Protein Structural Change at the Cytoplasmic Surface as the Cause of Cooperativity in the Bacteriorhodopsin Photocycle

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ABSTRACT The effects of excitation light intensity on the kinetics of the bacteriorhodopsin photocycle were investigated. The earlier reported intensity-dependent changes at 410 and 570 nm are explained by parallel increases in two of the rate constants, for proton transfers to D96 from the Schiff base and from the cytoplasmic surface, without changes in the others, as the photoexcited fraction is increased. Thus, it appears that the pK_a of D96 is raised by a cooperative effect within the purple membrane. This interpretation of the wild-type kinetics was confirmed by results with several mutant proteins, where the rates are well separated in time and a model-dependent analysis is unnecessary. Based on earlier results that demonstrated a structural change of the protein after deprotonation of the Schiff base that increases the area of the cytoplasmic surface, and the effects of high hydrostatic pressure and lowered water activity on the photocycle steps in question, we suggest that the pK_a of D96 is raised by a lateral pressure that develops when other bacteriorhodopsin molecules are photoexcited within the two-dimensional lattice of the purple membrane. Expulsion of no more than a few water molecules bound near D96 by this pressure would account for the calculated increase of 0.6 units in the pK_a.

INTRODUCTION

In bacteriorhodopsin, the light-driven proton pump of the halobacteria, photoisomerization of the retinal initiates sequential proton transfers between the protonated retinal Schiff base and D85 located to the extracellular side, and D96 located to the cytoplasmic side and the deprotonated Schiff base (Mathies et al., 1991; Tittor, 1991; Ebrey, 1993; Krebs and Khorana, 1993; Lanyi, 1993). Studies of this protein have been greatly helped by the fact that it forms trimers within a two-dimensional hexagonal crystalline lattice (Blaurock and Stoeckenius, 1971), known as the purple membrane. In these membranes bacteriorhodopsin is the only protein, and the lipid content is unusually low (Kushwaha et al., 1975; Fisher and Stoeckenius, 1977; Blaurock, 1982). The monomer is active in proton transport (Dencher and Heyn, 1979), but because the rigid crystalline structure confers extraordinary stability on the protein, most investigations are carried out with purple membranes rather than with detergent-solubilized bacteriorhodopsin monomers. In one respect, however, the purple membranes are more problematic than the monomers, and this has been a matter of much recent dispute and controversy. In purple membranes, but not in the monomers, the photocycle, which describes the sequence of intermediate states (termed J, K, L, M, N, and O) after photoexcitation of the retinal chromophore with a light pulse, exhibits distinct kinetic differences when the intensity of the actinic laser beam is varied. The differences consist mainly of what appears to be an increase in the amplitude, although not the time constant, of the slower component in the biphasic decay of the M intermediate at pH near 9 (Tokaji and Dancsházy, 1992; Dancsházy and Tokaji, 1993; Tokaji, 1993, 1995; Hendler et al., 1994; Mukhopadhyay et al., 1994; Shrager et al., 1995). Thus, the excitation intensity can evidently influence not only the number of bacteriorhodopsins that are photoexcited but also the rate of at least one of the steps in the proton transport.

Inasmuch as the kinetic scheme of the photocycle itself has been disputed, the origin of this behavior has not been settled. In fact, it has been suggested that the ability to explain the intensity dependence of the kinetics might be a test for the validity of photocycle models and a way to decide among the various proposed schemes (Shrager et al., 1995). There are three alternatives. First, it was proposed that bacteriorhodopsin samples are heterogeneous with respect to the decay rate of the M state, and higher laser intensities excite with greater efficiency the subpopulation that produces the "slowly decaying" M (Shrager et al., 1995). Second, at high laser intensities the two proposed populations interact during the photocycle through a directional cooperative effect within the trimers, and whenever a neighboring bacteriorhodopsin molecule is excited the M decay is more likely to be of the slowly decaying kind (Tokaji, 1993, 1995; Komrakov and Kaulen, 1995). Third, there is only one bacteriorhodopsin population with respect to M decay, and because the two decay phases originate from equilibration of M with the N state, followed by decay of the M *plus* N mixture, the intensity dependence is through interaction with neighboring bacteriorhodopsin molecules that change the rate constants. As indicated in a recent review (Shrager et al., 1995), in the third alternative but not in the first two, not only the amplitudes but also the

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rate constants would have to change with the exciting light intensity.

This report is our attempt to explain the intensity dependence with the third alternative, the single photocycle model. It is true that several kinetic models can fit the data for the wild-type bacteriorhodopsin, and as usual, it is not always possible to decide purely on mathematical grounds which is correct. We are guided by the principle that, when several models are consistent with the observations the one more likely to be correct is that which a) does not require new and additional hypotheses to support it, b) explains more than one kind of data, and c) represents a reasonable physical process. The analysis of the kinetic data in terms of the single cycle model indicates that it is the two protonation equilibria for D96 that are affected by the excitation intensity. Higher intensities shift both of them in the direction of increased protonation of this aspartate. We argue that general cooperative interaction in the purple membrane, based on the conformational change at the cytoplasmic surface (Subramaniam et al., 1993) in the single photocycle model, is an adequate and complete explanation for this. Enlargement of the cytoplasmic surface due to the outward tilt of helix F, suggested by the crystallographic information, should cause compression in the plane of the membrane, and when sufficiently high numbers of bacteriorhodopsin molecules are photoexcited, should cause a loss of water molecules from the region of D96, with an ensuing rise in its pK_a. This model correlates the intensity dependence with a variety of other data: diffraction changes in the M state (Subramaniam et al., 1993), as well as the effects of increased hydrostatic pressure (Váró and Lanyi, 1995) and decreased water activity (Cao et al., 1991) on this part of the photocycle. It is consistent with the physical processes that cause proton transfers in the photocycle.

MATERIALS AND METHODS

Purple membranes were prepared from *Halobacterium salinarium* by a standard method (Oesterhelt and Stoeckenius, 1974). The D96N, T46V, and T46V/R227Q mutants used have been described before (Cao et al., 1991; Brown et al., 1994). Absorption changes were followed after photoexcitation with a Nd-Yag laser pulse (532 nm, 7 ns), as in earlier publications of ours (*e.g.*, (Váró et al., 1995). The intensity of the exciting light was varied with neutral density filters. The intensity denoted as 100% corresponds to 55 mJ/cm^2, which photoexcited an estimated 35% of the bacteriorhodopsin molecules. All other intensities are relative to this value. The temperature was regulated at 20°C throughout.

The various possible models were globally fitted (Nagle, 1991) to the kinetic data at 410 and 570 nm with the RATE program, written by G. Groma, using the extinctions of the M, N, and the unphotolyzed state at the two wavelengths (Zimányi et al., 1993; Váró and Lanyi, 1995). This program also calculated the time-dependent concentrations of the intermediates from the rate constants of the best fits.

RESULTS

The dependence of photocycle kinetics on the excitation energy is usually measured by following the absorption changes at 410 nm, which originate nearly entirely from the M intermediate, after the photoexcitation. The effects of excitation energy on the M kinetics are most evident at alkaline pH. We measured these changes and the changes at 570 nm that originate from depletion of BR associated with the accumulation of M, but in the millisecond time range they also indicate the presence of the N intermediate because N has a lower extinction than BR at this wavelength (Fukuda and Kouyama, 1992; Zimányi et al., 1993). In particular, at pH > 9 the kinetics at 570 nm contains a decay component slower than any of those at 410 nm. We had interpreted the spectroscopic data under these conditions with the scheme, $M_1 \rightarrow M_2 \Leftrightarrow N^{(-1)} \Leftrightarrow N^{(0)} \rightarrow BR$, where the $N^{(-1)}$ and $N^{(0)}$ states referred to the N state before and after reprotonation of D96, respectively (Zimányi et al., 1993). The high pH ensured that both $N^{(-1)}$ and $N^{(0)}$ could be observed and was advantageous not only for optimal cooperativity, but also because the O state did not accumulate to complicate the kinetics. Fig. 1 shows kinetics in wild-type bacteriorhodopsin at pH 9.5 at the two wavelengths, at 10% and 100% excitation intensities. As found before (Tokaji and Dancsházy, 1992; Dancsházy and Tokaji, 1993), the rise of the M state is unaffected but the decay at 410 nm is changed. Table 1 shows that, when fitted with two exponentials, the amplitude of the slower component increased at the greater excitation intensity, but the

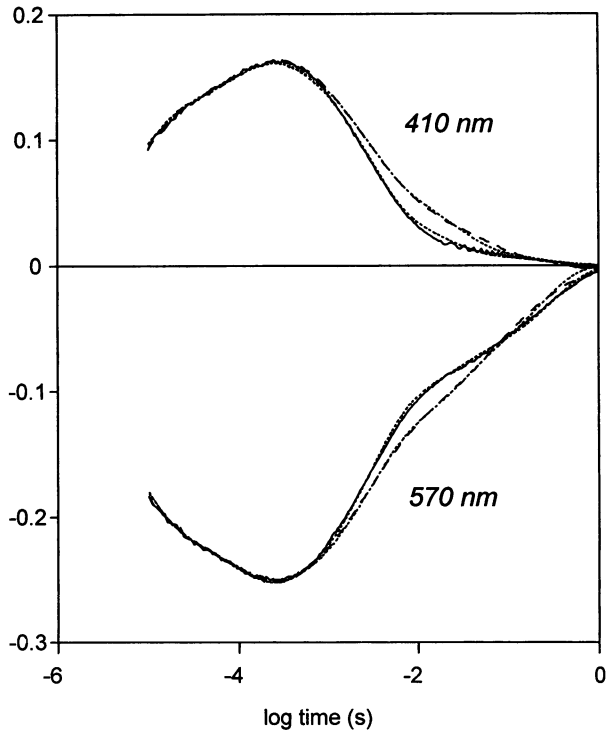

FIGURE 1 Absorption changes in bacteriorhodopsin, measured at 410 and 570 nm after photoexcitation at two different intensities. ——, 10% intensity, measured absorption change; – – –, 100% intensity, measured absorption change; ···, best fits of the linear model, as discussed in the text. The traces are normalized to the maximum change at 410 nm. Conditions: 16 μM bacteriorhodopsin, 100 mM NaCl, 50 mM bis-Tris-propane, pH 9.5.

TABLE 1 Amplitudes* and time constants of the exponentials in the 410-nm decay kinetics at two excitation light intensities,‡ in wild-type and mutant bacteriorhodopsins

Sample	Light intensity	First decay component		Second decay component	
		τ_1 ms	Amplitude	τ_1 ms	Amplitude
Wild type	Low	3.4	0.87	70	0.13
	High	3.1	0.70	55	0.30
D96N§	Low	304	1	—	—
	High	322	1	—	—
T46V§	Low	0.63	1	—	—
	High	0.93	1	—	—
T46V/R227Q	Low	1.8	0.95	5.1	0.05
	High	1.8	0.72	6.4	0.28

*Relative to the total amplitudes at 410 nm.
‡Low and high refer to 10% and 100% intensities (cf. Materials and Methods). Experimental conditions as in Figs. 1 and 4.
§The decay is described by one exponential phase.

time constants did not change. As observed by Dancsházy and Tokaji (1993) as well as Komrakov and Kaulen (1995), however, the last phase of decay at this wavelength became not slower but faster at the higher excitation intensity (Fig. 1).

Data such as in Fig. 1, but at numerous excitation intensities between 1% and 100%, were evaluated in terms of the $M_1 \rightarrow M_2 \Leftrightarrow N^{(-1)} \Leftrightarrow N^{(0)} \rightarrow BR$ scheme. The best fits at 10% and 100% intensity are shown with dotted lines in Fig. 1. The rate constants between M_2 and BR from the fits are shown as functions of the light intensity in Fig. 2. Significant change is seen only for the rate constants of the $N^{(-1)} \rightarrow N^{(0)}$ and $N^{(-1)} \rightarrow M_2$ reactions. Both refer to proton transfers to D96, from the cytoplasmic surface and from the Schiff base, respectively, and the rate constants increase linearly with excitation intensity. The model used predicts a third phase of small amplitude for the decay of M, and three exponentials do indeed fit the data better than two (not shown). Remarkably, however, when the decay of M is analyzed in terms of sums of two exponentials, these changes in the rate constants combine to produce changes mostly in the phenomenological amplitudes but not in the time constants (Tokaji and Dancsházy, 1992; Dancsházy and Tokaji, 1993 and Table 1).

The calculated kinetics for M_1 plus M_2, $N^{(-1)}$, and $N^{(0)}$ at 10% and 100% excitation intensities are shown in Fig. 3. The time-dependent concentrations of these states illustrate how the model with light-intensity-dependent rate constants predicts that the amplitudes will change more obviously than the rates. This is evident from the three decay components of M, where the amplitudes of the slower components seem to increase at the higher light intensity, but also from the kinetics of N, where the rates do not change but both $N^{(-1)}$ and $N^{(0)}$ accumulate in lesser amounts.

In many bacteriorhodopsin mutants the reaction sequence itself is unaffected but the rate constants are changed in ways that can reveal (or hide) some of the kinetic details (e.g., Lanyi, 1993). We examined three mutants in which the kinetics of M and N are sufficiently different to expect changed effects from the excitation intensity. In D96N, because the proton donor to the Schiff base is replaced, the decay of M is monoexponential under most conditions, greatly slowed, and inversely dependent on pH (Butt et al., 1989; Otto et al., 1989; Miller and Oesterhelt, 1990; Cao et al., 1991). Except at very low pH, where the M decay approaches that in wild type, the N state does not accumulate. In T46V the decay of M is also monoexponential (near neutral pH) but much more rapid than in wild type, whereas the decay of N is very slow (Brown et al., 1994). Proton uptake proceeds together with the decay of N, indicating

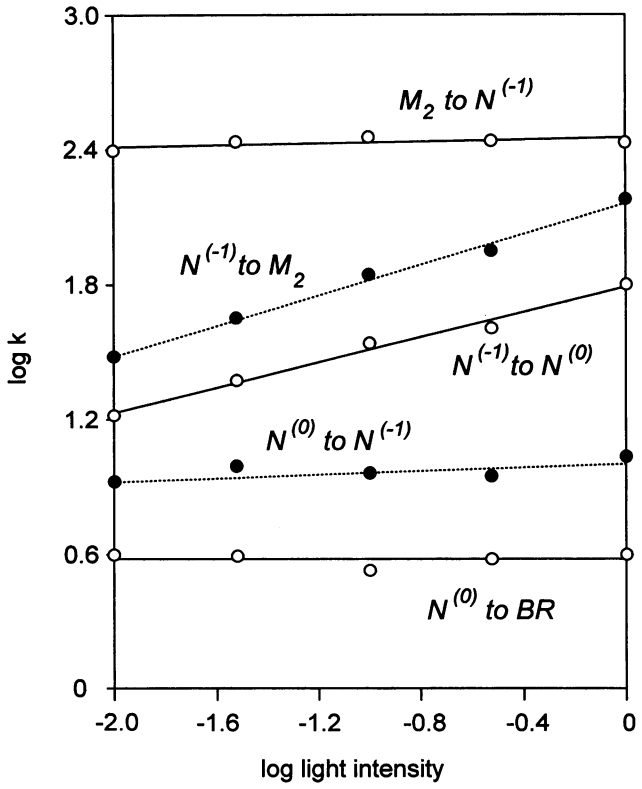

FIGURE 2 Rate constants of the $M_2 \Leftrightarrow N^{(-1)} \Leftrightarrow N^{(0)} \rightarrow BR$ model at different excitation intensities. Open circles and solid lines refer to forward reactions, closed circles and dotted lines to reverse reactions. The rate constants were obtained from the best fits of the model to data such as those in Fig. 1.

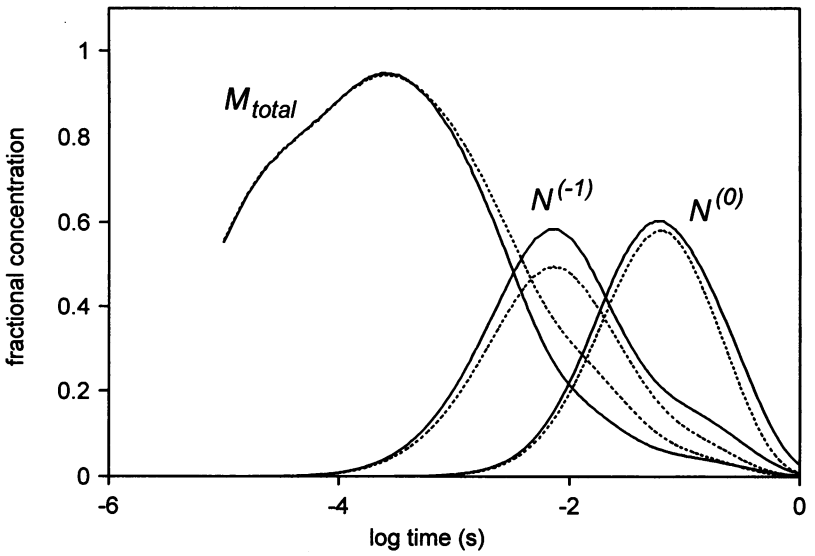

FIGURE 3 Time-dependent concentrations of M_1 plus M_2 (M_{total}), $N^{(-1)}$, and $N^{(0)}$ at two excitation intensities. The kinetics were calculated from the rate constants of the best fits for the model (cf. Fig. 2). ——, 10% intensity; ···, 100% intensity.

that this N is $N^{(-1)}$ (Brown et al., 1994). In T46V/R227Q the second mutation restores the biphasic M decay, and roughly its rates in the wild-type photocycle, although strongly biphasic decay is observed here not only at high but also at low pH (Brown et al., 1994). The proton kinetics indicated that the N is a mixture of $N^{(-1)}$ and $N^{(0)}$. The results with these mutants are given in Fig. 4, and their exponential fits in Table 1.

It is evident from Fig. 4 *A* that the excitation light intensity has no effect on the decay of M in D96N. This is consistent with the results for the wild-type protein, where the intensity dependence was linked to the two reactions that involve D96 (Fig. 2), because residue 96 is not an aspartate in this mutant. In T46V the rate of the monophasic M decay is slowed by about 50% at the higher excitation intensity (Fig. 4 *B* and Table 1). In this mutant the deprotonation of D96 is evidently hindered as the excitation intensity is increased. Such an effect on the time constant and not on the amplitude demonstrates that changing the excitation intensity can change a rate constant in the photocycle even in the case of a monophasic decay. Models that invoke heterogeneity and therefore predict that only the amplitudes but not the time constants can change (Shrager et al., 1995) do not explain the intensity dependence of the photocycle of this mutant. The decay of N (Fig. 4 *B*) is so far separated from the decay of M here that no kinetic analysis is necessary to resolve it. The more rapid decay of N (i.e., $N^{(-1)}$) at the higher excitation intensity also constitutes an effect on a time constant rather than on an amplitude. Its direction is consistent with the wild-type model, because the recovery of the initial state in this mutant will occur via the rate-limiting $N^{(-1)} \rightarrow N^{(0)}$ reaction, where a proton is taken up. These effects of high excitation intensity correlate well with the effects of high hydrostatic pressure on T46V, which slows the monophasic M decay and accelerates the decay of N (Brown et al., 1995). Fig. 4 *C* shows the kinetics of the T46V/R227Q double mutant. The partial return of the wild-type kinetics upon introducing the second mutation, as well as the much more

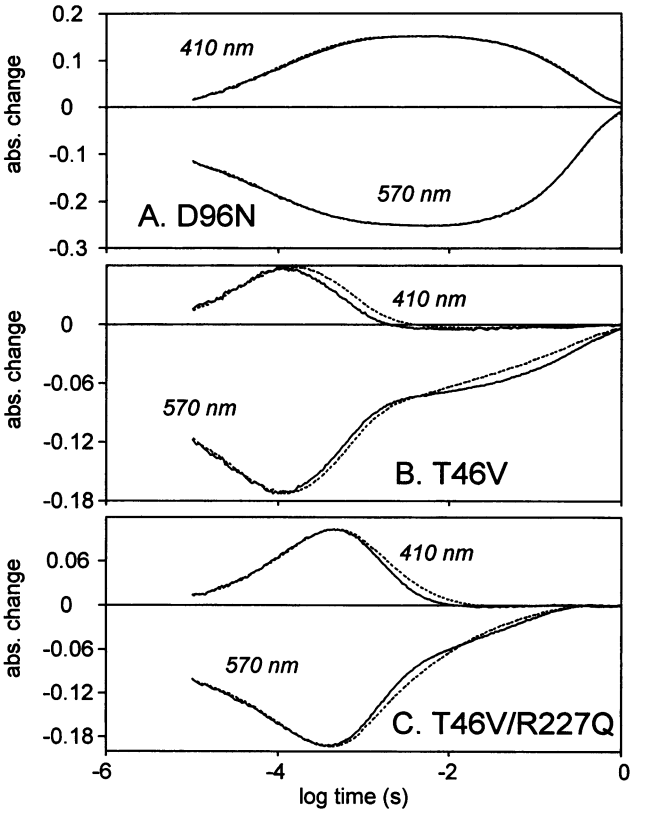

FIGURE 4 Absorption changes in the bacteriorhodopsin mutants D96N (*A*), T46V (*B*), and T46V/R227Q (*C*), measured at 410 and 570 nm after photoexcitation at two different intensities. ——, 10% intensity; ···, 100% intensity. The traces are normalized to the maximum changes at 410 nm. Conditions: 16 μM bacteriorhodopsin; for *A*: 100 mM NaCl, 5 mM acetate, pH 5; for *B* and *C*: 2 M NaCl, 50 mM bis-Tris-propane, pH 6.0.

rapid proton uptake (Brown et al., 1994), is accompanied by return to the wild-type-like intensity dependence (Table 1). Thus, the difference between the phenotypes of T46V and the wild type is probably not caused by any fundamental structural change due to the removal of T46, but by changed rate constants.

DISCUSSION

The kinetic changes with excitation intensity that have been interpreted earlier as evidence for interaction between two parallel photocycles or photoselection in a heterogeneous bacteriorhodopsin population within the purple membrane, can be satisfactorily explained also within the framework of the single photocycle model that contains reversible reactions. In this model the excitation intensity shifts the two protonation equilibria for D96, with the Schiff base ($N^{(-1)} \Leftrightarrow M_2$) and with the medium ($N^{(-1)} \Leftrightarrow N^{(0)}$), so as to favor greater protonation of this residue at higher intensities. Although this conclusion for the wild-type protein is the result of kinetic analysis based on the single photocycle model and thus is model dependent, the more rapid protonation of D96 from the medium at higher intensity is confirmed in a model-independent way in the T46V mutant. Likewise, the prediction that there will be no intensity dependence in the D96N is fulfilled. In a recent report the effect of excitation intensity was also suggested to be exerted on the $N \rightarrow M$ and $N \rightarrow BR$ reactions (Komrakov and Kaulen, 1995). Although interpreted in terms of a heterogeous bacteriorhodopsin population, this observation also implicated protonation of D96 as the reaction affected by cooperativity.

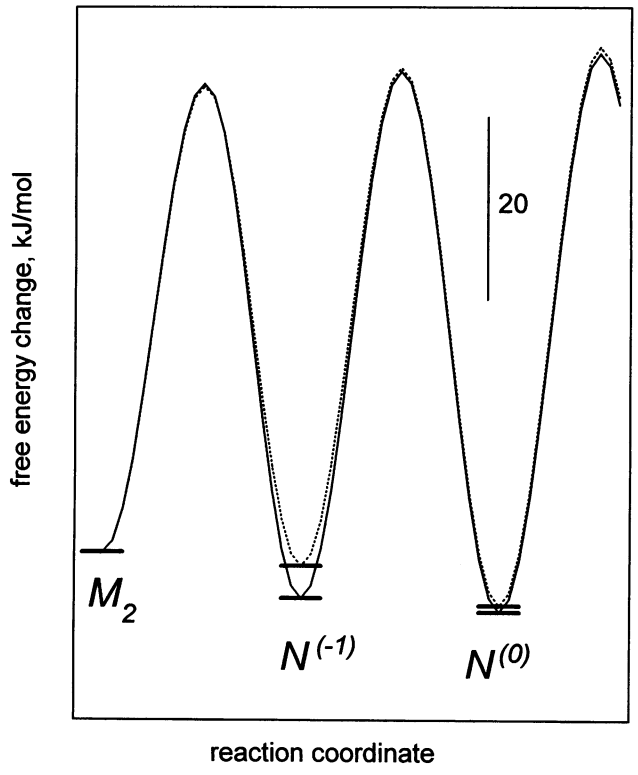

FIGURE 5 Free energy diagram for the photocycle segment containing the M_2, $N^{(-1)}$, and $N^{(0)}$ intermediates at two different excitation intensities. ——, 1% intensity; ···, 100% intensity.

Fig. 5 shows a free energy diagram for the $M_2 \Leftrightarrow N^{(-1)} \Leftrightarrow N^{(0)} \rightarrow BR$ segment of the photocycle at 1% and 100% excitation intensities. The free energies of activation are calculated from the rate constants at these intensities according to the relationship $\Delta G^{\ddagger}_i = -RT(\ln k_i + \ln k_B T/\hbar)$, where k_i is the first-order rate constant of reaction i, k_B is the Boltzmann constant, and $\hbar$ is the Planck constant. Where both forward and reverse reactions could be measured, the free energies of activation gave also the relative free energy levels. Because excitation intensity did not affect the formation of M, we assumed that the free energy level of M_2 is constant. Fig. 5 shows that neither the free energy of $N^{(0)}$ nor the free energies of any of the transition states were altered significantly. What the excitation intensity changed was the free energy of $N^{(-1)}$ relative to the other intermediates. This parameter increased by 3.5 kJ/mol between 1% and 100% excitation intensities, i.e., between essentially no cooperativity and where one in three bacteriorhodopsin molecules is photoexcited. Although the changes in the kinetics are fairly large (Figs. 1 and 3), the increase of free energy is rather small when compared to the activation barriers. Inasmuch as the $N^{(-1)}$ state is formed from M_2 or $N^{(0)}$ by protonation of D96, the free energy increase for $N^{(-1)}$ corresponds in effect to an increase in the pK_a of D96 by 0.6 pH units. This change in the pK_a appears to be caused by cooperativity in the purple membrane. Could photoexcitation of other bacteriorhodopsin molecules in the purple membrane patch raise the proton affinity of D96?

Bacteriorhodopsin molecules are immobilized as trimers in the hexagonal lattice of the purple membrane. A possible mechanism by which monomers can interact in such a rigid two-dimensional crystalline structure is through conformational changes that would result in the development of lateral pressure in the membrane. The density map for the M intermediate from electron diffraction had indeed suggested that structural changes occur, that they are localized mainly at the cytoplasmic region of the protein where D96 is located, and that they include prominently the outward tilt of the cytoplasmic end of helix F (Subramaniam et al., 1993). As expected from this, the lattice constant in the M intermediate is greater relative to the unphotolyzed state (Nakasako et al., 1991), indicating an area increase. As reported elsewhere (Váró and Lanyi, 1995), the effects of hydrostatic pressure on the photocycle kinetics suggest that there is a 32 cm^3/ml volume increase at the $M_1 \rightarrow M_2$ reaction. If this expansion reflects the structural change detected by diffraction, as we believe, it would be consistent with the entry of 10–20 water molecules and the approximately 12 $Å^2$ increase in the surface area of the cytoplasmic side calculated from the crystallographic data (Nakasako et al., 1991). The more bacteriorhodopsin molecules that

are converted to M_2, therefore, the greater the lateral pressure that would develop at the cytoplasmic surface. This would compress the cytoplasmic region of the protein, proportionally to the fraction of bacteriorhodopsin that photocycles, and by expelling some of the bound water raise the pK_a of D96. Such cooperativity would thus arise not in a directional sense, where the interaction is within a trimer and affects only one of the two neighbors of an excited bacteriorhodopsin (Tokaji, 1993), but isotropically, from a uniform lateral pressure in the purple membrane lattice.

Five independent lines of evidence support this idea. First, light-scattering changes indicate that the purple membrane sheets become curved during the photocycle, in the way one might expect if the area of one of the two surfaces increased preferentially (Czégé, 1987; Czégé and Reinisch, 1990). Second, osmotic agents affect the photocycle mainly at one reaction, proton exchange between D96 and the Schiff base (Cao et al., 1991). It appeared in these studies that decreasing the water activity, and thereby withdrawing water from the protein, removed some dipole stabilization of the anionic aspartate. Although the locations of the bound water molecules near D96 in the cytoplasmic domain are not known, this suggests that the hydration of the cytoplasmic region of the protein is a determining factor in the protonation reactions of this residue. Third, high hydrostatic pressure affects the photocycle kinetics somewhat similarly to high excitation intensity: the decay of M becomes slower and the decay of N more rapid (Váró and Lanyi, 1995). Fourth, the photocycle of monomeric bacteriorhodopsin does not exhibit any excitation intensity dependence (Danshina et al., 1992). In fact, brief (1-min) exposure of purple membranes to detergent greatly decreased the dependence, and this occurred even before the abolition of the trimeric structure (Mukhopadhyay et al., 1994). Fifth, recent molecular dynamical modeling of the protein suggested that changing hydration of the extracellular domain is a feasible mechanism for changing the pK_a of D96 in the photocycle (Scharnagl et al., 1995).

We suggest, therefore, that the effects of excitation intensity on the photocycle kinetics reflect cooperativity that depends on the rigid arrangement of bacteriorhodopsin in the purple membranes. The suggested outward tilt of the cytoplasmic end of helix F and possibly other structural changes in the M state (Subramaniam et al., 1993) increase the area of the cytoplasmic surface, and the lateral pressure that develops compresses the bacteriorhodopsin molecules. We note that D96 is located in a rather hydrophobic environment (Henderson et al., 1990; Greenhalgh et al., 1991), and its pK_a must depend on internal hydration. Compression will drive bound water into the bulk phase (Heremans, 1982), and the resulting lesser dielectric stabilization of the aspartic carboxylate will raise its pK_a. This effect alone can explain all of the observed changes in the photocycle kinetics in the wild-type protein. Because the first hydration shell of an aspartate in the aqueous medium contains about six water molecules (Kuntz, 1971), and in the unphotolyzed bacteriorhodopsin the absence of probably most of these raises the pK_a of D96 from its expected value near 4 to about 11 (Száraz et al., 1994), we estimate that the calculated rise in the pK_a even at the rather high excitation light intensity used requires the expulsion of at most only a few water molecules from the vicinity of D96.

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