

Consequences of amino acid insertions and/or deletions in transmembrane helix C of bacteriorhodopsin

(membrane protein/proton transport/protein structure/mutagenesis/kinetic spectroscopy)

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ABSTRACT Six bacteriorhodopsin mutants containing either single amino acid deletions ($\Delta A84$, $\Delta L87$), insertions ($V85A$, $V88A$), or both deletions and insertions ($\Delta A84/V88A$, $V85A/\Delta L87$) within the first two turns of transmembrane helix C, starting from the extracellular side, have been prepared. The mutant apoproteins refold in phospholipid/detergent micelles and display secondary structures similar to that of the wild type. However, the mutants $V88A$ and $\Delta A84/V88A$ do not form a chromophore with retinal. The regenerated chromophore of $V85A$ displays absorption maxima and retinal isomer compositions in the dark- and light-adapted states similar to those of the wild type. In $\Delta A84$, $\Delta L87$, and $V85A/\Delta L87$ these chromophore properties are altered, and the structures are less stable than that of the wild type, as shown by an enhanced rate of reaction with hydroxylamine in the dark, an increased pK_a of the denaturation at acidic pH, and a decreased pK_a of Schiff base deprotonation. Proton translocation is abolished in the $\Delta A84$ and $V85A/\Delta L87$ mutants, whereas in $V85A$ and $\Delta L87$ the activity is reduced to about 25% of the wild-type value at pH 6. The overall properties of the $V85A$, $V85A/\Delta L87$, and $\Delta L87$ mutants indicate that the deletions and/or insertions result in displacement of residues Arg-82, Asp-85, or Asp-96, respectively, which participate in proton translocation. The results are compatible with a helical structure for transmembrane segment C and emphasize the flexibility of intramolecular contacts in bacteriorhodopsin.

Bacteriorhodopsin (bR) is a retinal-based integral membrane protein that functions as a light-driven proton pump in *Halobacterium halobium* (1). The absorption of light by bR initiates a photochemical cycle that consists of at least five transient intermediates (K, L, M, N, and O) and is coupled to vectorial translocation of protons. Spectroscopic studies of site-directed mutants have shown that Asp-85, Asp-212, and Arg-82 participate in the proton release from the Schiff base to the extracellular side, whereas Asp-96 serves as a proton donor during reprotonation of the Schiff base from the cytoplasm (2–7). Mutagenesis experiments have also identified a number of amino acids that interact with the retinal chromophore (8, 9).

A structural model for bR (cf. Fig. 1) has been recently derived by electron diffraction (10). However, the proposed model lacks atomic resolution, and therefore a number of basic structural questions remain. For example, do the membrane-embedded segments indeed have α -helical structures? Are there specific helix-helix interactions that are involved in proton translocation? Predetermined displacements of amino acids in helices should severely affect the stability of the folded structure as well as proton transfer reactions. In the present work we took the following approach to address such questions: the positions of amino acids in helix C were altered

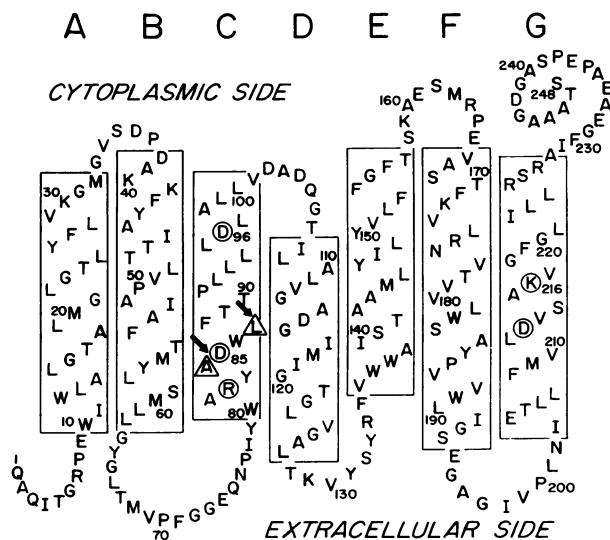


FIG. 1. Secondary structure model of bR. The alignment and length of the seven α -helical transmembrane segments A–G are as proposed by Henderson *et al.* (10). Residues that were deleted (Ala-84 or Leu-87) are indicated by triangles. Positions where a single alanine was inserted (between Ala-84 and Asp-85 or Leu-87 and Phe-88, respectively) are shown by arrows. The locations of residues involved in proton transport are marked by circles. Lys-216 is the site of attachment of retinal.

by introducing either deletions ($\Delta A84$, $\Delta L87$), insertions ($V85A$, $V88A$), or both deletions and insertions ($\Delta A84/V88A$, $V85A/\Delta L87$) of single alanine or leucine residues (Fig. 1). These amino acids were chosen because of their strong helix-forming tendencies (11). Transmembrane segment C contains several residues that participate in proton transport, and therefore it should be possible to identify the consequences of displacements. By studying the effects of the deletions and/or insertions on folding, chromophore formation, spectral properties, and proton transport, we aimed to establish how these changes in the primary structure are

Abbreviations: bR, bacteriorhodopsin; ebR, bR prepared by expression of a synthetic wild-type bR gene in *Escherichia coli*; ebO, the apoprotein of ebR; PSB, protonated Schiff base; SB, deprotonated Schiff base; DA, dark adapted; LA, light adapted; DMPC, dimyristoylphosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. $\Delta A84$ or $\Delta L87$ designates mutants in which Ala-84 or Leu-87, respectively, has been deleted (cf. Fig. 1). $V85A$ or $V88A$ designates mutants in which at position 85 or 88, respectively, of the wild-type sequence an alanine has been inserted. Thus, in $V85A$ the original Asp-85 is now residue 86, and in $V88A$ the original Phe-88 is now residue 89. $\Delta A84/V88A$ or $V85A/\Delta L87$ designates corresponding double mutants with a deletion and an insertion. All other bR mutants are designated by the wild-type amino acid (single-letter code) and its position number followed by the substituted amino acid. Thus, in D85N Asp-85 has been replaced by asparagine.

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reflected in the mutant proteins. Our results show that all mutants fold to bacterioopsin-like structures. The mutants V88A and Δ A84/V88A do not form chromophores, possibly due to a steric constraint in the retinal-binding pocket. The mutants Δ A84, V85A, Δ L87, and V85A/ Δ L87 regenerate bR-like pigments. Except for V85A, they display reduced stabilities and altered spectral characteristics, as shown by shifts in the absorption maxima and changes in the retinal isomer compositions. In addition, their proton-pumping activities at pH 6 are reduced to 0–28% of the wild-type activity. The overall phenotypes of several of the mutants are consistent with a helical displacement of specific functional residues located in transmembrane segment C of bR.

METHODS

Mutants containing deletions and/or insertions of single amino acids in helix C were constructed in a synthetic bacterioopsin gene and expressed in *Escherichia coli*; the proteins were purified using previously described methods (12). Chromophores were regenerated from the apoproteins (16 μ M) in 1% dimyristoylphosphatidylcholine (DMPC)/1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS)/0.2% SDS/1 mM sodium phosphate, pH 6.0, by the addition of all-*trans*-retinal (9). Retinal isomer compositions and extinction coefficients were determined as reported (9, 13). Hydroxylamine reactions were carried out in the dark at 22°C in lipid/detergent micelles (14). Spectrometric titrations were carried out as described (13), and the amount of titrated pigment was determined from difference spectra. For the transition to a deprotonated Schiff base (SB) at alkaline pH, the absorbance increase at 365 nm [for bR prepared by expression of a synthetic wild-type bR gene in *E. coli* (ebR), V85A, Δ L87, and V85A/ Δ L87] or at 395 nm (for Δ A84) was measured (13). Denaturation at acidic pH was measured by the formation of a 442-nm absorbing protonated Schiff base (PSB) devoid of retinal-protein interactions. The pK_a values and the number of protons (*n*) involved in the transitions were obtained as reported (13).

For proton-pumping assays, the mutants were reconstituted into soybean lipid vesicles by detergent dilution, and light-dependent pH changes were recorded in 2 M NaCl as described (12, 15). The photocycle was measured with a homebuilt flash photolysis spectrometer (7). CD spectra of the samples were obtained in 1-mm pathlength cuvettes at 25°C using an Aviv 60DS spectropolarimeter. Data were taken with a 1-nm step size and a 5-s average time; the results were averaged over five scans.

RESULTS

Mutant Δ A84. Chromophore formation of Δ A84 proceeds with a rate that is slowed down about 10-fold compared with that of the wild type (Table 1). The dark-adapted (DA) λ_{\max}

of Δ A84 in DMPC/CHAPS/SDS micelles is red-shifted by 16 nm relative to ebR, and the chromophore is predominantly in the all-*trans* conformation (Table 1). Whereas light adaptation of ebR causes a 10-nm red shift and essentially 100% conversion of the chromophore to all-*trans*-retinal, illumination of Δ A84 increases the proportion of *cis* isomers without a change in the λ_{\max} (Table 1). Spectrometric titrations reveal that the pK_a of the Schiff base is significantly decreased in this mutant. The chromophore of Δ A84 displays, between pH 5 and 9, a reversible transition from protonated to deprotonated species with λ_{\max} at 395 nm (Fig. 2A, dotted lines). The titration shows an isobestic point at 452 nm and involves a single proton (Table 2), presumably the proton at the Schiff base. A SB with λ_{\max} at 365 nm is formed above pH 10 in a cooperative way (*n* = 1.4). An analogous transition is also observed in ebR, where this species (λ_{\max} = 365 nm) directly arises upon deprotonation of the PSB (13). Analysis of the titration data for Δ A84 yields a SB pK_a value of 6.6, compared with 11.3 for ebR (Table 2). In the acidic pH range, the absorption spectrum of Δ A84 indicates the rise of a chromophore with a λ_{\max} at 442 nm (Fig. 2A, dashed line). This transition, which is generally observed for ebR and mutants in micelles (8, 13), represents the formation of a free PSB due to denaturation of the protein. The titration data show that the pK_a of this cooperative transition is increased from 2.2 in wild type to 3.6 in Δ A84 (Table 2). To probe structural perturbations in the mutant, the reactivity to hydroxylamine in the dark was measured (14). Fig. 3 shows that the chromophore of Δ A84 bleaches with an exponential decay time ($\tau_{1/e}$) of 2.76 h, compared with 9.4 h for ebR in micelles. After reconstitution into liposomes, no proton pumping is observed for Δ A84 between pH 5.5 and 7.5 (<1% of the wild-type activity).

Mutant V85A. The absorption maxima and retinal isomer compositions in the DA and light-adapted (LA) states of V85A are similar to those of the wild type (Table 1). In the presence of hydroxylamine, V85A bleaches with a rate comparable to that of ebR (Fig. 3). Titrations at alkaline pH show the formation of a deprotonated SB (λ_{\max} = 365 nm; Fig. 2B) with a pK_a of 11.2 (Table 2). In the presence of 2 M NaCl, the SB pK_a of V85A is lowered by 0.5 pH units. At acidic pH, a free PSB (λ_{\max} = 442 nm; Fig. 2B) is formed with a pK_a similar to that of ebR (Table 2). Proton pumping in 2 M NaCl shows a steady-state activity of 28% at pH 6.0. In the presence of 150 mM KCl, the level of pumping is 39% at pH 7.3, whereas at pH 6.0 it is reduced to 4% of the wild type. The photocycle of the mutant also displays pH-dependent changes. Fig. 4A shows a projection of the absorbance changes onto the (λ , *t*) plane at pH 7.3. On the whole, the photocycle of V85A is similar to that of ebR (9). The early absorbance increase above 600 nm reveals the presence of the K intermediate, which is followed by the rise of a normal M intermediate (τ = 17 μ s) with λ_{\max} at 410 nm. The decay of M (τ_1 = 1.5 ms; τ_2 = 27 ms), which correlates with the

Table 1. Spectral and functional properties of deletion and/or insertion mutants measured at pH 6.0

Mutant	<i>t</i> _{1/2} of chromophore regeneration*, min	Regeneration extent, %	ϵ , M ⁻¹ ·cm ⁻¹	λ_{\max} , nm		Retinal isomer composition, %					Steady-state proton pumping [†] , H ⁺ per bR
						DA		LA			
						13- <i>cis</i>	all- <i>trans</i>	9- + 11- <i>cis</i>	13- <i>cis</i>	all- <i>trans</i>	
ebR	1.0	84	52,000	551	561	60	40		3	97	1.00
Δ A84	13	70 [‡]	45,500 [‡]	567	566	16	84	6	22	72	<0.01
V85A	11	76	49,800	553	560	48	52		8	92	0.28
Δ L87	4.9	75	31,800	502	510	25	75	13	47	40	0.23
V85A/ Δ L87	69	63	61,700	560	563	54	46	25	30	45	<0.02

*Measured at 20°C with all-*trans*-retinal.

[†]The values reported are the average of five independent reconstitutions. The activity of ebR was 36 H⁺ per bR.

[‡]Measured at pH 5.0.

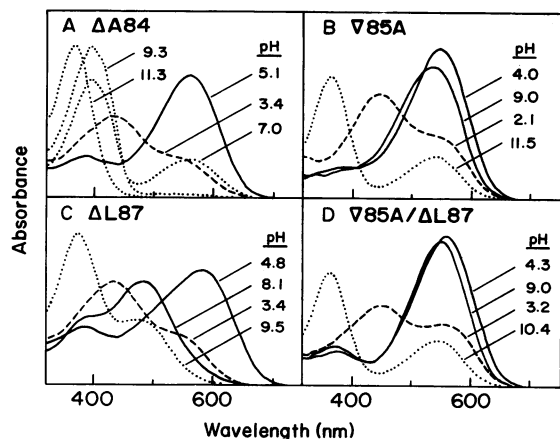


FIG. 2. pH dependence of the absorption spectra of deletion and/or insertion mutants. (A) $\Delta A84$. (B) $\nabla 85A$. (C) $\Delta L87$. (D) $\nabla 85A/\Delta L87$. At alkaline pH, the PSB chromophore converts to a deprotonated SB with λ_{max} near 365 nm (dotted lines). In $\Delta A84$ (A) a deprotonated SB with λ_{max} at 395 nm is formed, which subsequently converts to the 365-nm species. At acidic pH, a transition to a free PSB with λ_{max} near 442 nm is observed (dashed lines). At the intermediate pH values, the λ_{max} values are as follows: 560 nm at pH 5.1 for $\Delta A84$, 547 nm at pH 4.0 and 533 nm at pH 9.0 for $\nabla 85A$, 581 nm at pH 4.8 and 485 nm at pH 8.1 for $\Delta L87$, and 557 nm at pH 4.3 and 550 nm at pH 9.0 for $\nabla 85A/\Delta L87$.

return of the ground state, has an additional slow component ($\tau_3 = 2.4$ s). In contrast to ebR, the O intermediate is not observed. The overall photocycle in micelles is slowed down by one order of magnitude, which could explain the reduced pumping at pH 7.3 relative to ebR. Fig. 4B shows that the early absorbance changes at pH 6.0 are similar to those at pH 7.3. However, the decay of K is associated with the formation ($\tau = 7 \mu s$) of a long-lived intermediate with λ_{max} near 500 nm. The amplitude of this intermediate is reduced, and its apparent maximum is shifted to 450 nm, due to overlap with the strong depletion signal. This appears to be an L-like intermediate, as observed previously in the photocycles of blue membrane (16) or the D85N mutant (17). Its decay ($\tau_1 = 18$ ms; $\tau_2 = 2.2$ s) is coupled to the disappearance of the depletion signal. In agreement with the proton-pumping measurements, there is very little absorbance change at 410 nm corresponding to the M intermediate.

Mutant $\Delta L87$. $\Delta L87$ displays a highly blue-shifted λ_{max} at 502 nm at pH 6.0 (Table 1). The chromophore is broadened in the longer wavelength region and has an ϵ of $31,800 M^{-1}cm^{-1}$. In reversal of the retinal ratios observed for the wild type, the DA chromophore of $\Delta L87$ contains predominantly all-*trans*-retinal, whereas in the LA state cis isomers predominate (Table 1). This indicates that the isomer specificity of the retinal-binding pocket is perturbed. The rate of reaction with hydroxylamine is drastically enhanced relative to ebR (≈ 900 -fold; Fig. 3), suggesting greater water acces-

Table 2. pK_a values of the denaturation at acidic pH and of the deprotonation of the Schiff base at alkaline pH for deletion and/or insertion mutants

Mutant	PSB deprotonation					
	Denaturation		- NaCl			
	pK_a	n	pK_a	n	pK_a	n
ebR	2.20	2.62	11.26	2.49	11.20	2.77
$\Delta A84$	3.59	1.80	6.63	0.83	7.20	0.92
$\nabla 85A$	2.25	1.47	11.22	1.88	10.73	1.66
$\Delta L87$	3.53	2.42	9.21	1.16	9.22	1.29
$\nabla 85A/\Delta L87$	3.37	2.37	10.11	1.25	10.68	1.12

n , number of protons involved in the transition.

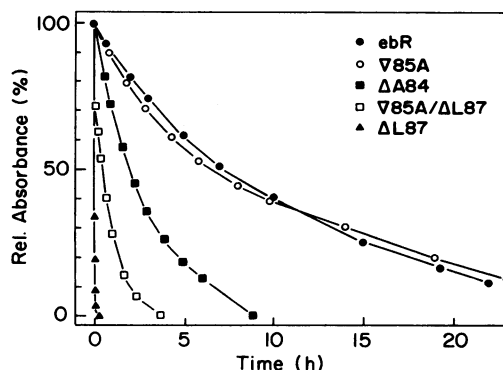


FIG. 3. Rates of reaction with hydroxylamine for ebR and deletion and/or insertion mutants. The reactions were carried out in the dark as described (14). The absorbance decrease at the λ_{max} of the DA chromophore is plotted versus the reaction time. The data points have been normalized. The $\tau_{1/e}$ values are 9.42, 7.73, 2.76, 0.77, and 0.01 h, respectively, for ebR, $\nabla 85A$, $\Delta A84$, $\nabla 85A/\Delta L87$, and $\Delta L87$.

sibility to the PSB in this mutant. Spectrometric titrations indicate that the PSB in the folded protein is stable over a narrow pH range. Thus, the transition to the 442-nm species at acidic pH is raised by 1.3 pH units and the SB pK_a is decreased by 2 pH units, compared with the wild type (Table 2). Between pH 8.1 and 4.8, the chromophore of $\Delta L87$ displays a transition from a blue-shifted ($\lambda_{max} = 485$ nm; Fig. 2C) to a red-shifted ($\lambda_{max} = 581$ nm) form. The latter chromophore is likely to have a retinal environment similar to that of the wild type, since a corresponding red shift ($\lambda_{max} = 588$ nm) is observed for ebR in micelles at pH 2.6. Steady-state proton-pumping measurements in 2 M NaCl

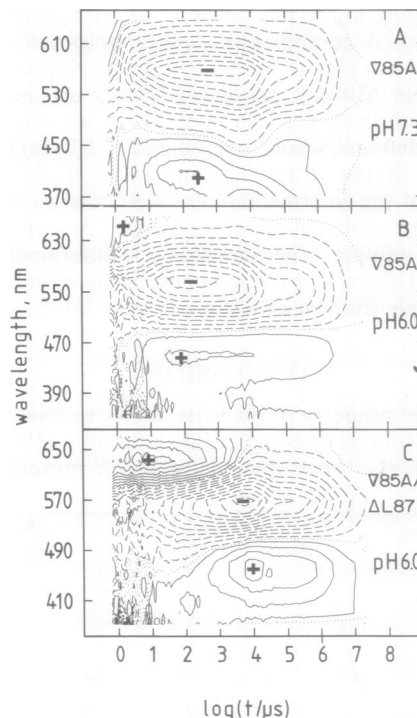


FIG. 4. Contour plots in the (λ , t) plane for photocycle absorbance changes of deletion and/or insertion mutants. (A) $\nabla 85A$ at pH 7.3. (B) $\nabla 85A$ at pH 6.0. (C) $\nabla 85A/\Delta L87$ at pH 6.0. Lines of equal absorbance change are plotted. Maxima and minima are labeled with "+" and "-", respectively. The photocycle was measured in steps of 20 nm. The excitation was at 590 nm (3 mJ, 10 ns), and the repetition rate was $<0.1 s^{-1}$. The samples were in 0.025% DMPC/1% CHAPS/150 mM KCl under DA conditions at 22°C.

show an increase in activity from 8% at pH 7.3 to 23% at pH 6.0. The inactive *cis* isomers present in the LA state obviously contribute to the reduced pumping level relative to ebR, as observed for other mutants (9).

Mutants V88A and Δ A84/V88A. The apoproteins of V88A or Δ A84/V88A do not form a chromophore with retinal (extent of regeneration, <2%). Altering the pH as well as the DMPC, CHAPS, or salt concentrations did not affect this result. To detect possible differences in secondary structure between the wild-type and mutant apoproteins, CD measurements were carried out in micelles (Fig. 5). The spectra exhibit strong negative ellipticities at 222 and 210 nm, indicating substantial α -helicity for the apoproteins of ebR (ebO) and the mutants. The magnitude of the molar ellipticity at 222 nm ($[\Theta]_{222}$) and the spectral shape are characteristic of renatured bacterioopsin, which is known to possess a helix content (\approx 70%) identical to that of native bR (18). The $[\Theta]_{222}$ values for ebO, V88A, and Δ A84/V88A are $-19,700$, $-20,100$, and $-19,600$ deg \cdot cm 2 \cdot dmol $^{-1}$, respectively. Based on the minimal deviations of the $[\Theta]_{222}$ values relative to the wild type, it is evident that the secondary structure is maintained in the two mutants.

Mutant V85A/ Δ L87. The kinetics of chromophore formation for this mutant are significantly slowed down (\approx 70 fold; Table 1) relative to the wild type. The DA chromophore has a λ_{\max} at 560 nm and shows a normal retinal isomer composition, whereas in the LA state the all-*trans* proportion is reduced to 45% (Table 1). Measurements of the rate of reaction with hydroxylamine (Fig. 3) and of the pK_a of the denaturation at low pH (Table 2) indicate that the mutant chromophore is destabilized relative to ebR. Titrations at alkaline pH (Fig. 2D) reveal that the SB pK_a of V85A/ Δ L87 is lowered to 10.1. Addition of 2 M NaCl leads to an increase of the SB pK_a by 0.6 units (Table 2). The mutant displays no proton pumping in the pH range 5.5–7.5 (<2% of the wild-type activity). A contour plot of the photocycle of V85A/ Δ L87 is shown in Fig. 4C. Initially a strong K intermediate is observed near 630 nm. Its decay ($\tau = 3$ ms) is slow and correlates with the formation of an L-like intermediate with a λ_{\max} near 460 nm, whose rise time ($\tau = 1.2$ ms) is delayed compared with that of the V85A mutant at pH 6.0 (Fig. 4B). The decay of the intermediate ($\tau_1 = 9.5$ ms; $\tau_2 = 1.6$ s) is coupled to the return of the ground state. As expected from the proton-pumping experiments, no M intermediate is observed near 410 nm. The overall photocycle resembles that of the acid-purple form of bR (19).

DISCUSSION

A remarkable property of bR is its ability to renature to the native state following complete denaturation (18, 20). This property has also been demonstrated for proteolytic frag-

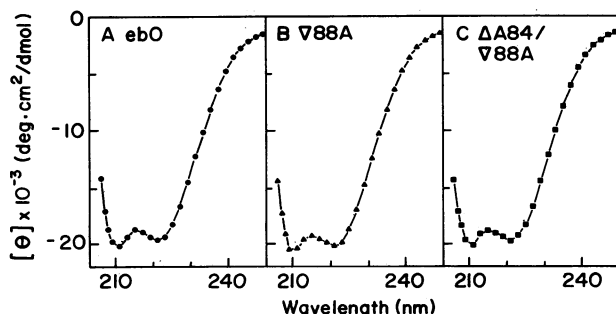


FIG. 5. CD spectra of ebO and deletion and/or insertion mutants. (A) ebO. (B) V88A. (C) Δ A84/V88A. The apoproteins (13.8 μ M) were prepared in 0.1% DMPC/0.1% CHAPS/0.18% SDS/1 mM sodium phosphate, pH 6.0.

ments (21, 22) and a large number of mutants (8, 9, 13, 23). Thus, it appears that specific interactions between the putative transmembrane helices play a dominant role in folding and stability of bR. In this work we have prepared six mutants that contain deletions and/or insertions of single amino acids in transmembrane helix C. This region was chosen since it contains Asp-85, Asp-96, and Arg-82, all of which are important for proton transport (2, 4, 7, 12). Within a helical conformation, the insertion and/or deletion of the helix-stabilizing alanine or leucine are expected to cause relative displacements of these functional residues. In the mutants this will be reflected in effects on folding, secondary structure, stability, absorption spectrum, and proton pumping.

To visualize possible impacts of the changes introduced in the mutants, linearized projection maps were constructed for an 18-residue segment of helix C (positions 79–96; Fig. 6). In the mutants V85A/ Δ L87 and Δ A84/V88A a small number of positional substitutions are expected, since an insertion is compensated after approximately one helical turn by a deletion and vice versa, respectively. Assuming that reorientation of helix C does not occur, positions 85–87 in V85A/ Δ L87 and 84–87 in Δ A84/V88A would be altered (Fig. 6). Indeed, the properties of V85A/ Δ L87 are consistent with the introduction of an alanine at position 85 (2, 13). Thus, (i) the mutant is inactive in proton pumping; (ii) deprotonation of the PSB during the photocycle is not observed (Fig. 4C); (iii) the DA λ_{\max} is red-shifted relative to ebR; and (iv) the SB pK_a is lowered to 10.1; however, in the presence of salt it is increased by 0.6 units (Table 2). The consequent displacement of Asp-85 into the position of residue 86 in the wild type could further account for the higher SB pK_a and the blue-shifted λ_{\max} of V85A/ Δ L87 relative to the D85A mutant, if the carboxylate group remains ionized and exerts electrostatic influence on the PSB.

Chromophore regeneration was abolished in Δ A84/V88A, which again renders helix C out of phase for about one turn. The defect is likely to originate from a structural perturbation. Assuming that the orientation of helix C remains unchanged, Trp-86 would be rotated by 100° into the position of Asp-85 in bR (Fig. 6). A bulky hydrophobic residue in the position of the PSB counterion could interfere with the access of retinal to its binding pocket or the formation of a PSB linkage. This interpretation is supported by the observation that replacement of the secondary counterion Asp-212 by valine also abolishes chromophore formation (T.M. and H.G.K., unpublished work).

Several observations indicate minimal structural alterations in the V85A mutant, relative to the wild type. Thus, the dark-light adaptation behavior is normal (Table 1), the SB pK_a and the pK_a of the denaturation at low pH are identical to the wild type (Table 2), the rate of reaction with hydroxylamine is comparable with that of ebR (Fig. 3), and the photocycle (Fig. 4A) as well as the pumping activity at pH 7.3 are relatively normal. These results strongly suggest that the position of Asp-85 remains unchanged in V85A. If, as is likely, helix formation is not affected by insertion of alanine, the consequent result would be the displacement of Arg-82 from its proposed location in the proton channel (Fig. 6). Indeed, some additional properties of V85A are consistent with the presence of a neutral residue at position 82. First, the SB pK_a is lowered by 0.5 units in the presence of salt (Table 2), indicating increased surface charge effects compared with ebR, as previously noted for R82Q (13). Second, in the photocycle of V85A, deprotonation of the PSB is blocked near pH 6 (Fig. 4B), thereby explaining the observed loss of proton pumping. This suggests that in this mutant the pK_a of protonation of Asp-85 is raised relative to the wild type, as in the case of R82Q and R82A (15, 24). Thus, we conclude that the insertion of alanine at position 85 is accommodated by

	80	91	84	95	88	81	92	85	96	89	82	93	86	79	90	83	94	87
bR	W	P	A	L	F	A	L	D	D	T	R	L	W	Y	T	Y	L	L
∇85A/ΔL87	W	P	A	L	F	A	L	A	D	T	R	L	D	Y	T	Y	L	W
ΔA84/∇88A	W	P	D	L	F	A	L	W	D	T	R	L	L	Y	T	Y	L	A
∇85A	A	P	A	L	F	R	L	D	D	T	Y	L	W	W	T	A	L	L
∇88A	A	P	D	L	F	R	L	W	D	T	Y	L	L	W	T	A	L	A
ΔA84	Y	P	Y	L	F	W	L	D	D	T	A	L	W	I	T	R	L	L
ΔL87	W	L	A	D	T	A	L	D	L	T	R	L	W	Y	P	Y	L	F

FIG. 6. Linearized projection maps of helix C for bR and deletion and/or insertion mutants. The primary sequence was transformed to a helical projection map, assuming 3.6 residues per turn (8, 12). Linearization was done for an 18-residue segment starting at Tyr-79. The numbering solely indicates the position of the residue in the wild-type sequence. The proposed alignment for the mutants is based on their phenotype and assumes that a helical structure is maintained (see Discussion). Altered positions relative to bR are marked by squares. The various contact faces of the helix are indicated at the bottom.

displacement of the preceding residues in helix C toward the extracellular side.

In contrast to ∇85A, insertion of a residue one helical turn above in ∇88A prevented chromophore regeneration. The CD spectrum of the mutant is unaffected relative to the wild type (Fig. 5 A and B). Accommodation of the insertion as for ∇85A (i.e., by backrotation of the preceding residues by 100°) would place Trp-86 into the position of Asp-85 in bR (Fig. 6). On the other hand, a corresponding forward rotation of all residues subsequent to the insertion would place Phe-88 into the position of Thr-89. As suggested for ΔA84/∇88A, these bulky substitutions are likely to interfere with the proper orientation of the chromophore in the binding pocket (cf. ref. 9). Alternatively, substitutions at the interhelical contact sites could prevent formation of the seven-helix bundle.

The mutants ΔA84 and ΔL87 regenerate chromophores whose properties are significantly altered relative to ebR. ΔA84 displays a phenotype that is compatible with the presence of a protonated aspartate or a neutral residue at position 85 (12, 24). Thus, (i) the mutant shows no proton-pumping activity; (ii) the DA λ_{max} is red-shifted relative to ebR (Table 1); and (iii) the SB pK_a is reduced by 4.6 units (Table 2), as observed for D85N (2, 13). It is uncertain whether these effects originate from the displacement of Asp-85 or from the disruption of intramolecular interactions, for example between Arg-82 and Asp-85. The mutant ΔL87 shows a highly blue-shifted λ_{max} near neutral pH (Fig. 2C). Its chromophore is destabilized relative to ebR, as is evident from an enhanced reactivity to hydroxylamine (Fig. 3), an increased pK_a of the denaturation at acidic pH, and a decreased SB pK_a (Table 2). This suggests that the protein-retinal interactions are weakened and allow an enhanced access of water to the PSB. Although the position of Asp-96 relative to Asp-85 is altered by deletion of Leu-87, the mutant retains proton-pumping activity. This indicates that Asp-85 functions as an obligatory acceptor of the Schiff base proton, and thus the residue is likely to remain in its native environment. Assuming a helical structure is maintained in ΔL87, all residues in helix C subsequent to the deletion would be backrotated by 100° (Fig. 6). The presence of a leucine at position 96 could explain the increased pumping activity observed upon lowering the pH from 7.3 to 6.0. Previous studies have indicated that mutants in which Asp-96, the internal proton donor to the Schiff base, is neutralized show an increased rate of pumping at acidic pH, presumably because the SB is directly reprotonated from the cytoplasm (6, 7). Thus, it is likely that the deletion of Leu-87 results in shortening of helix C at the cytoplasmic side.

In summary, deletions and/or insertions of single residues in helix C do not affect the helical content in the resulting mutant apoproteins. While structural differences among the assembled seven-helix bundles clearly arise due to alterations of specific intramolecular contacts, the system shows re-

markable flexibility. For example, it was surprising that ΔL87 regenerated a chromophore. Judging from the properties of ΔA84/∇88A, ∇85A/ΔL87, ∇85A, or ΔL87, the mutations are accommodated with a minimal number of positional changes, thus retaining a maximum number of native intramolecular interactions. The phenotypes of several of the mutants can be rationalized in terms of the displacement of specific functional groups in a helical structure.

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