Study of the Photocycle and Charge Motions of the Bacteriorhodopsin Mutant D96N

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ABSTRACT Absorption kinetic and electric measurements were performed on oriented purple membranes of D96N bacteriorhodopsin mutant embedded in polyacrylamide gel and the kinetic parameters of the photointermediates determined. The rate constants, obtained from fits to time-dependent concentrations, were used to calculate the relative electrogenicity of the intermediates. The signals were analyzed on the basis of different photocycle models. The preferred model is the sequential one with reversible reaction. To improve the quality of the fits the necessity of introducing a second L intermediate arose. We also attempted to interpret our data in the view of reversible reactions containing two parallel photocycles, but the pH dependencies of the rate constants and electrogenicities favored the model containing sequential reversible transitions. A fast equilibrium for the $L_2 \leftrightarrow M_1$ transition and a strong pH dependence of the M_2 electrogenicity was found, indicating that the M_1 to M_2 transition involves complex charge motions, as is expected in a conformational change of the protein.

INTRODUCTION

Bacteriorhodopsin (BR) is a light-driven proton-pumping protein in the cell membrane of the *Halobacterium halobium*. The energy of the absorbed light quantum partially is stored in the protein by isomerization of the retinal. This energy is used during a complex photocycle to transport the proton from the cytoplasm to the extracellular phase. The photocycle consists of several principal intermediate states, labeled J, K, L, M, N, and O and characterized spectroscopically by different absorption spectra reviewed recently by Mathies et al. (1991) and Lanyi (1992). Early absorption kinetic measurements carried out at a few wavelengths indicated a unidirectional sequential model of the photocycle (Lozier et al., 1975). Later the kinetics in the rise and decay of the M intermediate were found to be more complex. This could be explained in two ways: by introducing parallel photocycles with slightly different unidirectional kinetic steps (Groma and Dancsházy, 1986; Diller and Stockburger, 1988; Balashov et al., 1991; Tokaji and Dancsházy, 1992; Drachev et al., 1992; Eisfeld et al., 1993); or by one photocycle and introducing reversible reactions between the intermediates (Chernavskii et al., 1989; Otto et al., 1989; Váró and Lanyi, 1990; Ames and Mathies, 1990; Gerwert et al., 1990; Váró and Lanyi, 1991b).

The model with parallel photocycles explains some extra phenomena, for example the dependence of the kinetics on the exciting light intensity (Groma and Dancsházy, 1986), but the reason for which it was introduced, the complex rise and decay of the M, cannot be understood with parallel unidirectional photocycles (Váró and Lanyi, 1990). This suggests that even in the case of the parallel photocycle concept,

the reversible reactions between the intermediates should be considered (Váró and Lanyi, 1990; Drachev et al., 1992). The large number of independent parameters in this case allows a good quality fit to almost any kinetics.

In the case of a single photocycle, based on kinetic criteria the M intermediate was decomposed into several optically indistinguishable states, placed sequentially in the model (Váró and Lanyi, 1991b; Zimányi et al., 1992). In special, extreme conditions, like detergent solubilization of the wild-type BR or in the D115N mutant, the spectra of some of these intermediate M states could be distinguished (Váró and Lanyi, 1991a; Váró et al., 1992). Although the single photocycle model contains fewer independent parameters, still the number is so high that almost all kinds of kinetics can be readily fit.

The transition between the two M states is the so-called switch of the proton pump, which has an important role in proton transport by guaranteeing that the reactions proceed mainly in a forward direction. The active site moves from the intracellular to the extracellular part of the protein (Váró and Lanyi, 1991b; Zimányi et al., 1992). For this reason, it is important to understand these processes in detail. A good approach to achieve this is to use conditions where the intermediates after M are missing, giving the possibility of resolving many of the kinetic details of the photocycle. Two possibilities are: the mutant D96N (Holz et al., 1989) or dried samples (Váró and Keszthelyi, 1983).

Initially the BR mutant with Asp-96 to Asn replacement was expressed in *Escherichia coli* (Mogi et al., 1988; Holz et al., 1989), but later this mutation was also successfully expressed in *H. halobium* strains (Soppa et al., 1989; Váró and Lanyi, 1991a). The D96N mutant was intensively studied, as the Asp-96 has an essential role in the proton pumping mechanism of the BR, being the proton donor for the Schiff base (Holz et al., 1989; Butt et al., 1989; Cao et al., 1991). The kinetics of the absorption changes were measured and a photocycle model was derived (Mogi et al., 1988; Miller

and Oesterhelt, 1990; Váró and Lanyi, 1991a; Zimányi et al., 1992). The electric signals corresponding to the charge motions during the photocycle were also measured (Holz et al., 1989; Butt et al., 1989), but no absorption change measurements could be performed under the conditions these authors used. For this reason, the detailed analyses of the electric signals and their relation to the photocycle intermediates of the mutant are missing.

In the present work, we have attempted to complete this missing part for the mutant D96N by measuring the absorption changes at three wavelengths and the corresponding electric signal under the same conditions. The optical signals were used to calculate the time dependent concentration changes of the photocycle intermediates. These concentration changes were analyzed and further used to calculate the electrogenicity of the intermediates from the electric signals. The data were fitted with several photocycle models and from these conclusions drawn on the charge motions taking place inside the protein.

MATERIALS AND METHODS

The purple membrane suspension of the mutant D96N, expressed in *H. halobium* strain L-33, were supplied by J. K. Lanyi. The degree of orientation of the membrane fragments in a DC electric field was compared to that of the wild-type BR and they were similar. Oriented purple membrane samples were immobilized in polyacrylamide gel following the method developed by Dér et al. (1985). The gel samples were kept overnight in a solution of 100 mM NaCl, 50 mM phosphate buffer for every pH value between 4 and 8. These conditions allowed us to avoid the buffer effects discussed by others (Liu, 1990; Liu et al., 1991). Azide accelerates the M decay in the mutant D96N by replacing the function of Asp-96 as a proton donor (Tittor et al., 1989). In our measurements the solution contained 4 mM azide at pH 8.0 and, at lower pH, its concentration was decreased to assure that the decay of the M intermediate was roughly the same at every pH, slow enough not to allow the accumulation of the later intermediates and fast enough to complete the photocycle in about 1 s. The pH of the sample was checked before each measurement. The electric and optical measurements were performed at 20°C with the setup described elsewhere (Váró and Keszthelyi, 1983). Optical absorption kinetic data at different wavelengths together with the corresponding electric signal were recorded after laser excitation of the sample on a logarithmic time scale, covering the 100-ns to 1-s interval. With the aid of the spectral data from Zimányi and Lanyi (1993) the extinction coefficients were estimated (see Table 1). These were used to convert our optical kinetic data to time dependent concentration changes of the photocycle intermediates. The resulting concentration changes were fitted with different models and the kinetic parameters of these fits were used to calculate the electrogenicity corresponding to every intermediate. The response function of the measuring system was approximated by a simple resistance capacitance with a time constant of about 6.10^{-8} s. The electrogenicity is defined following Trissl (Trissl, 1990). The dipole magnitude of the jth intermediate is

$$D_j = A \cdot \sum_i \mu_{ji}(\epsilon_{ji})$$

where A is a constant and μ_{ji} are the dipole moments existing in that intermediate depending on ϵ_{ji}, the dielectric constant of the protein around the dipole. The electrogenicity of the intermediate is

$$E_j = D_j - D_0,$$

where D_0 is the ground state dipole magnitude. The electric current generated in the measuring circuit by the change of the electrogenicity is

$$i(t) = B \cdot \sum_j E_j \cdot dC_j/dt,$$

TABLE 1 Extinction coefficients of the intermediates at the wavelengths used in calculation for D96N mutant

Wavelength (nm)	Extinction coefficient (mol^{-1}·cm^{-1}) of			
	K	L	M	BR
414	8,700	11,000	34,000	10,000
530	22,000	46,600	0	40,000
630	46,600	1,500	0	17,500

where B is a constant determined by the electric circuit and C_j is the concentration of the jth intermediate. From the last equation it can be seen that, if an intermediate is missing or its concentration is constant throughout the whole photocycle, it has no electric signal. It is interesting to note that as the charge of the protein depends on the external conditions (pH, ionic strength, etc.) the electrogenicity of the intermediates could depend also on these conditions.

As established earlier, the following working hypothesis concerning the sign and relative amplitude of the electrogenicity was used (Keszthelyi and Ormos, 1989): the sign of the fast unresolved electric signal, which is attributed to the primary charge separation, the isomerization of the retinal, is negative and is the same in the mutant and wild-type BR. Its relative value was taken to be equal to −1.

RESULTS

One complete set of data consisted of optical absorption kinetic measurement at three wavelengths (Fig. 1) and the integral of the electric signal (see Fig. 4, *continuous line*). Every wavelength was chosen to characterize mainly one of the intermediates. For data analysis, the integral of the electric signal reveals more information compared to the measured current, as the very small currents induced by slow but significant charge motions can be seen. Data was acquired at five different pH values between 4 and 8. Different photocycle models were fit to the time course of the concentration of the intermediates calculated from the absorption changes (Fig. 2, *continuous line*). As has already been shown (Zimányi et al., 1992), unlike at pH 7 (Fig. 2 *B–D*), at lower pH (pH 5, Fig. 2 *A*), a considerable amount of the L intermediate remains during the whole photocycle.

The model containing two M intermediates: K ↔ L ↔ M_1 ↔ M_2 → BR (Fig. 2 *B*) had the same problems as the fits to the data published earlier (Zimányi et al., 1992). Clearly the largest deviation between the data and fit takes place at the L intermediate (Fig. 2 *B*). The fits were improved substantially by introducing another L intermediate (model I): K ↔ L_1 ↔ L_2 ↔ M_1 ↔ M_2 → BR (Fig. 2 *C*). Another possibility, which gave a fit of the same quality, was with L_2 as a "cul-de-sac" branching from M_1 proposed by others (Zimányi and Lanyi, 1993), but was dismissed from the discussion as no interpretation could be given for it.

The unidirectional two parallel photocycles model: K1 → L1 → M1 → BR1, K2 → L2 → M2 → BR2, had to be dismissed for simple kinetic reasons. The ratio of the fast and slow part of the K, L, and M intermediates were totally different, which was also manifested in the bad quality of the fit (not shown). The two parallel photocycles, with reversible reactions (model II): K1 ↔ L1 ↔ M1 → BR1, K2 ↔ L2 ↔

FIGURE 1   Kinetics of the absorption change measured at different wavelengths for D96N mutant at pH 7.0.
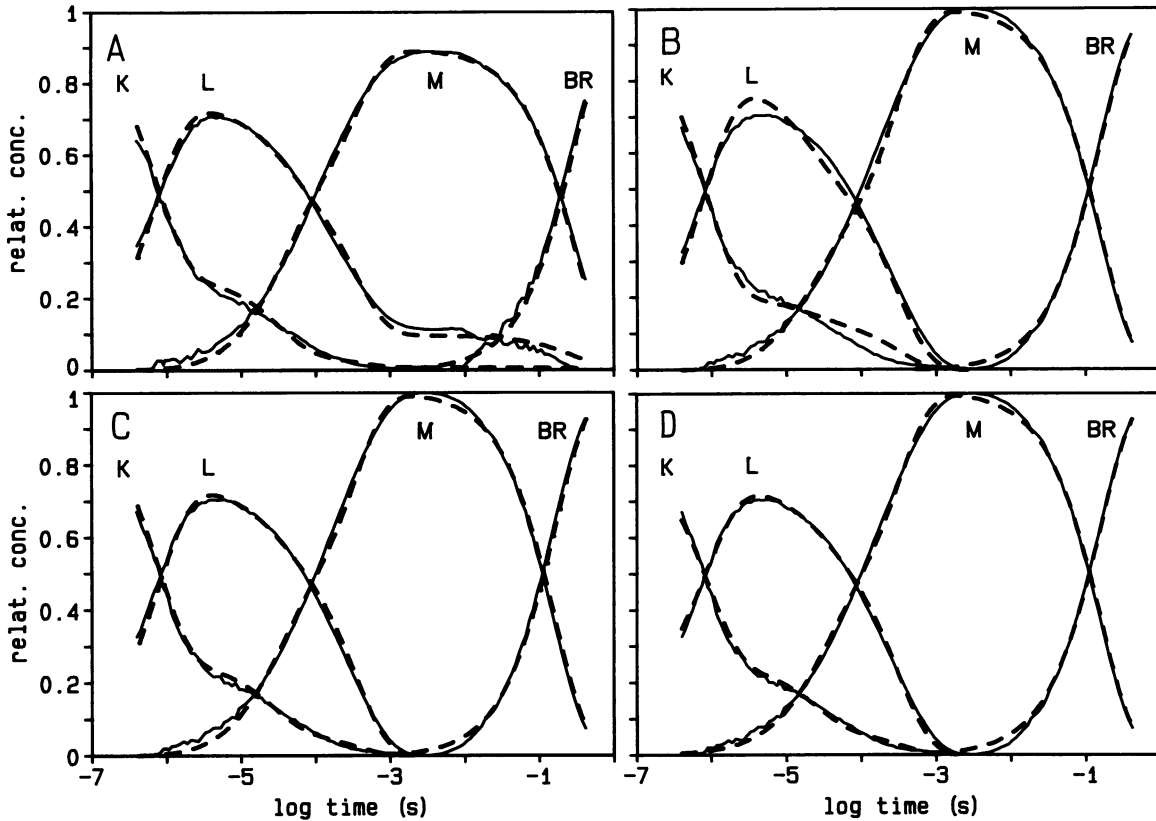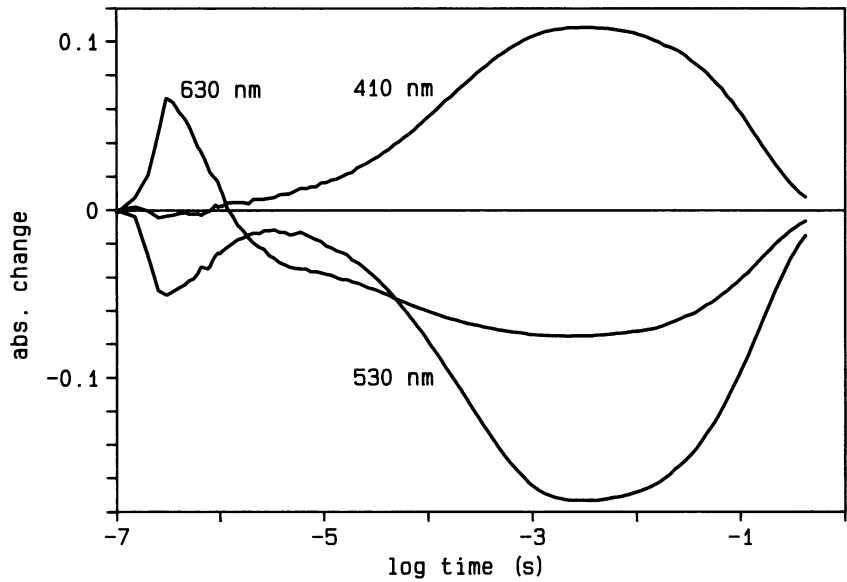

FIGURE 2   The concentration of the intermediates calculated from optical measurements (*continuous line*) and the fits (*dotted line*) for D96N mutant: model $K \leftrightarrow L_1 \leftrightarrow L_2 \leftrightarrow M_1 \leftrightarrow M_2 \rightarrow BR$ for pH 5.0 (*A*) and pH 7.0 (*C*); model $K \leftrightarrow L \leftrightarrow M_1 \leftrightarrow M_2 \rightarrow BR$ for pH 7.0 (*B*); model $K1 \leftrightarrow L1 \leftrightarrow M1 \rightarrow BR1$, $K2 \leftrightarrow L2 \leftrightarrow M2 \rightarrow BR2$ for pH 7.0 (*D*).

$M2 \rightarrow BR2$ fit the data as well as model I (Fig. 2 *D*). Model I contains 10 independent fitting parameters, and model II contains 11. Some other models with the same number or more parameters were checked, but the fits were not improved. Hence in the following discussion, the model I and II were considered in more detail as the simplest models with equally good fit.

The remaining small differences between the fit and measured data can be explained by the fact that the excited protein exists in different conformations, caused by thermal fluctuations, which give slightly different time constants in the photocycle.

The kinetic constants of the two models were plotted as a function of pH (Fig. 3). Model I gave similar dependence
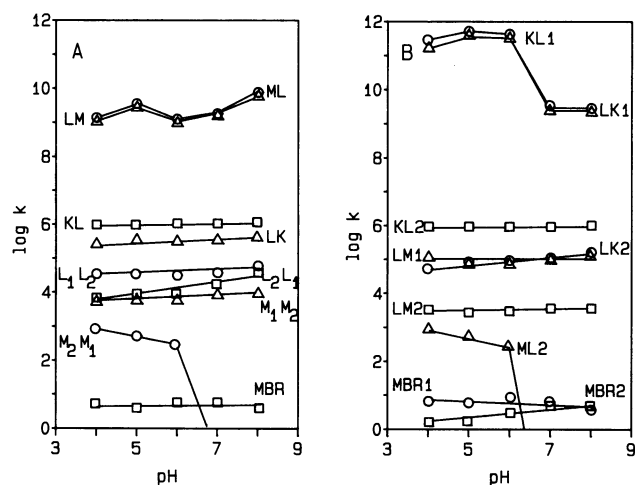
FIGURE 3   Dependence of the photocycle rate constants on pH. The values were obtained from fitting the concentrations of the intermediates with model $K \leftrightarrow L_1 \leftrightarrow L_2 \leftrightarrow M_1 \leftrightarrow M_2 \to BR$ (A) and with $K1 \leftrightarrow L1 \leftrightarrow M1 \to BR1$, $K2 \leftrightarrow L2 \leftrightarrow M2 \to BR2$ model (B). The errors are $<\pm5\%$, with exception of the two fastest constants in both cases (see text).

FIGURE 4   The integrated electric signal (*continuous line*) and its fit (*dotted line*) with model $K \leftrightarrow L_1 \leftrightarrow L_2 \leftrightarrow M_1 \leftrightarrow M_2 \to BR$ for mutant D96N at pH 5.0 and 7.0.

FIGURE 5   pH dependence of the calculated relative electrogenicities for D96N mutant. The values were obtained from fitting the electric signals with model $K \leftrightarrow L_1 \leftrightarrow L_2 \leftrightarrow M_1 \leftrightarrow M_2 \to BR$ (A) and with $K1 \leftrightarrow L1 \leftrightarrow M1 \to BR1$, $K2 \leftrightarrow L2 \leftrightarrow M2 \to BR2$ model (B). The errors are $<\pm10\%$.

(Fig. 3A) as that published earlier (Zimányi et al., 1992). The only difference appears at the second L intermediate. The transition between the L_1 and L_2 intermediates in our case is similar to that of L to M and back reactions published in the above mentioned paper. In model I, the time constants of L_2 to M and back reactions are equal and extremely fast, showing a real equilibrium between the two states. The value of these fast kinetic constants could be lowered by two orders of magnitude, and no change in the quality of the fit occurred. By fixing one of them within its range during the fit, the other became automatically equal to it. This supports the idea that the corresponding reactions are much faster than all the others, but their exact time constants are uncertain and the apparent pH dependence has no real molecular meaning. The kinetic constants of model II show very similar pH dependence, but in this case the equal and fast rate constants with the same properties occur between the K and L intermediates in one of the photocycles.

The kinetic constants obtained at every pH value for both models were used to calculate the electrogenicity value for the corresponding intermediates. The fits were within the error of measurements. Fig. 4 contains two representative signals and their fits with model I at pH 5 and 7, showing a strong pH dependence. Model II gives similar good fits (not shown). The relative electrogenicity plotted in function of pH (Fig. 5) reveals that in both cases only the value corresponding to the second M form depends strongly on proton concentration. This dependence is monotonic only for model I. The electric signals measured on the D96N mutant expressed in *E. coli* showed no pH dependence within this time range (Holz et al., 1989). The difference can be explained may be because in the *E. coli* mutant the BR is not produced in the natural two dimensional crystalline form or that the measuring conditions are different.
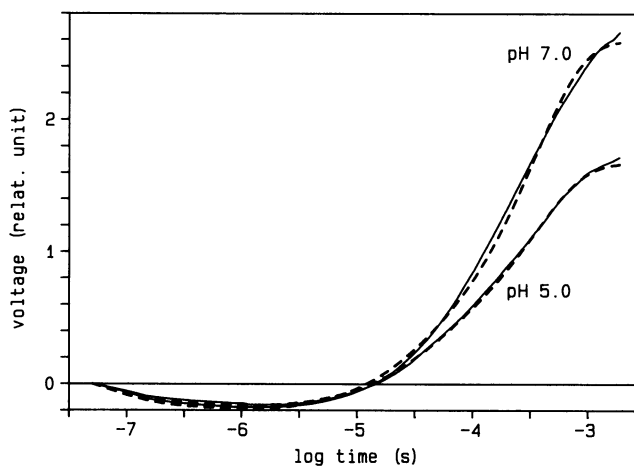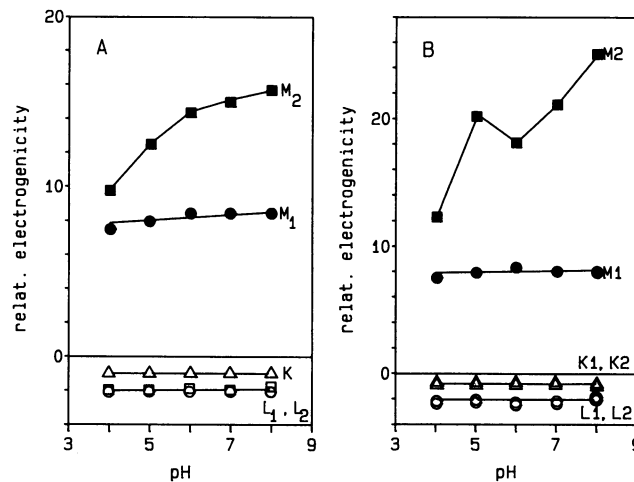
DISCUSSION

The kinetic and electrogenic properties of the mutant D96N bacteriorhodopsin were studied between pH 4 and 8, so all the conclusions has to be considered valid mainly in this pH interval. The results presented above are very similar when the two models being considered for the D96N mutant are compared. However, some differences exist which can provide the possibility to decide in favor of one or the other. An important difference is the position of the reversible reaction in the photocycle with high and equal rate constants. In model II this is located between the K and L intermediates in one of the parallel photocycles. The K to L transition is a relaxation of the retinal and its surrounding after the all-*trans* to 13-*cis* isomerization, which occurs before the K intermediate (Mathies et al., 1988; Lohrmann and Stockburger, 1992). This is very improbable to complete so fast because

it is accompanied by a small conformation change, which limits the speed of the reaction. The system transforms from a stressed conformation to a relaxed one, which means a transition from a higher energy level to a lower one, but the calculated equilibrium means two equal energy levels. A difference of one order of magnitude exists between the direct and reverse reactions in the other branch of the model II, meaning a real energy loss.

In model I, the reactions in question are between the L_2 and M_1 intermediates. If the K to L reaction is a relaxation process of the retinal and its surrounding (Mathies et al., 1988; Lohrmann and Stockburger, 1992), then it is very probable that this relaxation propagates further in the protein giving rise to another intermediate. This would mean some changes in the protein without affecting the position of the charges which determine the color of the retinal, giving rise to the L_2 form with the same spectrum as for L_1. This possibility explains the very good resemblance of the spectra corresponding to the L and N intermediates in the D85N and D212N mutant BR (Needleman et al., 1991). The L_2 to M_1 transition is a proton transfer from the Schiff base to the Asp-85 (Braiman et al., 1988). The protonated Schiff base and deprotonated Asp-85 are coupled via a hydrogen bond (Rothschild, 1992), which allows this transition of the charge to happen quickly between two equal energy levels (Brickmann, 1976). The existence of an extra L-like intermediate between the L and M (Hofrichter et al., 1989; Dér et al., 1992) or as a "cul-de-sac" branch from the M_1 (Zimányi and Lanyi, 1993) was already postulated on kinetic bases for BR. Another possible explanation of the very fast transition between the L and M states was recently calculated theoretically by molecular dynamic simulation (Zhou et al., 1993). It was shown that the all-*trans* retinal in the ground state BR after photoexcitation goes to 13,14-*dicis* configuration and this persists during the K and L intermediates. In the M intermediate the retinal is in 13-*cis* configuration. The calculations described in the above mentioned paper prove that this 13,14-*dicis* to 13-*cis* transition has a lifetime of a few ps, causing a free energy change of this transition to be close to zero (Zhou et al., 1993), which means an equilibrium between the two states.

Another difference between the two models is in the pH dependence of the electrogenicity of the intermediates. Model I gives monotonic increase with pH for the M_2 electrogenicity and an almost constant value for all the other intermediates (Fig. 5 A). Among the rate constants only the M_2 to M_1 transition shows pH dependence (Fig. 3 A). The relation between the electrogenicity and the rate constants is not direct and they do not necessarily depend from the same pK as the corresponding rate constant. The pH dependence of the rate constant is related to the titration of mainly one amino acid, participating directly in the proton release (Zimányi et al., 1992). The titration of some other amino acids, far from the proton release site and with different pKs, should have only a slight perturbation on this process. The electrogenicity in the present case, related not only to the proton release but to the conformational change of the whole

protein as well (Váró and Lanyi, 1991b), involves all the moving charged residues with different pKs. The charge motions taking place in the first part of the photocycle are independent of the external pH, while a part of the charges involved in the transition between M_1 and M_2 are influenced by it. The electrogenicity of M_2 can be related only partially to the titration of an X amino acid assumed earlier (Zimányi et al., 1992).

The pH dependence of the electrogenicity values calculated for model II (Fig. 5 B) raises some interpretation problems. The electrogenicities of the K and L intermediates are equal for both photocycles, but those corresponding to the two M forms are different. In addition, only one of them shows a non-monotone pH dependence. If it is accepted that the L to M transition belongs to the proton transfer from the Schiff base to Asp-85 (Braiman et al., 1988), then these behaviors of the electrogenicities are hard to explain. One obvious way to explain them is to consider this transition composed from more elementary steps as proton transfer, conformation change, etc. This assumption would approach model II to model I, but even in this case the different behavior of the two M forms is difficult to explain.

This study corroborates the validity of a sequential photocycle model with reversible reactions between the intermediates. It does not exclude the existence of the parallel photocycles appearing, for example, due to the above mentioned heterogeneity induced by the fluctuations of the protein, or at higher pH. A very important finding is the significant difference in the electrogenicity of the two M intermediates and their pH dependencies. The M_1 to M_2 transition involves the movement of more charges, as it is expected in a conformation change of the protein. This and the relaxation process between the two L intermediates, as discussed above, shows the active participation of the whole protein in the photocycle.

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