Structural Studies on Transmembrane Proteins. 1. Model Study Using Bacteriorhodopsin Mutants Containing Single Cysteine Residues†

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ABSTRACT: In developing new approaches to structural studies of polytopic transmembrane proteins, we have prepared bacteriorhodopsin mutants containing single cysteine residues at selected sites in different topological domains. Four such mutants were prepared: Gly-72 → Cys and Ser-169 → Cys in the presumed looped-out regions on the opposite sides of the membrane bilayer and Thr-90 → Cys and Leu-92 → Cys in the membrane-embedded helix C. The four mutants folded and regenerated the characteristic chromophore in detergent/phospholipid micelles and pumped protons like the wild-type bacteriorhodopsin. After reconstitution in asolectin vesicles, the sulfhydryl groups in the mutants Gly-72 → Cys and Ser-169 → Cys reacted with iodo[2-^{3}H]acetic acid, while the sulfhydryl groups in the membrane-embedded mutants, Thr-90 → Cys and Leu-92 → Cys, did not. The sulfhydryl groups in all four mutants could be derivatized in the denatured state by reaction with iodoacetic acid or 6-acryloyl-2-(dimethylamino)naphthalene. Of these derivatives, the two from the mutants Gly-72 → Cys and Ser-169 → Cys folded like the wild-type bacterioopsin, whereas of the two from the helix C mutants, Thr-90 → Cys and Leu-92 → Cys, only the latter folded normally. However, the folding of Leu-92 → Cys was also impaired when treated with the bulky 5-(iodoacetamido)fluorescein. The reactivity and the folding behavior of the cysteine mutants can thus report on the topographic domain as well as on the orientation of the helices within the membrane.

Transmembrane proteins perform a wide variety of important biological functions. Examples of such proteins are the cytochrome oxidases (Wikstrom et al., 1985), ATPases [e.g., Futai et al. (1987) and Brandl et al. (1986)] and other transport proteins [e.g., Kaback (1988), Kopito and Lodish (1985), and Mueckler et al (1985)], ion channels (Numa et al., 1987; Unwin, 1984), a number of light-transducing proteins such as bacteriorhodopsin (Stoeckenius, 1979) and visual pigments (Applebury & Hargrave, 1986; Nathans, 1987; Findlay & Pappin, 1986), and several families of transmembrane receptors (Changeaux et al., 1984; Dohlman et al., 1987). Currently, there is great interest in the structure and function and regulation of this broad class of membrane proteins. While cDNA sequencing has enabled the derivation of the amino acid sequences of many membrane proteins, structural information on them is very limited. Hydropathy algorithms (Kyte & Doolittle, 1982; Engelman et al., 1980; Guy, 1985; Eisenberg, 1984) have been used to deduce secondary structural models based on membrane-embedded domains. Independent evidence on the following basic structural questions is essentially nonexistent. (1) What are the sizes and boundaries of the individual helical segments that are embedded in the bilayer? (2) What are the sizes and structures of the polypeptide regions looped out of the membrane into the aqueous medium? (3) What are the specific interactions between the membrane-embedded helices? The latter presumably form the functional domains where the response to the signals (e.g., from retinal isomerization in bR[1] and visual pigments or ligands in receptors) begins. (4) What are the orientations of the helices within the membrane, and how are their interactions with the membrane lipids determined? We are interested in developing new chemical and molecular biological approaches to these structural problems in the transmembrane proteins.

Studies of soluble proteins have benefited greatly from the use of the sulfhydryl groups in cysteine residues as structural probes. In this paper, we investigate the use of cysteine residues introduced in selected positions in bR in structural studies. We chose bR as a model for the present study because this is the best-studied transmembrane protein. bR is presumed to contain seven α-helical segments (Henderson & Unwin, 1975; Engelman et al., 1980), and a proposed secondary structure model is shown in Figure 1. bR is also a particularly suitable choice for the present work because it can be renatured efficiently following complete denaturation.

bR does not contain any cysteine residue. Starting with this zero background, we have introduced one cysteine residue at a time in bR at the selected positions shown in Figure 1. These replacements, Gly-72 → Cys, Thr-90 → Cys, Leu-92 → Cys, and Ser-169 → Cys, were chosen according to the following considerations: (1) the mutations should cause minimal perturbation of the native structure and thus be as conservative as possible; (2) they should be in different topographical locations, i.e., aqueous vs membrane-embedded regions; and (3) they should be suitably located on different faces of the same

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[1] Abbreviations: 5-IAF, 5-(iodoacetamido)fluorescein; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; bO, bacterioopsin; bR, bacteriorhodopsin; DMPC, L-α-1,2-dimyristoylphosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane.

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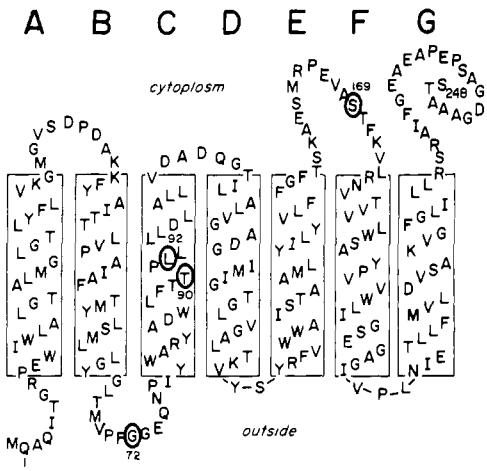

FIGURE 1: Secondary structure model for bR showing the locations of amino acid residues that were changed to cysteines. The boxed regions A–G are the proposed seven α-helical membrane segments (A–G). The carboxyl-terminal region is on the cytoplasmic side of the membrane, while the amino terminus is outside.

helix, e.g., Cys-90 and Cys-92, to determine the influence of the orientation of this membrane-embedded helix on the behavior of the sulfhydryl groups. The experiments reported below demonstrate the usefulness of cysteine residues in topological studies of transmembrane proteins, especially in identifying the membranous domains and looped-out water-accessible polypeptide chains, as well as the orientation of the helices in the membrane-embedded domain.

Materials and Methods

L-α-1,2-Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and octyl β-D-glucopyranoside were obtained from Boehringer Mannheim. The purity of octyl β-D-glucopyranoside was checked by thin-layer chromatography. If necessary, it was purified according to published procedures (VanAken et al., 1986). *all-trans*-Retinal was from Sigma or Kodak; iodoacetamide and iodoacetic acid were from Fluka. 1,2-Di[1-^{14}C]myristoyl-L-α-phosphatidylcholine, iodo[2-^{3}H]acetic acid, and iodo[1-^{14}C]acetamide were from Amersham. Acetone-washed soybean lipids were a gift from Dr. Debra Thompson of this laboratory. 6-Acryloyl-2-(dimethylamino)naphthalene (acrylodan) and 5-(iodoacetamido)fluorescein (5-IAF) were from Molecular Probes. Fluorescence measurements were performed on a GREG PC spectrofluorometer (ISS) using an SFA-11 rapid kinetics accessory (Hi-Tech Scientific).

Cloning Procedures. The cloning plasmid pSBO2 and the expression plasmid pPL1 for the synthetic gene for bacterioopsin (bO) have been described previously (Nassal et al., 1987). *Escherichia coli* strains JM109 and DH1 were used for the cloning vector, while expression of the gene was performed in the strain C600/pC$_{1857}$.

Single-stranded oligonucleotides for the required synthetic restriction fragments were synthesized by the phosphite ester method using a 380A DNA synthesizer (Applied Biosystems) and were purified by polyacrylamide gel electrophoresis (Nassal et al., 1987). The restriction fragments containing the cysteine codon replacements are indicated in Figure 2. For the preparation of the bO mutants Thr-90 → Cys, Leu-92 → Cys, and Gly-72 → Cys, the appropriate restriction fragments were excised out of the *bop* gene, and the duplexes (Figure 2) were inserted by using T4 ligase. For the mutant Ser-169 → Cys, a three-component ligation method using a third restriction site (*Pvu*I in pSBO2) was used (Hackett et al., 1987). All the restriction fragments were purified on low melting point agarose gels and recovered by phenol extraction and ethanol precipitation.

DNA Sequence Analysis. Plasmid DNA was isolated from the *E. coli* expression system by the alkaline extraction method (Birnboim & Doly, 1979). The sections of the mutant bO genes containing the replaced synthetic fragments (Figure 2) were sequenced by using the method of Maxam and Gilbert (1980).

Expression and Purification of bO Mutant Proteins. The mutants were expressed in *E. coli* as described (Hackett et al., 1987) and were purified by the two-step procedure previously described (Braiman et al., 1987). Mercaptoethanol (15 mM) or dithiothreitol (DTT) (5 mM) was added to all solvents.

Renaturation of the bR Cysteine Mutants and Regeneration of the bR-like Chromophore. The refolding and chromophore regeneration were according to the previously described method (Huang et al., 1981). However, for samples that were subsequently purified by size-exclusion chromatography a modified procedure was used: The protein in SDS (0.2%) was diluted into a lipid/detergent mixture containing 3.3 mg/mL protein, 1% DMPC, 1% CHAPS, 1.6% SDS, 10 mM sodium phosphate, pH 6.0, 0.025% sodium azide, and 1.5 molar excess over the protein of *all-trans*-retinal [lipid/protein ratio (w/w) 3:1 compared to 25:1 in the previously described procedure (Huang et al., 1981)]. Excess SDS was precipitated as the potassium salt by adding potassium chloride to give an excess of 150 mM over SDS concentration. The regeneration mixture was kept overnight in the dark, and the precipitated potassium dodecylsulfate was removed by centrifugation.

Size Exclusion Chromatography. High-pressure liquid chromatography was performed by using Bio-Sil TSK-125 columns (600 × 21.5 mm for preparative and 300 × 7.5 mm for analytical work) equipped with Bio-Sil TSK guard columns (75 × 21.5 mm for preparative work and 75 × 7.5 mm for analytical work) supplied by Bio-Rad Laboratories. They were connected to a 6000A solvent delivery system (Waters Associates, Inc.), and the eluate was optically analyzed at 280 and 548 nm with a Model 440 absorbance detector (Waters Associates). Flow rates were usually 3 mL/min for the preparative column and 0.5 mL/min for the analytical column. The buffered solvent systems used were (A) 0.8% CHAPS, 0.2 M sodium chloride, and 50 mM sodium phosphate (pH 6.4) and (B) 1% octyl β-D-glucopyranoside, 0.2 M sodium chloride, and 50 mM sodium phosphate (pH 6.4). For preparative work 1.5-mL samples containing 1–5 mg of the protein and for analytical work 0.1-mL samples containing 10–100 μg of protein were injected.

Reconstitution of bR Mutants into Soybean Lipid Vesicles and Proton Pumping Measurements. The cysteine mutants were regenerated in detergent/lipid mixtures and then purified by HPLC using buffer system A, followed by buffer system B consecutively on the same column, as described above. The final concentration was 0.1 mg/mL protein in buffer B. This solution was mixed with a solution of soybean lipids (100 mg/mL in 10% CHAPS) to give a lipid concentration of 4 mg/mL. The mixture was dialyzed for 2 days against 150 mM potassium chloride containing 30 mM sodium phosphate (pH 6.0), 0.025% sodium azide, and 4 mM mercaptoethanol (three buffer changes), followed by dialysis against 200 mM potassium chloride (1 day, three buffer changes). Proton pumping measurements were performed after the vesicles were diluted into 150 mM potassium chloride as has been described (Huang et al., 1980).

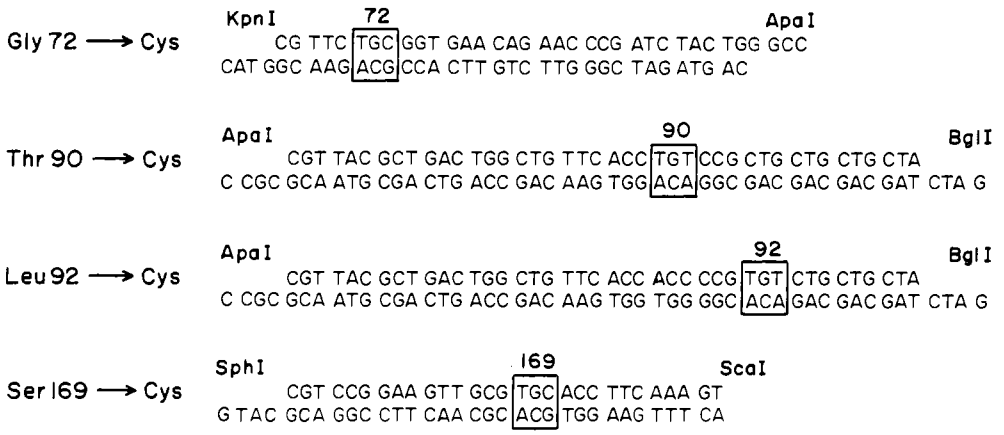

FIGURE 2: Synthetic restriction fragments that were used to construct cysteine-containing *bop* mutants.

Table I

	chromophore regeneration[a] $t_{1/2}$ (s)	λ_{max} of regenerated chromophore[b]		proton pumping activity[c]	
		light adapted	dark adapted	steady state (H^+/bR)	rate [(H^+/bR)/s]
wild type	110	559	552	20	1.5
Gly-72 → Cys	115	559	548	18	1.6
Thr-90 → Cys	80	546	542	20	1.6
Leu-92 → Cys	310	559	549	19	2.1
Ser-169 → Cys	140	559	548	17	1.7

[a] Half-life of chromophore regeneration when bO in a solution of 1% DMPC/1% CHAPS/0.2% SDS and 10 mM sodium phosphate (pH 6) was mixed with *all-trans*-retinal at 20 °C (Hackett et al., 1987). [b] Absorption maxima of chromophores after overnight dark adaptation (dark adapted) followed by 5 min of irradiation from a 300-W projector lamp equipped with a 470-nm filter at 4 °C (light adapted). [c] Proton pumping activity of bR proteins reconstituted into asolectin liposomes by the dilution method (Braiman et al., 1987).

Derivatization of Thiol Groups in Cysteine-Containing bO Mutants with Iodoacetamide and Iodoacetic Acid. bO mutants (2 mg/mL) in 2% SDS, 6 M urea, 10 mM EDTA, and 0.1 M Tris-HCl (pH 8) were incubated with dithiothreitol (0.38 mM) for 2 h at 37 °C and then treated with either iodoacetamide (1 mM) or iodoacetic acid (1 mM). The mixture was incubated under argon in the dark for 1 h and the reaction quenched by adding 50 mM DTT. The reaction mixture was dialyzed against 0.2% SDS and 50 mM sodium phosphate (pH 6) for 1 day (two buffer changes).

Derivatization of Reconstituted Cysteine Mutants with [1-^{14}C]Iodoacetamide and [2-^{3}H]Iodoacetic Acid. A mixture of 0.5 mL of bR in soybean lipid vesicles (0.1 mg/mL in 0.2 M potassium chloride) was mixed with 15 μL of DTT (0.32 mM), 50 μL of Tris-HCl (1 M, pH 8.0), and 25 μL of EDTA (0.05 M) and incubated for 30 min at 37 °C. Either 30 μL of iodo[2-^{3}H]acetic acid (38 μmol/mL, 131 mCi/mmol in H_2O) or 60 μL of iodo[1-^{14}C]acetamide (7.8 μmol/mL, 53 mCi/mmol in H_2O) was added and the mixture incubated for 2 h at 37 °C in the dark under argon. It was quenched by adding 5 μL (0.3 M) of DTT, washed two times with 20 mM sodium phosphate, pH 6.9 (1 mL) centrifuged (100000*g*, 1 h), resuspended in 100 μL of 1% Triton X-100 and 20 mM sodium phosphate (pH 6.9), and left overnight in the dark. After solubilization, the samples were analyzed by size exclusion chromatography.

Derivatization of Thiol Groups in Cysteine-Containing bO Mutants with Acrylodan and 5-IAF. Denatured bO mutants (2 mg/mL) in 2% SDS, 6 M urea, 10 mM EDTA, and either 0.1 M Tris-HCl (pH 8, for 5-IAF) or 0.1 M sodium phosphate (pH 7, for acrylodan) were incubated with dithiothreitol (0.38 mM) for 2 h at 37 °C, and acrylodan (1 mM; from a 44 mM stock solution in acetonitrile) or 5-IAF (1 mM; from a 19 mM stock solution in 0.1M Tris-HCl, pH 8) was added. The mixtures were incubated for 20 min in the dark at room temperature, quenched with 20 mM glutathione, and dialyzed against 0.2% SDS, 10 mM sodium phosphate (pH 6), and 0.025% sodium azide (2 days, four buffer changes).

Results

Mutagenesis and Expression of the bop Gene and Purification of the Mutant bO Proteins. Four *bop* mutants containing single cysteine codons were prepared by cassette mutagenesis of the synthetic gene (Hackett et al., 1987) using the synthetic duplexes listed in Figure 2. The mutant genes were expressed in *E. coli* and purified by organic solvent extraction and ion-exchange chromatography (Braiman et al., 1987). The average yields of the mutant proteins were 20 mg from 100 g of wet cells.

Chromophore Regeneration, Spectral Properties, and Proton Pumping Behavior of the Cysteine Mutants. The cysteine mutants (Figure 1) folded and regenerated the bR-like chromophore at rates that were comparable to that of the wild type, although the rate of regeneration was slightly lower for Leu-92 → Cys (Table I). The absorption spectra in the light- and dark-adapted states for Gly-72 → Cys, Leu-92 → Cys, and Ser-169 → Cys were identical with those of the wild-type protein, while Thr-90 → Cys showed a slightly shifted (10 nm) absorption maximum (Table I). All four mutants showed normal proton pumping activity after reconstitution in phospholipid vesicles (Table I).

Purification of Regenerated bR and Its Mutants by HPLC on TSK Gel Filtration Columns. Folding and chromophore regeneration of denatured bR mutants usually proceed to a high degree (60–80%) but not quantitatively (Braiman et al., 1987). Since pure samples were required for the present experiments, a method was developed for the purification of regenerated bR and cysteine mutants from nonregenerated bO, lipids, detergents, and excess retinal. The method also allows removal of excess sulfhydryl reagents after the sulfhydryl group was labeled (see below).

The method involves size exclusion chromatography on TSK HPLC columns in two successive steps. In the first step, bR is eluted isocratically with a buffer system containing CHAPS

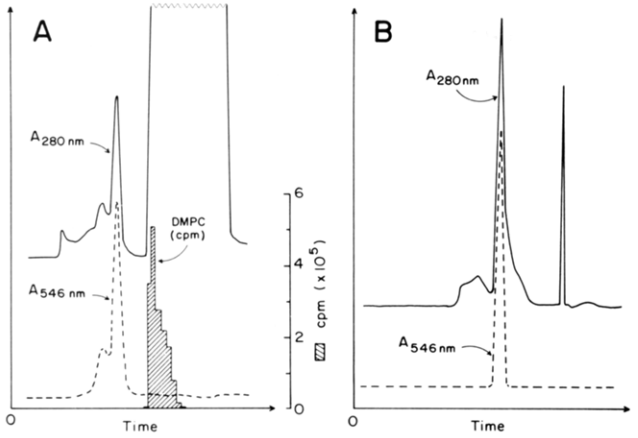

FIGURE 3: Elution profiles of the two-step HPLC purification procedure using a TSK-125 size exclusion column (600 × 21.5 mm). (A) bR (3 mg/mL; 1.6 mL) in 1,2-di[1-^{14}C]myristoyl-L-α-phosphatidylcholine/CHAPS/SDS mixed micelles was solubilized with 1% Triton X-100 and eluted isocratically in buffer system A (0.8% CHAPS, 0.2 M NaCl, 50 mM sodium phosphate, pH 6.4). (B) All fractions containing material with A_{546} from step A were pooled and rechromatographed on the same column by using buffer system B (1% ocytl β-D-glucopyranoside, 0.2 M NaCl, 50 mM sodium phosphate, pH 6.4). The absorbance of eluate was measured at A_{280} and A_{546}, fractions were collected, and radioactivity was determined by liquid scintillation counting.

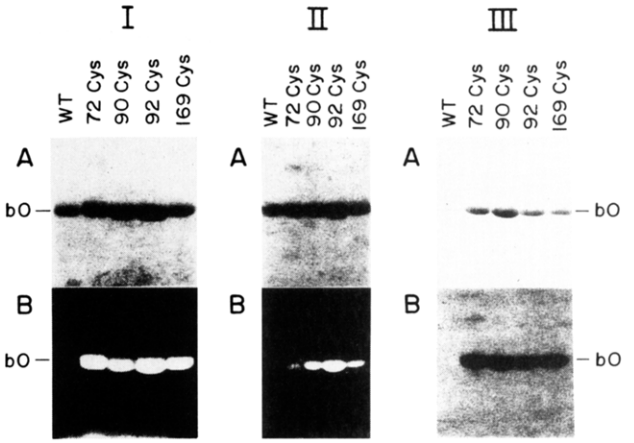

FIGURE 4: Analysis of bO and cysteine containing bO mutants by SDS–polyacrylamide gel electrophoresis after treatment of unfolded protein with sulfhydryl reagents (see Materials and Methods). (I) 5-IAF. (II) Acrylodan. (III) Iodo[2-^{3}H]acetic acid. (A) Gels stained with Coomassie blue. (B) (I, II) Fluorescence upon UV irradiation of gels shown in panel A; (III) fluorograph of gel shown in panel III,A.

as the detergent (buffer A under Materials and Methods). CHAPS was the better detergent for separation of the protein from lipids because of its small micelle size (Huang et al., 1980). Elution of the lipid was followed by using ^{14}C-labeled DMPC. The profile of the radioactivity eluted in different fractions is shown in Figure 3A. After the first step, the protein fraction was essentially lipid free (99%) as judged by the radioactivity associated with it. However, the protein peak was broad (Figure 3A) and was mixed with nonregenerated material. To separate the latter, all fractions with absorbance at 546 nm were rechromatographed on the same column by using buffer B, which contains octyl β-D-glucopyranoside (Materials and Methods, step 2). The folded regenerated protein eluted as a single sharp peak (Figure 3B) with a A_{280}/A_{546} ratio of close to 1.6, a value that agrees with the presence of folded protein only.

The protein preparation thus obtained was then reconstituted into soybean lipid vesicles by using the dialysis method (Huang et al., 1980).

The Sulfhydryl Groups in All Four Cysteine-Containing bO Mutants Are Selectively Derivatized under Denaturing Conditions. bO and the four cysteine-containing mutants were treated in 2% SDS and 6 M urea with iodo[2-^{3}H]acetic acid, acrylodan, and 5-IAF in parallel experiments. Samples of the proteins were analyzed by polyacrylamide gel electrophoresis, and the radioactivity or fluorescence in the derivatized samples was visualized by autoradiography or illumination with UV light, respectively. Figure 4 shows that all four mutants were equally labeled, whereas wild-type protein, the control, showed no labeling with any of the reagents.

The Derivatized Mutant Thr-90 → Cys Does Not Fold into the bR-like Structure. Wild-type bO and the four mutants were treated in parallel experiments with iodoacetamide in 2% SDS and 6 M urea. After isolation of the pure derivatized proteins, their behaviors in regeneration of the chromophore were compared. Figure 5 shows the absorption spectra of both bR and Thr-90 → Cys before and after iodoacetamide treatment. Neither the rate (Figure 5A) nor the extent of chromophore regeneration of the wild-type protein was affected by iodoacetamide treatment. Similarly, the three cysteine mutants, Cys-72, Cys-92, and Cys-169, also regenerated the typical bR-like chromophore after iodoacetamide treatment (data not shown). On the other hand, iodoacetamide treatment of the mutant Thr-90 → Cys abolished the ability of the latter to form a bR-like chromophore (Figure 5B).

The Sulfhydryl Groups in the Folded Cysteine Mutants, Thr-90 → Cys and Leu-92 → Cys, Are Not Accessible to Iodoacetic Acid. The four mutants Gly-72 → Cys, Thr-90 → Cys, Leu-92 → Cys, and Ser-169 → Cys were treated under standard conditions for regeneration of the chromophore. The folded proteins were purified and reconstituted into soybean lipid vesicles (Materials and Methods). They all were then treated with iodo[2-^{3}H]acetic acid. The extent of labeling of the folded proteins was determined by the two-step gel filtration procedure (Figure 3) after the proteins were solubilized in Triton X-100. Figure 6 shows the elution profiles of the second gel filtration step as monitored by A_{280}, A_{546}, and radioactivity in the proteins after the second gel filtration step. It is seen that only the mutants Gly-72 → Cys and Ser-169 → Cys were radioactively labeled, whereas for Thr-90 → Cys and Leu-92 → Cys no radioactivity collected with the folded protein. Parallel experiments with iodo[1-^{14}C]acetamide gave similar results (data not shown).

Study of the Folding of the 5-IAF- and Acrylodan-Labeled Cysteine Mutants. The four cysteine mutants, after 5-IAF or acrylodan labeling, were treated with the standard detergent/phospholipid chromophore regeneration mixture. The time course for fluorescence changes in every case was monitored as follows. The excitation wavelength for acrylodan label was 390 nm and that for 5-IAF was 490 nm, and the changes were observed in the emission bands at 440 and 520 nm, respectively. The quenching of fluorescence of both labels should parallel the retinal binding and chromophore regeneration process due to energy transfer from the label to retinal (London & Khorana, 1982).

This was indeed observed for the three acrylodan-labeled mutants Gly-72 → Cys, Leu-92 → Cys, and Ser-169 → Cys (Figure 7A). No fluorescence quenching was observed for the acrylodan-labeled Thr-90 → Cys mutant. Therefore, this mutant cannot fold or bind *all-trans*-retinal to form the bR-like chromophore, which is consistent with the above results from iodoacetamide labeling.

Similar experiments with the 5-IAF derivatives of the four cysteine mutants (Figure 7B) show that now only the derivatives of Gly-72 → Cys and Ser-169 → Cys are able to refold

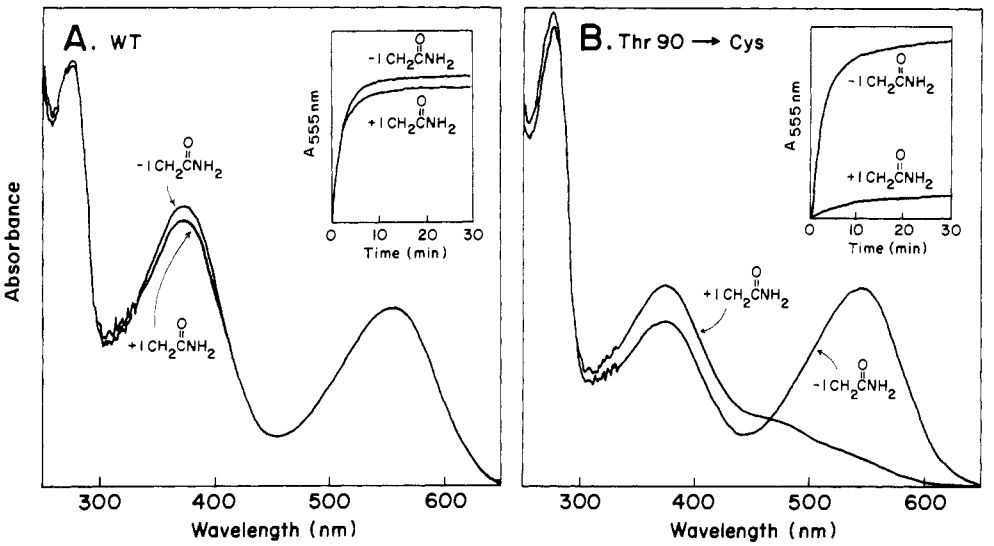

FIGURE 5: UV-vis spectra of wild-type (A) and mutant Thr-90 → Cys (B) before and after treatment with iodoacetamide in the unfolded form (see Materials and Methods). (Inserts) Kinetics of chromophore regeneration after addition of *all-trans*-retinal to the protein in DMPC/CHAPS/SDS micelles (T = 20 °C).

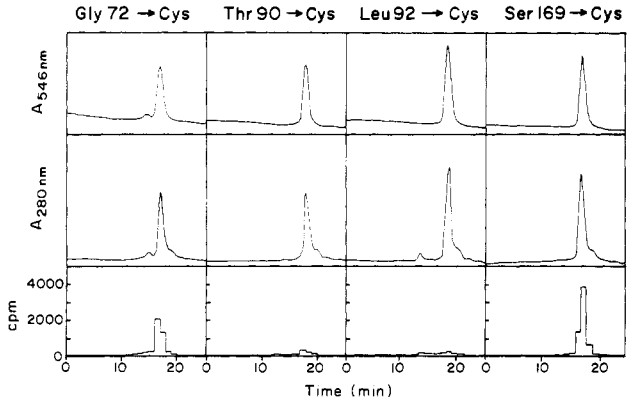

FIGURE 6: HPLC elution profiles of cysteine containing bR mutants after reconstitution into soybean lipid vesicles, treatment with iodo-[2-^{3}H]acetic acid and purification by size exclusion HPLC with buffer system A (see Materials and Methods). The proteins were eluted isocratically from a TSK-125 size exclusion column (300 × 7.5 mm) by using buffer system B (see Materials and Methods); 0.5-mL fractions were collected, and the radioactivity was determined by liquid scintillation counting.

and form the chromophore, whereas the labeled Thr-90 → Cys and Leu-92 → Cys are not.

Discussion

Development of new chemical, biochemical, and molecular biological methods is highly desirable for the study of membrane proteins. This is especially so because it has so far proved difficult to crystallize this class of proteins. In this paper we have developed one approach to the study of topography in transmembrane proteins. By using site-specific mutagenesis, we have introduced single cysteine residues at locations representative of different structural domains. The sulfhydryl group in the cysteine residues is unique in reactivity among amino acid side chains, and it has provided an extremely useful handle for introducing different labels in structural studies of many soluble and membrane proteins (DeGrip et al., 1975; Karlin, 1980; Falke & Koshland, 1987). The results reported in this and an accompanying paper (Altenbach et al., 1989) show that judiciously introduced cysteine residues provide a highly promising approach for the study of the hydrophobic membrane proteins.

Four mutants each containing one cysteine residue were prepared as shown in the secondary structure model of Figure

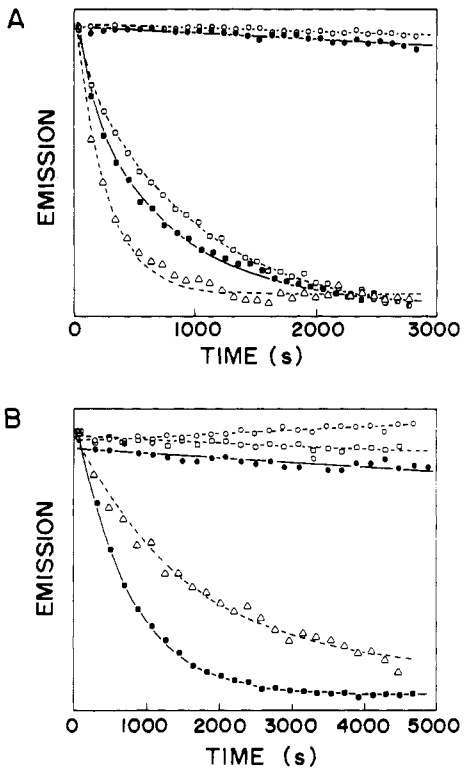

FIGURE 7: Changes in 450- (A) and 520-nm emission (B) of labeled cysteine mutants upon addition of *all-trans*-retinal to the protein in DMPC/CHAPS/SDS mixed micelles. Protein was labeled with (A) acrylodan or (B) 5-IAF. (●) Wild type; (△) G72C; (○) T90C; (□) L92C; (■) S169C.

1. To probe their environments two sets of experiments were carried out. First, the mutants were reconstituted into vesicles in the folded form and the sulfhydryl groups were tested for reactivity with iodoacetic acid and iodoacetamide. The sulfhydryl groups in the mutants containing Cys-72 and Cys-169 showed reactivity, while the two containing Cys-90 and Cys-92 residues were unreactive (Figure 6). Clearly, the latter residues are shielded from the reagents. This suggests that the experiment distinguishes the cysteine residues present in the aqueous looped-out domain from those that are located in the membrane-embedded domain. Although bR is known to reconstitute asymmetrically (inside-out) in asolectin vesicles,

no selectivity of labeling between Cys-72 and Cys-169 was observed. (Cys-72 would be expected to be on the inside of the vesicles.) The result could be due to leakiness of the vesicles to iodoacetic acid or to the scrambled orientations of the vesicles. However, both mutant vesicles showed the same proton pumping behavior.

In the second set of experiments, the tests started with unfolded (denatured) bO mutants. All four cysteine mutants reacted, as expected, with iodoacetamide and iodoacetic acid as well as with the bulky fluorescent reagents acrylodan and 5-IAF (Figure 4A). On being subjected to the renaturation (folding) conditions, the derivatives of both Cys-72 and Cys-169 mutants underwent folding to regenerate the chromophore. This would be expected since these mutants are in the looped-out regions. Of the two membrane-embedded mutants Cys-90 and Cys-92, none of the derivatives of Cys-90 folded, while of the derivatives of Cys-92, the acetamide and acrylodan derivatives folded, but the bulky 5-IAF derivative did not. It seems that the difference lies in the different orientation of the two cysteine residues within the helix C. We interpret that the Cys-90 derivatives are unable to fold because this group is exposed to greater crowding within the interior of the helical bundle. The folding of the derivatized Leu-92 → Cys, however, is restricted only when a bulky ligand (5-IAF) is attached. This suggests that Cys-92 is pointing toward the less crowded or hindered lipid surface of the protein. A specific role of the hydroxyl (or thiol) group at position 90 for folding is unlikely, since the mutant Thr-90 → Val does regenerate a chromophore (Mogi and Khorana, unpublished results).

The initial experiments now reported indicate that useful procedures for determining topography as well as the orientations of α-helices in the transmembrane regions can be developed. Systematic studies, which probe polypeptide stretches within bR regions by replacing suitable amino acids consecutively by cysteines, are being currently pursued in this laboratory. Furthermore, the folding studies with fluorescent labels 5-IAF and acrylodan (Figure 7) demonstrate that spectroscopic reporter groups can be attached to bR mutants specifically (Figure 4). This should be useful for further biophysical investigations. A study employing spin resonance spectroscopy on spin-labeled bR mutants is described in the accompanying paper (Altenbach et al., 1989).

Finally, we draw attention to a new gel filtration HPLC procedure to prepare detergent-solubilized pure fully folded recombinant bR (Figure 3). The method is based on principles previously published (Huang et al., 1980; Pabst & Dose, 1985). It consists of two size-exclusion HPLC steps on the same column using the two detergents CHAPS and octyl β-D-glucopyranoside consecutively. The first step allows the removal of excess lipids, retinal, detergents, and labeling reagents (Figure 3) and the second step the separation of unfolded protein from folded bR. This method has proven to be very useful for preparing fully reconstituted bR and for quantitative analysis of cysteine mutants after labeling.

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