Probing of the Retinal Binding Site of Bacteriorhodopsin by Affinity Labeling†

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ABSTRACT: The position of the chromophore within bacteriorhodopsin has been identified by cross-linking a cysteine group, introduced by site-specific mutagenesis, with a chromophore suitably derivatized with an active leaving group. Since bacteriorhodopsin has no cysteines, a site-specific cysteine mutant will contain only one free sulfhydryl group capable of reacting with the retinal analog. Met118, Thr121, and Ser141 were selected to be mutated to cysteine. No pigment absorbing in the visible region was obtained for the Ser141Cys mutant. The Met118Cys and Thr121Cys mutants have similar absorption maxima, proton pumping efficiencies and photocycles to those of the wild-type pigment. 4-Bromoretinal, in which the reactive allylic halide readily undergoes nucleophilic displacement, was used as the reactive chromophore. Pigments were obtained on reaction of *all-trans*-4-bromoretinal with the apoproteins of Met118Cys, Thr121Cys, and wild-type bacteriorhodopsin (λ_{max} = 464–470 nm). Analysis of the denatured pigments on SDS–polyacrylamide gels showed incorporation of tritiated chromophore into the Met118Cys mutant but not into the wild-type or Thr121Cys pigments. Met118Cys apoprotein which was preincubated with the cysteine-specific reagent *N*-ethylmaleimide formed a pigment with 4-bromoretinal but no cross-linking was observed, providing evidence that the cross-linking of the chromophore is to the cysteine at 118. We conclude that Met118 is positioned in the chromophore binding pocket, proximal to the C-4 position of cyclohexyl ring of retinal.

Bacteriorhodopsin (bR),[1] a retinal-based light-transducing pigment, is found in the purple membrane of the archaebacterium *Halobacterium salinarium* (Oesterhelt & Stoeckenius, 1971). The protein contains a single polypeptide chain of 248 amino acids and a single covalently bound chromophore, *all-trans*-retinal, attached via a Schiff base to the ϵ-amino group of Lys216. bR transduces light energy into a proton gradient. Light absorption which causes isomerization of *all-trans*-retinal to the 13-*cis* isomer results in proton translocation from the inside to the outside of the *H. salinarium* membrane. The resulting electrochemical gradient is then used by the cell to drive ATP synthesis and other metabolic processes under anaerobic conditions.

bR is the only protein in the purple membrane. The electron density map at 7 Å suggests that each bR molecule is comprised of seven transmembrane α-helices (Henderson & Unwin, 1975; Henderson et al., 1990). From neutron diffraction studies, the chromophore of bR appears to be oriented toward helix F and inclined to the extracellular side of the membrane at an angle of 35–40° (Jubb et al., 1984; Heyn et al., 1988). The replacement of the native retinal chromophore by a suitably tailored synthetic analog has been used to synthesize analog pigments of bacteriorhodopsin, and this technique has elucidated several aspects of the interaction of the chromophore with the protein and the role of the chromophore in the physiological function of the pigment (Crouch, 1986; Ottolenghi & Sheves, 1989). Photoaffinity studies using derivatized retinals have previously reported that the *m*-diazirinophenyl retinal analog cross-linked with the Ser193 and Glu194 on helix F (Huang et al., 1982). Ding et al. (1990) using *all-trans*-3*S*- and 3*R*-diazoacetoxyretinal showed labeling of Thr121 and Gly122. Four other amino acids, Ala126, Leu127, Trp137, and Trp138, were labeled with *all-trans*-3-diazo-4-ketoretinal (Boehm et al., 1990) (Figure 1).

bR has no cysteines present in the native protein. This is especially convenient as a cysteine can be introduced by site-directed mutagenesis into a specific site, and the mutant will contain only one free sulfhydryl group capable of reacting with an appropriately derivatized retinal analog. Khorana and colleagues have introduced cysteines into both rhodopsin and bR and derivatized the mutants with a sulfhydryl-specific spin-labeling reagent in order to examine structural issues (Altenbach et al., 1990; Resek et al., 1993). Thiol chemistry has been extensively applied in protein chemistry for affinity labeling (Hartman, 1977). Selective alkylation of the thiol group is possible due to its high nucleophilic reactivity relative to other amino acid side chains. Selective alkylation in peptides has also been studied in model peptides and side chains (Or et al., 1991). This approach was used to identify a cysteine residue within the substrate binding site of glutathione S-transferase by using a reactive analog of glutathione containing a bromo group (Katusz et al., 1992).

In this study, we have explored the cross-linking of cysteine groups introduced into the protein by site-specific mutagenesis with a chromophore suitably derivatized with a good leaving group. From a model generated using the structure reported by Henderson et al. (1990), we have selected sites of mutation

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[1] Abbreviations: bR, bacteriorhodopsin; NEM, *N*-ethylmaleimide; PCR, polymerase chain reaction; YT, yeast–tryptone; LB, Luria–Bertani; RF, replicative form; ds, double-stranded; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; LA, light-adapted; DA, dark-adapted; WT, wild type; JL, Janos–Lanyi.

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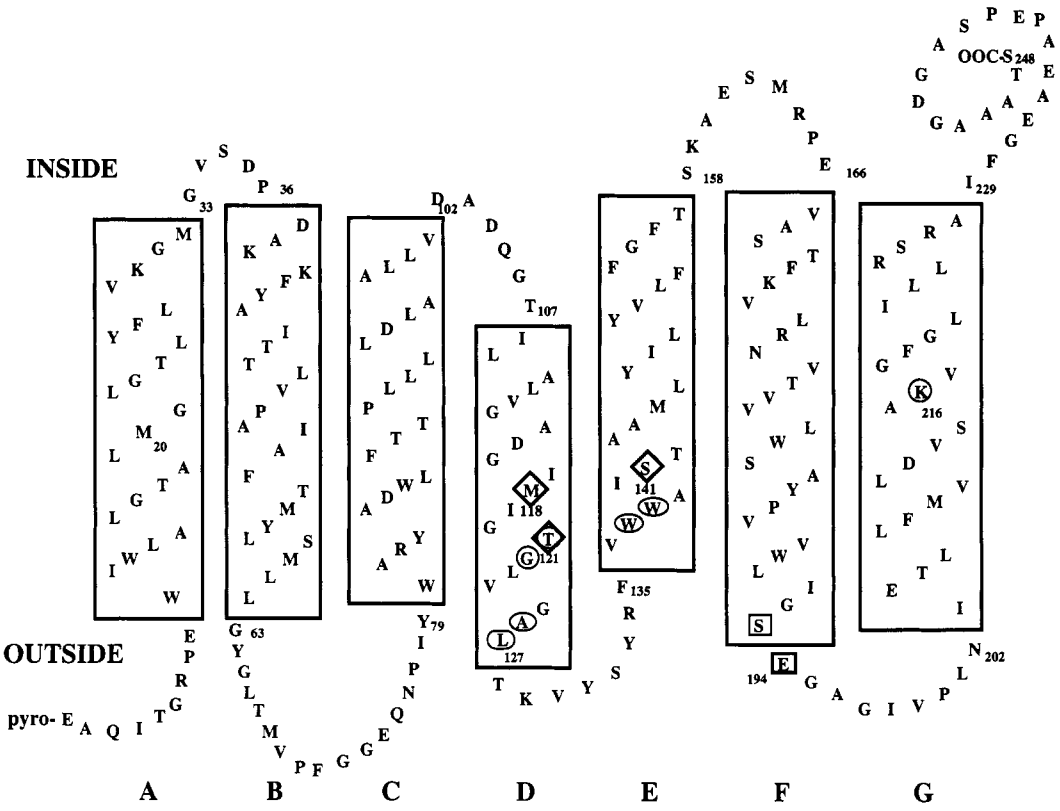

FIGURE 1: Affinity-labeled sites of bacteriorhodopsin. The seven transmembrane helices in a two-dimensional structure of bacteriorhodopsin are boxed and lettered A–G. Lys216, to which the retinal is bound by a Schiff base, is circled. Ser193 and Glu194 (Huang et al., 1982), Thr121 and Gly122 (Ding et al., 1990), and Ala126, Leu127, Trp137, and Trp138 (Boehm et al., 1990) were reported labeled in photoaffinity studies. Met118, Thr121, and Ser141 are predicted to be in close proximity to the C4 position of retinal (Beischel et al., 1994).

that are predicted to be in the retinal-binding pocket of bR, proximal to the cyclohexyl ring (Beischel et al., 1994). We report here that a cysteine at the Met118 position cross-links with *all-trans*-4-bromoretinal, in which the bromine is in the allylic position on the cyclohexyl ring, and conclude that Met118 lies within the chromophore binding site of bR.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Escherichia coli BMH71-18 *mutL* (Kramer et al., 1984) was used for site-directed mutagenesis. *E. coli* JM101 was used to propagate mutant phages, and competent *E. coli* NovaBlue (Novagen, Madison, WI) was used for transformation of the pT7Blue vector (Novagen, Madison, WI) containing PCR-generated fragments. All *E. coli* strains were grown in 2× YT or LB medium (Ausubel et al., 1989). The wild-type and all cysteine-containing mutant *bop* genes were expressed in *H. salinarium* IV-8 (a gift of Richard Needleman, Department of Biochemistry, Wayne State University School of Medicine, Detroit, MI), which contains a stable IS*H1* insert in the *bop* gene (DasSarma et al., 1984). *H. salinarium* IV-8 was grown on C medium (Needleman et al., 1991).

Site-Directed Mutagenesis of bR. Oligonucleotide-directed site-specific mutagenesis was performed essentially as described by Menick (1991). The 2.7-kb *Eco*RI–*Hin*dIII *bop* gene insert was restricted from the *H. salinarium* shuttle vector pMC-1 (a gift of R. Needleman) and ligated into M13mp19. The amino acid residues Met118, Thr121, and Ser141 were individually replaced with Cys by using the mutagenic primers which contained the cysteine genetic code TGC. Mutants were initially screened by colony hybridization and then plaque-purified, and the mutation was verified by dideoxy sequencing (Sanger et al., 1977). The entire coding region of the mutant *bop* gene was sequenced to insure that no additional changes occurred. The *Eco*RI–*Hin*dIII *bop* mutant gene was then restricted from the M13mp19 RF and religated into pMC-1 expression shuttle vector.

Expression of bR Cysteine-Containing Mutants. The Met118Cys, Thr121Cys, and Ser141Cys bR mutants and wild-type control were transformed into the *H. salinarium* bR-defective strain IV-8 which contains IS*HI* insert within the endogenous *bop* gene as described previously (Balashov et al., 1993). Aliquots of transformed spheroplasts were plated on JL growth medium agar plates (Needleman et al., 1991) and 15% sucrose and 10 mg/mL lovastatin (a gift of Merck Sharp & Dohme). After approximately 2 weeks of incubation, pigmented colonies were transferred onto a new JL growth medium agar plate with 10 mg/mL lovastatin. Isolated colonies were grown in liquid JL growth medium (Needleman et al., 1991) to allow subsequent characterization of each bR mutant.

Southern and Sequencing Analysis of the bR Mutants. Southern blot analysis was carried out as described (Ausubel et al., 1989). Genomic DNAs from *H. salinarium* IV-8 cells and IV-8 cells transformed with wild-type or the cysteine-containing mutant *bop* genes were digested with *Pst*I, transferred to Gene Screen Plus hybridization transfer membranes (New England Nuclear Research Products, Boston, MA), and hybridized with a ^{32}P-labeled 500-bp *Kpn*I *bop* gene fragment. In addition, the cysteine-containing *bop* genes from the Met118Cys-, and Thr121Cys-, and Ser141Cys-transformed IV-8 clones were amplified for sequencing by polymerase chain reaction. PCR was performed with sheared *H. salinarium* genomic DNA as template using the conditions described previously (Balashov et al., 1993). The 1150-bp

PCR product was gel-purified and sequenced by using the sDNA cycle sequencing system (BRL, Grand Island, NY).

Purification and Bleaching of the bR Pigment-Containing Purple Membranes. *H. salinarium* IV-8 cells transformed with wild-type and cysteine *bop* mutant genes were grown as described (Ni et al., 1991). The cells were pelleted and lysed in 0.1 N sodium acetate buffer, pH 4.5. The cell lysates were incubated at 37 °C with 25 units/mL DNase I for 6–8 h. Purple membranes were purified by centrifugation at 100000*g* for 17 h in a 30–60% continuous sucrose gradient. Bleached purple membrane was prepared by extensive irradiation with white light at 37 °C in a solution of 1 M hydroxylamine, 0.1 M NaCl, and 10 mM Tris buffer (pH 9.5) for 4–5 h. The mutant purple membranes were bleached under the milder conditions of 0.1 M hydroxylamine at 4 °C. Retinyloximes were removed by sonication and hexane extraction (Tokunaga et al., 1977).

Synthesis of ^{3}H-all-trans-4-Bromoretinal. [11,12-^{3}H]-*all-trans*-Retinol (2.4 nmol, 0.25 μCi; DuPont–New England Nuclear Research Products, Boston, MA) was oxidized to the aldehyde by manganese dioxide (0.1 g) in methylene chloride at room temperature for 18 h, and the product was filtered through celite. *N*-Bromosuccinimide (0.39 mg) (Aldrich, Milwaukee, WI) was added to ^{3}H-*all-trans*-retinal (0.5 mg) with stirring in 300 μL of methylene chloride and a crystal of benzoyl peroxide (Polysciences, Warrington, PA) at 0 °C. The reaction was allowed to continue for approximately 7 min. The solvent was evaporated under argon. Ethyl acetate (5%)/hexane (95%) was added, and after filtration through glass wool, the solvent was evaporated. The purity of this analog (λ_{max} in ethanol was 328 nm) was confirmed by NMR (Crouch et al., 1986).

Reconstitution of bR Pigment Analogs. For the pigment regeneration, ^{3}H-*all-trans*-retinal or ^{3}H-*all-trans*-4-bromoretinal in ethanol (<1% final volume) was added to the suspension of bleached membrane containing bR apoproteins (50 mM sodium phosphate buffer, pH 7.4) under dim red light (2:1 absorbance ratio of retinal/protein). Pigment formation was monitored and assessed by changes in absorption with bleached membrane being used as a reference. The reactions were shaken in the dark at 4 °C overnight. The unreacted retinal was removed by centrifuging with 1% bovine serum albumin in 50 mM sodium phosphate buffer, pH 7.4, at 100000*g* for 30 min. The stability of the pigments were confirmed by monitoring the absorption spectra before and after the addition of an excess (3×) of *all-trans*-retinal over a period of 2 h. To confirm the site of cross-linking, the pigments were incubated with excess ^{3}H-*all-trans*-4-bromoretinal (3×) for 2 h and washed with bovine serum albumin as above. The radiolabel associated with protein was resolved by gel electrophoresis.

N-Ethylmaleimide Treatment of bR Pigment Analogs. Bleached membranes containing the wild-type and mutant bR apoproteins were incubated with *N*-ethylmaleimide (NEM, 100-fold molar excess, Sigma, St. Louis, MO) in the dark at room temperature for 2 h. The procedure of pigment formation was allowed to continue with these mixtures as described above.

Spectroscopic and Proton Pumping Measurement of bR Mutants. Light- and dark-adapted absorption spectra were recorded in 1-cm quartz cuvettes at room temperature using a Cary-Aviv 14 spectrophotometer (Aviv Associates, Lakewood, NJ). Wild-type, Met118Cys, and Thr121Cys pigment samples (containing the native chromophore) were dark-adapted in KCl (1 M, 17 h, 22 °C). The samples were subsequently light-adapted by illumination for 8–10 min using a 250-W xenon lamp projector filtered at 500–550 nm and their absorption spectra were remeasured.

Flash-induced absorption changes at 410, 460, and 570 nm were measured using a special-design kinetic spectrophotometer previously described (Balashov et al., 1993). The actinic flash was provided by a Quanta Ray DCR-11 Nd–YAG laser (532 nm, 7 ns, 5–10 mJ/pulse; Spectra Physics, Mountain View, CA). Proton pumping was monitored using the pH-sensitive dye pyranine, which exhibits a decrease in absorbance at 460 nm upon protonation. Samples were in unbuffered KCl (100 mM, 20 °C). The pH was adjusted to 7 (±0.1) for the proton pumping experiment by the addition of sodium hydroxide (1.0 M).

Electrophoretic Analysis of bR Pigment Analogs. Pigment analogs (10 μg) were dissolved in sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 5% β-mercaptoethanol), sonicated for 15 min, heated at 80 °C for 5 min, and run on SDS–12% polyacrylamide precast minigels (Bio-Rad Laboratories, Hercules, CA). Each gel was then soaked in 1 M KCl solution for several seconds until protein bands appeared (Bergman & Jornvall, 1987). Gel slices containing protein bands, as well as slices along a lane as background, were cut and incubated in 1 mL of Solvable (New England Nuclear Research Products, Boston, MA) solution (1:1 dilution) at 55 °C for 3–4 h. The solutions were diluted with 10 mL of Aquasol (New England Nuclear Research Products, Boston, MA) and 50 μL of glacial acetic acid and counted in a liquid scintillation system.

RESULTS

The candidate amino acid residues in the retinal binding pocket were selected from models using the molecular modeling software SYBYL (Beischel et al., 1994) based on data from Henderson et al. (1990). The criteria for selection of residues for mutation to cysteine were proximity to C4 of retinal, size of the amino acid side chain, and similarity of electrochemical properties to cysteine. The possible mutation sites were selected by screening in three dimensions around the C4 position of the cyclohexyl ring of retinal. The selected amino acids were modeled as cysteines, and measurements were made from the sulfur atom of the cysteine to the C4 atom of retinal. The single cysteine-substituted mutants should cause minimal perturbation of the native structure. Being as conservative as possible on changes of the size and electrochemical properties, we chose sites Met118, Thr121, and Ser141 as candidates. The predicted distances between the sulfur atom on cysteine from these sites to C4 on retinal were Met118, 4.03 Å; Thr121, 3.35 Å; and Ser141, 3.60 Å (Figure 2).

The pMC-1 plasmid containing the wild-type or mutant *bop* gene can exist as an autonomously replicating form or integrated into the *H. salinarium* chromosome (Needleman et al., 1991). It is possible for the plasmid-borne wild-type or mutant *bop* gene to recombine at the *bop* chromosomal locus, or expression of the original chromosomal *bop* gene could occur by excision of the IS*HI* element. Therefore, Southern blot analysis was performed to insure that the IS*HI* element interrupting the chromosomal *bop* gene of strain IV-8 had not been excised and that no obvious recombination event had occurred between the plasmid-borne and chromosomal *bop* gene. Southern blot analysis of chromosomal DNA demonstrated that the wild-type and cysteine-containing mutant transformants maintained the chromosomal *bop*::IS*HI* insertion (data not shown). *H. salinarium* IV-8 has a single 5.9-kb *Pst*I fragment that corresponds to the chromosomal *bop* gene containing the 1.1-kb IS*HI* insert. The 5.9-kb

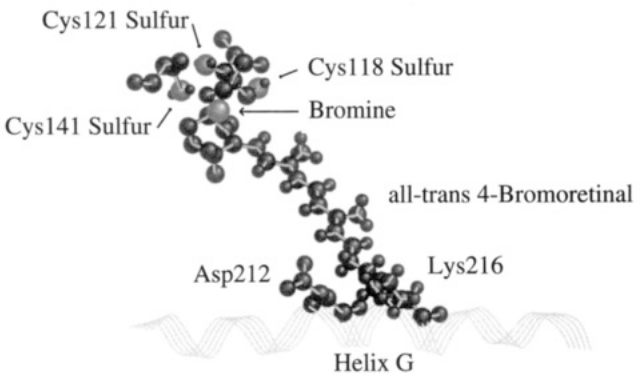

FIGURE 2: Molecular model of bacteriorhodopsin based on data of Henderson et al. (1990). Met118, Thr121, and Ser141 were changed to cysteines by site-directed mutagenesis. Distance measurements were taken from the sulfur of the cysteine to the C4 of retinal: Met118C, 4.03 Å; Thr121C, 3.35 Å; Ser141C, 3.60 Å.

Table 1: Absorption Maxima of Native bR Pigments and Regenerated Pigment Analogs[a]

	absorption maxima (nm)		
bacterioopsin	*all-trans*-retinal	*all-trans*-4-bromoretinal	NEM-treated bacterioopsin/*all-trans*-4-bromoretinal pigment
wild type	570	470	470
Met118Cys	563	464	470
Thr121Cys	560	470	470
Ser141Cys			

[a] All the pigments were suspended in 50 mM sodium phosphate buffer, pH 7.4.

fragment which hybridizes to *bop* is present in both wild-type and mutant transformants. The transformants contain the 4.8-kb *Pst*I fragment, which is identical to that of pMC1-*bop* plasmid (data not shown). The cysteine-containing *bop* genes were amplified by polymerase chain reaction, and the entire open reading frame was sequenced to ensure that the transformed mutant *bop* genes were fully intact and had only the desired sequence changes.

The Met118Cys and Thr121Cys pigments have absorption maxima which are slightly blue-shifted (5–10 nm) from that of the wild-type pigment (Table 1). Both undergo light–dark adaptation, and the light-dark spectra are similar to the wild-type (Figure 3a–c). The M-like intermediates in their photocycles are formed with kinetics similar to the M intermediate of the wild-type (Figure 3d–f). The kinetics of proton pumping in the two pigments show proton uptake and release similar to that of the wild type (Figure 3g–i).

While the Ser141Cys *bop* gene was expressed in IV-8 *H. salinarium*, the resulting cultures were pale in color and no colored pigment was observed when the membrane was purified on sucrose density gradients.

Pigment analogs were formed with *all-trans*-4-bromoretinal and the bleached membranes of the wild-type, Met118Cys, and Thr121Cys apoproteins. All three pigments had similar absorption maxima which were distinct from the corresponding *all-trans*-retinal pigments (Table 1). These three *all-trans*-4-bromoretinal pigments were stable when incubated with excess amounts of *all-trans*-retinal, confirming that *all-trans*-4-bromoretinal occupies the native retinal-binding site.

Cysteines have been placed individually in the protein at positions which are expected to be in close proximity to the retinal ring C4 position (Figure 2). The cross-linking reaction was designed to be between the *all-trans*-4-bromoretinal, which contains the potentially displaceable allylic halide, and the nucleophilic sulfhydryl group (Figure 4). It is anticipated that the reaction would occur *in situ* with the Schiff base being formed with the aldehyde and Lys216. Tritium labeling of the *all-trans*-4-bromoretinal allowed identification of the cross-linking by SDS–polyacrylamide gel analysis. On denaturation of the pigments in the gel sample buffer, the Schiff base is expected to cleave, but the thiol–ether linkage between the retinal ring and the protein is expected to remain intact. Analysis of excised bands from the SDS–polyacrylamide gel (Figure 5) showed the Thr121Cys and the wild-type pigment analogs to have a low background of labeling. In contrast, the Met118Cys pigment analog showed significant labeling (Figure 6).

NEM reacts with sulfhydryl groups to yield the stable ethyl ether derivative (Smyth et al., 1964); therefore, any cross-linking between the bromoretinal chromophore and the cysteine would be prevented. When the NEM-treated Met118Cys bR apoprotein was incubated with *all-trans*-4-bromoretinal, a pigment with an absorption maxima of 470 nm was obtained (Table 1). Analysis of the excised band from SDS–polyacrylamide gels showed that there was no significant incorporation of tritium (Figure 6). Therefore, formation of the ethyl thioether did not prevent the formation of pigment but did inhibit the cross-linking reaction. Pigment was also formed with the Thr121Cys apoprotein which had been incubated with NEM (Table 1), but the cross-linking results were unaffected, as expected.

In order to eliminate the possibility that cross-linking had occurred with *all-trans*-4-bromoretinal which was not in the chromophore binding pocket, the native pigmented membranes were incubated with excess amounts of ^{3}H-*all-trans*-4-bromoretinal. No significant increases in tritium incorporation were observed (Figure 6). This result emphasizes that the cross-linking between cysteine and *all-trans*-4-bromoretinal takes place only inside the chromophore binding pocket.

DISCUSSION

The use of molecular graphics allows insights and measurements on the bR molecule as well as building of accurate models of less complicated molecules such as retinal analogs (Beischel et al., 1994). On the basis of our modeling studies using the data of Henderson et al. (1990), we predicted three favorable sites, Met118, Thr121, and Ser141, to cross-link to *all-trans*-4-bromoretinal.

Interestingly, after mutant *bop* genes were expressed in IV-8 bR$^-$ *H. salinarium* cells, the Ser141Cys mutant did not have a pigmented membrane. Using bR monoclonal antibodies, we found that the bR membrane fraction from Ser141Cys mutant contained approximately an equal amount of bR protein compared to that of the wild type (data not shown). The events which could affect the appropriate expression of bR mutant pigment are (1) the retinal binding pockets of the mutants are structurally different from that of the wild type and the chromophore could be no longer effectively shuttled into its binding pocket, (2) the protonated Schiff base between the chromophore and apoprotein is destabilized in the mutant, or (3) the protein does not fold correctly. If the OH group of the Ser141 was contributing to a H-bond, the substitution of sulfhydryl hydrogen may weaken this bond since the optimal distance of a H-bond between a thiol group and a base is longer (3.5 Å) than that between a hydroxyl group and a base (Fersht et al., 1985).

The fact that good yields of functional pigment were obtained with both the Thr121Cys and the Met118Cys

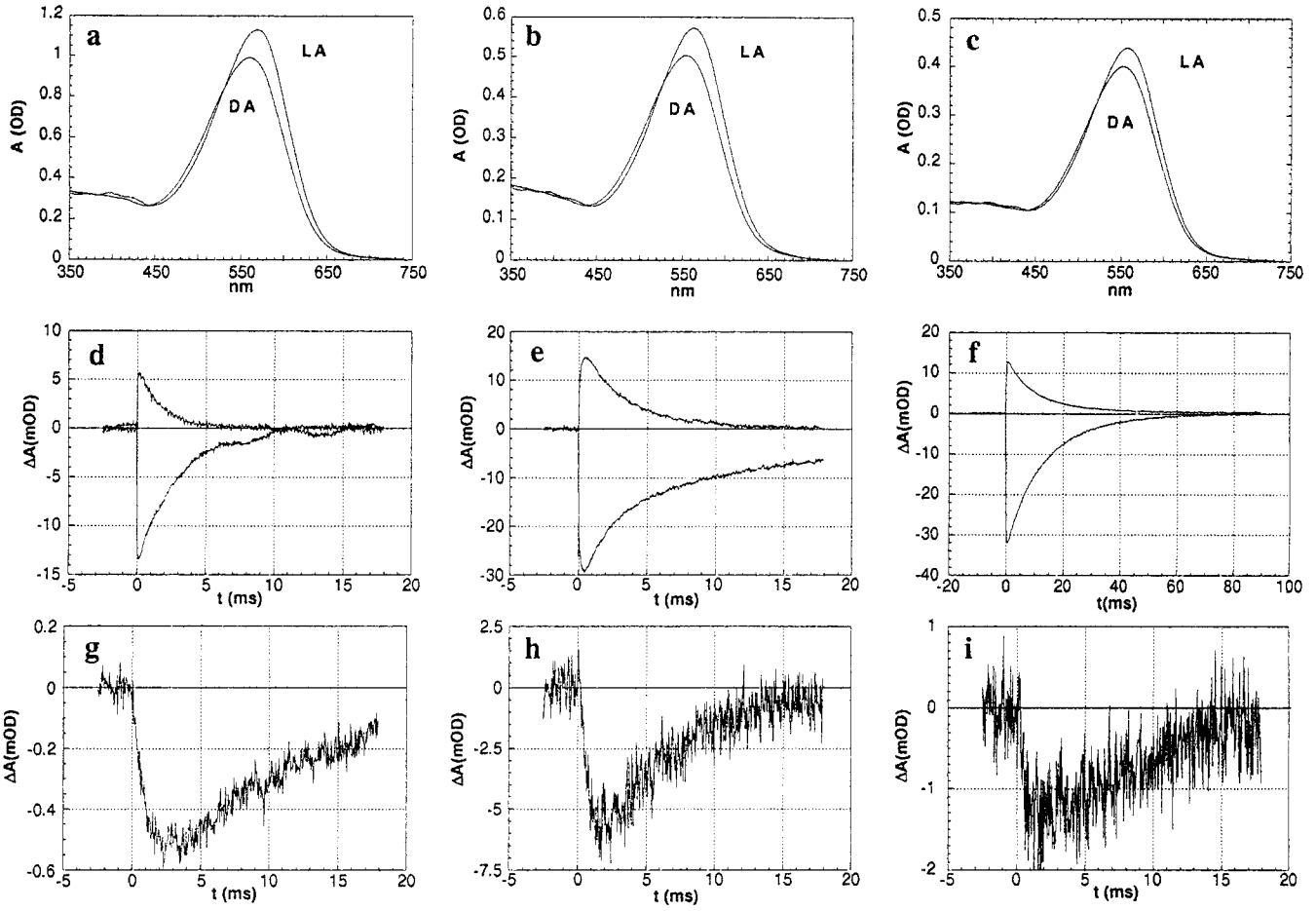

FIGURE 3: (a–c) Absorption spectra of light- and dark-adapted pigments containing the native chromophore. Light- and dark-adapted absorption spectra were recorded in 1-cm quartz cuvettes at room temperature using a Cary-Aviv 14 spectrophotometer (Aviv Associates, Lakewood, NJ). The wild-type, Met118Cys and Thr121Cys pigment samples (containing the native chromophore) in 1 M KCl were dark-adapted overnight before their absorption spectra were measured. The samples were illuminated for 8–10 min (with a filmstrip projector filtered at 500–550 nm) prior to measuring the light-adapted absorption spectra. Each sample was scanned from 350 to 750 nm in steps of 1 nm. (a) Wild type (maxima: LA 569 nm, DA 560 nm); (b) Met118Cys (LA 563 nm, DA 554 nm); (c) Thr121Cys (LA 558 nm, DA 554 nm). (d–f) Absorption change characteristics of the M intermediates and ground states of pigments containing the native chromophore measured using a homemade kinetic spectrophotometer as described in Balashov et al. (1993). The actinic flash was provided by a Quanta Ray DCR-11 Nd–YAG laser (532 nm, 7 ns, 5–10 mJ/pulse; Spectra Physics, Mountain View, CA). Wild-type (4 μM) or mutant (8 μM) samples were prepared in 100 mM KCl and the pH was measured just prior to each experiment. Samples were kept at 20 °C during the experiments. Each trace shown is the average of 64 flashes. The positive traces show absorbance changes at 410 nm and correspond to formation and decay of the M intermediate in each sample. The negative traces show absorption changes at the absorbance maximum of the light-adapted form of each sample and thus correspond to depletion and recovery of the ground state in each sample. (d) Wild-type bR, 4 μM, pH 6.6; (e) Met118Cys, 8 μM, pH 6.2; (f) Thr121Cys, 8 μM, pH 6.6. (g–i) Absorption changes of pyranine at 460 nm, indicating proton pumping. Experiments were performed using the kinetic spectrophotometer described above. Wild-type (5 μM) or mutant (20 μM) samples were prepared in 100 mM KCl with 20 μM (wild-type sample) or 1 mM (mutant samples) pyranine. pH was adjusted to about 7 (the pK_a of pyranine) and remeasured just prior to each experiment. Each trace shown is the average of 64 flashes. The initial decrease in absorption corresponds to proton release from the membrane, while the subsequent increase in absorption corresponds to proton uptake into the membrane. (g) Wild-type bR, 5 μM, 20 μM Pyr, pH 6.9; (h) Met118Cys, 20 μM, 1 mM Pyr, pH 7.0; (i) Thr121Cys, 20 μM, 1 mM Pyr, pH 7.1.

mutants indicates that our assessment of these mutations as being benign was correct. Models show that the distance from the Thr121 residue to the C4 of the cyclohexyl ring of retinal is less than that from Met118 residue. However, we did not observe the cross-linking in the Thr121Cys/4-bromoretinal pigment. The Thr121 residue has been cross-linked by photoaffinity labeling using *all-trans*-3-diazoacetoxyretinal (Ding et al., 1990). These results suggest that Thr121 residue is more likely to tilt toward the C3 position of the cyclohexyl ring of retinal than toward the C4 position.

The results from the experiments of Ding et al. (1990) and Boehm et al. (1990) are closer to the findings reported here than the findings of Huang et al. (1982). The experiments of Huang et al. showed the labeling of Ser193 and Glu194 with a photosensitive *m*-diazirinophenyl analog of retinal. However, the bR pigment analog formed with this photosensitive retinal analog has been reported to be nonfunctional (Boehm et al., 1990). When this analog was tested with two models of bR that had Ser193 and Glu194 built on as a helix and a random coil, these two amino acids were not among predicted labeling sites (Beischel et al., 1994).

The homologous expression of bR mutants in halobacteria is a promising approach for evaluating the chromophore binding site of bR. The absorption maxima of Met118Cys and Thr121Cys mutants are all slightly blue-shifted compared to that of the wild-type (Table 1). Spectrophotometric assays of Met118Cys and Thr121Cys bR mutants suggested that their binding sites have not been significantly perturbed from that of the wild type. The two pigments had light- and dark-adapted spectra and M intermediate kinetics similar to those of the wild type. The kinetics of the proton pumping as measured by pyranine are also similar to those of the wild type (Figure 3). All these results suggest that the chromophore binding pockets in these two mutants have not been signifi-

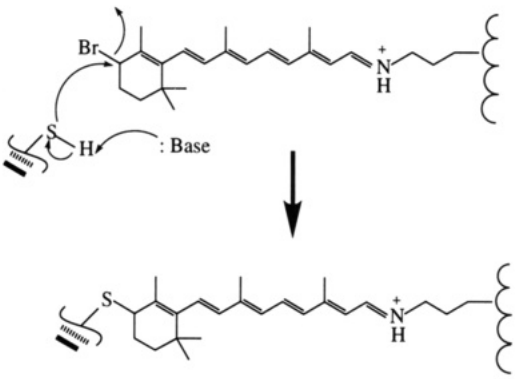

FIGURE 4: Affinity cross-linking: Model of the nucleophilic displacement by the cysteine sulfhydryl group of an active leaving group on retinal.

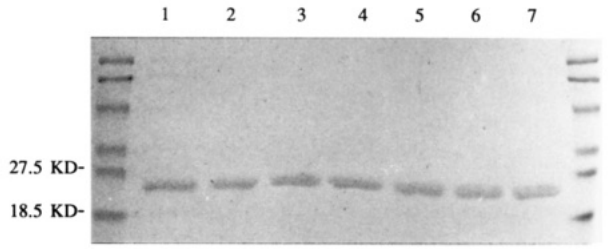

FIGURE 5: Electrophoresis gel of tritiated bR pigment analogs. The cross-linked pigments were run on 12% gradient SDS–polyacrylamide gels under denaturing conditions. Lane 1, WT/*all-trans*-retinal pigment; lane 2, Met118Cys/*all-trans*-retinal pigment; lane 3, WT/*all-trans*-4-bromoretinal pigment; lane 4, Met118Cys/*all-trans*-4-bromoretinal pigment; lane 5, WT pigment/excess *all-trans*-4-bromoretinal; lane 6, Met118Cys pigment/excess *all-trans*-4-bromoretinal; lane 7, NEM-treated Met118Cys/*all-trans*-4-bromoretinal pigment.

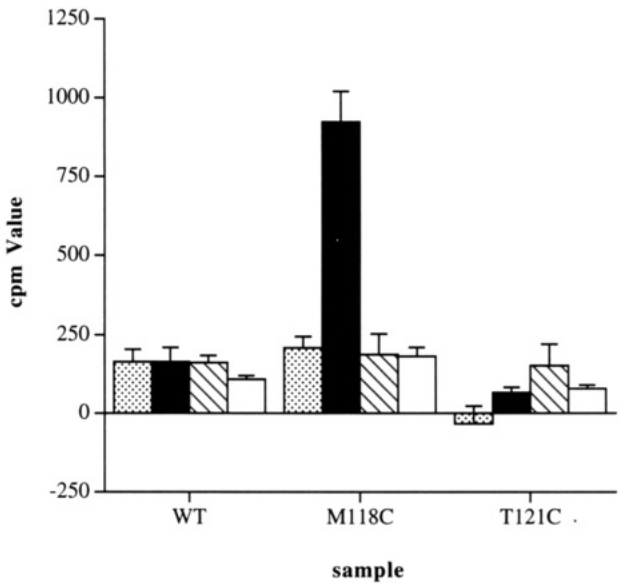

FIGURE 6: Distribution of radioactivity in the wild-type, Met118Cys and T121C bR pigments reconstituted with ^{3}H-*all-trans*-retinal or ^{3}H-*all-trans*-4-bromoretinal. Gel slices containing pigment protein bands were counted for tritium activity. All cpm values were corrected for background. Data shown are the mean values ± SEM (n = 3) for (stippled bars) ^{3}H-*all-trans*-retinal pigments; (solid bars) ^{3}H-*all-trans*-4-bromoretinal pigments; (hatched bars) NEM-treated pigments; and (open bars) native pigment/excess ^{3}H-*all-trans*-4-bromoretinal.

cantly altered from that of the wild type. Crouch et al. (1986) synthesized a series of 4-substituted retinal analogs for probing the bR binding site and found that the *all-trans*-4-chloro-, -4-bromo-, and -4-iodoretinals form pigments with the wild-type bacterioopsin but undergo an *in situ* displacement of the allylic halogen to form the 4-hydroxyretinal pigment several hours after pigment formation. The 4-hydroxyretinal pigment analog pumped protons efficiently, indicating that the *all-trans*-4-bromoretinal suitably fits the bR binding pocket. In order to eliminate the possibility that this reaction occurred in the process of cross-linking, the maximal absorptions of the *all-trans*-4-bromoretinal pigment analogs (at 460–470 nm; 4-hydroxyretinal pigment at 541 nm) were reconfirmed prior to the SDS–polyacrylamide gel analysis.

We predicted that cross-linking could occur *in situ* with *all-trans*-4-bromoretinal if a cysteine residue was introduced into the binding site. Sweet et al. (1972) synthesized 16α-(bromoacetoxy)progesterone and studied its reactions with various amino acids. This compound, which contains a bromine as an active leaving group, alkylated cysteine, histidine, methionine, and lysine under physiological conditions. It was found that cysteine was the fastest and methionine was the slowest reactant, but amino acids such as glycine, serine, and phenylalanine gave no evidence of alkylation (Sweet et al., 1972). Since bR has neither a cysteine nor a histidine residue present in the native structure, cysteine, the fastest reactant, was therefore selected to be introduced into the predicted cross-linking sites. In model studies, we have shown that a synthetic peptide containing cysteine reacts with *all-trans*-4-bromoretinal to produce a thioether as expected (John Oatis, unpublished data).

A significant amount of cross-linking is observed in the Met118Cys pigment analog. Moreover, upon treatment with the cysteine-specific reagent NEM, the cross-linking was eliminated. All three NEM-treated apoproteins, Met118Cys, Thr121Cys, and wild type, can still generate pigment with *all-trans*-4-bromoretinal, so the slight steric hindrance from the additional ethyl group on the cysteine mutants resulting from the modification did not hinder pigment formation. As expected, NEM had no effect on the incorporation of tritium into wild type or the Thr121Cys mutant. To exclude the possibility that Met118Cys was labeled by *all-trans*-4-bromoretinal that was outside the retinal-binding pocket, we performed a cross-linking experiment with *all-trans*-retinal in the binding site and showed background levels of radioactivity. We have therefore demonstrated that Met118 is in the chromophore binding site, near the C4 position of the cyclohexyl ring, and that Thr121 is not in close proximity to this site. These results are in agreement with the helical wheel model presented by Henderson et al. (1990). Interestingly, the Met118 residue is conserved in a number of bacterial rhodopsins (Otomo et al., 1992).

Photoactivatable retinal analogs are difficult to synthesize and resultant cross-linking is only obtained in low yields. We have shown that *in situ* cross-linking based on thiol chemistry is a convenient and convincing method to probe the chromophore binding site in bR. The combination of the technique of protein site-specific mutagenesis with the use of retinal analogs presents an excellent opportunity to further elucidate the structure of the retinal-binding site of bR.

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REFERENCES

Altenbach, C., Marti, T., Khorana, H. G., & Hubbell, W. L. (1990) *Science 248*, 1088–1092.

Ausubel, F. M., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1989) *Current Protocols in Molecular Biology*, Wiley, New York.

Balashov, S. P., Govindjee, R., Kono, M., Imasheva, E., Lukashev, E., Ebrey, T. G., Crouch, R. K., Menick, D. R., & Feng, Y. (1993) *Biochemistry 32*, 10331–10343.

Beischel, C. J., Gray, K., Hazard, E. S., Knapp, D. R., & Crouch, R. K. (1994) *Biochem. Mol. Biol. Int. 32*, 933–939.

Bergman, T., & Jornvall, H. (1987) *Eur. J. Biochem. 169*, 9–12.

Boehm, M. F., Gawinowicz, M. A., Foucault, A., Derguini, F., & Nakanishi, K. (1990) *J. Am. Chem. Soc. 112*, 7779–7782.

Crouch, R. K. (1986) *Photochem. Photobiol. 44*, 803–807.

Crouch, R. K., Scott, R., Ghent, S., Govindjee, R., Chang, C., & Ebrey, T. G. (1986) *Photochem. Photobiol. 43*, 297–303.

DasSarma, S., RajBhandary, U. L., & Khorana, H. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 125–129.

Ding, W., Tsipouras, A., Ok, H., Yamamoto, T., Gawinowicz, M. A., & Nakanishi, K. (1990) *Biochemistry 29*, 4898–4904.

Fersht, A. R., Wilkinson, A. J., Carter, D., & Winter, G. (1985) *Biochemistry 24*, 5858–5861.

Hartman, F. S. (1977) *Methods Enzymol. 46*, 130–153.

Henderson, R., & Unwin, P. N. T. (1975) *Nature 257*, 28–32.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.

Heyn, M. P., Westerhausen, J., Wallat, I., & Sieff, F. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2146–2150.

Huang, K., Radhakrishnan, R., Bayley, H., & Khorana, H. G. (1982) *J. Biol. Chem. 257*, 13616–13623.

Jubb, J. S., Worcester, D. L., Crespi, H. L., & Zaccai, G. (1984) *EMBO J. 3*, 1455–1461.

Katusz, R. M., Bono, B., & Colman, R. F. (1992) *Biochemistry 31*, 8984–8990.

Kramer, B., Kramer, W., & Fritz, H. J. (1984) *Cell 38*, 879–887.

Menick, D. R. (1991) in *Methods in Nucleic Acids Research* (Karam, J. D., Chao, L., & Warr, G. W., Eds.) pp 45–59, CRC Press, Inc., Boca Raton, FL.

Needleman, R., Chang, M., Ni, B., Varo, G., Fornes, J., White, S., & Lanyi, J. (1991) *J. Biol. Chem. 266*, 11478–11484.

Ni, B., Chang, M., Duscht, A., Lanyi, J., & Needleman, R. (1990) *Gene 90*, 169–172.

Oesterhelt, D., & Stoeckenius, W. (1971) *Nature 233*, 149–152.

Or, Y. S., Clark, R., & Luly, J. (1991) *J. Org. Chem. 56*, 3146–3149.

Otomo, J., Tomioka, H., Urabe, Y., & Sasabe, H. (1992) in *Structures and Functions of Retinal Proteins* (Rigaud, J. L., Ed.) pp 105–108, Colloque INSERM/John Libbey Eurotext Ltd., Montrouge, France.

Ottolenghi, M., & Sheves, M. (1989) *J. Membr. Biol. 112*, 193–212.

Resek, J. F., Farahbakhsh, T., Hubbell, W. L., & Khorana, H. G. (1993) *Biochemistry 32*, 12025–12032.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Smyth, D. G., Blumenfeld, O. O., & Konigsberg, W. (1964) *Biochem. J. 91*, 589–595.

Sweet, F., Arias, F., & Warren, J. C. (1972) *J. Biol. Chem. 247*, 3424–3433.

Tokunaga, R., Govindjee, R., Ebrey, T. G., & Crouch, R. K. (1977) *Biophys. J. 19*, 191–198.