Functional Significance of a Protein Conformation Change at the Cytoplasmic End of Helix F During the Bacteriorhodopsin Photocycle

Leonid S. Brown,* György Váró,* Richard Needleman,‡ and Janos K. Lanyi*
*Department of Physiology and Biophysics, University of California, Irvine, California 92717, and ‡Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201, USA

ABSTRACT The second half of the photocycle of the light-driven proton pump bacteriorhodopsin includes proton transfers between D96 and the retinal Schiff base (the M to N reaction) and between the cytoplasmic surface and D96 (decay of the N intermediate). The inhibitory effects of decreased water activity and increased hydrostatic pressure have suggested that a conformational change resulting in greater hydration of the cytoplasmic region is required for proton transfer from D96 to the Schiff base, and have raised the possibility that the reversal of this process might be required for the subsequent reprotonation of D96 from the cytoplasmic surface. Tilt of the cytoplasmic end of helix F has been suggested by electron diffraction of the M intermediate. Introduction of bulky groups, such as various maleimide labels, to engineered cysteines at the cytoplasmic ends of helices A, B, C, E, and G produce only minor perturbation of the decays of M and N, but major changes in these reactions when the label is linked to helix F. In these samples the reprotonation of the Schiff base is accelerated and the reprotonation of D96 is strongly retarded. Cross-linking with benzophenone introduced at this location, but not at the others, causes the opposite change: the reprotonation of the Schiff base is greatly slowed while the reprotonation of D96 is accelerated. We conclude that, consistent with the structure from diffraction, the proton transfers in the second half of the photocycle are facilitated by motion of the cytoplasmic end of helix F, first away from the center of the protein and then back.

INTRODUCTION

The proton transfer pathway in the light-driven proton pump bacteriorhodopsin consists of the anionic D85 near the extracellular side of the protein, the centrally located protonated retinal Schiff base, and the undissociated D96 near the cytoplasmic side (reviewed by Mathies et al., 1991; Oesterhelt et al., 1992; Rothschild, 1992; Lanyi, 1992, 1993; Ebrey, 1993; Krebs and Khorana, 1993). Sequential proton transfers along this pathway initiated by photoisomerization of the retinal, and proton exchanges with the extracellular and cytoplasmic bulk phases, describe the trajectory of the transported proton. However, the transport proceeds unidirectionally (from the cytoplasmic toward the extracellular side) and will occur against a transmembrane proton gradient. Thus, like any other pump (Lanyi, 1995), this active transport system depends, additionally, on the alternating access of a proton binding site during the transport cycle from one side to the other. In bacteriorhodopsin this reaction was termed the "reprotonation switch" (Nagle and Mille, 1981; Kalisky et al., 1981; Fodor et al., 1988; Henderson et al., 1990; Váró and Lanyi, 1991b) and refers to the change of the connectivity of the Schiff base from D85 to D96 between its deprotonation and reprotonation.

The photoreaction cycle was described with a scheme that contains two states with unprotonated Schiff base, M_1 and M_2, that correspond to the pre- and post-switch configurations: BR $\rightarrow$ K $\Leftrightarrow$ L $\Leftrightarrow$ M_1 $\rightarrow$ M_2 $\Leftrightarrow$ N $\Leftrightarrow$ O $\rightarrow$ BR (Váró and Lanyi, 1991b,c; Váró et al., 1992; Zimányi et al., 1992a; Druckmann et al., 1992). The L $\Leftrightarrow$ M_1 equilibrium refers to the proton transfer from the Schiff base to D85, and the M_2 $\Leftrightarrow$ N equilibrium to the proton transfer after the switch, from D96 to the Schiff base.

The molecular details of what determines the connectivity of the Schiff base are not clear. In principle, the M_1 $\rightarrow$ M_2 reaction could be based on a change of the hydrogen-bonding geometry in this region, or on a change of the proton affinities of the two aspartates relative to the Schiff base (Kalisky et al., 1981). Significantly, however, diffraction of the two-dimensional bacteriorhodopsin array in purple membranes had revealed distinct protein conformation changes during the lifetime of the M state (Dencher et al., 1989; Nakasako et al., 1991; Koch et al., 1991; Subramaniam et al., 1993; Han et al., 1994; Kataoka et al., 1994). They are greater on the cytoplasmic side of the protein and involve helices F and G (Nakasako et al., 1991; Subramaniam et al., 1993). Helix F seems to tilt away from the center of the protein somewhat, so as to expose the retinal binding cavity to the aqueous phase. A structural change at helix F in the N state is suggested also by the FTIR spectrum of Y185 labeled in its carbonyl group (Ludlum et al., 1995). An increase of the surface area was suggested by a somewhat greater lattice constant in the M state (Nakasako et al., 1991). These structural changes are consistent with the effects of hydrostatic pressure on the rate constants of the photocycle reactions (Váró and Lanyi, 1995). It appears

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Address reprint requests to Dr. Janos K. Lanyi, Department of Physiology and Biophysics, University of California, Irvine, CA 92717. Tel.: 714-824-7150; Fax: 714-824-8540; E-mail: jlanyi@orion.oac.uci.edu.

The permanent address of Dr. Váró is Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, Hungary.

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from these that the $M_1 \rightarrow M_2$ reaction is associated with an increase of volume by about 30 cm^3/mol, and the recovery of the initial volume occurs during the decay of the N state that follows.

Structural changes can have additional roles in the photocycle. A large activation volume, $\Delta V^{\ddagger}$, detected in the $M_2 \Leftrightarrow N$ equilibrium by the effects of increased hydrostatic pressure on the forward and reverse rate constants, is consistent with the effects of lowered water activity, both in films upon reducing humidity (Váró and Lanyi, 1991a) and membrane suspensions upon adding osmotically active solutes (Cao et al., 1991). Proton exchange between the Schiff base and D96 is strongly inhibited under these conditions, suggesting that hydration is necessary for passage of protons through the cytoplasmic half of the protein. An increase of volume and therefore a high activation volume would be part of the barrier for the proton transfer if it required an increase in the binding of water, and this would account for the observed steep pressure dependence.

The suggested relationship between what appears to be a transient tilt of the cytoplasmic end of helix F, and the photocycle reactions that include reprotonation of the Schiff base and the ensuing events, makes a self-consistent model, but it has not been directly demonstrated. Testing such a relationship is difficult because the structure is not known to a resolution that would describe the conformational changes at the residue level near the Schiff base and D96, nor can the structural changes be linked to individual photocycle reactions with a time resolution sufficient to decide among the complex kinetic alternatives during the decay of M. We have taken a different approach instead. We introduced structural perturbations at various locations, 1) by covalently binding bulky molecules to engineered cysteine residues with the idea of easing the opening of a cleft between the helices but hindering its closing and 2) by cross-linking one of these labels with the idea of immobilizing the helices. Examination of the consequences of these modifications on the photocycle reactions would allow testing of hypotheses concerning the structural changes. We did not study the M_1 to M_2 conversion because, except in the D96N mutant at low pH (Zimányi et al., 1992b), dissecting the kinetics to reveal the equilibration process in this reaction is difficult. Furthermore, the activation volume for the $M_1 \rightarrow M_2$ step is not particularly high (Váró and Lanyi, 1995). Instead, we examined the proposition that structural changes on the cytoplasmic surface affect the reprotonation of the Schiff base and the reactions that follow.

Fig. 1 shows a view of the seven helices of bacteriorhodopsin (labeled A–G) from the cytoplasmic side. The F27C, L100C, F156C, T170C, and I222C residue replacements were chosen to introduce single cysteines, one at a time, near the cytoplasmic ends of helices A, C, E, F, and G, respectively, oriented, as far as the structure predicts, toward the center of the interhelical cavity. Helix D was not labeled because the first residue at its cytoplasmic end that is directed toward the center of the protein, I111, seemed too deeply buried. Wild-type bacteriorhodopsin contains no

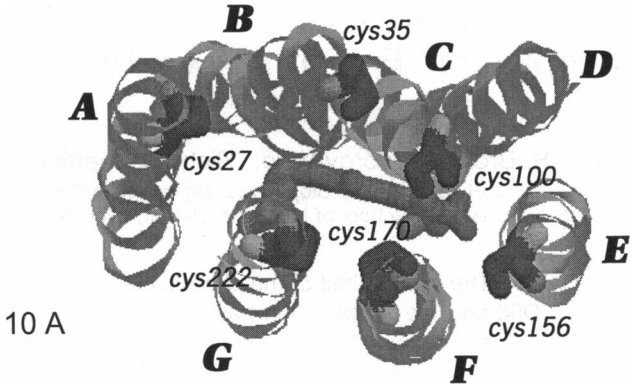

FIGURE 1 View of the seven helices of bacteriorhodopsin (A through G) from the cytoplasmic side, with six engineered cysteines edited into the structure. The source of the coordinates was the structure from cryo-electron microscopy (Henderson et al., 1990).

cysteines. The sixth (S35C) mutation places a cysteine on the short cytoplasmic loop between helices A and B, near the end of helix B. As described in this report, we found that the decays of the M and N intermediates are affected strongly when bulky groups are attached to C170 on helix F, weakly when they are attached to C156 on helix E, and virtually not at all when they are attached at the other locations. The effects of cross-linking on M decay are similarly far more effective at helix F than at others, although cross-linking at helices C, E, and G has some effects.

Cross-linking at the seventh location, at C163 on the E-F interhelical loop, caused effects on the decay of M to an extent between those observed at helices E and F, arguing for a general immobilization of helix F rather than any specific influences. These results identify the interhelical region bounded by helices E, F, and G as the most sensitive to perturbation, as expected if the motion of helix F has functional consequences. The decay rates for M and N after these modifications changed in the directions expected if the outward tilt of helix F promotes proton transfer to the Schiff base, and reversal of its tilt promotes proton uptake from the cytoplasmic surface. The results are therefore consistent with the diffraction changes that implicate helix F in the structural change (Subramaniam et al., 1993) and confirm that the detected opening of the cytoplasmic interhelical region near helix F is the functionally important conformational change in the second half of the photocycle.

MATERIALS AND METHODS

The site-specific mutations F27C, S35C, L100C, F156C, M163C, T170C, and I222C were introduced into the *bop* gene, the changed gene was inserted into a newly developed nonintegrating vector (to be described elsewhere), and *Halobacterium salinarium* was transformed as before (Ni et al., 1990; Needleman et al., 1991). The mutated and wild-type proteins were purified from *H. salinarium* as purple membrane sheets according to a standard method (Oesterhelt and Stoeckenius, 1974), but in the presence of 5 mM dithiothreitol to prevent oxidation of SH groups. Unless otherwise mentioned, all experiments were done at 22°C. In some cases the spectroscopy was with polyacrylamide gel-encased samples.

For covalent modification of the engineered cysteines, 15–30 nmol of purple membranes containing the recombinant bacteriorhodopsins were washed free of dithiothreitol and reacted in the dark in either 0.1 M NaCl, 0.1 M bis-tris-propane, pH 8, or 0.1 M phosphate and 1 mM EDTA, pH 8, by incubating overnight with various labels. Maleimidyl, iodoacetamido, and acryloyl derivatives and mercurials were used at 0.1 to 0.25 mM concentrations, and disulfides at 1 to 5 mM. After the reactions, the membranes were recovered by centrifugation, in some cases in a discontinuous sucrose gradient. Cross-linking with benzophenone derivatives (Dormán and Prestwich, 1994) was accomplished by exposure to "long" wavelength illumination (>340 nm) with a Mineralight UVSL-25 lamp (1 cm distance). The UV illumination was continued until the consequences of the cross-linking on the photocycle no longer increased in magnitude (2–4 h).

Transient spectroscopy at single wavelengths was as described before (Cao et al., 1993). Determination of pH changes during the photocycle was made with and without pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) (Grzesiek and Dencher, 1986; Heberle and Dencher, 1992; Brown et al., 1994; Cao et al., 1995). Absorbance changes at varying hydrostatic pressures were determined as described elsewhere (Váró and Lanyi, 1995).

RESULTS

Introduction of covalently linked labels to the cytoplasmic ends of six of the helices

The cysteine residues at the helical ends of the six recombinant bacteriorhodopsins in Fig. 1, and on the E-F interhelical loop, were tested for reactivity with many commonly used sulfhydryl-specific reagents. For the maleimide labels, pyrene maleimide and maleimidyl anilinonaphthalene sulfonic acid were the most suitable for determining the extents of the reactions because they have high extinctions at wavelengths where the chromophore of bacteriorhodopsin does not interfere. Table 1 shows the molar stoichiometries of covalently linked maleimidyl pyrene and anilinonaphthalene sulfonic acid at the seven locations. Purple membranes of wild-type bacteriorhodopsin retained virtually no label, as expected from the lack of cysteine and the good specificity of maleimides for SH groups at pH not far from neutral (van Iwaarden et al., 1992). Labeling was between about 30 and 100%, depending on the location, but was less dependent on whether the label was hydrophobic or polar. Observations of samples preincubated without label (not shown) identified the cause of the incomplete reactions at some of the locations as rapid oxidation of the cysteine that competed with the maleimide reaction. Tris(2-carboxyethyl) phosphine (Han and Han, 1994) did not prevent oxidation of the cysteines. Adding EDTA (van Iwaarden et al., 1992) improved the yields, but did not protect fully.

These reactions, and all others discussed below, left the maxima of the absorption bands of the retinal chromophore unchanged, as did the seven amino acid replacements with cysteine. Figs. 2 and 3 show the consequences of maleimidyl anilinonaphthalene sulfonic acid labeling at the locations shown in Fig. 1 on the photocycle. Absorbance changes after laser pulse photoexcitation were measured at two wavelengths, 410 and 570 nm. Absorbance increase at 410 nm reveals the accumulation of the M state. The decrease at 570 nm originates from depletion of the BR state, and thus reflects partly the formation of M also, but once M has decayed the remaining negative absorbance is from the presence of the N intermediate. Although data for wild-type bacteriorhodopsin are not included in Figs. 2 and 3, we

TABLE 1 Modification yields (in mol/mol) for different cysteine mutants of bacteriorhodopsin, using two maleimide labels

Mutant	*N*-(1-Pyrene) maleimide	2-(4′-Maleimidylanilino) naphthalene-6-sulfonic acid
F27C	0.29	0.29
S35C	0.47	0.61
L100C	0.34	0.37
F156C	0.67	1.06
M163C	1.05	1.13
T170C	0.70	1.02
I222C	0.60	0.34

Reaction conditions as described in Materials and Methods. The yield was calculated from the additional absorbance of pyrene and anilinonaphthalene sulfonic acid in centrifuged, washed purple membrane samples, at 339 and 327 nm, respectively. Wild-type bacteriorhodopsin was labeled to a negligible extent.

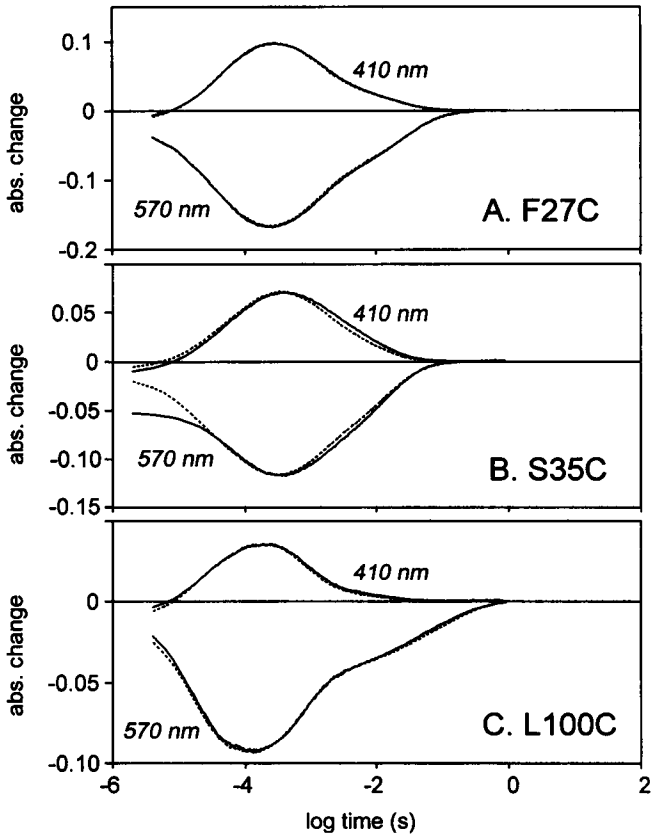

FIGURE 2 Kinetics of the photocycles of F27C, S35C, and L100C bacteriorhodopsins, before (*dotted lines*) and after (*solid lines*) reaction with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid. The absorbance changes at 410 and 570 nm reveal the accumulation of the M, and in some cases also the N photointermediates, and their formation and decay. Conditions: 20–25 μM bacteriorhodopsin, 0.1 M sodium phosphate, 1 mM EDTA, pH 8.

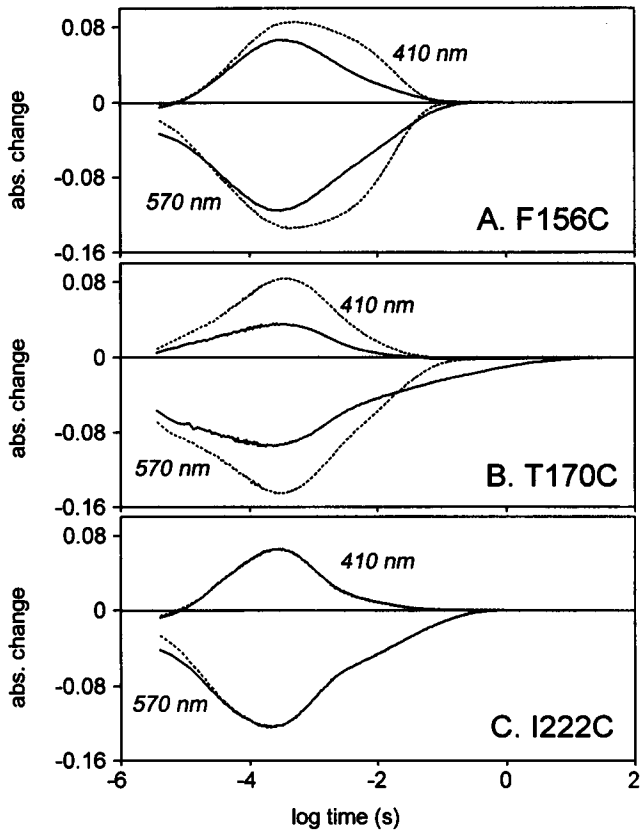

FIGURE 3 Kinetics of the photocycles of F156C, T170C, and I222C bacteriorhodopsins before (*dotted lines*) and after (*solid lines*) reaction with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid. The absorbance changes at 410 and 570 nm reveal the accumulation of the M, and in some cases also the N photointermediates, and their formation and decay. Conditions as in Fig. 2.

ascertained that when unlabeled (dotted lines), three of the the six cysteine-containing mutants (at residues 27, 35, and 170) had nearly unperturbed photocycles, two mutants (at residues 100 and 222) had somewhat more rapidly decaying M and more slowly decaying N, and F156C had somewhat slower decaying M. At position 100 (helix C) the changed decay for M and N is consistent with perturbation of the environment of D96 that would affect its protonation reactions. Changes near this location during the photocycle were revealed by spin labels linked to residue 101 (Steinhoff et al., 1994).

After the reaction with 2-(4′-maleimidylanilino) naphthalene sulfonic acid, the M decay of T170C became somewhat more rapid, and the N decay much more slow (by about 2 orders of magnitude). Similar changes, but to much lesser extents, occurred in F156C, where the wild-type kinetics were regained after the labeling. In the other four mutants the label had no detectable effect, even when the lesser extents of labeling (Table 1) are considered. Virtually the same results as in Figs. 2 and 3 were obtained after reaction of these proteins with *N*-phenylmaleimide (not shown). In fact, the size of the maleimide labels had little influence on the perturbations. Reaction of C170 with *N*-ethylmaleimide, *N*-(1-pyrene)maleimide, or benzophenone-4-maleimide produced very similar changes in the kinetics. It appeared, therefore, that the effects of labels at position 170 originated mainly from the maleimide ring, and not the rest of the molecule.

These effects on the photocycle were examined therefore for a larger number of chemical modifications at C170. Qualitatively, M decay was faster in most cases than in the unmodified sample (not shown), but the presence of several ambiguous exponential components precluded a rigorous kinetic analysis. They must have been caused by incomplete reactions in some of the cases (cf. Table 1). The analysis of N decay was somewhat simpler. Table 2 gives the time constants for the last decay component of the absorption change at 570 nm, which refers to the conversion of N to BR. Small groups linked to the cysteine at position 170, such as acetamido or *S*-methyl, produced about 10-fold slowing of the decay of N, whereas maleimides produced about 100-fold slowing with little further influence from the size of the attached group. We conclude that whatever interference is caused by these covalent modifications, the location of the perturbation is indeed very near the engineered cysteine. Presumably, when a large group is linked to the distal part of the maleimide ring, it assumes a position near the surface that does not conflict with residues in the interior of the protein.

Properties of *N*-phenylmaleimide-labeled C170 bacteriorhodopsin

At a pH of >8.5, the uptake of the transported proton on the cytoplasmic side was earlier described as the $N^{(-1)} + H^+ \rightarrow N^{(0)}$ reaction in the scheme $M_2^{(-1)} \Leftrightarrow N^{(-1)} \Leftrightarrow N^{(0)} \rightarrow$ BR (Zimányi et al., 1993), where the superscripts represent the net protonation of the protein relative to the unphotolyzed state. The reisomerization of the retinal to all-*trans* appears under these conditions in the $N^{(0)} \rightarrow$ BR reaction because the O state does not accumulate. Usually $N^{(-1)}$ is the N state seen, but depending on the relative rates of proton uptake and reisomerization, under some conditions,

TABLE 2 Decay time constant for the N intermediate after covalent labeling of T170C bacteriorhodopsin with various agents

Modification	Time constant, s
Control	0.044
Methyl-methane thiosulfonate	0.36
Iodoacetamide	0.43
4,4′-Dithiopyridine	1.4
N-(1-Pyrene) maleimide	3.5
N-Phenylmaleimide	3.5
N-Ethylmaleimide	3.7
6-Acryloyl-2-dimethylaminonaphthalene	4.0
Phenylmercuric acetate	4.05
Benzophenone-4-iodoacetamide	5.2
Benzophenone-4-maleimide	8.2

Modification and assay in 100 mM NaCl, 100 mM bis-tris-propane, pH 8.

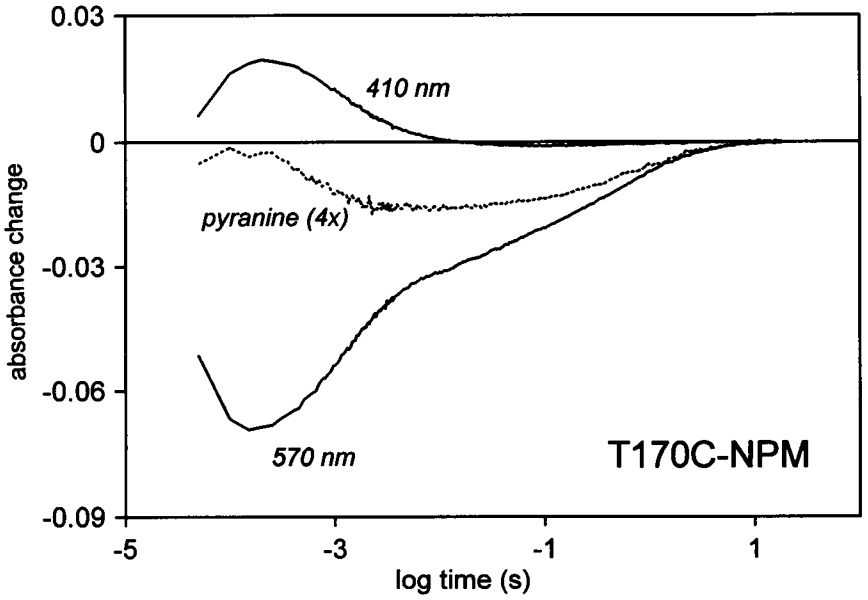

FIGURE 4 Kinetics of the M and N intermediates and proton release and uptake in T170C bacteriorhodopsin modified with *N*-phenyl maleimide. The trace labeled as pyranine was obtained by subtracting the signal at 457 nm without pyranine from one with 50 μM pyranine. Conditions: 15 μM bacteriorhodopsin, 2 M NaCl, pH 7.2.

for example increasingly at a pH of >9, $N^{(0)}$ accumulates also (Zimányi et al., 1993). Which of these processes is affected by the chemical modification of C170, which slows down the decay of N by as much as 100-fold? Fig. 4 shows absorbance changes after photoexcitation, measured at 410 and 570 nm, as in Figs. 2 and 3, for the *N*-phenylmaleimide derivative of C170, as well as the net absorbance change of the pH indicator dye pyranine, which detects protons released into the bulk (Grzesiek and Dencher, 1986; Zimányi et al., 1993; Cao et al., 1993; Brown et al., 1994). The protons released from the extracellular side are detected at about 1 ms as in the wild type, but their uptake on the cytoplasmic side is slowed from about 10 ms to several seconds, which is similar to the decay of N. If the proton uptake itself were the rate-limiting step in the modified protein, the decay of N would be pH dependent, which is similar to the second component of M decay (which proceeds with the rate of the $N^{(-1)} + H^+ \rightarrow N^{(0)}$ reaction at pH > 8) in the wild-type protein. This was not observed. The decay of N was nearly independent of proton concentration (slope of log *k* vs pH was 0.13, not shown) between pH 4.8 and 8.6. Slowed proton uptake and pH independence were observed also after acryloyldimethylaminonaphthalene and pyrene maleimide labeling (not shown). We conclude that the chemical modification at C170 affected N decay by inhibiting a process after the reprotonation of the Schiff base but before the uptake of a proton at the cytoplasmic surface.

In a recent study of the effects of hydrostatic pressure on the photocycle rate constants (Váró and Lanyi, 1995), we found that although there was almost no volume difference between the M and N intermediates, the interconversion of these states was dependent on a very high (about 50 cm^3/mol) activation volume. Thus, proton transfers from D96 to the Schiff base and from the Schiff base to D96 were both strongly inhibited by increasing the pressure. The nature of the volume increase in the transition state for the proton exchange is not clear, but one possibility is that it is related to the opening of a cleft at the cytoplasmic surface already in the M state, suggested by electron diffraction (Subramaniam et al., 1993). Because a bulky group linked to helix F should interfere with the motion of this helix implicated in this opening (and the closing), we measured the photocycle reactions of *N*-phenylmaleimide-labeled T170C at increased hydrostatic pressures. Fig. 5 shows absorbance changes after photoexcitation, measured at 410 and 570 nm as before, at 1 bar, 0.5 kbar, and 1 kbar in three kinds of samples. In Fig. 5 *A* the slowing of the M decay with pressure is illustrated in wild-type bacteriorhodopsin. As described elsewhere in more detail (Váró and Lanyi, 1995), the approximately 10-fold slower rate at 1 kbar is consistent with the large volume increase in the transition state. Although Fig. 5 *A* contains information also about the decay of N, this cannot be extracted without a complete kinetic analysis. Under conditions where the proton uptake is limited by proton concentration (i.e., at pH 10), the decay of N is not very pressure sensitive (Váró and Lanyi, 1995). However, an acceleration of the N decay process at increased pressure is illustrated directly in the T46V mutant, which is shown in Fig. 5 *B*. In this mutant, proton uptake and the decay of N are limited by structural influences (Brown et al., 1994), and unlike in the wild type they are so well separated in time from the decay of M as to be kinetically independent of it. The traces at 410 and 570 nm demonstrate both the slowed M decay and the accelerated N decay at the higher pressures. Thus, the pressure dependencies of the decays of M and N are consistent with opening and closing of the cytoplasmic region of the protein. In view of these results, the pressure dependencies of these processes in the *N*-phenylmaleimide derivative of C170 bacteriorhodopsin (Fig. 5 *C*), where the

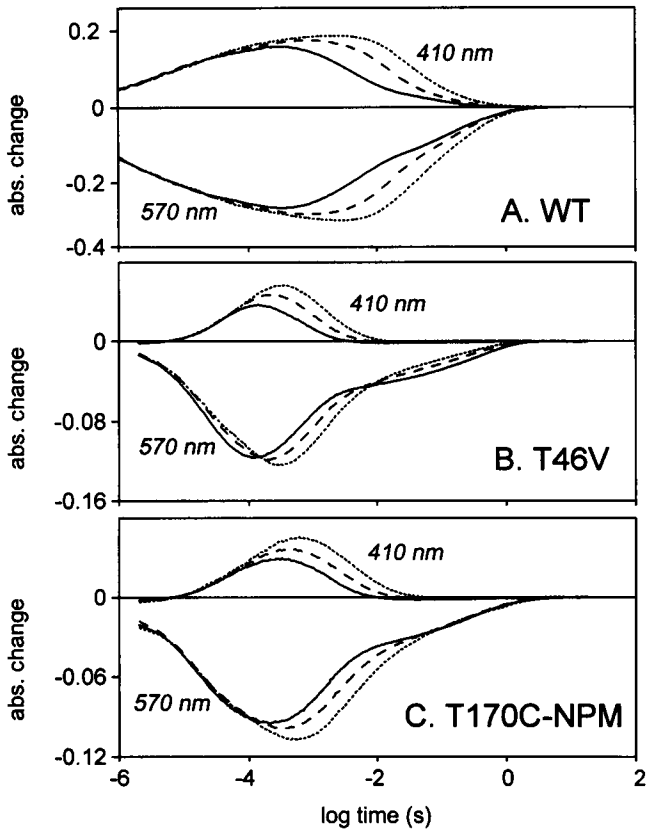

FIGURE 5 Effect of increased hydrostatic pressure on the photocycles of wild-type bacteriorhodopsin (*A*), the T46V mutant (*B*), and T170C bacteriorhodopsin labeled with *N*-phenyl maleimide (*C*). Pressures: ——, 1 bar; – – –, 0.5 kbar; · · ·, 1 kbar. Conditions: for (*A*), 0.1 M NaCl, 50 mM bis-tris-propane, pH 9.5; for (*B*) and (*C*), 0.1 M NaCl, 0.1 M bis-tris-propane, pH 6.4. Bacteriorhodopsin, 15 μM; the temperature was controlled at 20°C.

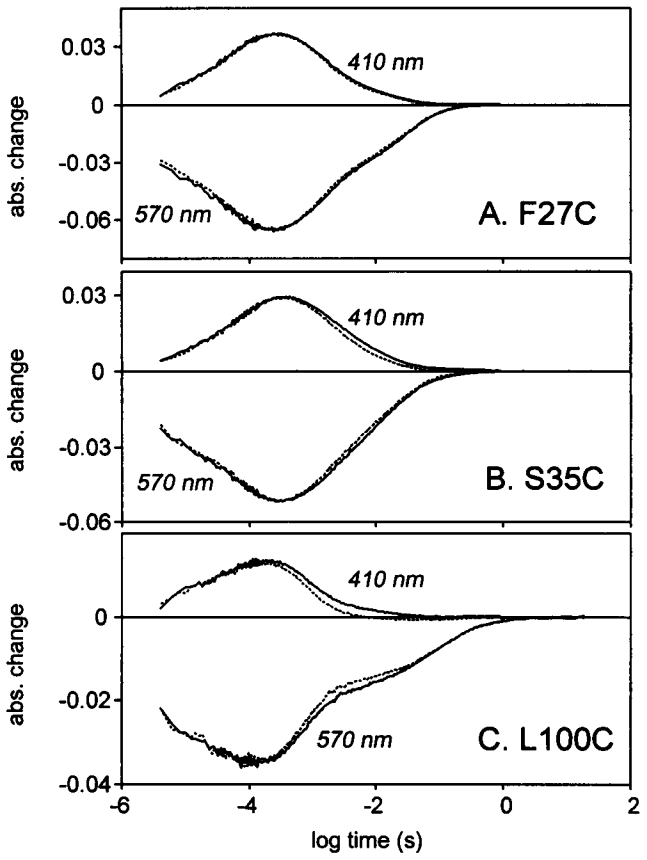

FIGURE 6 Kinetics of the photocycles of F27C, S35C, and L100C bacteriorhodopsins labeled with benzophenone-4-maleimide, before (*dotted lines*) and after (*solid lines*) irradiation with near-UV that results in cross-linking. The absorbance changes at 410 and 570 nm reveal the accumulation of the M, and in some cases also the N photointermediates, and their formation and decay. Conditions as in Fig. 2, but bacteriorhodopsin between 8–12 μM.

relationship of M and N is otherwise quite similar to the relationship of M and N in the T46V mutant, are significant. According to these results M decay is slowed, as in the wild type and T46V, but N decay is virtually unaffected by pressure.

Cross-linking with covalently linked labels at the cytoplasmic ends of six of the helices

The chemical labeling also offers an opportunity for achieving the opposite effect: immobilizing the helices through cross-linking. Labeling of cysteines at the six locations in Fig. 1 with benzophenone-4-maleimide results in the same kind of effect on the photocycle as any of the other maleimide labels (Figs. 6 and 7, dotted lines, and Table 2). Irradiation of this agent with near-UV light (>340 nm) causes production of a free radical at the carbonyl group, however, and nonspecific cross-linking by reaction with any neighboring C-H bond (Dormán and Prestwich, 1994). Figs. 6 and 7 (solid lines) shows that virtually no differences were seen after such cross-linking at residues 27 and 35 (helices A and B), and only minor slowing of the decay of M at residues 100, 156, and 222 (helices C, E, and G). However, very significant slowing of the decay of M (about 100-fold) and acceleration of the decay of N occur when the cross-linking is at residue 170 (helix F). Similar effects at C170 were observed after cross-linking of benzophenone iodoacetamide labeld samples (not shown).

Cross-linking with a covalently linked label at the cytoplasmic loop between helices E and F

Reaction of maleimide labels with M163C introduced bulky groups to what appears from the structure (Henderson et al., 1990) to be a location well into the hydrophilic loop between the cytoplasmic ends of helices E and F. The decay of M was made more rapid, as described above. However, comparison of three labels, *N*-phenylmaleimide, maleimidyl anilinonaphthalene, sulfonic acid, and benzophenone maleimide indicated (Fig. 8) that, unlike at the other locations (Figs. 2, 3, 6, 7), here the size of the label has a strong influence on the photocycle reactions. The decay of M is nearly unaffected by the phenyl group, somewhat acceler-

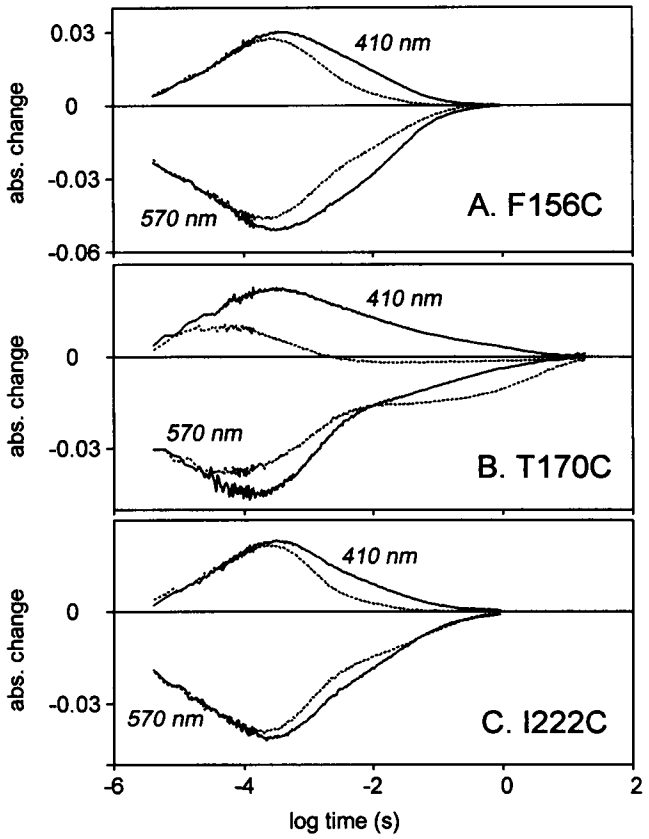

FIGURE 7 Kinetics of the photocycles of F156C, T170C, and I222C bacteriorhodopsins labeled with benzophenone-4-maleimide, before (*dotted lines*) and after (*solid lines*) irradiation with near-UV that results in cross-linking. The absorbance changes at 410 and 570 nm reveal the accumulation of the M, and in some cases also the N photointermediates, and their formation and decay. Conditions as in Fig. 6.

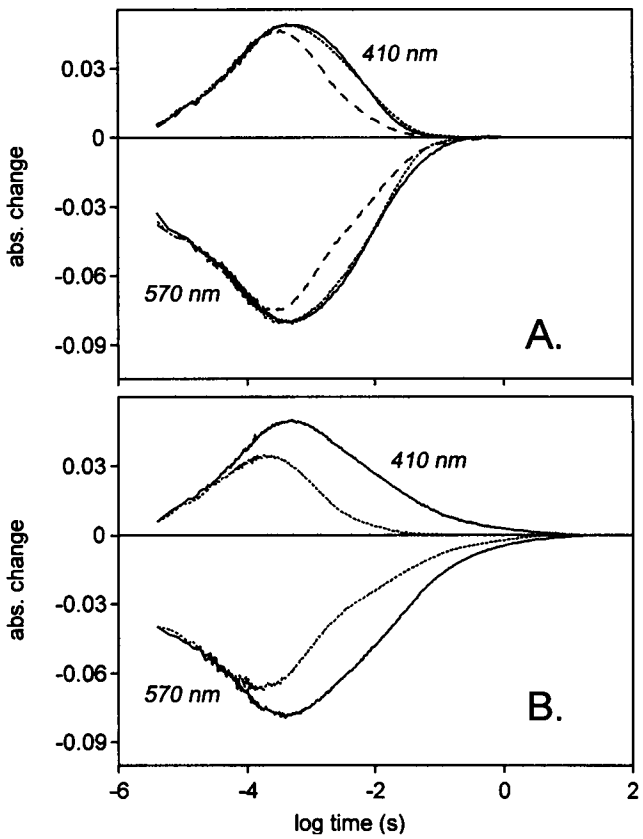

FIGURE 8 Kinetics of the photocycles of M163C bacteriorhodopsin, (*A*) unlabeled, and labeled with *N*-phenyl maleimide, maleimidyl anilinonaphthalene sulfonic acid, and (*B*) benzophenone-4-maleimide. (*A*) ——, control; · · ·, *N*-phenyl maleimide; – – –, maleimidyl anilinonaphthalene sulfonic acid. (*B*) Before (· · ·) and after (——) irradiation with near-UV that results in cross-linking. The absorbance changes at 410 and 570 nm reveal the accumulation of the M, and in some cases also the N photointermediates, and their formation and decay. Conditions as in Fig. 6, but with 15 μM bacteriorhodopsin.

ated by anilinonaphthalene sulfonate, and considerably accelerated by benzophenonone. The perturbation is caused therefore not by the maleimide ring but the labeling group. Cross-linking by irradiation of the benzophenone derivative caused slowing of the decay of M by about an order of magnitude (Fig. 8 *B*). The magnitude of this effect is between those of cross-linking at helices E and F (Fig. 7, *A* and *B*), consistent with a general immobilization of helix F, depending on the distance of the site of cross-linking from its cytoplasmic end rather than any specific effects of the benzophenone label.

DISCUSSION

We have studied the consequences of introducing bulky groups and cross-linking at selected locations at or near the cytoplasmic surface of bacteriorhodopsin. The intention was to test the hypothesis that there are functionally relevant conformational changes in the photocycle that consist of an "opening" and "closing" of the interhelical cavity to the aqueous medium, related to the outward tilt of helix F suggested by diffraction changes (Subramaniam et al., 1993) in the M intermediate. Results with osmotically active solutes (Cao et al., 1991) and high hydrostatic pressure (Váró and Lanyi, 1995) suggested that the proton transfer from D96 to the Schiff base, and perhaps also the reprotonation of D96, are controlled by the changing dipole environment provided by a changing hydration of the cytoplasmic domain. We would expect therefore that an "opening" would allow an influx of water into the protein and trigger the reprotonation of the Schiff base from the cytoplasmically located D96, and the "closing" should in turn expel the water and raise the pK_a of D96 to facilitate the proton uptake from the cytoplasmic surface. Placing a bulky group at an engineered cysteine residue on helix F that faces the interhelical region where the putative "opening" occurs would interfere with the association of this helix with the rest of the protein, and thereby predispose it toward tilting out and against tilting back. Thus, derivatization at this location, but not at others, should result in more rapid reprotonation of the Schiff base (decay of the M state), but slower reprotonation of the cytoplasmic region (decay of the

N state). By the same token, immobilization of the protein by cross-linking with a label at helix F (but not at other helices) should hinder the motion of this helix, and thus slow the reprotonation of the Schiff base. These expectations were confirmed.

This kind of approach to assessing the functional consequences of a conformational change during the reaction cycle of a protein is admittedly crude and could produce misleading results. Perturbing the structure at any location may result in unpredictable changes at other locations, and for unambiguous interpretation of the results it is essential to have confidence in the integrity of the protein and the reaction kinetics. However, the simple seven-helical structure of bacteriorhodopsin is robust, and the spectroscopically measurable properties of the chromophore provide some assurance that, near the retinal at least, the protein is not greatly perturbed by the modifications. We regard it as important that the effect of the modifications at various locations is nearly all or nothing (Figs. 2 and 3). Thus, most locations at the cytoplasmic ends of the helices examined are not at all sensitive to the reagents used. Varying the size and charge of the reacting group has relatively little effect on the slowing of the decay of the N intermediate (Table 2), suggesting that the process is affected by bulk fairly near the reacting C170 residue. In fact, because *N*-ethylmaleimide modification produces as great an effect as reaction with any of the larger labels, the maximal distance at which the protein is perturbed is not more than about 6 Å from the SH group of the cysteine on helix F and must originate from the maleimide ring itself. In contrast, when the label is on the E-F interhelical loop, the location of the perturbation is at a greater distance from the cysteine (Fig. 8), as might be expected.

The effects of hydrostatic pressure on the photocycle of the *N*-phenylmaleimide-modified C170 protein (Fig. 5 *C*) provide additional confirmation of the "opening/closing" hypothesis. Although the slowing of the decay of M is as in the wild type and suggests that the outward tilt of helix F occurs in this case also, the decay of N is unaffected by pressure. We visualize this as the consequence of a stringent requirement for the "closed" conformation for proton uptake, which can be poorly satisfied when the cytoplasmic end of helix F is forced away from the interhelical cavity. One might expect that the compressibility of the structure that contains the maleimide ring wedged into this space would be small, and even increased pressure would not cause the normal closing of the cytoplasmic region in the photocycle.

Whereas reaction with benzophenone-4-maleimide produces the same behavior as any of the maleimides (compare Figs. 2 and 3 and Table 2 with Figs. 6 and 7), cross-linking of this label at helix F to another (as yet unknown) part of the protein produces just the opposite result. Thus, reprotonation of the Schiff base is slowed by 2 orders of magnitude, consistent with a requirement for helix F to move away. This slowing of the decay of M by cross-linking is dependent on the distance of the benzophenone label from helix F. It is greatest at helix F (Fig. 7), less at the E-F interhelical loop (Fig. 8), even less at helices E, G, and C (Fig. 7), and nearly absent at the other helices (Fig. 6), suggesting that the cross-linking affects helix F, and in a nonspecific way.

The results support the involvement of structural changes at the cytoplasmic surface in the photocycle reactions. In particular, they are consistent with the idea that the outward displacement of helix F triggers the protonation of the Schiff base by D96, and its reverse motion creates the conditions where D96 can be reprotonated from the cytoplasmic surface.

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