Thr-90 Plays a Vital Role in the Structure and Function of Bacteriorhodopsin*

The role of Thr-90 in the bacteriorhodopsin structure and function was investigated by its replacement with Ala and Val. The mutant D115A was also studied because Asp-115 in helix D forms a hydrogen bond with Thr-90 in helix C. Differential scanning calorimetry showed a decreased thermal stability of all three mutants, with T90A being the least stable. Light-dark adaptation of T90A was found to be abnormal and salt-dependent. Proton transport monitored using pyranine signals was ~10% of wild type for T90A, 20% for T90V, and 50% for D115A. At neutral or alkaline pH, the M rise of these mutants was faster than that of wild type, whereas M decay was slower in T90A. Overall, Fourier transform infrared (FTIR) difference spectra of T90A were strongly pH-dependent. Spectra recorded on films adjusted at the same pH at 243 or 277 K, dry or wet, showed similar features. The D115A and T90V FTIR spectra were closer to WT, showing minor structural differences. The band at 1734 cm\(^{-1}\) of the deconvoluted FTIR spectrum, corresponding to the carboxylate of Asp-115, was absent in all mutants. In conclusion, Thr-90 plays a critical role in maintaining the operative location and structure of helix C through three complementary interactions, namely an interhelical hydrogen bond with Asp-115, an intrahelical hydrogen bond with the peptide carbonyl oxygen of Trp-86, and a steric contact with the retinal. The interactions established by Thr-90 emerge as a general feature of archaean rhodopsin proteins.

The purple membrane patches of Halobacterium salinarum cells contain a single protein, bacteriorhodopsin (BR),\(^1\) which translocates protons from the interior to the exterior of the cell upon photon absorption by a retinal molecule (1). The native structure of bacteriorhodopsin consists of a bundle of seven densely packed α-helices forming a para-crystalline arrangement of BR trimers (2–8). Protein-protein and lipid-protein interactions as well as the lateral pressure exerted by the lipid chains on the protein molecules have important roles in this arrangement (9–11). Because of the high purple membrane density, the protein, in turn, should comply with some characteristics to allow the formation of the hexameric arrangement. Helix-helix interactions as well as retinal protein interactions are important elements involved in the BR compactness. When a relaxation of the protein structure occurs, the hexagonal arrangement appears impaired. One example refers to the bleached membrane, where the retinal absence compels the helices to lose several interactions (12, 13). Another case corresponds to the triple or quadruple mutants E9Q/E194Q/E204Q and E9Q/E74Q/E194Q/E204Q, which exhibit a more relaxed conformation as compared with WT (14, 15).

The mutant T90A shows some characteristics indicative of a certain degree of softening of both the para-crystalline arrangement and the interactions within the seven-helix bundle. This is demonstrated by the decreased cooperativity and temperature of the differential scanning calorimetry pre-transition, as well as by a decreased temperature of the main transition (16). Because Thr-90 most probably does not form part of the proton transport chain (6, 7), it is likely that its importance relies on structural aspects. An earlier work already demonstrated the inability of the iodoacetic acid-derivatized T90C to fold normally in detergent/phospholipid micelles (17), indicating that the –OH function and/or the side chain bulkiness is an important structural feature. According to the high-resolution BR structures, Thr-90 (helix C) and Asp-115 (helix D) form a hydrogen bond (Refs. 6 and 7, and see Fig. 1). These two amino acids, which are conserved among various archaeobacterial strains (18, 19), may be important in structural terms. Another significant structural interaction may be the hydrogen bond formed by the –OH group of Thr-90 with the peptide carbonyl oxygen of Trp-86, as bends and twists in transmembrane helices are induced by Thr through formation of an hydrogen bond with the backbone (20, 21). The BR structural models effectively show a kink in helix C at this level (Fig. 1). On the other hand, Pro residues are known as helix-breaking residues, and, thus, the adjacent Pro-91 may contribute to bending the helix. Again, sequence comparison shows that Pro-91 is conserved (18). Finally, there are van der Waals contacts between the methyl group of Thr-90 and the retinal chain that may also be important for the correct location of retinal.

In this work, we analyze in detail the behavior of the T90A, T90V, and D115A mutants to determine the structural and functional consequences of the disruption of the interactions involving the Thr-90 side chain. In the T90A mutant, we describe a chromoprotein with very different properties as compared with wild type BR, including a decrease in the efficiency of the proton transport and some critical alterations in the...
photocycle. In addition, the D115A and T90V mutations provide further knowledge about the role of the set of interactions involving Thr-90.

EXPERIMENTAL PROCEDURES

The construction and expression of T90A and D115A mutants in Halobacterium salinarum was carried out as described (14). The mutant T90V was a generous gift from Dr. J. K. Lanyi.

UV-visible spectra of dark- or light-adapted purple membrane suspensions (1.5 \( \times \) 10^{-5} M, and 3.5 \( \times \) 10^{-5} M in the case of T90A) were recorded with a Cary Bio3 spectrophotometer using an integrating sphere when necessary. The difference spectra were obtained by subtracting light-adapted minus dark-adapted samples.

Flash-induced transient absorbance changes were monitored using a LKS50 instrument from Applied Photophysics. A Q-switched neodymium-yttrium-aluminum-garnet (Nd:YAG) laser (Spectron Laser Systems; pulse width \( \approx 9 \) ns; \( E = 5 \) mJ/pulse/cm²; repetition frequency 0.5 Hz) at 532 nm was used for light excitation. Transient pH changes in the bulk medium were followed by measuring the absorbance changes of 50 \( \mu \)M pyranine at 460 nm in a purple membrane suspension in 1 M KCl, pH 7. Preparation of membrane films and spectra acquisition were done as described (23) with a Bio-Rad FTS6000 spectrometer at 2 cm⁻¹ resolution. At least three cycles of 350 scans were averaged (i.e. at least 1050 interferograms were accumulated per spectrum). Difference spectra were calculated by subtracting unphotolyzed BR from the corresponding photointermediate. Absorption spectra were Fourier self-deconvoluted using the Kauppinnen algorithm (24), with a Lorentzian band shape, a full width at a half-height of 10 cm⁻¹, and a band narrowing factor \( k \) of 2. When necessary, films were illuminated with blue light to drive any remaining intermediate back to BR. Differential scanning calorimetry (DSC) experiments were performed as described previously (16).

RESULTS

Light-Dark Adaptation—Light-dark adaptation was found to be abnormal in T90A. Fig. 2 shows the absorbance and difference spectra (light-adapted minus dark-adapted) in water, 150 mM KCl, and 1 M KCl (pH 7, room temperature). In H₂O, a decrease of the extinction coefficient and virtually no shift of the maximum upon illumination are observed. When the ionic strength is increased, a more normal behavior is observed (increase of the extinction coefficient and red shift upon light adaptation), due probably to the partial recovery of the normal isomer ratio in dark-adapted form. Under the conditions presented in Fig. 2, dark adaptation is very slow, taking at least 1 month to complete. In contrast, T90V and D115A show a normal dark adaptation, although all mutants present a shifted absorbance maxima as compared with WT (see Table I).

Photocycle Reactions and Proton Uptake and Release—Fig. 3A shows kinetic traces of the light-induced absorption changes for T90A, T90V, D115A, and WT in 1 M KCl, pH 6.5 (room temperature) normalized to the M amplitude. All mutants...
present a faster M rise and a slower M decay than WT (see Table I). Fig. 3A also shows that D115A presents a higher amount of O intermediate than T90A, T90V, or wild type and that T90A and T90V exhibit a longer-living O intermediate. At pH 10, like at neutral pH, the M rise of T90A and of T90V is faster and the M decay slower than those of the WT, whereas D115A has a faster M decay (Fig. 3B and Table I). On the other hand, at neutral or at alkaline pH, both the amplitude of the M intermediate (maximal signal at 410 nm) and the signal at the \( \lambda_{\text{max}} \) of 555 nm (disappearance of the BR form) of T90A are ~20% of the WT signal, whereas they are ~40% for T90V and ~50% for D115A. At pH 4, no signal was detected for T90A at

|                  | WT     | D115A  | T90V   | T90A   |
|------------------|--------|--------|--------|--------|
| Max. Abs. DA (nm)| 558    | 551    | 545    | 548    |
| Max. Abs. LA (nm)| 568    | 557    | 551    | 553    |
| M rise pH 6.5 (µs)| 4.1 (0.10) | 8.2 (0.30) | 4.5 (0.50) | 1.5 (0.70) |
| M rise pH 10 (µs)| 70 (0.90) | 50 (0.70) | 40 (0.50) | 60 (0.30) |
| M decay pH 6.5 (ms)| 3 (1.0) | 7.2 (0.90) | 4.5 (0.80) | 9 (0.70) |
| M decay pH 10 (ms)| 30 (0.20) | 20 (0.25) | 20 (0.35) | 20 (0.40) |
|                  | 51 (0.60) | 51 (0.60) | 155 (0.55) | 235 (0.60) |

**Fig. 3. Kinetics of photocycle intermediates.** The traces show the absorption changes corresponding to the M intermediate (410 nm; top), BR depletion and recovery (555 nm for T90A, T90V and D115A and 570 nm for WT; middle), and the O intermediate (660 nm; bottom). Column A, pH 6.5, 1 M KCl, 293 K. Column B, pH 10, 1 M KCl, 293 K. The absorption changes at 410, 660, and 570/555 nm, were normalized to the amplitude of the M intermediate for each pigment at the respective pH.
any of the wavelengths analyzed, indicating the absence of photocycle intermediates under these conditions, whereas both T90V and D115A showed small signals (data not shown).

Fig. 4 shows the pyranine signal for T90A, T90V, D115A, and WT at pH 7. As is apparent, a weak signal is obtained for T90A and T90V, amounting to −10% of the WT signal for T90A and −20% for T90V. This is in keeping with the low accumulation of the M intermediate obtained at neutral pH and with the decreased pumping efficiency of T90A incorporated into liposomes, which was found to be <20% (16). The signal of D115A also appears decreased, to −50% of the WT signal, again in accordance with the amplitude of the M intermediate for this mutant.

Fourier Transform Infrared Spectra—A general characteristic of the infrared difference spectra of T90A films, obtained by continuous illumination, is their low intensity in accordance with the small signal obtained in flash photolysis experiments. On the other hand, we have found that the difference spectra depend mainly on the pH. As shown in Fig. 5A, the spectra taken at pH 10, at either 243 or 277 K, dry or wet, are similar to each other and relatively similar to the WT M1 intermediate spectrum (25). Asp-85 appears protonated (band at 1762–1763 cm⁻¹), and a small negative band appears at 1747 cm⁻¹ that is not present in the WT difference spectrum. The amide I is also somewhat changed in comparison to the WT M1 intermediate. The pair of bands at 1640 cm⁻¹ (negative) and 1624 cm⁻¹ (positive) indicate the presence of a protonated Schiff base in the unphotolyzed pigment and a deprotonated Schiff base in the M1-like intermediate. In the amide II, the C = C stretching band appears at 1530 cm⁻¹, in keeping with the known inverse relationship with the λmax of the visible absorption spectrum, which is 550 nm at pH 10. On the other hand, the retinal negative peaks at 1201 and 1167 cm⁻¹ indicate that the retinal is in the all-trans configuration in the unphotolyzed state (26). It is interesting to note that the small negative peak at 1276 cm⁻¹ is not present, indicating that the conformational changes of the T90A photocycle do not affect Tyr-185 (27).

At pH 7 (Fig. 5B), the difference spectra depend more on the particular conditions, especially on the water content. The carboxylate band of Asp-85 is less evident, and it shifted to 1764–1768 cm⁻¹, depending on the temperature and the state of the sample. The negative band at 1698 cm⁻¹, which is particularly evident in the wet samples, probably corresponds to the band at 1692 cm⁻¹ in WT and T90A at pH 10. This shift may indicate that the corresponding reverse turns of the unphotolyzed protein have a different structure in wet samples at pH 7 from those at pH 10. The positive band at 1509 cm⁻¹ is also mainly seen in wet samples (Fig. 5B). It may be reminiscent of the band at 1506 cm⁻¹, seen in the O difference spectra (28). The most striking feature observed at pH 7 is the positive 1222 cm⁻¹ peak, which again is more intense in wet samples and is accompanied by a low intensity of the 1200 cm⁻¹ band. These features indicate that at neutral pH, the retinal adopts a distorted configuration in T90A as compared with WT. Difference spectra for D115A and T90V were more similar to WT, with only some deviation appearing in the amide I and in the fingerprinting region of the retinal (data not shown).

Fig. 6A presents FTIR difference spectra collected under conditions of the N intermediate. D115A shows an N-like intermediate, whereas the main feature of T90A and T90V is an M-like intermediate (compare with the spectrum of WT). In D115A, the peak of protonated Asp-85 is shifted to a more M-like position (1761 cm⁻¹), and the 1650 cm⁻¹ peak corresponding to helical conformational changes is absent, as in...
pared with WT (main transition at 98 °C). T90V and D115A show an intermediate behavior. They have the same main transition temperature (92 °C), but T90V shows a lower cooperativity. The mutations affect the pre-transition even more. As compared with WT, there is a decrease in the temperature of the pre-transition of 12 °C for D115A, 20 °C for T90V, and ~30 °C for T90A. Additionally, an important decrease in cooperativity is observed, especially for T90V and T90A. Overall, these data indicate a more relaxed structure of the BR mutants, giving rise, in turn, to a decreased stability of the paracrystalline arrangement.

**DISCUSSION**

The study of the BR mutants T90A, T90V, and D115A presented in this paper provides the means to evaluate the scope of the three interactions held by the Thr-90 side chain, namely the steric interaction with the retinal at the level of C_{11}-C_{13} and the hydrogen bonds established with Asp-115 and the carbonyl oxygen of Trp-86. In accordance with our previous conclusions (16), the results reveal Thr-90 as a key element in the structure of BR. Focusing on proton pumping, mutagenesis on Thr-90 yields a protein with a clear decrease in the proton-pumping ability. Thus, substitution of Thr-90 with Ala, which avoids all the interactions of residue 90, decreases the pumping efficiency to only ~10% of the pyranine signal in comparison with WT. Similarly, substitution of Thr-90 with Val, which keeps the steric interaction with the retinal but loses the hydrogen bonds, shows a proton pumping ~20% of that of WT. In accordance with this trend, the D115A mutant, which only loses the hydrogen bonding of Thr-90 with Asp-115, shows a proton pumping of ~50% as compared with WT. Previous data on T90V expressed in *Escherichia coli* and reconstituted into liposomes also showed a somewhat decreased proton-pumping activity, although not as important as in our case (~70%; Ref. 30), whereas T90C showed similar proton pumping as WT (17).

This discrepancy may be due to a more relaxed conformation of the protein in the monomeric state that is obtained by reconstitution, as compared with the crystalline lattice of purple membrane.

The kinetics and yield of the photocycle intermediates constitute a valuable appraisal of the disruption of the function of BR produced by the mutations. First of all, the amount of M intermediate is much decreased in T90A compared with WT and is decreased to half of the value of WT in T90V and D115A, suggesting a back-reaction to the purple form from one of the

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**Fig. 7.** DSC thermograms of wild type and mutants D115A, T90V, and T90A. Purple membrane patches were suspended in H_{2}O at a concentration of 2.0 mg/ml, pH 7. The curves were corrected with the instrumental and chemical baselines. Scans were taken at 1.5 K/min. Scale bar represents an apparent heat capacity of 5.10^{-4} cal/°C.

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**Fig. 6.** N-like FTIR difference spectra and carboxylic region of the FTIR deconvoluted spectra of T90A, T90V, D115A, and WT. A, comparison of T90A, T90V, D115A, and WT FTIR difference spectra under conditions for WT to yield the N intermediate (pH 10, wet sample, at 277 K). WT; T90A; T90V; and D115A. B, carboxylic region of the deconvoluted spectra of purple membrane dry films at 293 K obtained from membrane suspensions in 150 mM KCl, pH 7. The curves were corrected with the instrumental and chemical baselines. Scans were taken at 1.5 K/min. Scale bar represents an apparent heat capacity of 5.10^{-4} cal/°C.
intermediates preceding M. On the other hand, the M rise is faster than WT in all mutants at neutral or alkaline pH, whereas the M decay is slower in all mutants at both pH conditions, except for D115A, which shows faster M decay kinetics at alkaline pH. This indicates that the deprotonated state of the Schiff base, i.e., the M intermediate, is favored in T90A and T90V, in agreement with FTIR data (see Fig. 6A). On the other hand, the absorbance maximum and dark-light adaptation properties give information on the environment of the retinal chromophore. As described under “Results,” all three mutants show shifted absorbance maxima, but only T90A has abnormal dark-light adaptation kinetics. This means that the steric interaction between the methyl group of Thr-90 and the retinal participates in the dark-light adaptation process. Anomalous percentage of the all-trans and 13-cis isomers in the mutant, as was described for T90V expressed in E. coli and reconstituted into liposomes (30), can be another expression of this altered environment of the retinal chromophore.

FTIR difference spectra on T90A reinforce the idea of a strong distortion of the photocycle due to the mutation and depend essentially on the pH. The fact that a dry sample at 243 K gives rise to a spectrum nearly identical to that of a wet sample at 277 K, both at the same pH (see Fig. 5), is a clear demonstration of the insensitivity of T90A on hydration level and temperature. On the other hand, under the experimental conditions for which the WT films produce an almost pure N intermediate, D115A gives rise to an N-like intermediate with some alterations and T90V yields a mixture of M and N intermediates, whereas T90A does not show any N-like intermediate. At neutral pH, T90A appears to behave in an even more abnormal manner as detected especially through the positive charge of the retinal. This is due to the protonation of one or more groups abolishes the essential structural design of the protein characteristic of WT. Because this effect does not occur in WT or in either D115A or T90V, the clear and dominant effect of pH must be due to a change in the pK_a of one or more side chains due to the changed conformation in the mutant.

To check if the altered functional behavior of the mutants has any correspondence with their conformational properties in the resting state, we have performed DSC experiments. In parallel to the decrease in proton-pumping efficiency, the mutations studied give rise to a decrease in the protein stability. T90A, which has lost all interactions created by Thr-90, shows a remarkable decrease in thermal stability (16). The intermediate behavior of T90V and D115A, as compared with WT and T90A, is in keeping with the maintenance of some of the interactions held by Thr-90 in the WT. Both T90V and D115A have lost the hydrogen bond established between Thr-90 and Asp-115, and both show the same decrease in the temperature of the main transition, although D115A has a more cooperative transition. On the other hand, T90A has lost the interaction with the retinal as compared with T90V and it is certainly less stable. Therefore, we are tempted to deduce that both the hydrogen bond between Thr-90 and Asp-115 and the steric interaction of Thr-90 with the retinal contribute similarly to the BR conformational stability. However, this is a very simple description of the effects of the mutations, as other factors such as the volume occupied by the side chains and changes in the hydrophobic effect or the side chain conformational entropy will also contribute to the final structural stability. Denaturation experiments using aliphatic alcohols have already revealed the importance of polar interactions for the formation of the tertiary structure of BR (31). Therefore, although a variety of forces and interactions contribute to achieve the seven-helix functional bundle (32–36), polar interactions seem prominent. On the other hand, it appears that the para-crystalline arrangement of BR trimers in the membrane depends, in a more subtle way, on the interactions formed by Thr-90, as indicated by the gradual decrease of the pre-transition temperature among the mutants studied (see “Results”). Thus, it seems that the formation of the hexameric structure requires not only the presence of protein-protein and lipid-protein interactions but also a BR compact structure that some mutations are not able to preserve, giving rise to a less stable para-crystalline arrangement.

The importance of Thr-90 in assuring the correct location of key amino acid side chains of helix C involved in proton transport provides a basis for discussing the role of the central part of helix C. Two nearby side chains, Pro-91 and Trp-86, which are fully conserved among 25 archaeal rhodopsins (18), may also contribute to the precise location of helix C. Previous data obtained in detergent/phospholipid micelles showed that a Pro-91 mutation alters several photocycle and proton-pumping properties (37, 38), indicating that it is important for the proton-pumping function. Therefore, it is plausible that the three conserved residues at positions 86, 90, and 91 contribute through different ways to the same structural purpose, that is, to provide the correct location of the functional side chains located on helix C as well as that of the retinal. It is interesting to point out that Trp-86 and Pro-91 are conserved not only among the 25 archaeal rhodopsins (bacteriorhodopsins, halorhodopsins, and sensory rhodopsins) but also in five fungal chaperones and proteorhodopsin, whereas Thr-90 is conserved only among archaeal rhodopsins (18, 19). Although Trp-86 forms part of the retinal environment, it may have as well other more general structural functions, as Pro-91 does, related to the formation of the seven-helix bundle. Thr-90, in contrast, may be necessary especially for retinal-containing transmembrane proteins. For example, in halorhodopsin Asp-141 (homologous to Asp-115) is also within hydrogen-bonding distance of Thr-90 (39), indicating the same possible function as in BR.

The importance of Thr-90 may come from the necessity of accurate conformational changes affecting helix C during the photochemical cycle that are linked to the retinal movements. Such subtle conformational changes have been described recently in the K to L transition in which a local bending of helix C toward the proton translocation channel is anticipated as playing a central role in setting the stage for proton transfer (40).

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Thr-90 Plays a Vital Role in the Structure and Function of Bacteriorhodopsin
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