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Biophysical Chemistry 56 (1995) 105–112

Biophysical Chemistry

Evidence for parallel photocycles and implications for the proton pump in bacteriorhodopsin

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Abstract

In order to account for the large variety of kinetic phenomena in the light-induced reactions of bacteriorhodopsin's retinal chromophore (BR), a scheme of parallel photocycles has been proposed [W. Eisfeld, C. Pusch, R. Diller, R. Lohrmann and M. Stockburger, Biochemistry, 32 (1993) 7196–7215]. In the present study an experimental test for the validity of this model is described which is based on the fact that in the alkaline region the longest-living intermediates M^f, M^S or N in each of the proposed cycles have significantly different lifetimes. A condition for the existence of parallel cycles would be that the population of M^f, M^S or N is accompanied by a respective depletion of BR in each individual cycle. Dual-beam laser experiments were performed which showed that this condition is fulfilled. It is concluded that those proton transfer steps which are important for the function as a proton pump are the same for all cycles.

Keywords: Bacteriorhodopsin; Parallel photocycles; Optical transient spectroscopy

1. Introduction

The light-induced cyclic reaction (photocycle) of the retinal chromophore in bacteriorhodopsin (bR), which controls its function as a proton pump, is still a matter of debate. — During the photocycle, following the primary photoisomerization step, the chromophore runs through a series of intermediate states (K_{590}, L_{550}, M_{412}, N_{560}, O_{640}), before its parent state BR_{570} is reconstituted. It was found that after the stage of L the formation and decay of the intermediates do not follow monoexponential functions in all cases. This is best known for the intermediate M_{412} whose decay can only be described by the superposition of a 'fast' (M^f) and a 'slow' (M^S) component. — But also the decay of L and the formation of N cannot be fitted by a monoexponential function [1].

Two different concepts were proposed in the literature to account for such deviations. In the first one, it is 'tacitly' assumed that the protein has a completely homogeneous structure which would imply that all bR molecules undergo identical cyclic reactions. A linear reaction sequence BR, K, L, M, N, O, BR was proposed and deviations from the monoexponential decay were explained by introducing back-reactions from the respective product to its direct precursor [2,3].

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SSDI 0301-4622(95)00021-6

Subspecies of bR	Reaction Sequence of the Chromophore (pH > 8)
bR(α,1)	BR ··· L → M^f → BR
bR(α,2)	BR ··· L → M^s → BR
bR(β,1)	BR ··· L → N → BR
bR(β,2)	BR ··· L → M^f → N → BR

BR ··· L ≡ BR $\overset{l_0}{\rightleftarrows}$ K ⟶ L

Scheme 1.

The second, 'heterogeneous' concept is based on the assumption that different classes or 'subspecies' of bR molecules exist which undergo independent cyclic reactions. In this model the various kinetic phenomena are attributed to different reaction steps and rate constants in parallel cycles [1,4].

In our earlier work a specific scheme of parallel cycles was proposed. This was inferred from a quantitative analysis of the various reaction steps of the chromophore over a wide pH range (pH 3–12) using time-resolved resonance Raman (RR) and optical transient spectroscopy [1,4]. The assignment to parallel cycles was essentially based on the finding that the observed pH-dependent kinetic phenomena in most cases did not affect the entire manifold of bR molecules but only certain fractions of it [4]. In the light of this analysis it is suggested that Scheme 1 describes the cyclic reactions of bR for pH > 8. Under such conditions the intermediate O_{640} is not accumulated. — The reaction sequence L → M^f → BR of Scheme 1 in an independent cycle, which was not involved in the earlier work [4], can be inferred from our recent RR-spectroscopic studies.

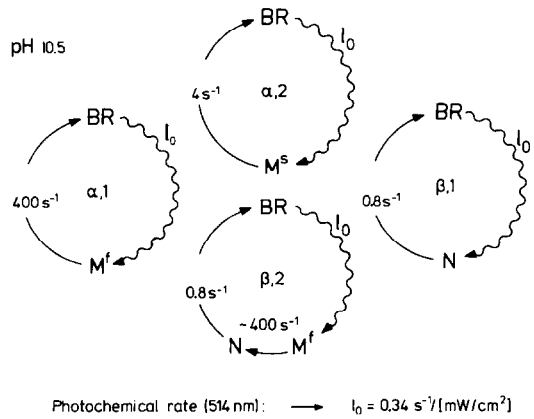
In Scheme 1 the four subspecies are distinguished according to the different transfer reactions of the Schiff base proton (L → M, L → M → N) and/or the different ways in which BR is reconstituted (M^f → BR, M^S → BR, N → BR). The notation 'bR' refers to the protein as a whole, whereas the capitals designate the chromophore as it is probed selectively by optical or RR spectroscopy. Those subspecies which involve M as the longest-living intermediate in their cyclic reactions are denoted by bR(α), whereas bR(β) is used for subspecies in which N takes in the respective role.

The kinetic heterogeneity of bR in Scheme 1 should be related to a structural heterogeneity. However, the RR spectra which very sensitively indicate structural variations at the chromophoric site do not give any evidence for a structural heterogeneity. This suggests that the expected structural variations are small or are located at some distance from the chromophoric site. Thus in this context we are concerned only with those subspecies which can be identified by their different kinetic behavior. This means that we do not 'see' the manifold of potential subspecies which are kinetically invariant.

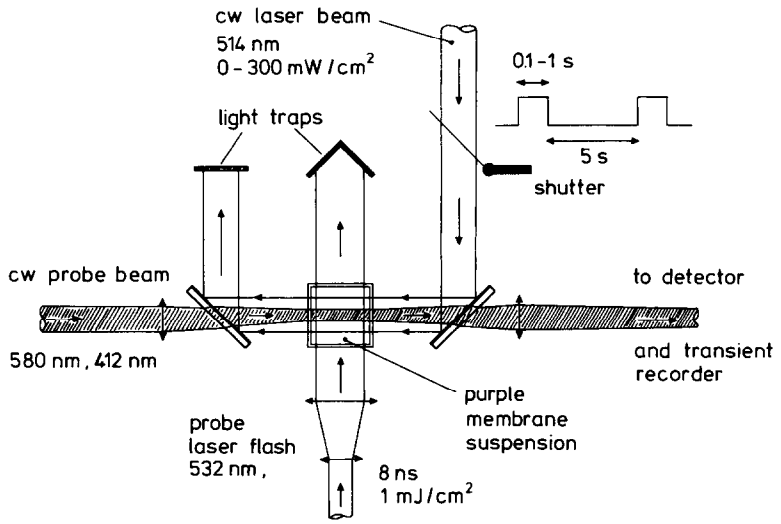
In the present study new experiments are described which provide evidence for the existence of parallel photocycles. These are based on the observation that in the alkaline region the lifetimes of the longest-living intermediates in Scheme 1 are significantly different. Under the condition that the rate of photoisomerization in the primary step

$$l_0 = \gamma \cdot \sigma \cdot I_0 \qquad (1)$$

(γ: quantum yield, σ: cross section for absorption of a photon, I_0: photon flux density) is small compared with the subsequent thermal rate constants (k_{KL}, k_{LM}, k_{LN}), Scheme 1 can be transformed into Scheme 2 in which the individual cycles are simply determined by l_0 and the rate constants for the decay of N, M^S and M^f which are identical with the rate for the recovery of BR in the respective cycles. In Scheme 2 these rates are given for pH 10.5. It follows from Scheme 2 that the various cycles can be populated in a 'stepwise' way.



Scheme 2.



Scheme 3.

When a sample of bR is illuminated over a period Δt, it follows from Scheme 2 that the population of the long-lived intermediates $I(\equiv \mathrm{N}, \mathrm{M}^{\mathrm{S}}, \mathrm{M}^{\mathrm{f}})$ is given by

$$I(i) = \mathrm{BR}_0(i) \cdot \frac{l_0}{l_0 + k_i} \cdot \left[1 - \mathrm{e}^{-(l_0 + k_i)\Delta t}\right] \qquad (2)$$

where 'i' refers to one of the four subspecies in Scheme 1 and k_i to the rate constants for the recovery of BR in the individual cycles.

Scheme 2 is valid if the two following conditions are fulfilled:

(1) The population of N, M^{S} and $\mathrm{M}^{\mathrm{f}}(\alpha, 1)$ as a function of l_0 after an illumination period Δt is given by Eq. (2).

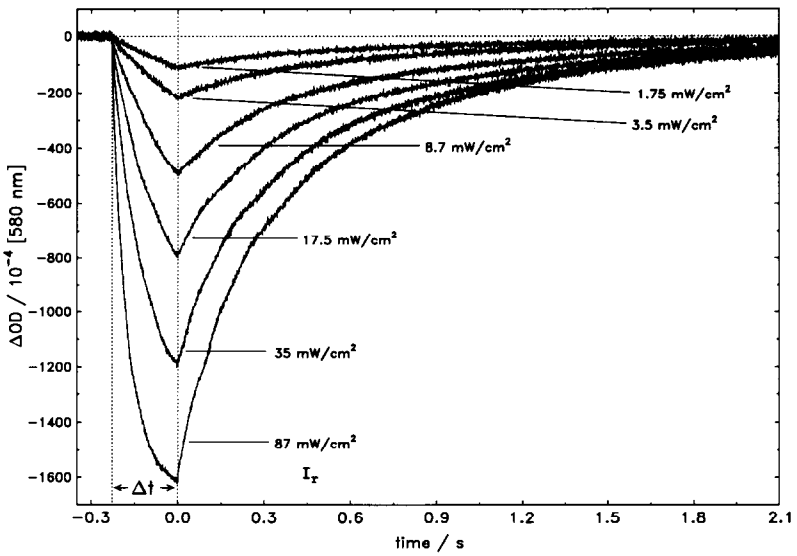


Fig. 1. Bleaching and recovery of BR probed at 580 nm for different values of I_r (514 nm) and $\Delta t = 250$ ms.

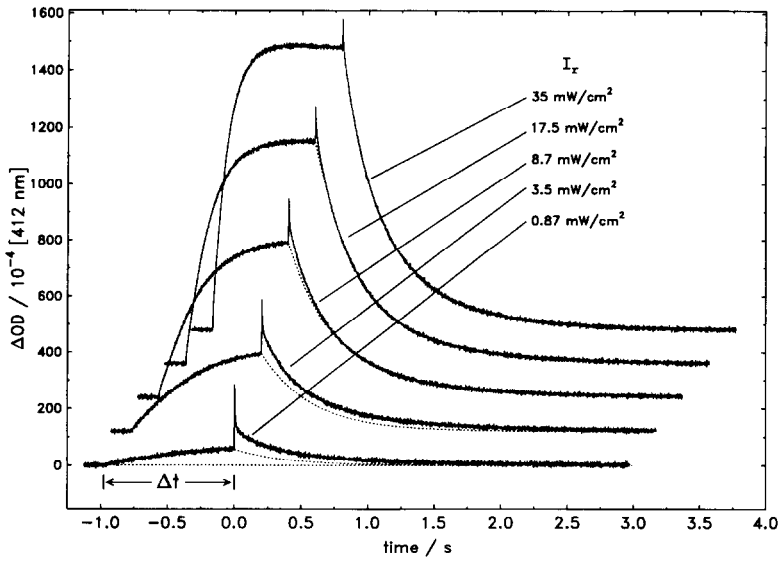


Fig. 2. Formation and decay of M, probed at 412 nm for different values of I_r (514 nm) and $\Delta t = 1$ s. The sharp positive signal immediately after the illumination period is caused by an additional short laser flash (cf. *Experimental*).

(2) The population of N, M^S or $M^f(\alpha, 1)$ in an individual cycle is correlated with an adequate depletion of BR in the same cycle.

The following experiments were designed to test these conditions.

2. Experiments (Scheme 3)

The optical layout of the experiments is shown in Scheme 3. A diluted aqueous suspension of purple membranes [1] at pH 10.5 (buffer 0.2 M Na-borate + 0.1 M NaOH) is illuminated periodically by a cw laser beam at 514 nm. The population of N and M is probed by bleaching BR as a function of I_0 and monitoring its recovery at 580 nm. I_0 is proportional to the irradiance I_r at 514 nm which in this text is given in mW/cm^2. The relative amplitudes of N and M^S (A_N, A_{M^S}) are deduced from the recovery signal by their different decay times in Scheme 2. The relative concentrations are given by

$$[N]/[M^S] = (A_N/A_{M^S}) \cdot [\varepsilon_{BR}/(\varepsilon_{BR} - \varepsilon_N)]_{580} \quad (3)$$

where the second factor has a value of 4.55 [5].

In order to assign the total amount of BR which is photolyzed during a certain period of illumination to the individual cycles, the residual fraction of BR is photolyzed immediately after the illumination period by a second short flash (532 nm, 8 ns) which is aligned perpendicularly. The relative amounts of M^f and M^S in the flash-induced M signal probed at 412 nm are determined as a function of I_0. In this way it is possible to deduce the depopulation of BR for each of the three cycles in Scheme 2 which involve an M intermediate.

3. Results

Fig. 1 shows transient signals for the bleaching of BR by the 514-nm beam ($\Delta t = 250$ ms) and its

[1] Purple membranes were a gift from the laboratory of D. Oesterhelt at the Max-Planck-Institute of Biochemistry which is gratefully acknowledged.

subsequent recovery. For low levels of irradiance (I_r) the recovery signal is dominated by the N-to-BR and for high levels by the M^S-to-BR conversion. For still higher levels of I_r (not shown) the $M^f \rightarrow BR$ recovery component is also observed. This behavior is in line with a 'stepwise' population of N, M^S and $M^f(\alpha, 1)$ as predicted by Scheme 2. The signals were analyzed as described in Experimental.

The formation of M during the illumination with the 514-nm light ($\Delta t = 1$ s) and its subsequent decay are shown in Fig. 2 for different values of I_r. The sharp positive signal, which appears immediately after the illumination period, is induced by the short 532-nm laser flash and probes the composition of residual BR via the different M components. For the lowest value of I_r the flash-induced M signal consists of an apparently single M^f and an M^S component. With increasing I_r one part of M^f rapidly decreases, leaving back a residual fraction which then only slowly decreases with I_r. In this way two components $M^f(1)$ and $M^f(2)$ can be distinguished kinetically although their lifetimes are the same within the limits of error. Between these two components M^S decreases with I_r. The 'stepwise' decrease of $M^f(1)$, M^S and $M^f(2)$ reflects the depopulation of BR in the cycles of $bR(\beta, 2)$, $bR(\alpha, 2)$ and $bR(\alpha, 1)$ of Scheme 2. Hence $M^f(1) \equiv M^f(\beta, 2)$, $M^f(2) \equiv M^f(\alpha, 1)$ and $M^S \equiv M^S(\alpha, 2)$.

Fig. 3 shows flash-induced transient signals of M obtained from a sample which is continuously illuminated with different levels of 514-nm light. In this series of signals the sequential decay of the three M components can also be nicely distinguished. It must be noted that under continuous illumination the apparent rate constants for the M decay are given by $l_0 + k_i$. This leads to a significant acceleration of the decay of M^S with increasing I_r.

The results from a quantitative analysis of data as described in Figs. 1 and 2 are displayed in Fig. 4. The upper part shows the relative concentrations (with respect to the photolysis rate ΔBR) of N, M^S and M^f as a function of I_r. In the saturation limit of N a certain fraction of BR is completely converted to N. By comparison with our RR data one obtains a value of 0.55 for this fraction. For the fraction of BR which is convertible to M^S this leads to a value of 0.2.

The lower part of Fig. 4 shows the composition of M as inferred from the flash-induced transient signals in Fig. 2. All values refer to the initial amplitudes in the transient signals. The calibration of the signals is again obtained by comparison with our RR

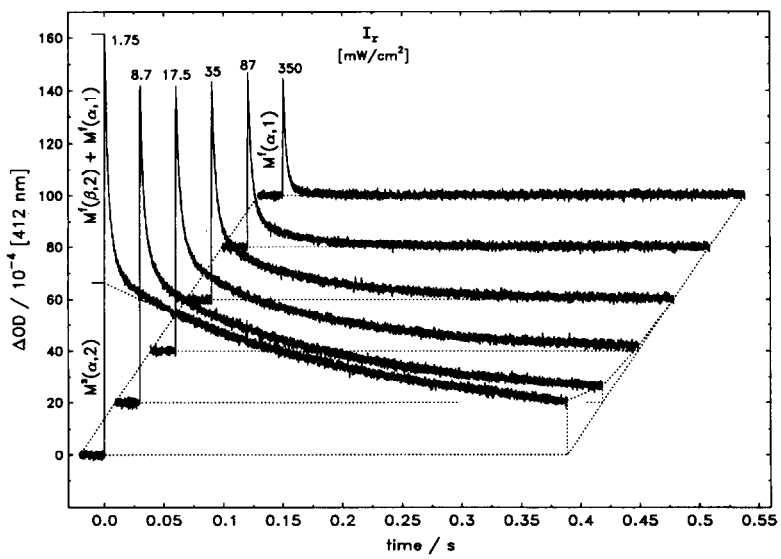


Fig. 3. Flash-induced formation of M from a continuously illuminated sample for different values of I_r (514 nm).

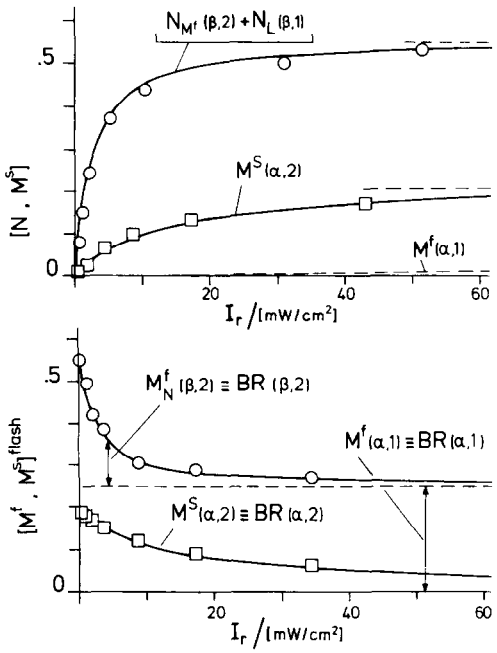


Fig. 4. Upper part: population of N, M^s and M^f as a function of I_r (514 nm), inferred from experiments as described in Fig. 1. Lower part: composition of residual BR, inferred from the flash-induced M signal in Fig. 2, as a function of I_r (514 nm).

and optical transient data at pH 10.5 which give $[M^f + M^S] = 0.75$ and $[M^S] = 0.2$.

From the data in Fig. 4 the relative population of the four subspecies at pH 10.5 can be obtained:

$$[bR(\alpha, 1)] = [M^f(\alpha, 1), I_0 = 0] = 0.25$$

$$[bR(\alpha, 2)] = [M^S(\alpha, 2), I_0 = 0] = 0.2$$

$$[bR(\beta, 1)] = [N(\beta, 1), I_0 \to \infty] = 0.25$$

$$[bR(\beta, 2)] = [M^f(\beta, 2), I_0 = 0] = 0.3$$

It can be seen in Fig. 4 that the population of $N(\beta, 2)$ is directly correlated with the decrease of $M^f(\beta, 2)$. This implies that in the cycle of $bR(\beta, 2)$ the population of N is coupled with the depletion of $BR(\beta, 2)$. The same conclusion holds for M^S and $BR(\alpha, 2)$. — Most obvious is the correlation between $M^f(\alpha, 1)$ and $BR(\alpha, 1)$. It can be seen in Fig. 4 (upper part) that up to $I_r = 60$ mW/cm² $M^f(\alpha, 1)$ is only slightly populated which corresponds to the fact that in the lower part $BR(\alpha, 1)$ is nearly unchanged. However, at still higher values of I_r the depopulation of $BR(\alpha, 1)$ can be clearly observed. These findings demonstrate that the two conditions for the validity of Scheme 2 (cf. *Introduction*) are fulfilled.

4. Discussion and conclusions

One may first ask if the conclusions from our experiments at pH 10.5 are relevant under physiological conditions. In an earlier paper the existence of parallel cycles was essentially concluded from experiments at neutral pH [4]. Under such conditions one also finds the biphasic decay of M as well as the two different channels for the formation of N. — The changes in the reactions which are observed when going from neutral to pH 10.5 can be adequately explained by reduction of the proton concentration in the external phase and at the surface of the membrane. There is no evidence for global structural modifications of BR in this pH range. This strongly suggests that the principle reactive behavior of BR is preserved in this range.

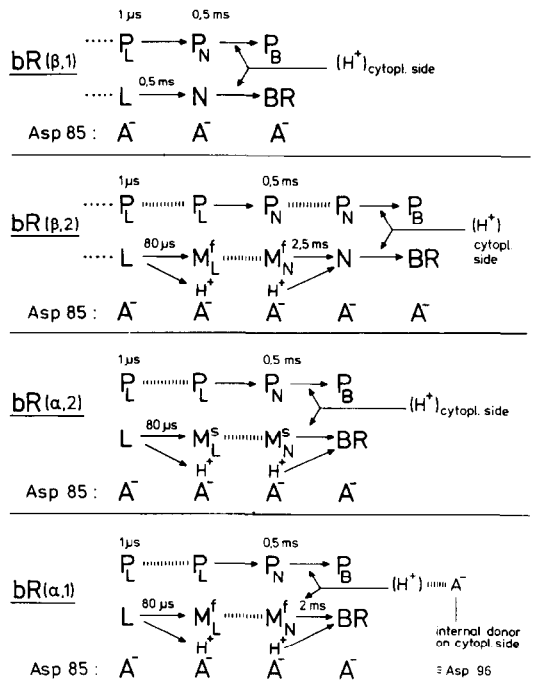
The existence of different cyclic reactions of bR raises further questions. So one may ask if all or only part of the cycles are active in proton pumping. A critical evaluation of the available data on the efficiency of the proton pump revealed that on the average one proton is pumped per each cycling bR molecule [6]. Under the reasonable assumption that the stoichiometry of the proton pump does not exceed unity it can be thus concluded that one proton is pumped in each of the four cycles.

Another important question concerns the mechanism of proton pumping. Is it different in the four cycles, as one would expect intuitively, or are there common features? At least a partial answer can be obtained from the inspection of the recovery phase of BR (from M or N) since during this phase the proton, which has been released earlier in the cycle to the outside, must be replaced from the cytoplasmic side. The recovery of BR from M involves two reaction steps, namely the reprotonation of the Schiff base and the reisomerization of retinal. However, only in the cycle of $bR(\beta, 2)$, which involves the sequence $M^f \to N \to BR$, are the two processes clearly resolved. From the finding that the decay rate of M^f is independent of pH, whereas that of N

strongly decreases with increasing pH, it can be concluded that the reprotonation of the Schiff base in the $M^f \rightarrow N$ step occurs from an internal donor, whereas the reisomerization of retinal in the $N \rightarrow BR$ step is controlled by the uptake of a proton from the external phase [4]. It thus turns out that in the recovery of BR from M two different proton transfer steps are involved. Consequently this should also be the case in the recovery processes $M^f \rightarrow BR$ and $M^S \rightarrow BR$ in the cycles of bR(β, 1) and bR(β, 2). It has been argued that in these processes the proton-induced isomerization is the rate-limiting step and precedes the reprotonation of the Schiff base [4]. In any case a common feature in the recovery process of all four cycles is the proton-induced reisomerization of retinal. This suggests that the respective proton transfer step is directly involved in the pump mechanism.

On this basis a new model for the proton pump mechanism has been proposed in which the protons during the cyclic reaction are transported through a hypothetical 'internal reactive site' [4]. In this model a proton would be released in the first phase of the photocycle from the 'reactive site' to the extracellular space. In the recovery phase the reactive site would be reprotonated from the cytoplasmic side inducing the reisomerization of retinal. In this model it is further proposed that in those cycles in which M is formed the Schiff base would be directly reprotonated from the counterion Asp85. This would mean that in the N state Asp85 is deprotonated.

This model could explain proton pumping in all four cycles. However, it is in contradiction with the presently favored model in which the Schiff base acts as a proton switch ('switch model') [7]. This model, which was inferred from a single reaction cycle with the $L \rightarrow M^f \rightarrow N$ sequence, involves as an essential step the reprotonation of the Schiff base from the cytoplasmic side via Asp96 as an internal donor. — An essential argument in favor of the 'switch model' was the statement in FT-IR studies that Asp85 is protonated in the N state which would imply that in the $M^f \rightarrow N$ transition the Schiff base cannot be reprotonated from Asp85 so that a different donor must be involved [8]. The FT-IR statement was based on the assignment of a strong band at 1755 cm^{-1}, which appears in the infrared difference spectra under conditions where N is accumulated, to the C=O stretch of Asp85. The deviation from the frequency of the C=O stretch of Asp85 in M which lies at 1762 cm^{-1} was attributed to structural changes in the vicinity of Asp85 during the $M^f \rightarrow N$ transition. However, until now no direct physical evidence (e.g. site-specific labeling) for the assignment of the 1755-cm^{-1} band to Asp85 is available. — On the other hand we had proposed in our earlier work that Asp85 is not protonated in N [4]. This was based on a comparison of the resonance Raman spectra of the intermediates L and N which reveals that the structure of the Schiff base and its counterion complex must be identical in both species. — Since in L the counterion Asp85 is definitively deprotonated this should also be the case for N. In order to test the protonation state of Asp85 in a direct way we have started recently a program of time-resolved infrared measurements in the spectral region of the C=O stretching vibrations in collaboration with other laboratories. As one of the results, obtained at pH 10, it turned out that the C=O stretch at 1762 cm^{-1} which reflects protonated Asp85 in the M state decays synchronously with the optically probed M^f and M^S



Scheme 4.

states. — This indicates that at least for pH 10 the Schiff base is reprotonated from the counterion Asp85. — In summary it can be stated that the model which has been proposed in our earlier work [4] can explain proton pumping in all of the four cycles and is in agreement with the observed spectroscopic phenomena. The results and conclusions in the present paper are summarized in Scheme 4.

In addition to the reactions of the chromophore and its interactions with the counterion (Asp85), conformational transitions of the protein backbone are taken into account. It has been concluded from spectroscopic studies that at the stages of L and N the protein is in two different conformational states we call P_L and P_N [9]. It has been proposed that the $L \rightarrow N$ transition in bR(β, 1) is induced by the protein transition $P_L \rightarrow P_N$ [4]. It is assumed that this transition takes place in the same way for all subspecies (ca. 0.5 ms at room temperature) and opens up a proton conduction channel to the cytoplasmic surface. The P_L–P_N transition would also define two M states (M_L, M_N). In the cycle of bR(β, 2) this could explain the reprotonation of the Schiff base ($M_N^f \rightarrow N$). In the cycles of bR(β, 1), bR(β, 2) and bR(α, 2) the recovery of BR is controlled by the uptake of a proton from the cytoplasmic side. In contrast to this the recovery step $M^f \rightarrow BR$ in the cycle of bR(α, 1) is independent of pH. In our experiments with the mutant D96N a fast recovery step $M^f \rightarrow BR$ could not be observed. This suggests that in wild-type bR the residue Asp96 which is located close to the cytoplasmic surface acts as the proton donor.

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