Structure-Function Studies of Bacteriorhodopsin XV
EFFECTS OF DELETIONS IN LOOPS B-C AND E-F ON BACTERIORHODOPSIN CHROMOPHORE AND STRUCTURE

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Bacteriorhodopsin mutants containing deletions in loop B-C, ΔThr67–Glu74 or ΔGly66–Gln76, or a deletion in the loop E-F, ΔGlu161–Ala168, were prepared. Following their expression in Escherichia coli, the mutant proteins were purified to homogeneity and refolded with retinal in detergent-phospholipid mixtures. The mutants containing deletions in the loop B-C were normal at 4°C but showed the following changes at 20°C: 1) The λmax, shifted from 540 to below 510 nm; 2) the rates of bleaching by hydroxyamine in the dark increased; and 3) the rate and steady state of proton pumping decreased. Deletion of the eight amino acids in loop E-F did not affect wild-type behavior. However, all the mutant proteins were more prone to thermal and sodium dodecyl sulfate denaturation than the wild-type bacteriorhodopsin. These observations show that the structures of the B-C and E-F loops are not essential for correct folding of bacteriorhodopsin, but they contribute to the stability of the folded protein.

EXPERIMENTAL PROCEDURES

Materials

T4 DNA ligase was purified from Escherichia coli lysogenized with phage λ expressing T4 ligase (19). Restriction endonucleases were from New England Biolabs and Boehringer Mannheim. The Klenow fragment of E. coli DNA polymerase I was purchased from Bethesda Research Laboratories. The “slow” form of nuclease Bal-31 was obtained from International Biotechnologies, Inc. Radiolabeled nucleotides were purchased from Amersham Corp. Nitrocellulose BSA5 filter was from Schleicher and Schuell. Media for the growth of bacteria were supplied by Difco. DEAE-Trisacryl was obtained from Bio-Rad Laboratories. L-[14C]glucose was from Amersham Corp. RNase A was from Boehringer Diagnostics; DMPC was from Avanti Polar Lipids, Inc. Nucleotides, ampicillin, Nonidet P-40, phenylmethylsulfonyl fluoride, lysine, antifoam C, and RNase A were obtained from Sigma. All-trans retinal and 13-cis retinal were from Kodak.

Methods

General Recombinant DNA Methods—Digestions with restriction enzymes were performed as recommended by Fuchs and Blakesley (11) or by the commercial suppliers of the endonucleases. Both small- and large-scale plasmid preparations were done by alkaline sodium dodecyl sulfate lysis procedures (12, 13). In large-scale preparations, plasmid DNA was purified by equilibrium centrifugation in cesium chloride gradients containing ethidium bromide and gel filtration on Bio-Gel A-50m. Restriction digestions were monitored on 0.6–1.7% agarose gels containing 0.5 μg/ml of ethidium bromide. Restriction fragments for ligations were purified from SeaPlaque agarose gels (14).

Construction of Deletions in bop Gene—The pXH/Gal 101 plasmid (15) was digested with either KpnI or SpeI to completion. An aliquot of DNA (1 μg) was treated with 0.16 units of slow Bal-31 in 10 μl of 20 mM Tris-HCl (pH 8.0), 0.6 M NaCl, 12.5 mM MgCl2, and 12.5 mM CaCl2 at 0°C for 15–60 s. The reaction was stopped with 0.1 M EDTA. Pure DNA was recovered and treated with the Klenow fragment of DNA polymerase I, in the presence of all four deoxynucleotide tri-
phosphates, and then treated with T4 DNA ligase. The ligation mixture was used directly to transform the E. coli Δlac strain LG90 by the procedure of Hanahan (16, 17). The transformants were initially screened after 16 h of growth at 37 °C on lactose McConkey plates supplemented with ampicillin (35 μg/ml). Plasmids from lac colonies were isolated by the above method (25). The size of each deletion was determined by further restriction analysis of plasmid DNA from each lac' clone and demonstration of the changed electrophoretic mobilities of two bop AvaII fragments. The loop B-C deletions were transferred to M13 and sequenced by the dyeoxy chain termination method (18). The loop E-F in-frame deletions were sequenced as described previously for bop DNA (19). The size of each deletion was determined by further restriction analysis of plasmid DNA from each lac' clone and demonstration of the changed electrophoretic mobilities of two bop AvaII fragments. The loop B-C deletions were transferred to M13 and sequenced by the dyeoxy chain termination method (18). The loop E-F in-frame deletions were sequenced as described previously for bop DNA (19).

Expression, Purification, and Immunological Identification of ebO Deletion Mutants—E. coli C600 (pC857) strain (20) was transformed with the pPLB05-CTA vectors (Fig. 2). Expression of the ebO mutants by temperature induction, their solvent extraction, and DEAE-Trisacryl purification were done as described previously for ebO (21, 22). The purified proteins (2 mg/ml) were dissolved in 1% SDS (w/v) and stored at -20 °C as lyophilized powders of these solutions. Immunodetection was essentially as described (21).

UV-visible Absorbance Measurements and Protein Determination—UV-visible spectra were taken on a Beckman DU-7 or Perkin-Elmer Lambda 1 spectrophotometer in 1-cm path length quartz cells. The temperature was controlled to within 0.1 °C with jacketed cell holders connected to a circulating bath (NENSCAL ENDOCAL). Retinal concentrations were based on molar extinction of 45,000 for all-trans retinal and 35,000 M⁻¹ cm⁻¹ for 13-cis retinal in absolute ethanol at 380 nm. Protein concentration was based on 280-nm extinction coefficients of 68,000 M⁻¹ cm⁻¹ for ebO and 79,000 M⁻¹ cm⁻¹ for DMPC/CHAPS/SDS at pH 6.0 (23). Extinction coefficients for the chromophores of the mutants were calculated from the initial slopes of the retinal binding curves of the proteins (24). The chromophores of the mutants were calculated from the initial slopes of the retinal binding curves of the proteins (24). The chromophores of the mutants were calculated from the initial slopes of the retinal binding curves of the proteins (24). The chromophores of the mutants were calculated from the initial slopes of the retinal binding curves of the proteins (24). The chromophores of the mutants were calculated from the initial slopes of the retinal binding curves of the proteins (24). The chromophores of the mutants were calculated from the initial slopes of the retinal binding curves of the proteins (24). The chromophores of the mutants were calculated from the initial slopes of the retinal binding curves of the proteins (24).

Circular Dichroism Measurements—Reconstitution of the mutants was essentially as described, with minor modifications to accommodate their instability (25). Protein (0.8 mg) was dissolved in 1.0 ml of 0.1 M to a quartz cuvette containing regeneration mixture prepared as described previously (26). The samples were equilibrated at each temperature in the spectrophotometer chamber, in the dark. Scans (250-750 nm) lasting 25 s or less were taken at the indicated times after addition of hydroxylamine.

RESULTS

Production of bop Deletion Mutants

We aimed to delete only portions of the amino acids presumed to be in the loops B-C and E-F of bO without introducing new amino acid changes (Fig. 1). Our strategy was to cut the plasmid carrying a bop:lacZ gene fusion (Fig. 2) at restriction sites KpnI or SpIh, corresponding to loops B-C and E-F, respectively. The cuts were extended on both sides by limited treatment with Bal31 exonuclease at 0 °C, and the DNA was religated. We screened for in-frame deletions of the appropriate size in three steps, as follows. 1) We transformed an E. coli Δlac strain with the mutated plasmids, expecting in-frame lac' clones at a frequency of 33%. 2) We checked that lac' clones had lost the KpnI or SpIh site, to distinguish in-frame deletions from parents. 3) We estimated the sizes of the deletions from the altered mobilities of two bop AvaII fragments on sequencing gels. Ten percent, rather than 33% of the transformants were lac'. It is possible that the remaining molecules were merely blunt-ended by mild exonuclease treatment and religated out of frame. Virtually no parental bands were found, and approximately 40% of the clones had dele-

SDS Denaturation—The kinetics of SDS denaturation were determined by mixing, in the dark, 250 μl of a regeneration mixture (26) pre-equilibrated at 20 °C and 75 μl of 10% SDS (w/v). The sample was quickly inverted three to four times, and recording of its 250-750-nm UV-visible spectrum was begun within 30 s. During the first 5 min the UV-visible spectrum was taken at 1-min intervals. Then the spectra were recorded less frequently, until only the absorbance of retinal remained, near 385 nm.

For equilibrium studies of the extent of denaturation in varying concentrations of SDS, 125-μl aliquots of regeneration mixture (26) were mixed in the dark with solutions of SDS, calculated to give final SDS concentrations ranging from 0.1 to 4.0% in a total volume of 250 μl. They were allowed to equilibrate 20-24 h at 30 °C, in the dark, and their dark-adapted UV-visible spectra were recorded the following day.

Hydroxylamine Bleaching of Regenerated ebO Mutants—Hydroxylamine hydrochloride (pH 6.0) was added to a final concentration of 0.1 M to a quartz cuvette containing regeneration mixture prepared as described previously (26). The samples were equilibrated at each temperature in the spectrophotometer chamber, in the dark. Scans (250-750 nm) lasting 25 s or less were taken at the indicated times after addition of hydroxylamine.

FIG. 1. A secondary structure model for bacteriorhodopsin. The seven helices embedded in the membrane are designated by letters A-G. The amino acids deleted in loops B-C and E-F are indicated by solid lines.
The deletion mutations in bop were transferred unidirectionally to the expression vector pPLBO5-CT (21) for temperature-inducible expression. The SphI and KpnI sites in the native bop gene are in sequences encoding loop E-F and loop B-C, respectively. The scheme is shown here for deletions in the loop E-F.

**Temperature-inducible Production of the Mutated Proteins**

The deletion mutations of bop were transferred unidirectionally to the expression vector pPLBO5-CT (21), downstream of the λ Pp promoter (Fig. 2). Since the pXB/Gal 101 plasmid (15) lacks suitable unique sites for transfer of the bop gene, a smaller vector, pLBB-Nae, was constructed with unique NheI and BanII sites. The subsequent construction of pPLBO5-CTΔ is shown in Fig. 2. For bop expression, the λ cI857 temperature-sensitive repressor was provided in trans so that induction could be carried out by a temperature shift.

**Characterization of the Bacteriorhodopsin Deletion Mutants**

**Electrophoretic Mobilities and Immunoblotting**—The purified mutant eBo’s were identified by Western blot analysis, using the monoclonal antibody BR114, specific for the bR carboxyl terminus (27). The increased electrophoretic mobility of the mutated proteins further confirmed the deletions (Fig. 3). The yield of purified protein was about 0.1 mg/g of wet E. coli cells in all cases.

**Rates of Chromophore Regeneration**—All the mutated proteins folded, bound retinal, and regenerated bR-like chromophores. Regeneration, on the whole, was slower (Fig. 4). The half-times of regeneration for eBo, ΔThr<sup>70</sup>-Glu<sup>75</sup>, ΔGly<sup>69</sup>-Gln<sup>75</sup>, and ΔGlu<sup>61</sup>-Ala<sup>68</sup>, measured at their wavelength of maximal absorption at 20 °C, were, respectively 84, 120, 150, and 210 s with all-trans retinal. The extent of regeneration varied, being 76, 60, 73, and 52% for eBo, ΔThr<sup>70</sup>-Glu<sup>75</sup>, ΔGly<sup>69</sup>-Gln<sup>75</sup>, and ΔGlu<sup>61</sup>-Ala<sup>68</sup>, respectively.

**UV/Visible Absorption Characteristics**—The deletion mutant ΔGlu<sup>61</sup>-Ala<sup>68</sup>, following regeneration, was like eBo (Fig. 5B) (22). Its chromophore showed normal light-dark adaptation (shift from 554 to 560 nm). The absorption characteristics were unaffected by temperature, changing less than 2 nm as the temperature was increased from 4 to 30 °C (Fig. 6D).

The mutants ΔGly<sup>69</sup>-Gln<sup>75</sup> (Fig. 5A) and ΔThr<sup>70</sup>-Glu<sup>75</sup> showed little or no light adaptation, and the absorption of their chromophore was very dependent on temperature (Fig. 6D). The dark-adapted chromophore absorptions were 541 and 533 nm, respectively, for mutants ΔThr<sup>70</sup>-Glu<sup>75</sup> and ΔGly<sup>69</sup>-Gln<sup>75</sup> at 4 °C. As the temperature was raised to 30 °C, their absorption broadened and shifted toward shorter wavelengths by more than 30 nm (Fig. 6D). The temperature effects were completely reversible for both mutants at up to 50 °C.

**Thermal Denaturation, as Measured by Changes in Circular Dichroism**—Large negative ellipticity at 208 and 222 nm in
for all three mutants (Fig. 6C). This transition was completely reversed on cooling. During heating, the negative ellipticity of the mutated proteins decreased more than eBR, indicating some loss of secondary structure (Fig. 6C). These thermal effects agreed well with the results of thermal denaturation monitored for the loop B-C mutants by UV-visible spectroscopy (Fig. 6D).

Proton Pumping after Reconstitution in Vesicles—ΔGlu₆¹-Ala₁₆₈ pumped protons like eBR. The initial rate of pumping was 3-5 H⁺/chromophore/s, and the steady-state level pumped was 30-50 H⁺/chromophore, under the standard conditions (Fig. 7) (22). Temperature dependence effects were studied under the conditions described under “Methods.” We obtained initial rates comparable with those measured under the standard conditions (the steady-state levels rose approximately 10-fold for each protein). ΔGlu₆¹-Ala₁₆₈ functioned as well as or better than eBR over the entire range of 4-35 °C (Fig. 6A).

ΔThr₆⁷-Glu₇₄ and ΔGly₆₅-Gln₇₅ pumped protons at initial rates 10 times lower and plateau levels three times less than eBR, under the standard conditions (Fig. 7). In contrast to the loop E-F mutant, temperatures exceeding 15 °C caused a reduction in these mutants’ initial rates of pumping (Fig. 6A).

Denaturation in Sodium Dodecyl Sulfate—The course of the denaturation was followed spectroscopically after adding 2.5% SDS (w/v) to protein which had been regenerated in DMPC/CHAPS/SDS micelles. At 20 °C, a denaturation intermediate at 600 nm and another at 440 nm were readily observed for eBR before the release of free retinal (Fig. 8). The 600-nm intermediate appeared first and decayed into the

the CD spectra indicated substantial α-helicity for eBR and the deletion proteins. The 222-nm values of spectra taken over a temperature of 4-40 °C obeyed the shape of a melting curve, with a midpoint temperature (T_m) value close to 22 °C
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440-nm species with a $t_0$ of 2 min. For early spectra, an isosbestic point could be seen at 490 nm. The disappearance of the 440-nm species left only the absorbance of free retinal. In sharp contrast to the wild-type behavior, all of the deletion mutants immediately denatured to the 440-nm species without the 600-nm transition (Fig. 8). Free retinal was released directly from their shorter-lived 440-nm species, with an isosbestic point at 420 nm.

The general stabilities of the ebR mutants were also compared by their extents of denaturation at equilibrium (16 h of incubation) in concentrations of SDS ranging from 0.1 to 4.0% (w/v). ebR was more stable to SDS than any of the mutants (Fig. 9). Their order of stability, determined by the SDS concentrations at which half of the original chromophore remained, was ebR (1.9%) $>\Delta$Thr$^{67}$-Glu$^{74}$ (1.4%) $>\Delta$Gly$^{65}$-Gln$^{75}$ (1.1%) $>\Delta$Glu$^{61}$-Ala$^{68}$ (0.78%) (Fig. 9).

Bleaching by Hydroxylamine—Irradiation is required to render the Schiff base of br accessible to water-soluble reagents like hydroxylamine (28). We followed the reaction of the Schiff base of the mutants with hydroxylamine in the dark as a measure of their chromophores’ environment. Hydroxylamine did not significantly bleach ebR in the dark, and mutant $\Delta$Glu$^{61}$-Ala$^{68}$ behaved identically to ebR (Fig. 6B). Rates of bleaching four to ten times higher were observed for $\Delta$Thr$^{67}$-Glu$^{74}$ or $\Delta$Gly$^{65}$-Gln$^{75}$ at 30°C. The activation energies for this transition were +14.6 kcal/mol for $\Delta$Thr$^{67}$-Glu$^{74}$ and +16.5 kcal/mol for $\Delta$Gly$^{65}$-Gln$^{75}$ (obtained from the Arrhenius relation $ln k = (-E_a/kT) + ln A$, where $k$ is the rate constant obtained at temperature $T$, $T$ is the temperature in Kelvin degrees, $R = 1.987$ cal/mol·K, and $A$ is also a constant).

4.0% (w/v). ebR was more stable to SDS than any of the mutants (Fig. 9). Their order of stability, determined by the SDS concentrations at which half of the original chromophore remained, was ebR (1.9%) $>\Delta$Thr$^{67}$-Glu$^{74}$ (1.4%) $>\Delta$Gly$^{65}$-Gln$^{75}$ (1.1%) $>\Delta$Glu$^{61}$-Ala$^{68}$ (0.78%) (Fig. 9).

DISCUSSION

Bacteriorhodopsin was the first transmembrane protein for which a structural model containing seven membrane-spanning $\alpha$-helices was proposed (6). A number of similar models has been advanced, for this protein, that differ only in the sizes of the individual helices and of the loops protruding from the lipid bilayer (7, 8). In particular, the location and size of the B-C and E-F loops in these models are based on enzymatic digestion studies including chromotryptic cleavage (B-C loop) and photoaffinity labeling (E-F loop) (7). Other information from spin labeling and proteolysis lends further support to the loop identification (8, 30). Most recently, Henderson et al. (9) used electron microscopy to reconstruct a three-dimensional image of bR. Their interpretation of this image, incorporating a comprehensive body of structural data, has led to the most detailed bR model to date. This model includes atomic structures for six of the helices. Fig. 1 shows the helix and loop assignments proposed in this work (9).

What are the contributions of the individual loops shown in Fig. 1 to folding and stability? Are their lengths required for optimal helix-helix interactions in the membrane? Are any biochemical functions served by the loops? We chose to study the effect(s) of reductions in the sizes of the two larger loops (B-C and E-F) on the structure and function of bR. We created two deletions in the B-C loop and one in the E-F loop to approach the questions raised above (Fig. 1).

Each of the three deletion mutants was able to fold; however, their stabilities toward specific denaturants were found to be different. The helices pack to give reasonable rates of retinal binding (Fig. 4), form characteristic chromophores (Fig. 5), and show proton pumping (Fig. 7). The general stability, as measured by SDS and thermal denaturation was diminished in a similar way for all the mutants. The effects of deletions in the two loops differ in their influence on the stability of the retinal environment. Thus, in the B-C loop mutants, proton pumping is greatly reduced, and the chromophore is more easily accessible to hydroxylamine than in the E-F mutant.

We drew the following interpretations from our results. The basic structure of the bR molecule is not dictated by the detailed arrangement of the loop structures, but the organization of the loops contributes to stability. If the extramembranous loops were completely disordered, one would expect
that shortening them would add to the stability of the protein by reducing the entropic term in folding, since the loops connect adjacent helices in the structure. Therefore the finding of destabilization supports a more ordered loop structure. Our CD data may be interpreted as supporting helical structure in the loops. The latest structural map of bR suggests that the loop regions are compact and that they may form short extramembranous α-helices (9).

Why is there such a dramatic difference between the stability of the mutants toward hydroxylamine and their stability toward SDS? From the rate of SDS denaturation, we would conclude that the E-F mutant was more denatured; yet hydroxylamine bleaching indicates the B-C mutants are more denatured. We may account for this apparent discrepancy by noting that the hydroxylamine bleaching rate is sensitive to highly localized perturbations of the structure and the retinal environment, whereas SDS denaturation acts more generally on the structure.

The very efficient refolding of bR from the denatured state for each of the mutants shows that strong specific packing interactions between the helices are retained and play a dominant role in bR folding. Thus, even bR cleaved through the loops will refold (7, 25, 26). For these interactions to be retained in the mutants, the deletions must be wholly contained in the loops, that is, not include helix initiation regions. In particular, loop E-F must be large enough to completely retain the eight amino acids deleted, since deletion has no significant effect on refolding or proton pumping. Therefore, we would like to propose that Henderson’s model (Fig. 1) should be adjusted, within his own estimated uncertainty of a few amino acids at the loop boundaries, so that Val\(^{167}\) and Ala\(^{168}\) are included in loop E-F. Photoaffinity labeling experiments also support this modification (7).

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