Locations of Arg-82, Asp-85, and Asp-96 in helix C of bacteriorhodopsin relative to the aqueous boundaries
(proton pump/site-specific mutagenesis/membrane protein/spin label/electron paramagnetic resonance)

DUNCAN A. GREENHALGH1, CHRISTIAN ALTENBACH†, WAYNE L. HUBBELL†, AND H. GOBIND KHORANA†

1Departments of Biology and Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139; and †Jules Stein Eye Institute and Department of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, CA 90024-7008

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ABSTRACT The amino acids Asp-96, Asp-85, and Arg-82, which are important for proton transport by bacteriorhodopsin, are located in helix C. Site-directed spin labeling has been used to map their positions relative to the aqueous boundaries of the membrane. Selected amino acids in helix C, in the B–C loop on the extracellular side, and in the C–D loop on the intracellular side of the membrane were replaced by cysteine residues and derivatized with a sulfhydryl-specific spin label. The topographical locations of the nitroxide groups were determined by electron paramagnetic resonance spectroscopy in terms of both motional restriction and collision frequencies with dissolved molecular oxygen and membrane-impermeable chromium oxalate. The results show that in dark-adapted bacteriorhodopsin, Tyr-79 is at the aqueous–protein interface on the extracellular side of helix C whereas Val-101 is close to the aqueous boundary on the intracellular side of the protein. Further, Asp-96 is estimated to be within 7 Å of the aqueous medium on the intracellular side of the membrane, whereas Arg-82 and Asp-85 are within 5 Å and 9 Å, respectively, of the aqueous boundary on the extracellular side of the membrane.

Bacteriorhodopsin (bR) is an integral membrane protein present in the purple membrane of Halobacterium halobium that functions as a light-driven proton pump (1, 2). Upon absorption of a photon, bR cycles through a series of photo-intermediates (3) that lead to the release of a proton into the extracellular medium and the uptake of a proton from the intracellular side (4–7). Site-directed mutagenesis studies of bR have identified several amino acid residues that are involved in proton transport (8–10). Asp-85, Asp-212, and Arg-82 mediate the release of a proton from the Schiff base to the extracellular side of the membrane whereas Asp-96 is involved in the reprotonation of the Schiff base (6, 11–14). The photocycle is completed by the reprotonation of Asp-96 from the cytoplasmic side of the membrane. The residues Arg-82, Asp-85, and Asp-96 are all located in helix C of bR (15), and information concerning their location relative to the aqueous boundaries should be useful in further understanding the mechanism of proton transport by bR. The purpose of the present work has been to determine the locations of the above residues in helix C relative to the aqueous boundaries.

Recently, the technique of site-directed spin-labeling was applied to a partial structural investigation of bR (16, 17). We have now used this method to investigate the boundaries of helix C and the locations of Arg-82, Asp-85, and Asp-96 in this helix (Fig. 1). Mutants of bR containing single cysteine substitutions were constructed at positions Gln-74, Asn-76, Tyr-79, Arg-82, Asp-85, Asp-96, Ala-98, Val-101, Ala-103, Gln-105, and Leu-109; and each one was derivatized with the sulfhydryl-specific methanethiosulfonate spin label (Compound I), shown below (18).

The nitrooxide lineshapes and collision frequencies of the spin labels with membrane-permeant molecular oxygen or the impermeant chromium oxalate (CROX) were used to deduce topographical locations of the labeled residues relative to the water–membrane boundaries. We find that in dark-adapted bR, Arg-82 and Asp-85 are about 5 Å and 9 Å, respectively, from the aqueous surface on the extracellular side of the membrane, whereas Asp-96 is within 7 Å of the aqueous boundary on the intracellular side of the membrane.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized by using an Applied Biosystems 380A DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis. Dimyristoylphosphatidylcholine (DMPC) and egg phosphatidylcholine (PC) were from Avanti Polar Lipids, all-trans-retinal was from Kodak, and octyl β-glucoside and 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) were from Boehringer Mannheim. The methanethiosulfonate spin label (Compound I) was from Reanal (Budapest, Hungary), and CROX was obtained from ICN.

Construction, Expression, and Purification of Mutant Opsins. The cysteine mutants were constructed by the replacement of appropriate restriction fragments in the synthetic gene with alternate duplexes containing the desired codon changes (19). The wild-type restriction fragments containing the changed codons (either TGT or TGC for the cysteine codon) were shown in Table 1. The DNA sequences of the mutant genes were confirmed by direct plasmid sequencing using Sequenase according to the manufacturer's instructions. The opsins were expressed in Escherichia coli (20) and purified to homogeneity by solvent extraction in the presence of 2-mercaptoethanol and by ion-exchange chromatography (21).

Derivatization, Regeneration, and Purification of Mutants. The mutant cysteine apoproteins were regenerated with all-trans-retinal in 30 mM phosphate buffer (pH 6) containing

Abbreviations: bR, bacteriorhodopsin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; CROX, chromium oxalate (potassium trioxalatocromate); DMPC, dimyristoyl-phosphatidylcholine; PC, phosphatidylcholine. Amino acid substitutions in bR mutants are designated by the wild-type amino acid residue (single-letter code) and its position number in the sequence followed by the substituting amino acid residue. Cysteine substitution mutants derivatized with the spin label are designated with an *.

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Fig. 1. Secondary structure of bR proposed by Henderson et al. (15). The boxed regions A–G represent the seven transmembrane α-helical segments. The region of the D–E loop is modified according to previous spin-labeling experiments (17). The residues that were changed to cysteine are circled, and the site of attachment of retinal, Lys-216, is boxed.

1% DMPC, 1% CHAPS, and 0.2% SDS and formed bR-like chromophores. The absorption maxima of the regenerated proteins were measured after overnight dark-adaptation. The kinetics of chromophore regeneration at 20°C were monitored by following the time-dependent absorption changes at the absorption maxima values for the particular mutant chromophores.

Derivatization of the mutant cysteine opsins with the spin-labeling reagent (Compound I) was performed on the unfolded protein as previously described (16). Following the removal of the denaturant, the chromophores were regenerated as described above. Regeneration was not quantitative, and folded proteins were separated from the unfolded counterparts by a two-step HPLC gel-filtration procedure as described (22). The derivatized bR mutants were reconstituted into asolectin vesicles for EPR spectroscopy (16).

Steady-State Proton Pumping Measurements. Mutant bRs were reconstituted into lipid vesicles as described (22, 23). Proton pumping activities were measured under saturating illumination (495 nm) from a 1000-W xenon/mercury arc lamp (Oriel, Stamford, CT).

**RESULTS**

Characterization of the Bacterioopsin Mutants Containing Single Cysteine Substitutions. All the cysteine mutants (Table 1) regenerated to give bR-like chromophores in DMPC/CHAPS/SDS micelles following the addition of all-trans-retinal. The only significant differences in the $\lambda_{max}$ values for the mutant chromophores relative to wild-type bR were in the mutants Y79C, R82C, and D85C (Table 2). The $\lambda_{max}$ of the mutant Y79C was blue-shifted to 524 nm, whereas the mutants D85C and R82C were red-shifted, with $\lambda_{max}$ values of 590 nm and 574 nm, respectively. The $\tau_{ij}$ values of chromophore formation for all the mutants were the same as for the wild-type bR except for D85C and R82C, which were 80-fold and 14-fold slower, respectively (Table 2). All of the cysteine mutants pumped protons at levels similar to that of the wild-type bR, except for the mutants D96C and D85C, which had 15% and 0% (±0.3%) of wild-type activity, respectively (Table 2).

Derivatization of the Cysteine Mutants with the SpinLabeling Reagent. After modification with the reagent (Compound I), all the opsins renatured and formed bR-like chromophores in DMPC/CHAPS/SDS micelles following the addition of retinal. The presence of the spin label did not slow down the rates of chromophore formation significantly except in the mutants D96C* and A98C* (an * indicates derivatization of the cysteine substitution mutant with the spin label), where the $\tau_{ij}$ values were slowed down 7- and 3-fold, respectively, relative to wild-type bR (Table 2). Although the $\tau_{ij}$ values for the chromophore formation in the mutants D85C* and R82C* were 8- and 4-fold slower, respectively, than wild-type bR, their rates were significantly faster when compared to their underivatized forms (Table 2). After HPLC purification, all the regenerated proteins, except for the mutant D96C*, had $\lambda_{max}$ values similar to the underivatized counterparts (Table 2). The mutant D96C* was unstable and bleached during purification, precluding a $\lambda_{max}$ determination or proton pumping measurement for this protein. The presence of the spin label had no effect on the proton pumping of the other mutants except for R82C*, which had 25% of wild-type activity (Table 2).

Effect of Lipid Composition on the Collision Frequencies Between Spin-Labeled bR and Paramagnetic Relaxers. The collision frequencies of the spin labels with membrane permeant O$_2$ or membrane-impermeant CROX are proportional to the quantity $\Delta P_{ij}$ (16). CROX is anionic and so its intrinsic collision frequency with the spin label, and hence $\Delta P_{ij}$, is expected to be influenced by the local electrostatic potential at the protein surface. To investigate this effect, the spin-labeled mutant A103C* was reconstituted into both negatively charged asolectin vesicles and neutral egg PC vesicles. The spectral lineshapes of the label at A103C* (shown for asolectin vesicles in Fig. 2) and their collision frequencies with O$_2$ (Table 2) were identical within experimental error in both systems, indicating that the local conformation of the protein was independent of the charge density of the lipid bilayer. However, the collision frequency of CROX with the nitroxide at A103C* in egg PC vesicles was 3-fold higher than for the same labeled protein in the asolectin vesicles, confirming that electrostatic interactions influence the collision frequencies of CROX at the membrane surface (Table 3).
Table 2. Properties of the cysteine mutants before (native) and after (modified) derivatization with the methanethiosulfonate spin label

<table>
<thead>
<tr>
<th>Sample</th>
<th>( t_{1/2} ) of chromophore formation</th>
<th>( \lambda_{max}, \text{nm} )</th>
<th>Rate of proton pumping</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Modified</td>
<td>Native</td>
</tr>
<tr>
<td>ebR</td>
<td>1.0</td>
<td>1.0*</td>
<td>551</td>
</tr>
<tr>
<td>E75C</td>
<td>1.8</td>
<td>0.9</td>
<td>551</td>
</tr>
<tr>
<td>N76C</td>
<td>1.0</td>
<td>0.9</td>
<td>552</td>
</tr>
<tr>
<td>Y79C</td>
<td>2.2</td>
<td>1.6</td>
<td>525</td>
</tr>
<tr>
<td>R82C</td>
<td>14.1</td>
<td>3.8</td>
<td>574</td>
</tr>
<tr>
<td>D85C</td>
<td>77.7</td>
<td>8.3</td>
<td>590</td>
</tr>
<tr>
<td>D96C</td>
<td>1.4</td>
<td>6.7</td>
<td>551</td>
</tr>
<tr>
<td>A98C</td>
<td>0.8</td>
<td>3.4</td>
<td>551</td>
</tr>
<tr>
<td>V101C</td>
<td>0.9</td>
<td>1.0</td>
<td>551</td>
</tr>
<tr>
<td>A103C</td>
<td>0.9</td>
<td>1.0</td>
<td>552</td>
</tr>
<tr>
<td>Q105C</td>
<td>1.0</td>
<td>1.0</td>
<td>552</td>
</tr>
<tr>
<td>L109C</td>
<td>0.9</td>
<td>0.8</td>
<td>552</td>
</tr>
</tbody>
</table>

bR-like chromophores were formed in DMPC/CHAPS/SDS micelles, and absorption spectra were recorded on the proteins in their dark-adapted states. To measure the kinetics of chromophore formation, excess all-trans-retinal was added to each mutant apoprotein, and the absorbance changes were measured at 550 nm (574 nm for R82C, 524 nm for Y79C, and 590 nm for D85C). The rates were normalized to wild-type values before and after derivatization (\( t_{1/2} \) values for wild-type ebR were 1.6 min and 3.3 min, respectively). Proteins were reconstituted into asolectin vesicles for proton pumping measurements, and the steady-state levels were normalized to wild-type activity. ND, Not detectable.

*As a control for the labeling specificity, the modification reaction was performed on bR prepared from the expression of a synthetic wild-type gene in E. coli (ebR), which contains no cysteine residues. No EPR signal could be detected in this protein following HPLC purification.

**Chromophore bleached during HPLC purification.

The proton pumping assays were performed on bR mutants reconstituted into asolectin vesicles since higher activities are obtained in these vesicles relative to bR reconstituted into PC vesicles (24). In order to measure the intrinsic collision frequencies between CROX and the protein-bound nitroxide groups in the asolectin vesicles, the electrostatic effects of the charged lipids were reduced by their dilution with the uncharged detergent octyl \( \beta \)-glucoside. It has been previously shown that solubilization in this detergent does not significantly alter the local conformation of bR (17). The collision frequencies of CROX with the label at A103C* were found to be similar in each lipid system following the addition of octyl \( \beta \)-glucoside (Table 3). The increase in exposure to CROX upon the addition of octyl \( \beta \)-glucoside is consistent with a reduction in electrostatic interaction.

EPR Spectroscopy of bR Mutants. On the extracellular side of helix C, the labels at R82C* and D85C* are immobile (Fig. 2), and the low collision frequencies with both oxygen and CROX indicate that these residues are buried in the protein interior (Fig. 3). The labels on Y79C*, N76C*, and E74C* show increasing relative collision frequencies with CROX, indicating that these nitroxides become sequentially more exposed to the aqueous environment (Fig. 3).

At the intracellular side of helix C, it was not possible to derive structural information from the nitroxide on the mutant D96C* because this mutant bleached during HPLC purification and the EPR spectra were probably derived from the mutant in an unfolded conformation. The nitroxide on A98C* has a very immobile lineshape (Fig. 2), and its low accessibilities to both \( O_2 \) and CROX demonstrate its buried location within the protein interior (Fig. 3). The nitroxides on V101C* and A103C* have intermediate mobilities (Fig. 2) and relatively high collision frequencies with water-soluble CROX, indicating that these residues are present in the aqueous environment (Fig. 3). Although \( O_2 \) has a preferential

Table 3. Effect of lipid composition on the \( \Delta P_{1/2} \) values for spin-labeled A103C* in the presence of oxygen and CROX

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oxygen</th>
<th>CROX</th>
<th>10% OG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asolectin vesicles</td>
<td>0.88</td>
<td>0.37</td>
<td>1.50</td>
</tr>
<tr>
<td>Egg PC vesicles</td>
<td>0.80</td>
<td>1.10</td>
<td>1.96</td>
</tr>
</tbody>
</table>

OG, octyl \( \beta \)-glucoside.
solubility in membranes, it is also present in the aqueous phase, allowing collisions to occur between water-exposed spin labels and oxygen, as in the case of A103C*.

The spin label on L109C* is mobile (Fig. 2), with high and low collision frequencies with O2 and CROX, respectively, and clearly faces the fluid interior of the bilayer (Fig. 3). The label at Q105C is also mobile (Fig. 2), indicating that it faces the lipids but has intermediate collision frequencies with both O2 and CROX (Fig. 3). Thus, Q105C* is close to the membrane–solution boundary on the intracellular side of helix D. The nitroxide on L109C* is more deeply buried in the membrane than Q105C* since the nitroxide experiences a higher collision frequency with O2 relative to the nitroxide at position 105.

**DISCUSSION**

A large number of polytopic integral membrane proteins have been discovered. These include the cytochrome oxidases, ATPases, transport proteins, ion channels, and many families of receptors. Although structural information on such membrane proteins is limited, notable exceptions include the photosynthetic reaction centers that have been crystallized from *Rhodopseudomonas viridis* (25, 26) and from *Rhodobacter sphaeroides* (27, 28). In addition, structural studies of bR have been facilitated since it naturally forms two-dimensional crystalline lattices in the purple membrane of *H. halobium* (29). A model for the structure of bR derived by electron cryomicroscopy has been recently proposed (15). However, structural information regarding the boundary of the membrane-embedded segments in integral membrane proteins and the segments looped out into the aqueous environments has been minimal. Site-directed spin labeling has been recently developed for structural studies on membrane proteins and has been successfully used in the topographical studies of bR, colicin E1, and melittin (16, 17, 30, 31). In the case of bR, topographical information was obtained for amino acid residues 125–142, and the results identified membrane-embedded α-helical domains as well as the membrane boundaries within this sequence (17).

The amino acids Asp-85, Arg-82, and Asp-96, all located in helix C of bR, have been shown to be important for the transport of protons by the protein (8, 10, 13). To more completely understand the mechanism of proton transport, it is desirable to know the locations of the above residues with respect to the aqueous boundaries. We therefore substituted a number of amino acids presumed to be near the ends of helix C, one at a time, with cysteine residues, and the latter were derivatized with a sulfhydryl-specific spin label (Compound I). All the mutants folded to give bR-like pigments (Table 2). Most of the mutants did not perturb either the structure or the function of the protein, even after derivatization with the spin label (Compound I). In particular, the derivatized cysteine mutants R82C*, D85C*, and D96C*, which are all buried in the protein interior, refolded to give chromophores in the DMPC/CHAPS/SDS micelles, suggesting that the presence of the spin label could be accommodated within the packing arrangement of the protein. The mutant D96C has a low proton pumping activity (15% of wild type) and is consistent with other neutral substitutions at this position where it has been shown that the decay of the M intermediate is slowed down by one order of magnitude, resulting in reduced proton pumping (6, 11, 14). After modification, D96C* was unstable to oxygenation/oxidation and could not be studied. However, it was observed that the mutant D85C was inactive in proton transport, consistent with previous studies that have shown that the loss of a proton-accepting moiety at this position completely inhibits proton pumping (8). The mutant R82C* had reduced proton pumping activity, suggesting some structural perturbation by the spin label. These effects were not surprising since these three residues are located in the putative proton pathway and play key roles in proton transport.

The results presented above show that in dark-adapted bR the nitroxide on Y79C* in helix C is located at the protein–aqueous interface on the cytoplasmic side of the membrane (Fig. 3). In the recent structural model (15), Tyr-79 is shielded from the phospholipids by the surrounding helices but is still accessible to water. This is consistent with the immobilized lineshape, significant CROX accessibility, and low O2 accessibility of the nitroxide group on Y79C*. Arg-82 and Asp-85 are removed by three and six amino acids, respectively, in the primary sequence from Tyr-79. Assumming that the region between these residues is in a regular α-helical configuration approximately perpendicular to the bilayer surface, the α-carbon atoms of these residues would be 5 and 9 Å, respectively, from the α-carbon of Tyr-79, the residue that defines the aqueous interface. The distances of the Arg-82 guanidino and the Asp-85 carboxyl groups from the aqueous interface depend on the conformation of the side chains. For the conformations assigned in the recent structural model (15), these groups would be about 5 and 15 Å, respectively, from the water-exposed nitroxide on Y79C*.

On the intracellular side of the membrane, Val-101 and Ala-103 have been shown to be in a short water-exposed loop between the C and D helices (Fig. 3), where the nitroxide on Val-101 is the first on the carboxyl-terminal side of helix C that can be detected in the aqueous environment. Asp-96 is thus five residues in the primary sequence from the aqueous interface. Assuming a regular helix, the α-carbon of Asp-96 is then estimated to be about 7 Å from the interface. Again, the position of the Asp-96 carboxyl group relative to the interface will be determined by the conformation of the side chains. However, the Asp side chain is short, and the above estimate for the α-carbon is also reasonable for the carboxyl group. Since Asp-96 is protonated in the ground state bR (32,
33), it is believed that this residue is buried within the hydrophobic interior of the upper proton channel and is probably not in direct contact with the bulk water.

The present work shows that in helix D the nitroxide on Q105C* is located at the intracellular boundary, whereas previous work has shown that T127C* is located on the extracellular boundary (17). Assuming that the region between these two residues is α-helical, we conclude that the membrane thickness in the vicinity of the protein is equivalent to 23 amino acids of the polypeptide sequence. This would make the bilayer thickness about 35 Å, a result that is consistent with previously estimated values (34).

Although the above locations of the amino acids in helix C and adjoining loops have been deduced by EPR spectroscopy on br in the dark-adapted state, their positions may alter during the conformational changes that occur during the photocycle (35–37). Recent studies using hydroxyxylamine as a structural probe for br conformation indicate a transient increase in water accessibility to the Schiff base at the L stage of the photocycle (38). Asp-85 and Arg-82 are buried within the protein interior in dark-adapted br; however, we anticipate that there will be environmental changes in the vicinity of these residues during the photocycle. Thus it would be desirable to use new structural probes to study these changes in protein conformation. Since EPR spectroscopy is sensitive to local environmental changes that occur on the time scale of milliseconds, it may be possible to map the domains of the protein that are mobile during the photocycle by using site-directed spin labeling. Such a method could be extended to study protein conformational changes that occur during signal transduction in other members of the seven-helix bundle protein superfamily.

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