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Review

Chemical and physical evidence for multiple functional steps comprising the M state of the bacteriorhodopsin photocycle

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Abstract

In the photocycle of bacteriorhodopsin (bR), light-induced transfer of a proton from the Schiff base to an acceptor group located in the extracellular half of the protein, followed by reprotonation from the cytoplasmic side, are key steps in vectorial proton pumping. Between the deprotonation and reprotonation events, bR is in the M state. Diverse experiments undertaken to characterize the M state support a model in which the M state is not a static entity, but rather a progression of two or more functional substates. Structural changes occurring in the M state and in the entire photocycle of wild-type bR can be understood in the context of a model which reconciles the chloride ion-pumping phenotype of mutants D85S and D85T with the fact that bR creates a transmembrane proton-motive force.

Keywords: Bacteriorhodopsin; Ion transport; M intermediate; Photocycle; Protein dynamics

1. Development of the concept that the M state of bacteriorhodopsin has either sequential or alternative substates

1.1. A working definition of the 'M state'

Bacteriorhodopsin is the sole protein component of the purple membrane of *Halobacterium salinarum* [1]. The function of bR in vivo is to convert solar energy into a proton-motive force which the organism uses to drive ATP synthesis [2]. For every proton it pumps out of the cell, bacteriorhodopsin (bR) undergoes a single deprotonation and reprotonation cycle at the Schiff base [3,4] that connects the side chain of Lys216 to the retinal molecule buried within the core of the apoprotein [1,5]. The term 'M intermediate' or 'M state' (we use these terms interchangeably) simply describes the normal photoproduct of bR in which the Schiff base is deprotonated.

If one imagines bR bisected by the plane where the two leaves of the lipid bilayer meet, its retinal moiety, including the Schiff base, would be near that plane in the center of the protein [5]. In the $L \rightarrow M \rightarrow N$ portion of the photocycle (Fig. 1A), the initially protonated Schiff base loses a proton to Asp85 in the extracellular domain, and it is subsequently reprotonated by Asp96 in the cytoplasmic domain [6–8], thus achieving unidirectional proton pumping. This change in access to the Schiff base from one side of the membrane to the other is likely to occur through changes in protein conformation rather than by alteration of retinal geometry [9]. Movement of the 13-*cis* retinal into a new chemical environment during the lifetime of the M state has not been detected in either Raman spectroscopy [10,11] or linear dichroism [12] experiments.

Deprotonation of the retinal group results in a peak absorption at about 412 nm rather than at

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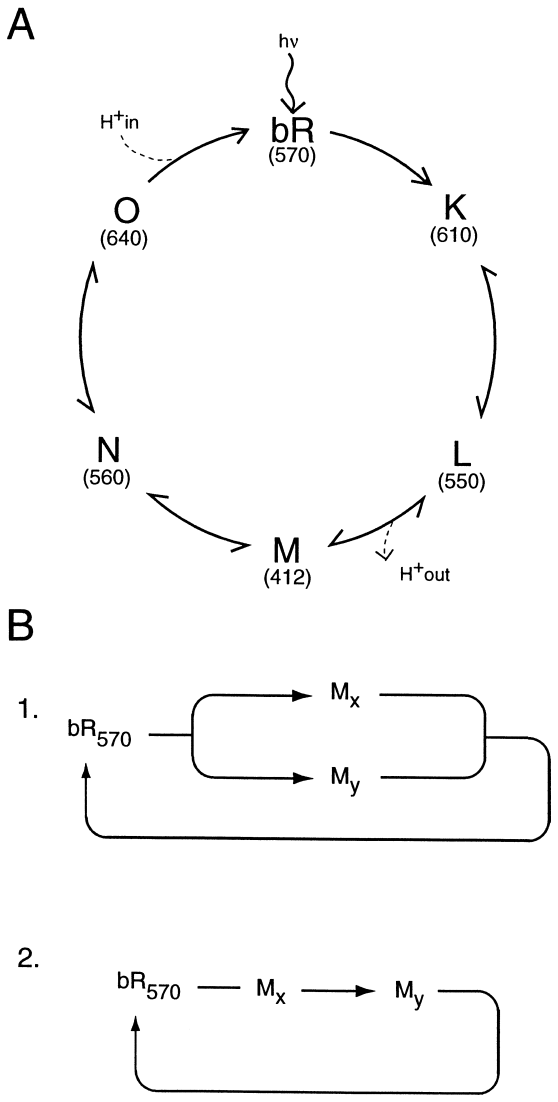

Fig. 1. (A) Essential features of the photocycle of bacteriorhodopsin determined by Lozier et al. [13] are shown, but are modified by the inclusion of thermal backreactions where they are believed to be significant [14,30]. Photoexcitation of the light-adapted resting state (bR_{570}) of the molecule initiates the photocycle, represented by the five major photointermediates. The optical absorbance maximum of each intermediate is given in parentheses; the conventional understanding of proton exchange with the bulk aqueous phases on either side of the membrane is also indicated. (B) Multiple substates of the M intermediate, represented by M_x and M_y, can occur as parallel steps in a branched photocycle (1) or as sequential steps in a linear photocycle (2).
←

550 nm or above, as is the case for the other intermediates and for the resting state, bR_{570} [13]. Hence, the M intermediate is the only step in the photocycle in which the color of bR is yellow rather than blue or purple. Because this yellow color is quite faint, bR in the M state is sometimes said to be 'bleached' or 'photolyzed', but strictly speaking this is incorrect, since the chromophore is never detached from the protein during a normal photocycle.

1.2. *Spectrally silent substates can explain the complexity of bR photocycle kinetics*

Absorption spectra in the visible range are far more sensitive to the protonation state of the Schiff base than they are to nuances in protein conformation. Hence, the photocycle may actually be subdivided into many more steps than the number of spectra from visible spectroscopy would suggest. Indeed, global analyses of the kinetics of the entire photocycle show that at least some photointermediates must be composed of spectrally silent substates [14–16].

The M intermediate has long been thought to encompass two or more substates. Numerous flash spectroscopy experiments have established that the time course of absorbance at 412 nm is biphasic following an initial flash of actinic light, whether the rise [10,17–20] or decay [10, 17, 18, 21–28] of the signal is measured. This simply means that both the rise and decay of the M state are better described by two exponentials.

Two populations of the M intermediate were inferred from the biphasic nature of M state kinetics; these were dubbed 'M_f' (fast decay or rise) and 'M_s' (slow decay or rise). The relationship between the multiexponential kinetics and the physically distinct intermediates has been controversial, though, and mutually exclusive hypotheses have arisen to explain the biphasic kinetics of M rise and decay. (Further confounding efforts to reconcile these data is the fact that the fast and slow components of the rise of the 412 nm signal have not generally been distinguished from the fast and slow components of the signal's decay.) Some authors proposed that M_f and M_s denote sequential steps in the photocycle [11,23,29,30], while others put forth models in which two forms of M are parallel steps either in a branched photocycle or in entirely separate photocycles [19,26,27]. Fig. 1B

illustrates the basic difference between the branched and linear models. Although the linear model is depicted as a series of unidirectional steps, Nagle et al. have shown that the single-pathway model of the bR photocycle fits their and others' data if and only if back reactions are allowed [14].

1.3. Nomenclature of M substates

The analysis of time-resolved visible spectra recorded over the range of 10^{-7}–10^{-1} s by Varo and Lanyi [29] has ultimately provided compelling evidence for sequential, discrete M substates. The authors represented the time course of the visible absorbance spectrum following a flash of actinic light as the linear combination of spectra for intermediate species that became populated at different times and with different amplitudes. This analysis, which fully allowed for reversible reactions if they were present, was consistent with the existence of two sequential M states, assigned the names 'M_1' and 'M_2', whose visible absorption spectra could to some extent be resolved and characterized by varying experimental conditions (e.g., [30–32]). M_1 and M_2 were proposed to be in series rather than in parallel because linking them by an essentially irreversible reaction was the simplest model that fit the kinetics of L decay.

The M_1 and M_2 nomenclature has come into widespread, but occasionally facile usage. As a result, one cannot always be sure that the 'M_1' or 'M_2' species referred to by other groups are the same as those identified by Varo and Lanyi in 1991. More recently it has become convenient to refer to an 'early M' and a 'late M'. Although M_1, in the kinetic definition of Varo and Lanyi, appears before M_2, that is not sufficient to guarantee that the 'early M' detected by another method is the same species as M_1, or that a particular 'late M' is the same as M_2.

To illustrate this last point, we cite the example of the 'M_N' intermediate [33], since this term has also gained general acceptance. First described for the D96N mutant of bR, the M_N substate has the protein conformation of the N intermediate as determined by both IR spectroscopy [33] and diffraction methods [34], yet retains a deprotonated Schiff base. Thus, it is clear that M_N is a 'late M' species, but there is no direct way to know whether M_N is the same as M_2. Indeed, we shall later argue that it is not.

2. Characterization of discrete M substates of bacteriorhodopsin

A switch in access to the deprotonated Schiff base from the extracellular side of the membrane to the cytoplasmic side has been proposed to account for vectorial proton pumping in bR [35–38]. Accordingly, the characterization of M substates is a high priority. Investigators have two basic approaches at their disposal: they can either make time-resolved measurements of continuously varying parameters from bR molecules synchronized by a brief ($\ll 1$ μs) flash of actinic light; or kinetically accumulate one or more M substates and take physical measurements from the metastable or photostationary state(s).

2.1. Analysis of the transitions among M substates

Not long after the discovery of bR [1], Slifkin and Caplan found evidence for two M species when they measured the polarization of absorbance at 410 nm [39], and Ort and Parson reported the appearance of a two-step, light-induced volume increase in bR coinciding with the occurrence of the M state [40]. For the most part, however, the characterization of distinct M substates would wait more than a decade.

As mentioned previously, Varo and Lanyi used time-resolved visible spectra recorded at different temperatures, at pH 7, in order to determine thermodynamic parameters for the entire photocycle [30,31]. What emerged was the conclusion that only the $M_1 \rightarrow M_2$ and $O \rightarrow bR_{570}$ steps are associated with a large decrease in free energy, and are thus essentially irreversible. Similar experiments performed at varying hydration levels revealed that the $M_1 \rightarrow M_2$ transition is inhibited at low hydration [32,41] This result is consistent with a protein conformational change between the two [42].

An important early observation made by Zimanyi et al. was that the mutation of Asp85, in the extracellular domain of the protein, shifts the absorbance maximum of M_1 but not that of M_2; whereas a mutation at Asp96, in the cytoplasmic half, shifts

the absorbance maximum of M_2 only [43]. This result was perhaps the first demonstration of the long sought-after 'switch' in access to the Schiff base from the extracellular to the cytoplasmic side [35–38].

The $M_1 \rightarrow M_2$ transition has also been associated with the release of a proton into the extracellular medium [44]. In an earlier study, the slow component of M decay was also correlated with proton release [26]. Since M_2 decays more slowly than M_1 [29], these sets of observations cannot easily be reconciled.

The absorbance maximum of the M state is well separated from that of bR_{570} and from the other photoproducts of bR (Fig. 1A), allowing double flash experiments to be performed in which the photoreaction of M itself [45–47] is followed. By varying the time delay (Δt) between the initial flash (530 nm) to start the photocycle and the subsequent flash (337 nm) to drive M back to bR_{570}, Druckmann et al. found that the kinetic profile of the M state photoreaction at earlier times ($\Delta t = 10^{-5}$ s) was different from that triggered at later times (up to $\Delta t = 10^{-3}$s) [48]. They concluded that they had observed the photoreactions of two, consecutive M substates: M_1 at $\Delta t = 10^{-5}$ s and M_2 at $\Delta t = 10^{-3}$ s. Two later studies, by Dickopf and Heyn [49] and Hessling et al. [50], confirmed and elaborated upon these results. What Dickopf and Heyn found was that the backreaction of M_2 was not only slower than that of M_1, but also pH-dependent in such a way as to suggest that the proton release group (for recent discussions, see [51,52]) must become reprotonated in the first step of the backreaction. Hessling et al., on the other hand, followed the backreaction of M with time-resolved Fourier transform infrared (FTIR) spectroscopy [50], noting small but significant changes in the amide I region at $\sim$1660 cm^{-1}. It is clear from their IR spectra that neither of the M substates whose backreactions were being followed could have been M_N.

The phenomenon of blue light reversal of the M intermediate was also employed by Nagel et al. to study the voltage dependence of proton pumping by bR expressed in the cell membrane of *Xenopus* oocytes [53]. A fast-decaying component of M decay was always present, but Nagel et al. also detected a slow-decaying component found only at pump-inhibiting potentials. The authors' interpretation of the data was that an early substate of M, in which release of a proton into the extracellular medium had not yet occurred, is responsible for the slow component of M decay. This model explains why the fraction of slow-decaying M increased when the voltage was applied in a direction that opposed proton pumping. As was shown by Misra as well, the transition between M substates (referred to there as M2 and M2′) can be prevented by low pH [52] as well as by an opposing membrane potential.

Another technique that has deepened our understanding of M substates is time-resolved FTIR spectroscopy. In early attempts, M_1 and M_2 could not be resolved [54,55]. However, recent improvement in the time-resolution with which FTIR spectra are recorded has permitted Roedig et al. to collect convincing structural evidence that discrete, serial transitions occur between M substates [56]. These authors distinguished three substates on the basis of time-dependent intensity differences in IR bands at 1616 cm^{-1}, 1557 cm^{-1}, and 1545 cm^{-1}, the latter two bands lying within the amide II region of the IR spectrum (Fig. 2A). These measurements show that the earliest M state to appear, with a rise time of $\sim$3 μs, is structurally quite similar to the L intermediate. The authors go on to invoke two more distinct M substates, with approximate rise times of 40 μs and 130 μs, respectively. The conditions of their experiments precluded the accumulation of M_N.

Are the first and third M substates identified by Roedig et al. equivalent to Varo and Lanyi's M_1 and M_2, respectively? A comparison of data obtained at pH 7 suggests that they indeed are the same; in both reports, the first M substate dominates at $\sim$10 μs, and the last, at $\sim$300 μs. Furthermore, the earliest M substate detected by Roedig et al. is in equilibrium with the L state, an equilibrium that favors L, as is M_1 [29,57]. If Roedig et al. have indeed captured the IR signatures of M_1 and M_2, the implication is immediate: because neither has the IR signature of M_N [33], the M intermediate must comprise (at least) three substates altogether, in the order $M_1 \rightarrow M_2 \rightarrow M_N$.

2.2. *Physical studies of bR trapped in discrete M substates*

The recently published solid-state nuclear magnetic resonance (NMR) study by Hu et al. [58] is a wel-

come addition to the canon of experiments exploring the progressive change in the structure of the M state. By incorporating ^{15}N at the Schiff base nitrogen ([ε-N]Lys216), Hu et al. could observe the chemical shift that is characteristic of a deprotonated Schiff base when wild-type purple membrane, in 0.3 M guanidine at pH 10.0, was illuminated at low temperature. However, the chemical shift of the deprotonated Schiff base observed at −60°C was different from that seen at −10°C, indicating the formation of two distinct M substates. The authors designated the two substates Mo ('old M') and Mn ('new M') in reference to spectra obtained in their previous work. The species trapped at the lower temperature, Mo, is expected to form earlier than the one that accumulates at the higher temperature, Mn. Importantly, Mo can be made to relax in the dark to Mn, which further establishes their relationship as a sequential one.

Similar experiments in which bR was labeled with ^{13}C led to two further insights. First, data obtained after labeling residues near the Schiff base imply the existence of at least two distinct M substates at −60°C, i.e., Mo itself appears to be a mixture of two species. Furthermore, labeling backbone carbonyls with ^{13}C gave results suggestive of a large conformational change between Mo and Mn. The authors found that Mn coexists with the N state, hence it may well be identical to the M_N substate (see above).

Other groups, using mainly IR spectroscopy and/or diffraction methods to characterize specimens, have also reported that different M substates exist at different temperatures. Between −63°C and −13°C, wild-type bR at pH 7 or above yields a photostate whose IR signature is unmistakably that of the M intermediate (e.g., Fig. 2A) when the spectrum of bR_{570} is subtracted [59–66]. Toward the low end of this temperature range (roughly −63 to −33°C), researchers find an M substate characterized by (i) an amide I difference band at 1660 cm^{-1} (Fig. 2B, solid line) and (ii) structural changes in helices F and G indicated by difference peaks seen in 3.5 Å projection maps [62,66,67]. Between about −33°C and −13°C, depending on how well-hydrated the specimen is, an M substate is detected which is characterized by an amide I difference band at 1670 cm^{-1} (Fig. 2B, dashed line) along with further developed structural changes in helices F and G [62,66,67]. This form of

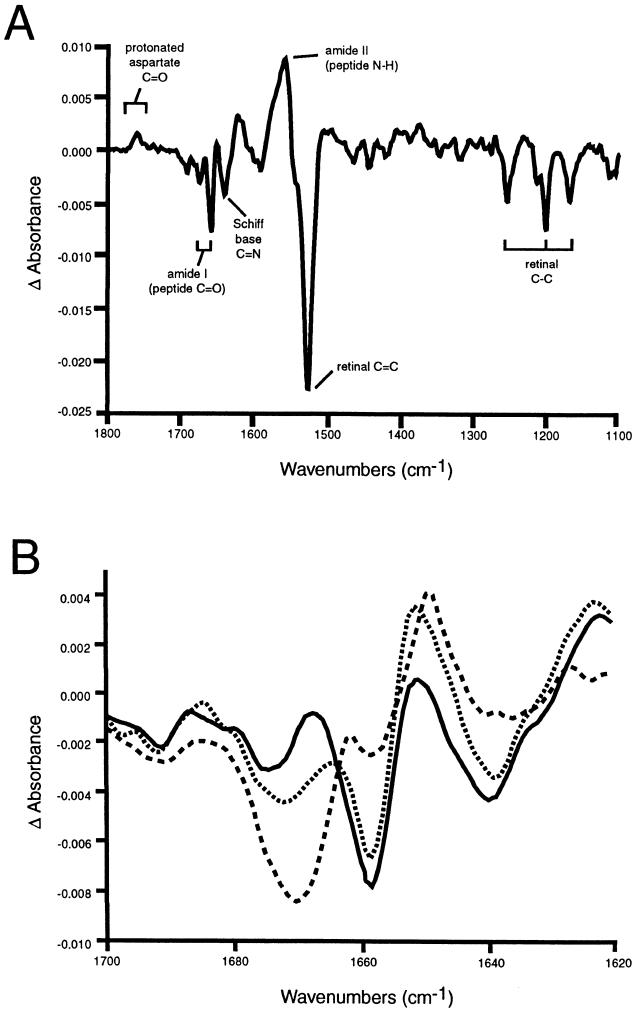

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Fig. 2. IR spectroscopy of M substates. (A) A difference spectrum (M-bR_{570}) obtained from a wild-type specimen equilibrated to 81% relative humidity and illuminated at −43°C. Annotations identify the chemical groups responsible for the salient features of the spectrum. The presence of a positive band at 1761 cm^{-1} signals the protonation of Asp85, and the absence of a positive peak at 1183 cm^{-1} reflects the unprotonated Schiff base. (B) Absorbance changes in the amide I region can distinguish among M substates. Spectra were collected at −43°C, pH 6.7 (solid line); −33°C, pH 6.7 (dotted line); and at −23°C, pH 10 (dashed line). A negative difference band at about 1670 cm^{-1} is typical of late M substates, for which M_N is a prototype. For other, presumably earlier substates, a negative peak at 1660 cm^{-1} is observed. At −33°C two or more subtypes appear to coexist, based on a partial shift of features of the spectrum from the solid line to the dashed line.

M is similar to the N state, with respect to both the amide I difference band and the projection map peaks, and may be equivalent to M_N [33].

Subramaniam and coworkers have taken a somewhat different approach by performing electron crystallographic analysis on wild-type bR specimens flash-frozen at various times between 1 and 35 ms after illumination at 5°C [68,69]. While strong differences in electron density near the cytoplasmic ends of helices F and G appear at each time point, progressive structural changes in this time range were not detected. The failure of the time-resolved approach to detect significant conformational differences among substates demands explanation. Perhaps, for the conditions employed by Subramaniam et al., the concentration of M_1 is already quite low even at the earliest time point (1 ms) (e.g. [30]). Since M_N is expected to have a vanishingly small half-life in the wild-type protein (Fig. 3), only 'middle-phase' substates, for which M_2 is a prototype, would be significantly populated in the time range of 1 to 35 ms.

Low temperature is one means of slowing down the photocycle of bR to facilitate the study of the M intermediate; another is low hydration [70]. Sass et al. have reported that below a relative humidity of approximately 60%, a form of the M intermediate accumulates which has an amide I difference band at 1660 cm^{-1} (Fig. 2B) but shows no structural changes as determined by X-ray powder diffraction at a resolution of about 8 Å [65]. A form of the M intermediate with the same characteristics as that trapped at low relative humidity was also found in fully hydrated membranes containing the bR mutant D38R [71] at neutral pH. Hence, the absence of structural change in the M state when it is formed in partially hydrated wild-type bR cannot be dismissed as an irrelevant artifact of drying.

The combination of high pH and the presence of guanidinium ion, either in the form of guanidine hydrochloride [72] or arginine [73], allows high levels of the M intermediate to accumulate as a photostationary state under continuous illumination. The M substate that accumulates under these conditions is very similar to M_N (which was originally produced at high pH in the D96N mutant [33]) with regard to both absorbance in the IR [71,74] and difference peaks in projection maps obtained by neutron [72] or X-ray powder diffraction [34,74–76]. The structural features observed are consistent with the movement of helices F and G, although the movement of helix F seems to be of less magnitude than that found in the D96N or D96G mutants at high pH [34,68,69]. In the most recent of these studies, Oka et al. [74] use heavy metal labeling to show that when M is formed in this way in the bR mutant I222C, the cytoplasmic end of helix G undergoes a large-scale conformational change. Thorgeirsson et al. previously used spin labels to demonstrate photocycle-dependent movement of the EF loop [77], which extends into the cytoplasm.

This brings us to some interesting questions: does the M_N substate exist in the photocycle of wild-type bR? More generally, are M substates described in bR

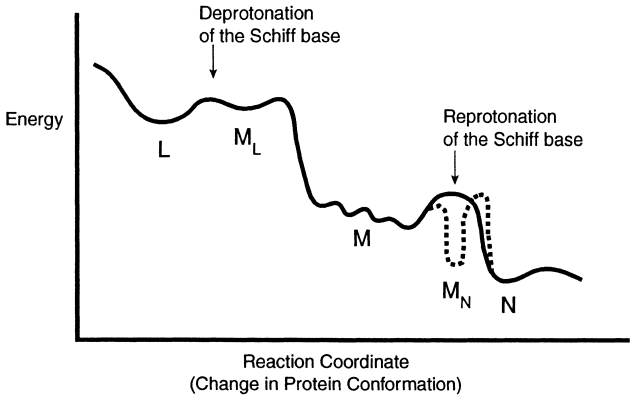

Fig. 3. Schematic diagram for the one-dimensional potential energy surface that would describe the existence of two or more substates in the M intermediate of the bacteriorhodopsin photocycle. Formation of the transition state between L and M must have a relatively low activation energy, as L can be trapped only at about −100°C. The earliest form of the M intermediate, designated here as M_L, is argued (on theoretical grounds) to have a protein structure similar to that of the L state. Except at low humidity or in some mutant proteins, the equilibrium between L and the earliest form of M (e.g., M_1) favors the L state; thus, the energy of M_L is shown to be slightly higher than that of L. The number of substate minima that make up the primary form of M is unknown; the predominant form of M is arbitrarily shown in this diagram as a region with 4 minima. The activation barrier between the M and the N intermediates is shown as being the highest one, because decay of the M state is known to be the rate-limiting step *up to this point* in the photocycle. Formation of an N-like state must occur in the transition between M and N. This state becomes a trappable intermediate, however, as is indicated by the fanciful dashed line, in the presence of guanidium ion at high pH, in glucose-embedded specimens, and in the D96G and D96N mutant proteins.

mutants necessarily relevant to the wild-type protein? No one has yet reported an M substate in wild-type bR with the same magnitude of structural change at helix F as that exhibited by Asp96 mutants at pH > 9 [34,68,69], although M_N-like projection maps have been reported for the mutants I222C [74] and D38R at high pH [71]. Subramaniam et al. [69] have addressed these questions with particular depth, studying structural changes in the M intermediate formed in wild-type, D96N, D38R, and other mutants under a wide variety of conditions. They deduce that each of these bR species forms a unique and single M-state conformation which is adopted in the second, 'post-switch' half of the photocycle, i.e., from M_2 to O640. Their rather spartan conclusion is not easily reconciled with evidence for multiple protein conformations associated with the M state of wild-type bR that has been gathered by diffraction methods and IR spectroscopy [66,67] and by NMR spectroscopy [58]. Two distinct M conformations have also been reported for the D96N mutant [78,79] at different hydration levels, and for the D38R mutant [71] at neutral and high pH. Ultimately, X-ray crystallographic data from bR crystals trapped in different M substates (e.g., [80]) should resolve this controversy.

We quite agree with Subramaniam et al. [69] in that some caution must be used in taking results of experiments done on any bR mutant and applying them to the photocycle of the wild-type molecule. A given bR mutant may encounter unique free energy minima in the course of its photocycle that allow it to adopt conformations not like those seen in other bR species (Fig. 3). However, the simplest assumption to make at this stage of our understanding is that all bR species follow the same reaction coordinate.

Bearing in mind the caveat that mutants may mislead us regarding the photocycle of wild-type bR, two studies of the long-lived, N-like M_N state of D96N are especially worthy of mention, as they demonstrate the important point that absorption changes in the IR amide I region do not correlate with large-scale changes in structure detected by diffraction methods [65,78]. Both groups report that specimens with 1670 cm^{-1} difference bands of widely varying intensity have essentially the same structure in projection. Their observations ineluctably lead to the conclusion that the appearance of the difference band at 1670 cm^{-1} in late M substates, such as M_N and those trapped at about −20°C in w bR [61,64] or at high pH in D38R [71], is unrelated to the large-scale conformational changes in helices F and G which seem to come earlier in the M state.

Newcomers to the field are likely to survey the teeming variety of M substates and wonder how they all relate to one another. In fact, virtually all of the M substates characterized by IR spectroscopy and/or diffraction methods can easily be assigned to one of three categories (Table 1) based on their IR absorbance changes in the amide I region (Fig. 2B) and structural changes seen in projection. In Section 3, we suggest that the M intermediate should be represented as a sequence of M substates, and we then propose a new interpretation of the key proton-transfer steps occurring throughout the photocycle.

3. Towards a functional synthesis of current experimental data

3.1. Two or more progressive substates are required a priori

We assume, as does current consensus in the field, that the major M substates have a sequential relationship rather than being in parallel, or even off-pathway, branches. A sequential model is necessarily characterized by progressive changes that occur along a single reaction coordinate, as is illustrated in Fig. 3. In reality, the potential energy surface must have more than the one dimension shown [81], which in turn formally requires that the pathway be continuously branched. Although we reject nonsequential models at this time, they are preferred by some investigators [82,83], and to date there has been no definitive experiment to rule them out.

The progressive development of two or more substates of the M intermediate seems to be required on purely physical grounds. The molecule enters the M state by means of one proton transfer, and exits by means of another. Since the inertia involved in even simple shifts in side chain positions is more than an order of magnitude larger than that involved in proton transfer, it follows that the first M substate must be structurally identical to the last stage of the L

Table 1
A tentative classification of M substates

bR species/pH/r.h.[a]/temperature	Ref.	Dominant difference band in amide I region of IR (cm^{-1})	Strong difference peaks seen in projection	Suggested type of M intermediate[b]
D96N/9.6/57%/rT[c]	[63]	n.d.[d]	No	I
D96N/9.6/38%/rT	[63]	1660	n.d.	I
D38R/6.7/100%/−6°C	[69]	1660	No	I
wt/6.7/81%/−33°C	[60]	1660	n.d.	II
wt/7.0/>95%/5°C	[66]	n.d.	F<G[e]	II
wt/6.7/81%/−33°C	[62,65]	1660	F<G	II
wt/10/100%/−53°C	[63]	1660	n.d.	II
wt/6.7/81%/−53°C	[64]	1660	F<G	II
wt/6/>95%/5°C	[67]	n.d.	F<G	II
wt/6.7/81%/−13°C	[62,65]	1660+1670	F=G	II+III
D96N/9.6/75%/rT	[63]	1660+1670	F<G	II+III
wt/6.7/81%/−33°C	[64]	1660+1670	F=G	II+III
wt/9.4/86%/6±1°C	[70]	n.d.	F=G	III
D96N/9.6/100%/rT	[73]	n.d.	F=G	III
wt/10.0/n.r.[f]/rT	[74]	n.d.	F=G	III
D96N/9.6/high[g]/1°C	[33]	1670	n.d.	III
D96G/8.5/>95%/25°C	[66]	n.d.	F=G	III
D96N/11/95%/5°C	[34]	n.d.	F=G	III
D96N/9.6/100%/rT	[63]	1670	n.d.	III
D38R/9.6/100%/6°C	[69]	1670	F<G	III
D38R/9.6[h]/100%/6°C	[69]	1670	F=G	III
D96N/9.4/86%/[i]	[77]	n.d.	F>G	III
I222C/9.5/84%/20°C	[72]	1670	F=G	III
D96G/6/>95%/5°C	[67]	n.d.	F=G	III

[a]r.h., relative humidity.
[b]In our rather arbitrary notation, type I substates are forms of early M, possibly equivalent to M_L or M_1; type II substates are examples of the 'principal' or middle M substate(s) discussed in the text; and type III substates are forms of late M, equivalent to M_N.
[c]rT, room temperature.
[d]n.d., measurement not done.
[e]The degree of structural change observed in helix F is less than (or equal to, or greater than) that observed in helix G, as indicated by the respective intensities of the positive difference peaks associated with these helices. Intensity is conventionally designated by contour levels; differences of only one contour level have been disregarded.
[f]n.r., not reported.
[g]Specimens were hydrated to 70% w/w (water/purple membrane).
[h]Guanidine hydrochloride present.
[i]Specimens were slowly cooled to −200°C during continuous illumination.

state. Similarly, the last M substate must have nearly the same atomic coordinates as the first N substate. Since the L intermediate and the N intermediate have markedly different conformations ([66] and [84], respectively), it follows that at least two conformationally distinct M substates must exist. Versions of the M intermediate that show little change in protein structure, which we would call M_L, have indeed been observed (Table 1; see also Section 2). As noted in Section 2, an N-like species of the M intermediate, M_N, has also been described [33,34,68].

The most widely studied form of the M state, however, has a difference band at 1660 cm^{-1} in the amide I band of the IR [59–66], and also shows numerous structural changes in projection maps produced by electron diffraction [66–68]. This principal form of M differs from the late, N-like state of M, which has a negative difference peak at 1670 cm^{-1}. It also differs from the postulated early, L-like intermediate since it has been established experimentally that the structural changes in L are very small [66]. We conclude that the M state must consist of at least three,

successive substates, as has been diagrammed in Fig. 3.

3.2. Much still remains to be learned about the molecular physics that drives the progression of substates within the M intermediate

Currently, one can only speculate on plausible events that drive the transitions between the three major substates of the M intermediate. (i) The change in the local electric field near the Schiff base, resulting from formation of the M_L substate, must alter the balance of torques and stresses applied to nearby polar residues, such as the adjacent peptide groups. Relaxation of the protein structure [85] to a new local minimum in the potential energy surface could result in (ii) a domino-like cascade of side chain and/or segmental movements, resulting in the structure we earlier called the predominant M substate.

The speculative picture described above still lacks the molecular detail needed to formulate testable hypotheses concerning the mechanism of M state progression. Completion of high-resolution X-ray crystal structures of bR in the L, early-M, and N state – work that is currently being pursued – will provide much of the missing information. In addition, molecular dynamics simulations that start with sufficiently accurate atomic coordinates for the L intermediate and the early M substate may ultimately refine the merely intuitive ideas pictured above.

3.3. Bacteriorhodopsin could be a hydroxyl-ion pump

A single amino acid substitution replacing Asp85 with threonine [86] or serine [87] has been shown to convert bR into a chloride pump. Neutralization of the same residue with HCl has the same effect [88]. What do these observations imply for the mechanism of the photocycle in general, and for our understanding of the structural changes that evolve between the L and N photointermediates in particular? The most reasonable hypothesis is that the neutralization of Asp85 by substitution or by acidification does nothing to change the molecular machine itself, while at the same time having a powerful effect on the ion-binding specificity of its 'active site'. A logical implication is that wild-type bR is a hydroxyl-ion pump rather than a proton pump.

The cartoon drawn in Fig. 4 represents a proposed scheme by which bR and its D85T/S mutants might carry out homologous OH^- and Cl^- ion-pumping photocycles, respectively, emphasizing in particular the role that M substates would contribute to ion transport in both cases. Since it is imagined in our proposed model that the changes in protein conformation are essentially the same in the two photocycles, we will also speak of the role of M-like intermediates in the Cl^- ion-pumping photocycle. By this we mean to propose that functional states of the mutant protein occur with the same protein conformation as that of the M intermediate formed by wild-type bR. This extension of nomenclature no longer conforms, of course, to our earlier definition of the M state, i.e., one in which the Schiff base is deprotonated.

A strong parallel between the two proposed cycles is seen most clearly if one starts first with the blue, O intermediate of bR, and the corresponding chloride-free state of the mutant protein. The blue proteins then form purple 'resting states' by binding OH^- and Cl^-, respectively. The situation for wild-type bR must be imagined to involve more than simply binding OH^- ion, however, in order to explain other well established facts. A proton must be taken up on the extracellular side of the protein to neutralize the proton release group. Since, in our model of the hydroxyl ion pump, this proton will be released again to the extracellular side of the membrane, we propose that it plays a role similar to the Bohr protons in hemoglobin. Binding of a hydroxyl ion in the active site is further envisioned as producing an unstable transition state rather than a true intermediate, since the OH^- ion would simply abstract the proton from the beta-carboxylic acid group of D85 (which is still neutral in the O intermediate) as soon as it entered the binding site. Abstraction of the proton from D85 by the OH^- ion would then produce a neutral water molecule in the active site, located at more or less the same place where a chloride ion would be proposed to be bound in the D85T/S mutants. Luecke et al. initially observed an ordered water molecule, W402, in this position in the wild-type resting state in their 2.3-Å model of bR_{570} [89]; this and other

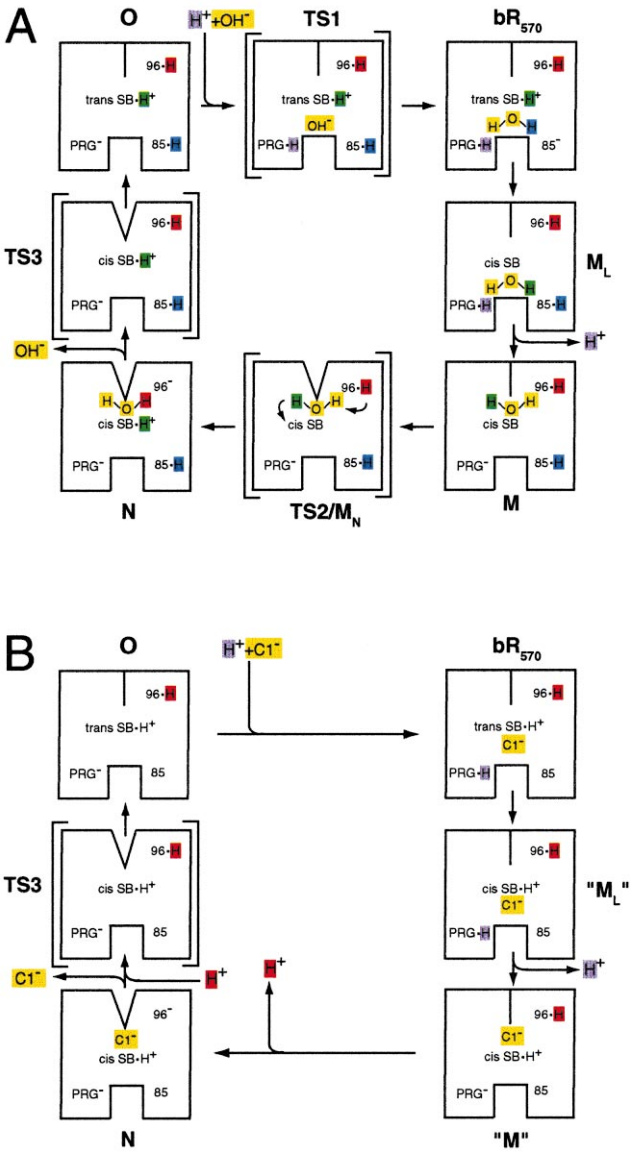

Fig. 4. A proposed scheme that describes wild-type bacteriorhodopsin as a hydroxyl ion pump rather than as a proton pump, and which allows the D85T or D85S mutant proteins to pump halide ions by the same mechanism that the wild-type protein uses to pump hydroxyl ions. Brackets are used to indicate structures that are expected to be transition states rather than trappable intermediates. The nomenclature TS1, TS2 and TS3 is used to designate proposed transition states; the light-adapted resting state is designated by bR_{570}; M, N, and O are meant to have their conventional meaning in designating intermediate states with distinct spectra in the visible; M_L and M_N are additional substates of the yellow, M intermediate discussed in the text. A vertical line on the hydrophobic, cytoplasmic side of the protein is used to indicate that access to the Schiff base is sealed, while a V-shaped indentation is used to indicate that this access is open. Since the extracellular side of the protein is relatively hydrophilic, it is shown as being open throughout the photocycle. Key residues are indicated by the following code: 85 and 96 represent those particular residues in the primary structure; SB and SBH represent the unprotonated and protonated Schiff base, respectively; PRG represents the as-yet unidentified 'proton release group'; and appropriate superscripts are used to indicate the charged state of these key residues. The well-known K and L intermediates have been left out intentionally. The primary emphasis in this cartoon is the role of successive substates of the M intermediate. (A) The mechanistically important steps that might be taken if wt bR functions as a hydroxyl ion pump; explanation for the details is given in the text. (B) Illustration of how the same steps would cause the protein to function as a chloride ion pump, provided that the substrate specificity of the active site were changed from hydroxyl ion to chloride ion by mutating residue 85 from aspartate to threonine or serine. The Schiff base does not become deprotonated during the M-like stage of the chloride-ion pumping cycle, a fact that we have indicated by the use of quotation marks.
←

waters were seen in greater detail in a 1.55 Å-resolution map of the resting state [90].

Photoisomerization of the all-*trans* retinal group must result in small displacements of the Schiff-base nitrogen atom and its accompanying water molecule (wild-type bR) or chloride counterion (D85T/S mutant). Indeed, small movements of the Schiff-base nitrogen, W402, and the sidechain of Asp85 were detected in the K intermediate by Edman et al. in their atomic model of that photoproduct [91]. These displacements are presumably still be present in the L intermediate. Furthermore, the FTIR studies of Maeda et al. [92,93] are consistent with a water molecule lying near the Schiff base and Asp85 that changes its hydrogen bonding patterns upon the formation of L, and again upon formation of the M intermediate. At some point, however, the changes in molecular distances and environment would allow transfer of the Schiff base proton to the ionized carboxylic acid group of D85 in wild-type bR, thus producing the first M intermediate, M_L. An equivalent transfer of the Schiff base proton to the chloride ion cannot occur, however, because Cl^- ion is an extremely weak base. In this case the protonated Schiff base and the chloride ion would remain a salt, similar to that formed by NH_4Cl.

The water molecule (in wild-type bR) or the chloride ion (in the mutants) is still imagined to be on the

extracellular side of the Schiff base at this point. However, later in the M state (or M-like state for the mutant), both must find their way to the cytoplasmic side of the Schiff base. It is also required that a proton would dissociate at this point from the proton release group, thereby returning the proton to the extracellular side of the membrane. This deprotonation event presumably plays a functional role in assisting the larger changes in protein conformation that must be produced in order for water or ion translocation to occur. Even after translocation has occurred, however, the water molecule (or chloride ion) is still retained deep within the protein, having moved to a location where it is no longer directly accessible to the extracellular side of the membrane. Since the environment on the cytoplasmic side of the Schiff base is hydrophobic, this water molecule might have no defined position and could thus be disordered in the electron density map. The absence of water 402 in the late M structure of Luecke et al. [80] could be explained by such a loss of order. A more electron-dense halide ion might still be visible, however, although it too would lack specific binding partners (apart from the protonated Schiff base) at this stage of the photocycle.

Later in the M state, there must be a further structural change, characterized by appearance of the amide I band at 1670 cm^{-1}, and by a further tilting movement of the cytoplasmic side of helix F. The result is the formation of the M_N species, which may be only a transition state in well-hydrated wild-type bR, although it is a metastable intermediate in D96 mutants at higher pH values and in glucose-embedded wild-type bR at a temperature of about −43°C. Increased solvation of the carboxylic acid side chain of D96, made possible by the movement of helix F, is proposed to stabilize the ionized form of the side chain of D96. Dissociation of the carboxyl group thus results in transfer of a proton to the Schiff base on wild-type bR, whereas the proton would be released to the cytoplasmic side of the membrane in the D85 mutant proteins. Reprotonation of the Schiff is the defining step for creation of the N intermediate in wild-type bR, but there is no corresponding event in the chloride pump.

The chloride ion in the D85 mutant proteins is free at this point to escape to the cytoplasmic side of the membrane. Helix F must then tilt back to its original position, resealing the cytoplasmic half of the pump. However, the hydrophobic channel is not likely to close as long as D96 remains ionized. Thus it is proposed that the carboxyl group of D96 abstracts a proton from a water molecule, as is shown in Fig. 4. In the case of wild-type bR the result is the release of a hydroxyl ion to the cytoplasmic side of the membrane, while in the D85 mutants the result can be understood as uptake of a Bohr-like proton that had been released earlier to the cytoplasmic side of the membrane.

The model outlined in Fig. 4 is unlikely to be the only one that can reconcile the chloride-ion pumping activity of the mutants (and of the homologous protein, halorhodopsin) with the ability of wild-type bR to generate a proton-motive force. Nevertheless, it is hoped that formal presentation of this still-crude scheme will stimulate new experimental work that will ultimately lead to a better understanding of the molecular biophysics of how this protein functions.

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