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Effect of Introducing Different Carboxylate-containing Side Chains at Position 85 on Chromophore Formation and Proton Transport in Bacteriorhodopsin*

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Duncan A. Greenhalgh, Sriram Subramaniam‡, Ulrike Alexiev§, Harald Otto§, Maarten P. Heyn§, and H. Gobind Khorana¶

From the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and the §Department of Physics, Freie Universitat Berlin, D-1000 Berlin 33, Germany

During the initial stages of the bacteriorhodopsin photocycle, a proton is transferred from the Schiff base to the deprotonated carboxylate of Asp^{85}. Earlier studies have shown that replacement of Asp^{85} by Asn completely abolishes proton transport activity, whereas extension of the side chain by an additional carbon-carbon bond (Asp^{85} → Glu) results in a functional proton pump. Here we show that extension of the Asp^{85} side chain by two additional bond lengths also results in a functional proton pump as long as the terminal group is a carboxylate moiety. These side chains were created by modification of the cysteine residue in the Asp^{85} → Cys mutant with either iodoacetic acid or iodoacetamide. *In vitro* chromophore formation studies show that the rate of Schiff base protonation in mutants that contain a carboxylate at residue 85 is invariably faster than in mutants that contain neutral substitutions at this position. We conclude that in bacteriorhodopsin, there is considerable tolerance in the volume of the side chain that can be accommodated at position 85 and that the presence of a carboxylate at residue 85 is important both for proton pumping and for stabilizing the protonated Schiff base.

Bacteriorhodopsin is a light-driven proton pump found in *Halobacterium halobium* (Stoeckenius *et al.*, 1979). Extensive studies of the mechanism of the proton translocation have shown that Asp^{85}, Asp^{212}, Arg^{82}, and Asp^{96} are directly involved in this process (Mogi *et al.*, 1988; Butt *et al.*, 1989; Otto *et al.*, 1989, 1990; Stern *et al.*, 1989; Holz *et al.*, 1989). Of these, only the mutants that have neutral substitutions at residue 85 are completely inactive in proton pumping (Mogi *et al.*, 1988). A number of spectroscopic studies have concluded that Asp^{85}, which is deprotonated in the ground state, functions as a proton acceptor in the early stages of the photocycle (Braiman *et al.*, 1988; Gerwert *et al.*, 1989; Otto *et al.*, 1990).

We reasoned that the substitution of Asp^{85} with other carboxylate-containing side chains would provide further insights into the role of this residue in bacteriorhodopsin function. Previously, Asp^{85} has been replaced by Glu, and the effect of this substitution on the photocycle and proton pumping has been studied (Otto *et al.*, 1990; Subramaniam *et al.*, 1990; Butt *et al.*, 1989; Heberle *et al.*, 1991; Lanyi *et al.*, 1992). Here, we compare the effects of substituting Asp^{85} with either carboxymethyl Cys (D85C(cm))[1] or carboxamidomethyl Cys (D85C(cam)) on chromophore formation and proton pumping. These side chains are formed by derivatization of Cys with either iodoacetic acid or iodoacetamide, respectively. Carboxymethyl Cys and carboxamidomethyl Cys side chains are two bond lengths longer than the native aspartate side chain and contain either a carboxylate or an amide as the terminal group.

The primary finding from this work is that the D85C(cm) mutant is active in proton transport and has a normal M intermediate in its photocycle. We also show that the carboxylate at residue 85 plays an important role in regulating the rate of *in vitro* chromophore formation in bacteriorhodopsin. Both results highlight the importance of Asp^{85} for the structure and function of bacteriorhodopsin as a proton pump.

EXPERIMENTAL PROCEDURES

Materials—[^{14}C]Iodoacetamide (21.1 mCi/mmol) and [^{3}H]iodoacetic acid (161.6 mCi/mmol) were purchased from Du Pont-New England Nuclear. Iodoacetamide and iodoacetic acid were purchased from Fluka and recrystallized prior to use. Dioleoyl phosphatidylcholine, dimyristoylphosphatidylcholine, and dilinoleic phosphatidic acid were obtained from Avanti Polar Lipids; all-*trans*-retinal was from Kodak; CHAPS and SDS were from Boehringer Mannheim.

Construction, Purification, and Chromophore Formation of Mutant Proteins—The construction of the mutants D85E and D85C in the synthetic bacterioopsin gene has been described previously (Mogi *et al.*, 1988; Greenhalgh *et al.*, 1991). The mutant proteins were expressed in *Escherichia coli* and purified as described (Braiman *et al.*, 1987). The chromophores were formed by the addition of all-*trans*-retinal to the apoprotein in 1% DMPC, 1% CHAPS, 0.2% SDS, 30 mM sodium phosphate, pH 6.2, and 0.025% sodium azide. The absorption spectra of the regenerated proteins were measured after overnight dark adaptation and after 2-min light adaptation (150-watt

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‡ Recipient of Fellowship DRG937 from the Damon Runyon-Walter Winchell Cancer Research Fund. Present address: Dept. of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205-2185.

¶ To whom correspondence should be addressed: Depts. of Biology and Chemistry, Rm. 18-511, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139. Tel.: 617-253-1871; Fax: 617-253-0533.

[1] The abbreviations used are: C(cm), carboxymethyl Cys; C(cam), carboxamidomethyl Cys; DMPC, 1-α-dimyristoylphosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HPLC, high performance liquid chromatography. Bacteriorhodopsin mutants are designated by the wild type amino acid residue (single-letter code) and its position number followed by the substituted amino acid residue. For example, in the mutant D85E, Asp^{85} is replaced by Glu.

lamp filtered through heat-absorbing and 495 long pass filters). The retinal isomer compositions were determined as described previously (Scherrer *et al.*, 1989).

The kinetics of chromophore formation were measured at 20 °C following the addition of a 3-fold molar excess of retinal. Under these conditions the rate of chromophore formation is independent of retinal concentration. The traces of absorbance *versus* time were fit to the sum of two exponential processes as described (Stern and Khorana, 1989).

$$Abs_t = Abs_{t=\infty}((1 - F)\exp^{(-t/\tau_f)} - (1 - F)\exp^{(-t/\tau_s)}) \quad \text{(Eq. 1)}$$

where Abs_t represents absorbance at a time t, F represents the fraction of the total absorbance change which occurred in the early phase of regeneration, and τ_f and τ_s represent the time constants of the two exponential processes obtained using a nonlinear least squares algorithm.

Derivatization of Mutant and Wild Type Opsin with Iodoacetic Acid and Iodoacetamide—Derivatization of the mutant D85C and wild type bacterioopsin (0.08 mM) was carried out following preincubation in 2% SDS, 6 M urea, 10 mM EDTA, 200 mM Tris-HCl, pH 8.2, and 0.24 mM dithiothreitol for 90 min at 20 °C. ^{3}H-Labeled iodoacetic acid and ^{14}C-labeled iodoacetamide were added to final concentrations of 20 mM (16.1 mCi/mmol) and 10 mM (16.1 mCi/mmol), respectively, and the reactions were carried out at 37 °C under argon in the dark. At selected time points the reactions were quenched by the addition of 150 mM dithiothreitol, and the derivatized proteins were separated from excess radiolabel by SDS-polyacrylamide gel electrophoresis (using a DATD cross-linker). The extent of modification was determined by liquid scintillation counting following excision of the protein band and solubilization in 25 mM periodic acid. The chromophores of the modified opsins were formed as described above following the removal of the denaturants by extensive dialysis against 0.2% SDS, 30 mM phosphate, pH 6.2, 0.025% sodium azide.

Steady-state Proton Pumping Measurements—The proteins were reconstituted into synthetic phospholipid vesicles by detergent dilution as described previously (Subramaniam *et al.*, 1990). The proton pumping activities were measured under saturating illumination filtered through heat-absorbing and 495 nm long pass filters in 2 M NaCl, 150 mM KCl at pH 7.2. After each measurement the system was calibrated by the addition of 2 nmol of HCl.

Time-resolved Visible Absorption Spectroscopy—The kinetics of the photocycle were measured at a number of wavelengths on samples in 0.1% CHAPS, 0.0024% DMPC, 150 mM KCl, pH 7.3 at 22 °C using a homemade flash photolysis spectrometer as described previously (Otto *et al.*, 1989).

RESULTS

Derivatization of the Mutant D85C with Iodoacetic Acid and Iodoacetamide—Fig. 1 shows the kinetics of derivatization of wild type bacterioopsin and the D85C mutant opsin with radiolabeled iodoacetic acid and iodoacetamide. The D85C mutant was derivatized to ~85% with iodoacetic acid after 2 h, whereas no incorporated label was detected in wild type bacterioopsin. On reaction with iodoacetamide the D85C mutant was derivatized to ~80% after 1 h; wild type bacterioopsin showed ~5% incorporation. Over longer periods of time, the extent of derivatization of wild type bacterioopsin with iodoacetamide slowly increased and probably represents nonspecific labeling of other residues. Further characterization of the mutants D85C(cm) and D85C(cam) were performed on mutant D85C apoprotein that had been derivatized with either 20 mM iodoacetic acid for 2 h or 10 mM iodoacetamide for 1 h, respectively.

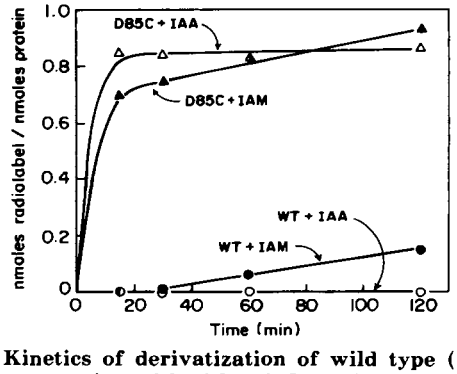

FIG. 1. **Kinetics of derivatization of wild type (*WT*) and D85C mutant opsins with either iodoacetic acid (*IAA, open symbols*) or iodoacetamide (*IAM, filled symbols*).** The modification conditions and the determination of the extent of derivatization are as described under "Experimental Procedures."

Kinetics of Chromophore Formation of Wild Type Bacteriorhodopsin and the Mutants—The kinetics of chromophore formation for wild type bacteriorhodopsin and the mutants D85E, D85C, D85C(cm), and D85C(cam) are shown in Fig. 2. The rate of chromophore formation represents a direct measure of the kinetics of protonation of the Schiff base in bacteriorhodopsin (Fisher and Oesterhelt, 1980). The rates of chromophore formation for wild type bacteriorhodopsin and the mutants D85E and D85C(cm) were fit to the sum of two exponential processes. The mutants D85C(cam) and D85C were fit to a single exponential process. The results of these fits are summarized in Table I.

During chromophore formation in wild type bacteriorhodopsin there are two kinetic components of $\tau_f = 1.8$ min and $\tau_s = 8.6$ min that contribute equally to this process (Table I). From Fig. 2, it is apparent that the initial rates of chromophore formation in mutants that contain a carboxylate at residue 85 (D85E or D85C(cm)) are faster than in mutants that contain neutral substitutions at this position (D85C or D85C(cam)). The faster initial rate in the D85E mutant results from a fast kinetic component (0.3 min) that contributes to ≃80% of the folding process. In the case of the mutant D85C(cm) the faster initial rate is caused by the fast component (τ_f = 0.06 min, 51%) which is approximately 30-fold faster than wild type bacteriorhodopsin. In the absence of a carboxylate moiety at this position the kinetics of chromophore formation in the mutants D85C and D85C(cam) were much slower than wild type bacteriorhodopsin and could be fit to a single exponential process. It is possible however that the slow regeneration rates for the mutants D85C and D85C(cm) could mask the biphasic nature of this process. This could explain the apparently monophasic kinetics presented in Table I for these mutants. We conclude that the presence of a carboxylate-containing side chain at position 85 plays an important role in regulating the rate of Schiff base

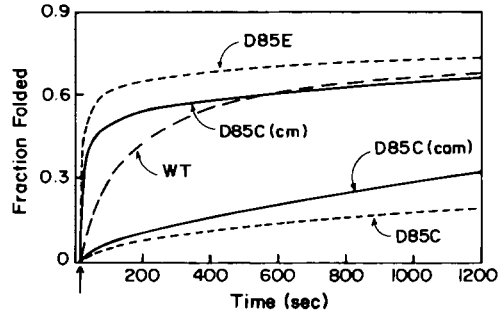

FIG. 2. **Kinetics of chromophore formation in wild type (*WT*) and mutant opsins.** Chromophore formation was measured by monitoring the change in absorbance at 550 nm as a function of time for wild type bacteriorhodopsin and the mutants D85E, D85C(cm), D85C(cam), and D85C. All of the opsins including the mutant D85C that had been derivatized with iodoacetic acid (20 mM for 2 h) and iodoacetamide (10 mM for 1 h) were extensively dialyzed against 0.2% SDS, 30 mM sodium phosphate, pH 6.2, 0.025% azide prior to chromophore formation in 1% DMPC, 1% CHAPS mixed micelles. The traces were rescaled after the end point of the folding process was determined.

TABLE I

Spectral and functional properties of wild type bacteriorhodopsin (bR) and the mutants

The visible absorption maxima of the chromophores were derived from samples in 1% DMPC, 1% CHAPS, 0.2% SDS, 150 mM KCl, and 30 mM sodium phosphate, pH 7.2. The kinetics of chromophore formation were measured as described under "Materials and Methods" where τ_f and τ_s represent the fast and slow time constants, and the numbers in parentheses represent their respective fractional contributions. The proton pumping assays were performed as described under "Materials and Methods," and the values reported are the average of three separate experiments.

Substitution	Kinetics of chromophore formation		λ_{max}		Proton pumping	
	τ_f	τ_s	DA[a]	LA	Initial rate	Steady state
	min	*min*	*nm*	*nm*	*(H^+/bR/s)*	*(H^+/bR)*
Wild type	1.76 (51)	8.6 (49)	548	557	1.95	148
D85E	0.31 (78)	8.5 (22)	546	546	0.69	51
D85C (cm)	0.06 (51)	13.3 (49)	536	523	0.52	40
D85C (cam)		33.4 (100)	584	584	ND	ND
D85C		50.8 (100)	584	584	ND	ND

[a] DA, dark-adapted form of the chromophore; LA, light-adapted form; bR, bacteriorhodopsin; ND, no proton pumping activity was detected (<0.3% of wild type activity).

protonation during *in vitro* chromophore formation.

Effect of Altering the Side Chain at Position 85 on the Color of the Pigment—The λ_{max} values of the dark- and light-adapted forms of wild type bacteriorhodopsin and the mutant chromophores are summarized in Table I. The dark-adapted chromophore of the mutant D85C has a λ_{max} of 584 and is red shifted by about 40 nm relative to wild type bacteriorhodopsin. Derivatization of this mutant with iodoacetamide results in a chromophore that is indistinguishable from that of the underivatized D85C mutant. In contrast, derivatization of D85C with iodoacetic acid results in a large shift in the λ_{max} of the pigment to 536 nm at pH 7.2. Unlike the wild type bacteriorhodopsin chromophore which shows a 9-nm red shift upon light adaptation, the chromophore of the mutant D85C(cm) displays a reversible 13-nm blue shift upon light adaptation. Extraction of the retinal isomers and their analysis by HPLC show that the dark-adapted form of the mutant D85C(cm) chromophore contains 58% 13-*cis* and 42% all-*trans*-retinal. Following light adaptation the chromophore contains 78% all-*trans*, 12% 13-*cis* and 10% of other *cis*-retinal isomers. Under similar experimental conditions the λ_{max} of the dark-adapted chromophore of the D85E mutant was 546 nm and did not change upon light adaptation (Table I). To exclude the possibility that nonspecific modification of the opsins could result in alteration of the chromophores, wild type bacterioopsin was also incubated with iodoacetic acid or iodoacetamide. As expected, no spectral perturbation of wild type bacteriorhodopsin was deleted following refolding in mixed micelles (data not shown).

The chromophores of both the mutants D85E and D85C(cm) are particularly sensitive to pH in the range of 4–7. These mutants exhibit purple to blue transitions like wild type bacteriorhodopsin. However, the p*K* values of these transitions are shifted from ~3 in wild type (Mogi *et al.*, 1989) to 4.8 in the mutant D85C(cm)[2] and 6.2 in the mutant D85E (Subramaniam *et al.*, 1990). Similar changes were *not* observed in the mutants D85C and D85C(cam) over the same pH range. These results demonstrate that the presence of the carboxylate moiety at position 85 has a role in regulating the color of the pigment.

[2] D. A. Greenhalgh, S. Subramaniam, and H. G. Khorana, unpublished observations.

Proton Pumping Activity of the Mutants—The proton pumping activities of wild type bacteriorhodopsin along with the mutants D85E, D85C(cm), D85C(cam), and D85C are shown in Fig. 3 and summarized in Table I. The proton pumping experiments were performed at pH 7.2 since the chromophores of the D85E and D85C(cm) mutants are predominantly in their purple forms at this pH.

Fig. 3 clearly demonstrates that the mutant D85C(cm) is active in steady state proton pumping (~27% of wild type bacteriorhodopsin) and that the level of pumping activity is comparable to that found in the mutant D85E (~34% of wild type bacteriorhodopsin). Thus, the chemical modification of the inactive mutant D85C with iodoacetic acid results in a functionally active protein. The absence of proton pumping in the mutant D85C(cam) further demonstrates that a carboxylate moiety at residue 85 is essential for function.

Photocycle Measurements of the Mutants—During the bacteriorhodopsin photocycle the only photointermediate that has a deprotonated Schiff base is the M_{410} species (Lozier *et al.*, 1975). The transient changes in absorbance at 410 nm for micellar solutions of wild type bacteriorhodopsin and the mutants D85E and D85C(cm) are presented in Fig. 4. In wild type bacteriorhodopsin the kinetics of M formation are biphasic with fast (0.8 μs, 32%) and slow (9.1 μs, 68%) components. In the mutant D85E, M rise is a multiphasic process containing a large very fast unresolved component (43%) as

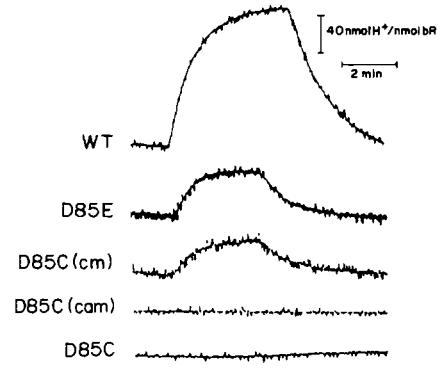

FIG. 3. **Steady-state proton pumping traces of wild type (*WT*) bacteriorhodopsin and the mutants D85E, D85C(cm), D85C(cam), and D85C.** The proteins were reconstituted into synthetic phospholipid vesicles, and proton pumping was measured at pH 7.2.

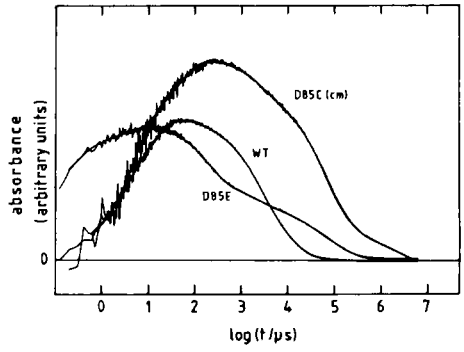

FIG. 4. **Time-resolved absorbance changes at 410 nm in wild type (*WT*) bacteriorhodopsin and the mutants D85E and D85C(cm) at pH 7.3.** For a better comparison of the kinetics the signal amplitudes of wild type bacteriorhodopsin and the mutant D85C(cm) are normalized such that the main components in the rise of M are equal. For the same ground state absorbance at 550 nm, the relative amplitudes of the M signal are 1:0.8:0.2 for wild type bacteriorhodopsin, D85E, and D85C(cm) respectively. The measurements were performed as described under "Experimental Procedures."

well as fast (0.2 μs, 38%) and slow (2.9 μs, 19%) components. M rise in the mutant D85C(cm) is more like wild type bacteriorhodopsin having a fast (5.7 μs, 68%) and a slow (61.6 μs, 32%) component. The amplitude of the absorbance change at 410 nm in this mutant is, however, much smaller than wild type. Since the amplitude of the depletion signal is likewise reduced by about the same factor, the quantum yield of cycling is apparently reduced. At pH 10 a very slow component develops in the decay of M of in the mutant D85C(cm) which is characteristic of the wild type bacteriorhodopsin photocycle. Measurements at other wavelengths show that the photocycle of D85C(cm) mutant has K- and O-like intermediates that are kinetically similar to wild type bacteriorhodopsin. Thus, under these experimental conditions, M rise in the mutant D85E is much faster than wild type bacteriorhodopsin, whereas M rise in the mutant D85C(cm) is comparable to wild type bacteriorhodopsin.

The mutants D85C and D85C(cam) which are inactive in proton pumping do not form any normal M intermediates during their photocycles in 1% DMPC, 1% CHAPS, 0.2% SDS, 150 mM KCl, 30 mM phosphate, pH 6.2 (data not shown). This is consistent with previous observations where other mutants containing neutral substitutions at this position (Asn, Ala, and His) do not form normal M intermediates in their photocycles and are inactive in proton transport (Mogi *et al.*, 1988; Otto *et al.*, 1990, Subramaniam *et al.*, 1992). In summary, only the proteins that have carboxylate-containing side chains at position 85 in bacteriorhodopsin have normal M intermediates in their photocycles and are active in proton transport.

DISCUSSION

During the photocycle of bacteriorhodopsin a proton is released to the extracellular side of the membrane, and another one is taken up from the cytoplasmic side. The proton release event occurs during the L⟶M stage of the photocycle (Grzesiek and Dencher, 1986). Previous studies have demonstrated that Asp^{85} plays a role in proton release since the replacement of this amino acid with neutral side chains results in proteins that do not form M intermediates in their photocycles and are completely inactive in proton pumping (Mogi *et al.*, 1988; Otto *et al.*, 1990; Subramaniam *et al.*, 1990; Stern *et al.*, 1989). However, replacement of Asp^{85} by Glu results in a functional proton pump (Mogi *et al.*, 1988). FTIR studies have shown that Asp^{85} which is deprotonated in the ground state becomes protonated during the L⟶M stage of the photocycle, suggesting that the carboxylate at position 85 serves as a proton acceptor for the Schiff base proton during M formation (Braiman *et al.*, 1988; Gerwert *et al.*, 1989).

We have shown here that, as expected, the mutant D85C is inactive, but that it is possible to chemically reactivate this mutant into a functional proton pump by modifying Cys^{85} with iodoacetic acid (Fig. 3). In a similar study with exotoxin A from *Pseudomonas aeruginosa*, Lukac and Collier (1988) found that the inactive cysteine mutant E553C could be reactivated following chemical modification with iodoacetic acid but not with iodoacetamide. Our studies show that a large variation in side chain volumes can be tolerated at position 85 in bacteriorhodopsin (aspartate ≃ 111 A^3 (Eggleston *et al.*, 1981a); glutamate ≃ 138 A^3 (Eggleston *et al.*, 1981b); and carboxymethyl Cys ≃ 159 A^3 (Mighell *et al.*, 1979)). In conclusion, these results, together with the previous studies on the mutants D85E, D85N, and D85A (Mogi *et al.*, 1988; Butt *et al.*, 1989; Otto *et al.*, 1990) confirm that the presence of a carboxylate-containing side chain at position 85 is obligatory for proton pumping activity.

The level of proton pumping activity in the mutant D85C(cm) is lower than in either wild type bacteriorhodopsin or the D85E mutant. This cannot be because of incomplete derivatization of the D85C mutant with iodoacetic acid since the chemical reaction was essentially complete under the experimental conditions used (Fig. 1). The lower activity may be caused by either a higher fraction of inactive *cis*-isomers in the light-adapted state or a lower apparent quantum yield in this mutant since the amplitude of the absorbance changes at 410 and 570 nm are significantly smaller than in wild type bacteriorhodopsin.

The absorption maxima of the light-adapted chromophores of wild type bacteriorhodopsin along with the mutants D85E and D85C(cm) in mixed micelles at pH 7.2 are 557, 546, and 523 nm, respectively. It can be seen from Table I that although the wild type bacteriorhodopsin chromophore becomes red shifted (548–557 nm) during light adaptation, the chromophore of the mutant D85C(cm) becomes blue shifted (536–523 nm). The HPLC experiments show that this blue shift is not caused by the generation of *cis*-isomers, since the proportion of all-*trans*-chromophore in the mutant D85C(cm) increases upon light adaptation like wild type bacteriorhodopsin.

Although these three carboxylate-containing side chains at position 85 are the only ones that result in Schiff base deprotonation, the kinetics of M formation are quite different in the three proteins. Previous experiments have demonstrated that the rise time of M in the D85E mutant is 5–20-fold faster than in wild type bacteriorhodopsin (Butt *et al.*, 1989; Otto *et al.*, 1990; Lanyi *et al.*, 1992). In contrast, the kinetics of M formation in the mutant D85C(cm) where the side chain at position 85 is one bond length longer than Glu is similar to wild type bacteriorhodopsin. It is likely that the rate of Schiff base deprotonation during the L⟶M transition will depend on the local dielectric constant, the distance between the donor and acceptor, the orientation of these groups, and their p*K* values in the L intermediate. It is possible to explain the accelerated kinetics of the M rise in D85E and the rather similar kinetics of wild type bacteriorhodopsin and the mutant D85C(cm) merely in terms of distance effects by positioning the three carboxylate moieties of the Asp, Glu, and carboxymethyl Cys side chains relative to the Schiff base at distances consistent with their dark- and light-adapted λ_{max} values. However, without more detailed information on the parameters mentioned above, any explanation for the different rates of M formation in wild type bacteriorhodopsin and these mutants would remain speculative.

There is evidence that Asp^{85} remains protonated well after the formation of the M intermediate (Gerwert *et al.*, 1990; Braiman *et al.*, 1991) and deprotonates only during the O⟶ bacteriorhodopsin transition (Muller *et al.*, 1991). The proton released into the extracellular medium during the L⟶M stage of the photocycle apparently comes from a group whose identity has not yet been established. FTIR experiments have suggested a role for Arg^{82} in this process (Braiman *et al.*, 1988). The reversal of release and uptake and the delayed proton release observed for the mutant R82A support this suggestion (Otto *et al.*, 1990). Furthermore, in the mutant D85E which shows a very fast M rise, proton release into the extracellular medium is delayed with respect to wild type bacteriorhodopsin, suggesting that Schiff base deprotonation and proton release are decoupled in this mutant (Heberle *et al.*, 1991). Our preliminary studies also show delayed proton release in the mutant D85C(cm)[3] suggesting an altered inter-

[3] H. Otto, U. Alexiev, and M. P. Heyn, unpublished observations.

action between the carboxylate moiety at position 85 and the group that releases the proton.

In addition to being involved in proton release, Asp^{85} is believed to be a major component of the complex counterion to the protonated Schiff base along with Asp^{212} and Arg^{82} in ground state bacteriorhodopsin (Otto *et al.*, 1990; Marti *et al.*, 1991, 1992). Previous studies have shown that the replacement of Arg^{82}, Asp^{85}, and Asp^{212} by other amino acids results in detectable changes in the rates of chromophore formation. For example, all the mutants D212N, D212A, D212E, D85N, R82Q, and R82C slow down the rates of chromophore formation (Mogi *et al.*, 1988; Stern and Khorana 1989; Greenhalgh *et al.*, 1991). In this respect, the mutants D85E and D85C(cm) are unique in that they are the only known mutations at these positions that increase the rate of chromophore formation in mixed micelles. The presence or absence of a carboxylate moiety at position 85 specifically influences the rate of chromophore formation in bacteriorhodopsin (Fig. 2). Consequently, Asp^{85} may be one of the two ionizable side chains previously predicted to play a role in the generation of the Schiff base linkage of the chromophore (Fischer and Oesterhelt, 1980). The large effects of Asp^{85} replacements on chromophore formation show that the interaction of the Schiff base with its counterion is an important step in determining the rate of protein folding.

Concluding Remarks—We have shown that residue 85 in bacteriorhodopsin can be replaced by side chains that vary considerably in size and that proton pumping activity is maintained only if a carboxylate moiety is present. Furthermore, the carboxylate at this position plays an important role in regulating the rate of Schiff base protonation during *in vitro* chromophore formation as well as serving as a major component of the complex counterion to the Schiff base in ground state bacteriorhodopsin. These results highlight the structural and functional importance of Asp^{85} in bacteriorhodopsin.

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