h incubation at 37 °C. The soluble tail and trypsinnized membrane were separated by centrifugation, and ESR spectra of the supernatant and pellet (after resuspension) were recorded at identical gain at 37 °C.

**DISCUSSION**

A combination of analytical procedures for spin-labeled membrane proteins has been applied to definitively show that at least two classes of carboxyl groups exist in bacteriorhodopsin: a strongly immobilized, buried membrane group and a weakly immobilized, surface set that includes carboxyls on the COOH-terminal tail. There is considerable theoretical interest in whether or not carboxylic amino acids, which are normally charged in aqueous solution, can be accommodated within stable hydrophobic membrane domains (34). Although models of bacteriorhodopsin structure have suggested the presence of buried carboxyl residues, this study provides the first evidence for such residues deep within hydrophobic domains of the protein. By utilizing paramagnetic ion interactions with spin-labeled stearic acids to measure molecular...
distances, we show that one or more of these carboxyls is ≥16 Å away from the aqueous interface.

Interpretation of ESR Data for Localization of Carboxyl Residues—To define carboxyl group topography in the protein, two types of paramagnetic interactions between ions in the aqueous phase and covalently attached nitroxides on carboxyl groups were employed: the spin exchange interaction and the dipole interaction. Spin exchange arises when electrons of two paramagnetic molecules or ions share orbitals and requires that there be an appreciable overlap of their electronic wave functions. This requirement is generally considered to be equivalent to the occurrence of direct collisions between the paramagnetic species (29) and results in line broadening of affected nitroxides. The occurrence of such broadening implies direct contact of spin-labeled groups with the aqueous phase, and these groups are considered to be surface residues. On the other hand, dipole interactions occur over much larger distances and can be used, in principle, to determine the distance of buried spin labels from the aqueous phase. Some transition metals, such as Cu²⁺, Mn²⁺, and Gd³⁺, are capable of a large through-space dipolar interaction with spin labels (38). This interaction varies as the inverse of the sixth power of the distance and has been used to estimate the distance between two spins (37, 38). The theoretical treatment of this problem developed by Leigh (30) demonstrated that the ESR signal of an immobilized spin label covalently bound to a protein was not broadened, but only decreased in amplitude as a result of a dipolar interaction with a paramagnetic ion at a fixed distance. Hyde and Rao (39) extended this type of calculation in order to consider a situation in which a number of metal ions could interact with each free radical. A main conclusion of their analysis was that, even though there is a wide range of distances, observed magnetic interactions will be determined primarily by the distance of closest approach.

We tested paramagnetic ions employed in this study to determine if they could be employed as spin exchange or dipole agents in the purple membrane preparations. Although it is a fairly straightforward matter to estimate the relative magnitudes of exchange interactions and dipole interactions between paramagnetic ions and nitroxides in aqueous solution (30), these properties can be markedly altered as a function of chelation or binding of paramagnetic ions. Therefore, a series of spin-labeled stearic acids bound to these membranes was used as an empirical molecular ruler to assess the extent to which a given paramagnetic ion interacts with intramembrane nitroxides as a function of their distance from the membrane surface. By this criterion, Fe(CN)⁶⁻⁻ could be used to identify surface spin labels, since there were no paramagnetic interactions between Fe(CN)⁶⁻⁻ and spin-labeled stearic acids bound to purple membranes. Fe(CN)⁶⁻⁻ would not be expected to bind significantly to membranes because of charge repulsion. On the other hand, nickel and other divalent cations have been shown to bind to phospholipid vesicles (37). We found that Cu²⁺ is concentrated at or near the surface of purple membranes using the spin label assay of Wagner et al. (41) and also observed that Cu²⁺ and Ni²⁺ are effective dipole reagents as determined with aqueous glycerol solutions (40). These observations are consistent with the low effective concentration of Cu²⁺ and Ni²⁺ required for dipolar interactions with nitroxides within hydrophobic membrane domains.

Cu²⁺ was found to be a strong dipolar agent since its interactions with the spin-labeled stearic acids extended to the depth of the 16NS nitroxide. One characteristic of the stearic acid spin label spectra in the presence of the Cu²⁺ was a decreased amplitude without apparent line broadening. The decrease in amplitude followed the pattern, 5NS > 10NS > 16NS, which is correlated with the depth of the nitroxide moiety from the membrane surface. Ni²⁺ was found to be a weaker dipolar agent on the basis of its interaction with spin-labeled stearic acids. The observations with purple membranes resemble previous data on Ni²⁺ interactions with the 5NS and 16NS spin labels in sarcoplasmic reticulum vesicles (42).

The effects of paramagnetic broadening agents showed that two distinct protein domains were spin-labeled, and spectral characteristics demonstrated that surface groups were considerably more mobile than buried groups. Paramagnetic broadening of the 2.1 TA-BR spectra by Fe(CN)⁶⁻⁻ selectively broadened the more mobile spectral characteristics. Referring to the stearic acid model system, we conclude that these mobile groups reside at the surfaces of the purple membrane. The presence of the large immobilized component in 2.1 TA-BR (60% of the total spin) that remained in the presence of high Fe(CN)⁶⁻⁻ concentrations is strong evidence for the existence of mobile label residues in a protein domain, presumably hydrophobic, which is inaccessible to Fe(CN)⁶⁻⁻. Denaturation of TA-BR caused increased spin mobility and increased accessibility to charged quenching agents, consistent with the transfer of previously buried immobilized spin residues to the aqueous environment.

The conclusion that both surface and buried groups are labeled is supported by double modification data. Blocking of surface groups with the permanently charged nucleophile AES showed that only immobilized spectral features could be observed upon subsequently spin-labeling of the pretreated BR. Comparison of the central line height reduction due to Cu²⁺ interaction with the 5NS, 10NS, 16NS, and AES/TA-BR shows that 16NS and AES/TA-BR are affected similarly and, hence, that the spin-labeled carboxyl group is deeply buried within the protein. Thus, to a first approximation, we would estimate the minimum distance of the buried carboxyl group(s) from the membrane surface to be 16.6 Å.

There are several possible sources of error in estimating accurately the effective intramembrane position of the buried carboxyl group(s). The first is that the presence of the nitroxide moiety may induce a local disordering effect on the acyl chain (43). Hence, in the distance calculation for 16NS, the use of 21° for the acyl chain segment from the 1–5 carbons and of 31° for the segment from the 5–10 carbons probably overestimates the tilt of those segments of the chain. This implies that the distance calculated for the position of the 16NS nitroxide group relative to the membrane surface should be considered to be a minimum value. A second possible error source is uncertainty about the surface structure of bacteriorhodopsin since this will determine the closest approach of quenching agents to the spin label. However, recent information from Zaccai and Gilmore (44) about the hydration areas of purple membranes suggests that the protein surfaces are co-planar with the lipid head groups to within about 1.5 Å. A third potential source of error is preferential association of copper ions with lipids rather than bacteriorhodopsin. This possibility is deemed to be unimportant because the quenching behavior of the buried carboxyl group was essentially the same at concentrations of copper between 0 and 100 mM. A fourth potential source of error is possible heterogeneity in labeling, both with respect to the number of carboxyls modified and their distribution within a given protein molecule. Labeled sites at different distances from the membrane surfaces would be differentially broadened by Cu²⁺ interactions. Greater Cu²⁺-quenching of groups closer to the surface would
bias the calculated distance of the more deeply buried groups such that their actual distance would be further from the surface than that which we calculated assuming a homogeneous population. In view of these considerations, we suggest that the 16.6-Å estimate for the minimum depth of the labeled carboxyl group(s) is conservative and includes reasonable allowances for errors.

**Mobility of the Carboxyl-terminal Tail**—Conversion of a weakly immobilized spectral component to a free component as a result of trypsin treatment indicates that the COOH-terminal tail in the native BR structure is immobilized to some extent. This is supported by fluorescence depolarization data from a dansyl-labeled carboxyl on the tail (45). Since the carboxyl-terminal tail has not yet been resolved in electron density maps (46), the position it may occupy on the cytoplasmic face of the protein and the nature of its interaction with the membrane is open to speculation. The immobilization seen with spin-labeling may be due to ion-pairing between the carboxyls on the tail and some of the positively charged groups of the 5 lysines or 4 arginines thought to be present on the cytoplasmic surface. The structure and function of the COOH-terminal tail in bacteriorhodopsin remains unknown, although it may play a role in the proton-pumping mechanism. Decreased H"+ release stoichiometries in purple membrane sheets after proteolysis of the tail have been reported, although its absence has no effect on photocycling activity (47, 48).

**Identification of Buried Carboxyl Residues in Bacteriorhodopsin**—One of the more important implications of this study concerns the placement of buried charges in models of BR structure. To date, BR models place some aspartic and glutamic carboxyl residues within the hydrophobic membrane phase, although the majority of such residues have been placed at the cytoplasmic and extracellular surfaces (8-11). The requirement that buried charges form ion pairs has been used as an important criterion in selecting among possible models meeting other criteria (9), but this was not directly shown. The ESR data suggest locations for the buried spin residue(s) that are consistent with three recent models of bacteriorhodopsin structure, each of which postulates 5 or 6 buried carboxyl groups with only a few residues located 16 Å or more from the membrane surface. We have determined the depths of the residue positions in the models based on a membrane thickness of 45 Å (8). Agard and Stroud (8) place Glu-9, Asp-85, Asp-96, Asp-115, and Asp-221 below the membrane surfaces, but only Asp-85, Asp-115, and Asp-221 are buried deeper than about 16 Å, Engelman et al. (10) combined evidence from free energy calculations for the insertion of bacteriorhodopsin into a lipid bilayer and chemical modification studies to derive a model which places five carboxyls, Asp-85, Asp-96, Asp-115, Glu-204, and Asp-202, in the hydrophobic membrane domain. Of these, only Asp-212 and Asp-115 are clearly buried deeper than 16 Å, while Asp-85 lies on the borderline. Huang et al. (11) have arranged the polypeptide chain based on the cross-linking data of a photoaffinity derivative of retinal and a recent proposal of Steitz et al. (49). The revised model includes the 5 buried residues of Engelman et al. (10) plus one additional residue, Glu-9. The deepest buried residue in this model is Asp-212, quite Asp-85 and Asp-115 are located about 15 Å from the surface. All three models (8, 10, 11) assign residues Asp-85, Asp-115, and Asp-212 as the most deeply buried carboxyl residues (d ≥ 15 Å). We consider these three residues as the most likely sites of labeling. Thus, our data are consistent with current models of bacteriorhodopsin structure.

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