Information and Energy Transduction in Biological Membranes,
pages 113–131

An Isomerization Model for the Pump Cycle of Bacteriorhodopsin

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INTRODUCTION

The primary form of energy driving the metabolism of most biological species is a chemiosmotic potential arising from a proton gradient across certain biological membranes [1]. Founded on this chemiosmotic potential is a versatile energy economy consisting of three kinds of processes: (1) the passive transport of protons by membrane proteins [2–4], (2) the active transport of protons to generate the chemiosmotic potential [5,6], and (3) the transformation of potential-driven proton currents into osmotic [7,8], biochemical [9], and mechanical [10] energy.

Now that the fundamental role of proton transport in bioenergetics has been revealed, attention has begun to focus on the molecular mechanisms underlying proton transport. In the quest for understanding, one is well advised to concentrate on the least complex systems, and in this respect, the membrane protein bacteriorhodopsin, which acts as a light-driven proton pump in the outer membrane of Halobacterium halobium [6], serves an important role. Bacteriorhodopsin consists of 7 α-helices that span the membrane, and its primary structure is known [11]. Bacteriorhodopsin combines the function of a passive [3] and an active [6] proton transport system. Its chromophore, a Schiff base of retinal that is buried inside the protein about 15 Å from the outer surface, is generally held to be the site of the proton pump. In this paper we consider a molecular model for the active transport in this protein.

The model to be presented has been developed on the basis of quantum-chemical calculations that reveal a strong coupling between the stereochemistry and the acid-base properties of the Schiff base of retinal [12–14] (see Fig. 1). The reasons for assuming the direct involvement of retinal in the vectorial transfer of protons are the following:

1) The pump cycle of bacteriorhodopsin is accompanied by strong spectral shifts of retinal [15]; these shifts show kinetic isotope effects upon deuteration [16].

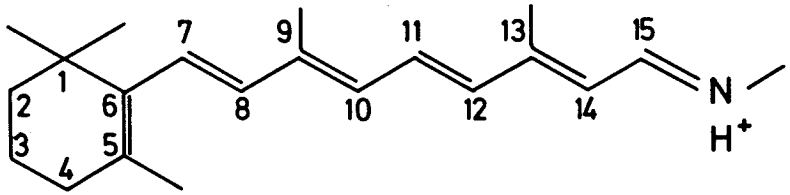

Fig. 1. The Schiff base of retinal. The chromophore is attached through the N-terminal to the protein.

2) During the pump cycle the terminal nitrogen of the retinal Schiff base, ie, the ϵ-amino group of a lysine residue, undergoes a cyclic protonation-deprotonation process [17,18]; a fast exchange of the Schiff base proton in light has been observed [17].
3) The active proton transport in bacteriorhodopsin depends on the formation of the blue, unprotonated intermediate M_{410} absorbing at 410 nm; eg, there is no transport in the photochemical cycles of $bR^{13\text{-cis}}$ (containing 13-cis rather than all-trans retinal) [19] nor at low pH, starting either from the bR_{610} or bR_{565} acidic forms of bacteriorhodopsin [20,21].
4) The observed photochemical interconversion of the intermediates in the pump cycle [eg, 19] indicates that retinal must control all stages of the cycle; shining blue light on the pumping bacteriorhodopsin reduces the pump efficiency [22].
5) Isomerization of retinal around its 13–14 double bond, a process which may enable a vectorial proton transport [see 12,13,15], has been observed [23,24].
6) Circular-dichroism spectral changes detected during the later stages of the pump cycle [25] indicate a stereochemical participation of retinal.

If one accepts retinal's direct participation in the proton transfer, then the following question immediately arises: How can retinal, at its binding site in bacteriorhodopsin under the action of light, perform as a gate, passing protons in one direction and preventing their backflow? In this article we would like to suggest an answer to this question. Our starting point is the isomerization model briefly presented in [12]. Using our recent quantum mechanical calculations [14,33] of the internal energies of retinal and making certain assumptions regarding the interaction between retinal and bacteriorhodopsin, we will show how the stereochemical changes in retinal can explain the above observations. These calculations also suggested conditions whereupon two protons instead of one could be transported during the pump cycle. Employing in addition the rate constants taken from the observed

kinetics of bacteriorhodopsin, we also established the thermodynamic feasibility of the resulting kinetic scheme according to an algorithm developed by Hill [26].

Before proceeding we would like to comment on the obvious objection that a theoretical approach to the minute details of bacteriorhodopsin may have to await further experimental observation or may be altogether useless, as only experiments can tell how the system works. Our answer is that the active and passive proton transport quite likely involves very minute motions of the protein skeleton and side groups that occur below the resolution limits of available methods of detection. An understanding of the mechanism will have to depend, then, on a matching of observations on one side and predictions of gross features of microscopic models on the other side. However, before any model can be presented as the solution to the puzzle of the proton pump in bacteriorhodopsin, a test of its thermodynamic feasibility must be applied, as we have provided here for our own model.

ACID-BASE CATALYSIS OF RETINAL SCHIFF BASE ISOMERIZATION

Retinal, the pigment of bacteriorhodopsin, belongs to the family of polyene dyes. The polyenes are well known for their characteristic structure of alternating single and double bonds. Double bonds derive their name from the property that ground-state bond rotation is prevented by very high barriers of activation, typically of about $E_a \approx 50$ kcal mol^{-1}. Single bonds, however, exhibit only very low barriers of rotation of about $E_a \approx 5$ kcal mol^{-1}. Single bond rotation proceeds readily within less than a nanosecond, whereas thermal double bond rotations are essentially unobservable. The coexistence of such different bond behaviour in linear conjugated π-electron systems appears quite remarkable. A perturbation of the π-system can, however, modify this bond pattern. In rhodopsin and bacteriorhodopsin such a perturbation is induced by the protonation of the Schiff base nitrogen of the pigment [27]. The effect of this protonation is most clearly witnessed by the strong bathochromic shift of the absorption maximum from about 380 nm for the unprotonated Schiff base [28] to values ranging between 430 nm [29] and 610 nm [30] for the protonated biological chromophore. This shift can be explained by arguing that the positive charge of the added proton attracts an electron out of the delocalized π-system towards the terminal nitrogen, thereby transforming the polyene moiety of retinal into a polyenylic cation [28,31]. Such cations exhibit, in fact, absorption bands strongly red-shifted with respect to those of the parent polyenes, eg, a red-shift of over 200 nm for a polyene isoelectronic with the conjugated π-system of retinal [32].

However, the transformation of a polyene into a polyenylic ion not only results in a lowering of the optically allowed excited state but also in a weakening of the ground-state bond alternation [31]. This change in the stereochemical properties of retinal is demonstrated in Table I, which presents the ground-state isomerization barriers of the Schiff base of retinal in its unprotonated and protonated forms. The calculations show that the unprotonated Schiff base, very much like a typical polyene, has an isomerization barrier of 46.8 kcal mol^{-1} for the 13–14 double bond and of 1.4 kcal mol^{-1} for the 14–15 single bond, whereas protonation alters the barriers to 11.5 kcal mol^{-1} for the "double bond" and 13 kcal mol^{-1} for the "single bond." The increase of the latter would slow down an all-trans $\rightleftarrows$ 14s-cis isomerization by about eight orders of magnitude. The complementary lowering of the double bond barrier would allow a thermal all-trans $\rightleftarrows$ 13-cis isomerization to proceed in a time as short as a few milliseconds. (For the numbering of the bonds see Fig. 1).

A thermal all-trans $\rightleftarrows$ 13-cis isomerization is indeed being observed in bacteriorhodopsin [35,24]. Such isomerization occurs during dark adaptation within about 40 min at pH = 7 and within about 1 min at pH = 4 [36]. Apparently, this isomerization is also part of the millisecond pump cycle of bacteriorhodopsin [24]. In our model for the pump mechanism presented below the "acid" catalysis of this isomerization by protonation of retinal and the complementary "base" catalysis of the single bond isomerization by the deprotonation of retinal serve a most essential role in retinal's function as a proton gate. We will argue in the next section that these two thermal isomerization processes are induced by a primary photochemical isomerization process of the pigment involving a simultaneous torsion about the 13–14 and 14–15 bonds.

Before proceeding we will illustrate for the case of the single bond isomerization that the strong dependence of the barrier height on the protonation state may give rise to stereochemical pK shifts of the Schiff base of retinal. Figure 2a presents a schematic diagram of the two potential curves governing the torsion around the 14–15 single bond of the Schiff base of retinal. In the unprotonated state there is a very small barrier of activation; in the protonated state the barrier amounts to about 13 kcal mol^{-1}. The diagram also reflects the assumption that the *planar* compound is a weak base relative to some (unspecified) proton donor; ie the protonated state lies below the unprotonated state. Figure 2b presents the potential owing to a possible external strain exerted on retinal, for example, by the apoprotein bacterioopsin. Figure 2c combines the potentials of Figure 2a,b to demonstrate that in the strained configuration the retinal Schiff base has been

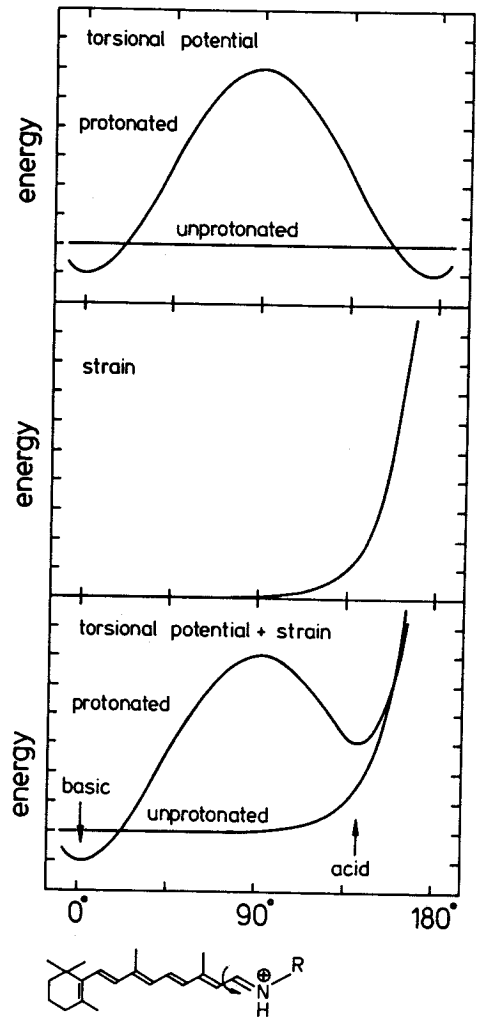

Fig. 2. Schematic representation of the torsionally induced acidification of Schiff base retinal.

converted to an acid, as in the corresponding equilibrium configuration (right-hand potential minimum) the protonated state comes to lie energetically above the unprotonated state. It can be estimated that the single bond torsion can induce a pK decrease of several units. Conversely, a torsion around a double bond can increase the pK value of the terminal nitrogen.

MODEL OF THE PRIMARY REACTION

The model for the mode of action of bacteriorhodopsin considered here is based on the cyclic isomerization of retinal: In a primary reaction retinal photoisomerizes upon illumination and reaches a "high"-energy configuration, which is to be identified with the K_{590} and L_{550} intermediates of the pump cycle. In a secondary "dark" reaction, the product of the photoisomerization is reversed thermally. It is during this stage that protons are actually being pumped by the coupling of the stereochemistry and the acid-base properties of the pigment as well as by the conformational dynamics of the protein. However, as the secondary reaction must involve the reversal of the primary reaction, a basic step in resolving the mechanism of bacteriorhodopsin lies in the nature of the primary photoprocess. In this section we suggest a stereochemical reaction path for the primary event.

The binding site of retinal in bacteriorhodopsin appears to be a tight cavity that permits only very limited motions of retinal's polyene moiety. This can be inferred from attempts to reconstitute the apoprotein with different retinal isomers [for a review, see 37]. These experiments demonstrated that only the all-trans and the 13-cis isomeric forms reconstitute the red-absorbing chromophore: other isomers—eg, 9-cis, 11-cis—do not reconstitute a functioning chromophore. From these observations we infer that retinal's freedom of isomerization is limited to rotations around its three terminal bonds, $C_{13}=C_{14}$, $C_{14}-C_{15}$ and the $C_{15}=N$ bonds. Rotations around the latter two bonds are likely to be sterically allowed, since the corresponding cis-isomers require less space than the 13-cis isomer. Unfortunately, the $C_{14}-C_{15}$ and $C_{15}=N$ rotations cannot be readily detected as a biochemical analysis [24] of the pigment cleaves the C=N bond in a stereochemically unspecific way and is also unlikely to fix the orientation of the C−C single bond. An exception, as far as the stereospecificity of the $C_{15}=N$ bond is concerned, is the reaction of the chromophore with hydroxylamin to form either syn- or anti-retinal oxims [35].

The question posed is, which of the rotations around the three terminal bonds is energetically feasible in the first excited singlet state of the protonated Schiff base of retinal. For an answer to this question one has to note that

the primary photoprocess proceeds very rapidly, within about 10ps. This time, however, is too short for any barrier of activation to be overcome and, therefore, the energetic feasibility implies that the photoreaction must be downhill on the S_1 potential surface along the path of the photochemical reaction.

The photobehaviour of an analogue of the chromophore of bacteriorhodopsin, a protonated Schiff base polyene with ten conjugated π-electrons has been studied in [14]. The excited-state energy difference between the planar all-trans configuration and the configuration with bonds twisted by 90° was determined by means of a quantum chemical MINDO4-CI calculation. These calculations showed that two photoisomerization routes are energetically open, the all-trans → 13-cis and the all-trans → 13-cis, 14s-cis routes. We suggest that the excited pigment ought to prefer the second route involving a concerted rotation about the adjacent 13-14 and 14-15 bonds. The reason is that such a rotation involves less motion of the molecular framework than a single 90° rotation about the 13-14 bond. In fact, as the hydrogen at C_{14} is moved out of the $C_{13}-C_{14}-C_{15}$ plane by the concerted rotation, the C_{14} electrons partially rehybridize from sp^2 to sp^3. The resulting shape of the twisted retinal conformer presented in Figure 3 is very similar to that of the all-trans chromophore. The difference mainly entails a motion of the C_{14} hydrogen and a slight downwards motion of the $C_{15}=N$ moiety. It appears then that even if the forces to induce a 13-14 bond photoisomerization are stronger than those governing the 13-14, 14-15 bond photoisomerization[14], the latter process involves up to the transition point (bonds twisted by 90° and C_{14} atom rehybridized) a motion of smaller masses and is less likely to collide with the protein cavity.

The behaviour beyond the transition point is illustrated in Figures 4 and 5. Figure 4 shows that the completion of the all-trans → 13-cis, 14s-cis photoisomerization beyond the transition point to reach the intermediates K,L involves a larg e-
sents a schematic view of the ground (S_0) and excited (S_1) state potential curves governing this photoprocess. We assume that the photoreaction near the transition point where the S_0 and S_1 potential surfaces come closest to each other follows a typical nonadiabatic route to the ground state. After crossing to the ground state the chromophore experiences strong restoring forces to either the all-trans or to the 13-cis, 14s-cis conformation. It is at this state of the photoprocess that a fraction of the light energy is stored by means of sterical and electrical interactions with the protein surroundings. Indeed, recent resonance Raman measurements have revealed that the protein holds the chromophore intermediate K in a distorted conformation after the trans → cis isomerization [41].

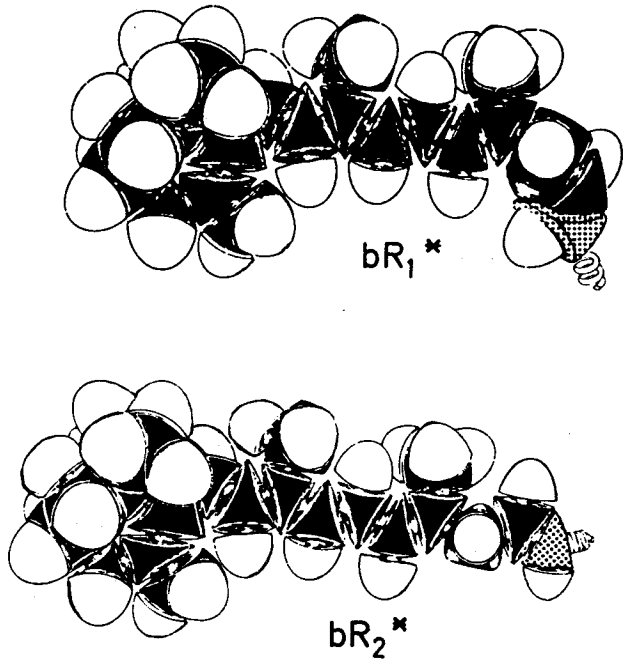

Fig. 3. Geometries of the transition state of the all-trans → 13-cis, 14s-cis photoisomerization of retinal assuming sp^2 orbitals at C_{14} (top) and assuming rehybridization to sp^3 orbitals at C_{14} (bottom).

The sum total of the "useful" work provided by the chromophore appears in the energy difference of Δ kcal/mol between the planar and isomerized conformers designated bR and K in Figure 5. It is this energy that drives the bacteriorhodopsin back to the initial conformation. However, this back-reaction cannot proceed directly along the photochemical reaction path, since along this path a very high ground-state activation barrier of $(35.2-\Delta)$ kcal mol^{-1} would have to be overcome. Rather, the system chooses to reisomerize along a different route by sequential single bond (14-15) and double bond (13-14) isomerizations that have, in the appropriate state of protonation, very low activation barriers (see Table I). The low barrier of 11.5 kcal mol^{-1} predicted for the rotation around the 13-14 bond of the protonated chromophore establishes a strong argument against an all-trans → 13-cis photoisomerization as the primary step in the pump cycle. Such a low barrier could not store a free-energy increment of $\Delta \approx 15$ kcal mol^{-1} attributed to the K

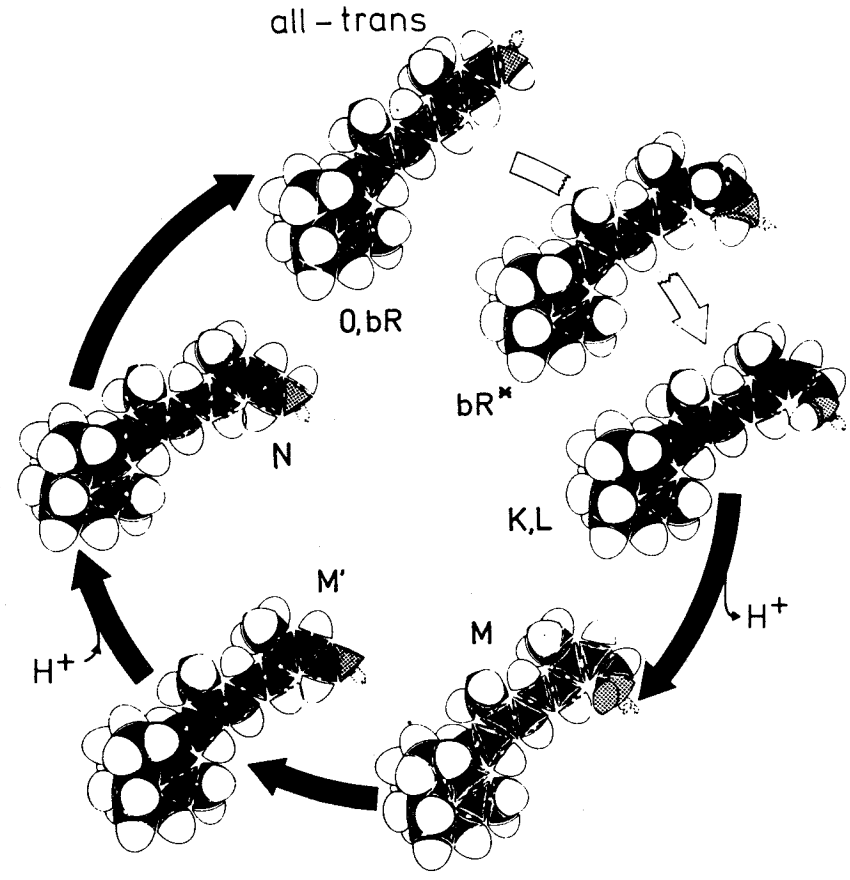

Fig. 4. Molecular model for the chromophore during the pump cycle of bacteriorhodopsin.

intermediate [Birge and Cooper, 1983], but rather it would allow leakage of such a high-energy intermediate state back to bR.

The energy that the excited chromophore stored initially can also be retrieved upon light excitation of the K intermediate. Upon excitation to S_1 the energy stored should drive the chromophore to the transition region, as indicated in Figure 5. One would expect then that the back-photoreaction should proceed even more rapidly than the forward photoreaction. This has been demonstrated indeed by picosecond spectroscopy [38]. At very low temperatures a step in the downhill process to the K intermediate may be frozen in. In such a situation which involves considerable sterical interactions

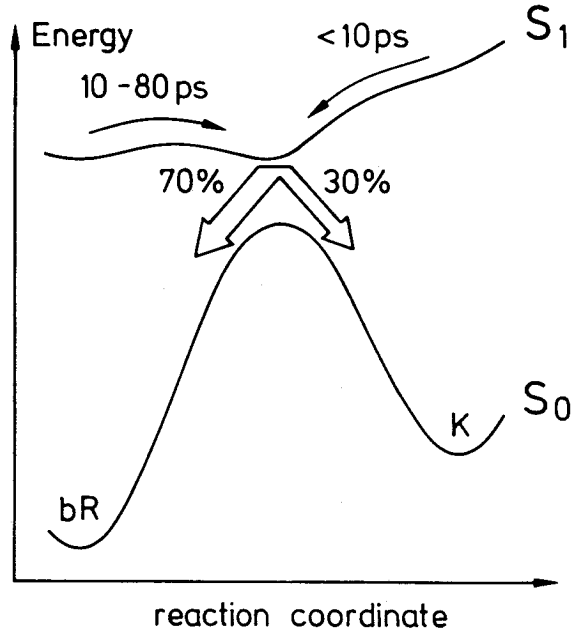

Fig. 5. Schematic potential along the reaction route of the all-trans → 13-cis, 14s-cis isomerization in the ground (S_0) and in the excited state (S_1).

with the protein, one may be able to observe an intermediate with a chromophore conformation close to that of bR_2^* in Figure 3.

NATURE OF THE RESTORING FORCES

A key question regarding the light-driven proton pump concerns the nature of the restoring forces that drive the K_{590} intermediate back to bR_{568}. These forces determine the reaction pathway and, thereby, the energy transformation. The restoring forces are determined by the free energy stored in the K_{590} intermediate $\Delta G = \Delta H - T\Delta S$ in that they are measuring the gradient of ΔG along the reaction path. ΔG entails an enthalpy contribution ΔH and an entropy contribution $-T\Delta S$. We suggest that ΔG contains different forms of energy which are released at different stages of the photocycle: Reaction processes are driven mainly by *sterical interactions at short times* (~ 1 ns), by *electrostatic interactions at medium times* (~ 1 μs) and by *entropic interactions at long times* (~ 1 ms).

TABLE I. Isomerization Barriers[a] E for the Rotations Ri, i = 9,...15 around the i—i + 1 Bonds of the Schiff Base of Retinal (See Fig. 1 for the numbering of the carbon atoms)[b]

=NX	Ri	E (90)[c]	E (t-c)[d]
Unprotonated			
NH	R13	46.8	0.2
	R14	1.4	0.0
Protonated			
NH_2^+	R9	25.6	1.5
	R11	19.1	−3.7
	R12	7.6	0.5
	R13	11.5	1.0
	R14	13.0	−3.0
$NH^+(CH_3)$	R15	17.2	−1.5
NH_2^+	R13 + R14	35.2	−3.7
$NH^+(CH_3)$	R13 + R15	19.5	−0.5

[a]Results from MNDOC/BWEN calculations [see 34]. Details of the quartine-chemical calculations will be published elsewhere [33].
[b]The molecule is characterized by the residue =NX at the N-terminal. All energies are given in kcal mol^{-1}.
[c]Barrier height, i.e. energy of the 90°C conformation relative to the lower of the two isomers.
[d]E (all-trans)-E (i-cis).

The origin of this suggested hierarchy of interactions lies in the differences in the potential slopes connected with the different forms of energy. Sterical interactions exhibit rather steep potential surfaces and induce fast local relaxation (reaction) processes. Electrostatic potential surfaces are comparatively shallow and the corresponding weak forces can be sustained by the protein for a longer time. This important feature of the electrostatic interactions has been pointed out by Warshel [39]. Finally, entropic interactions that accompany the refolding of a protein or originate from restricted mobilities of the motion of protein side groups imply by their very nature flat surfaces, the increment of the free energy being solely due to an increase of disorder of the protein and its aqueous environment. The corresponding relaxation involves chance processes and, therefore, should be rather slow.

Added to these forces is a corrugated structure of the multidimensional potential surface, which is due to the large number of degrees of freedom in the protein. As a result, even processes that are downhill in free energy on an overall scale can occur only upon thermal activation. Furthermore, the partitioning of the free energy between the three forms of energy—sterical, electrostatic, and entropic—is likely to change during the pump cycle. It appears very likely that all three forms of free energy play an important role

for the pump cycle of bacteriorhodopsin. The initial photoisomerization step occurring in a tight protein cavity can leave the chromophore in a sterically strained configuration, the energy being dissipated and partially transformed during the K_{590} and L_{550} stages of the photocycle. Also, as argued above, isomerization of the Schiff base of retinal is coupled to pK changes of the terminal nitrogen such that sterical relaxation can play a role in the proton translocation at later stages in the cycle [12–14] as well. The observation of a cis → trans isomerization after the M_{410} stage is also very indicative of such a role.

Electrostatic forces are particularly important for the translocation of protons during the pump cycle. The occurrence of charge relocation is witnessed by the spectral shifts of the pigment, as can be argued on the basis of the known spectral behaviour of retinal [see, for example, 27,28]. The rearrangement of charges can have a profound effect on the pKs of the amino acid side groups and of the Schiff base of retinal and can assist in steering the stereochemistry of retinal, since isomerization barriers depend sensitively on the charge environment of the chromophore [33].

A transient decrease of entropy at the ms stage of the pump cycle has been demonstrated by Ort and Parson [40]. This decrease may originate from structural transitions of bacteriorhodopsin, which could be particularly suited to the passive conduction of protons (see below). The structural transition could possibly involve a refolding of bacteriorhodopsin such that water enters the protein to constitute an aqueous pore (the water making contact with previously unexposed hydrophobic side groups becomes "structured" and, therefore, decreases the entropy content). However, it appears more likely that bacteriorhodopsin, rather than unfolding, partially tightens its structure by forming hydrogen bridges, a process that confines the mobility of the side groups and thereby decreases the entropy. The hydrogen bridges could be connected to form a chain that can conduct protons [4]. Assuming the ratio of the partition functions of a freely rotating side group and a hydrogen-bridged side group to be 100 (this value would be possible only if more than one degree of rotation would be hindered), one can estimate that nine hydrogen bridges have to be formed to explain the entropy change observed in [40].

THERMODYNAMIC SCHEME FOR A ONE-PROTON PUMP

In this section we analyze the efficiency of the molecular model of the proton pump cycle presented in Figure 4. We want to demonstrate that the barrier for the single bond isomerization, which depends on the protonation

state of the Schiff base, determines the efficiency of the pump. The pump mechanism considered here transfers only one proton during one cycle.

The complete set of rate constants and free energies employed in our analysis is provided in Figure 6. The data appearing in the figure were derived from the model in Figure 4 on the basis of the following assumptions:

1) The primary photochemical step bR → L occurs under a constant light level.

2) There are three contributions to the free energies in Figure 6: (A) The restoring forces of the protein provide 10 kcal mol^{-1} to both the L and the M intermediates which are in a 13-cis, 14s-cis conformation, and 7 kcal mol^{-1} to both the M′ and the N intermediates in the 13-cis conformation. (B) Sterical interactions of the retinal moiety alone according to the results of Orlandi and Schulten [14] contribute 0 (def.), 5, 2.2, 0.7, and 1 kcal mol^{-1} to the intermediates bR, L, M, M′, and N, respectively. (C) An energy release G_1 (G_2) is connected with the proton transfer between the chromophore and an adjacent group $X_1(X_2)$ in the L → M (N → M′) transition. The values assumed are $G_1 = G_2 = 0$ (case I) and $G_1 = 0$, $G_2 = -3$ kcal mol^{-1} (case II). Case II implies that the 14s-cis → 14s-trans isomerization of the chromophore in the M → M′ transition is accompanied by a charge redistribution in the protein which yields the Schiff base nitrogen more basic with a pK shift of about unity. The thermodynamic data corresponding to case II are provided in brackets.

The rate constants obey detailed balance, except that the rates L → M (M → L) are proportional to f_1 $(1 - f_1)$, where f_1 measures the fraction of X_1 groups that are unprotonated. Similarly, the rates N → M′ (M′ → N) were assumed proportional to f_2 (ie $1 - f_2$) where f_2 is the fraction of X_2 groups unprotonated. For the protonation state of the groups X_i we assumed very fast equilibrium with the external proton concentrations such that $f_i = 1/(1 + 10^{-pH_i+pK_i})$ where $pK_1 = 3$, $pK_2 = 10$, and pH_1,pH_2 are the pH values of the extracellular and cytoplasmic spaces.

3) For the reaction rates L → N and N → L an Arrhenius temperature dependence was assumed with activation energy E_a. The rate constants corresponding to $E_a = 7$ kcal mol^{-1} are given in Figure 6. In the following analysis E_a was varied in order to demonstrate its influence on the pump efficiency.

4) The frequency factors of the rate constants were then chosen such that the forward rates at pH = 7 on both sides of the membrane agreed with the observations on the kinetics of bacteriorhodopsin.

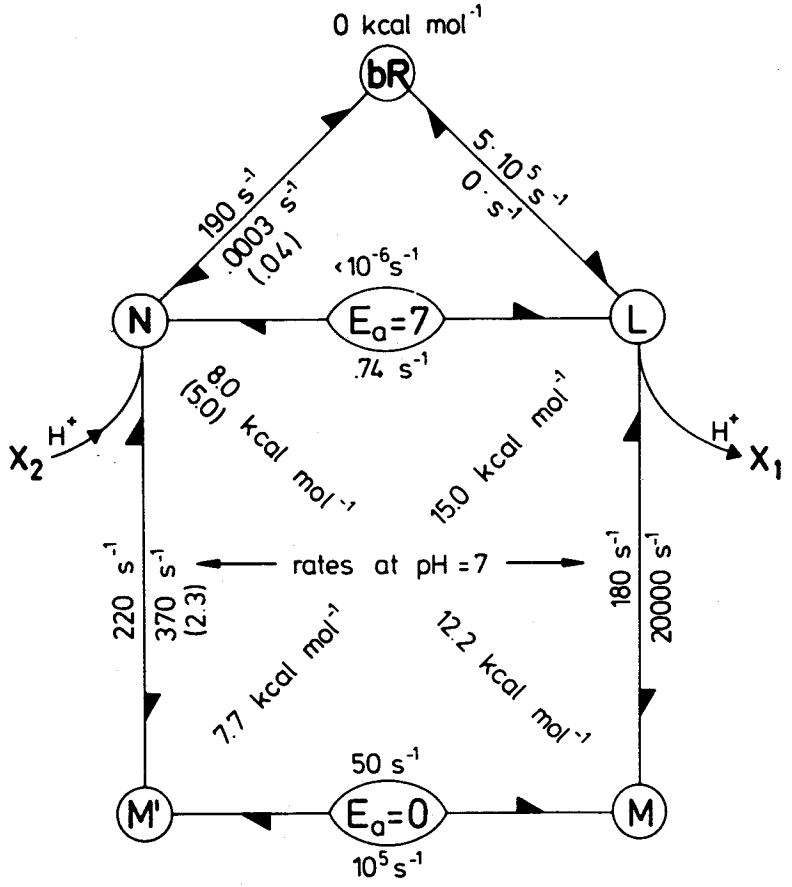

Fig. 6. Kinetic diagram corresponding to the molecular model of Figure 4 presenting free energy values and rate constants.

Figure 7 presents the pump yield resulting from Figure 6 for case I (top) and case II (bottom). The calculations of the yield followed Hill's diagrammatic algorithm [26]. The yield was determined for the situation that there was no load on the pump; ie, the proton gradient across the membrane vanishes, as well as for the situation with a load corresponding to a pH gradient of 4 units. Since no provision has been made for an electric field dependence of the rate constants, we cannot consider an effect of an electrical field gradient. However, an appropriate electrical field dependence could be incorporated through the functions f_i. The results in Figure 7 demonstrate

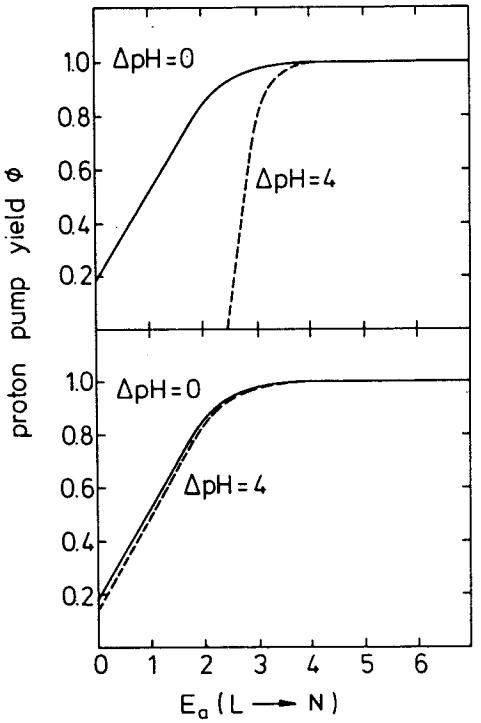

Fig. 7. Calculated yield of protons pumped for each transition bR → L in the kinetic diagram in Figure 6.

that the activation energy E_a for the L → N transition determines the pump yield, a large barrier forces the cycle to pass through the deprotonated intermediates M and M′ and, thereby, to transfer a proton through X_1 to the extracellular space and to receive a proton through X_2 from the cytoplasmic side. Since the activation energy for the M → M′ transition has been set to zero as a reference, the E_a values in Figure 7 have to be interpreted as the *increase* of the 14-15 single bond rotational barrier upon protonation of the Schiff base nitrogen. Hence, the results in Figure 7 demonstrate that such an increase, which according to Table 1 may measure about 12 kcal/mol, may be the key element of the mechanism of the pump cycle of bR. The mechanism of the chloride ion pump Halorhodopsin of Halobacterium can actually be explained by a similar argument [42].

TWO-PROTON PUMP

In the model of Figures 4 and 6 the chromophore acts like a switch that moves upon the 14s-cis → 14s-trans isomerization between groups X_1 and X_2, which are in contact with the extracellular and the cytoplasmic side, respectively. The contact is meant to imply passive proton transport on the time scale of the pump cycle. The mechanism in Figure 4 can be extended to allow the transfer of a second proton if one envokes a third side group, X_3, which as a result of the chromophore-induced protein conformational changes alternates between contacts with the extracellular and cytoplasmic sides. The transfer would be more efficient if the motion of X_3 would be accompanied by a favourable pK shift. Actually, if the chromophore should be denied any direct participation in the pump action, there could be two groups like X_3 active in bacteriorhodopsin. However, the chromophore might be closely involved also in the pumping of the second proton by interaction with X_3 through its Schiff base nitrogen. A possible sequence of steps is the following: the group X_3 is responsible for the protonation of the Schiff base nitrogen of the all-trans chromophore bR and in this resting state of the protein is in contact with the extracellular side. Upon photoisomerization retinal leaves the unprotonated X_3 behind, the protein conformational change brings the latter in contact with the cytoplasmic side. Under appropriate conditions (eg, intermediate or low pH), X_3 receives a proton at this stage. The chromophore reprotonated through X_2 returns from the 13-cis intermediate N to the all-trans conformation, relaxing the protein to its resting state. The situation of the protonated all-trans chromophore with an adjacent protonated X_3 group, the latter now again in contact with the extracellular side, might be identified with the O intermediate [42,43]. The proton on X_3 is then forced into the extracellular space and the initial state bR is reached. In the case that the group X_3 remains unprotonated throughout the cycle; eg, at high pH, the O intermediate would be bypassed [as reported in 19] and only a single proton would be transported. In case X_3 is not protonated before the N intermediate of the pump cycle is reached, the suggested mechanism with X_3 could also account for the blue light effect [22]. An explanation would be a photochemical return of the *unprotonated* chromophore in the M or in the M′ conformation to the all-trans conformation and a retrieval of a proton through X_3 from the extracellular side, the side to which the first proton had been conducted through X_1 after the L → M reaction. We point out that the suggested model implies a linkage between the appearance of the O intermediate and the vectorial transport of a second proton in the pump cycle.

PASSIVE PROTON CONDUCTION

To bridge the width of the entire membrane, passive proton conduction has to complement the active transport in bacteriorhodopsin. It appears likely that the larger portion of bacteriorhodopsin is bridged by passive transport. Current suggestions about the mechanism of passive proton conduction involve the sole participation of amino acid side groups capable of forming hydrogen bridges [4]. The transport is based on two elementary processes, jumps of protons between the two minima of a hydrogen bridge potential between two groups, and translocation of protons through rotations of the side groups. These processes could occur in alternate succession, ie, jump, rotation, jump, rotation, etc. However, in case that several side groups form a stable chain of hydrogen bridges it is more likely that conduction occurs in two phases. A fast initial phase involving only jumps of protons between rotationally fixed side groups is terminated by a proton release at the chain end. Since in this phase the protons are not conducted yet across the participating side groups, only a fractional proton charge is moved along the conductor although a whole proton is released. The apparent contradiction is resolved by noting that at this stage the hydrogen bridge chain is polarized with its dipole moment shifted against the direction of the conducted proton. This polarization is discharged and the chain returned to its initial state by means of side group rotations in the slow phase of the conduction. The slow phase is then characterized by a melting of hydrogen bridges with a correspondingly high entropy content. This picture of the passive proton conduction appears to be in agreement with the observation of two phases of charge shifts in the forward direction of bacteriorhodopsin [20,44].

The rate of proton conductance is optimal if the participating amino acid side groups have similar pK values. In the case of heterogenous side groups the conduction will be slowed down. However, a suitable sequence of acidic and basic side groups can endow a conductor with diodic properties, ie, support conduction mainly in a single direction. This feature would be favourable for bacteriorhodopsin and, hence, may be realized in this protein [45].

OUTLOOK

The strong bathochromic spectral shifts of the Schiff base retinal during the various functional stages of the rhodopsins and of bacteriorhodopsin have been the subject of intense study for a long time. The key issue of this paper is that the shift in the absorption spectrum of the chromophore induced by protonation and other electrical interactions is accompanied by changes in

the ground-state stereochemical properties of retinal. Since the functional role of retinal in rhodopsin and bacteriorhodopsin develops only after the primary photoprocess through their ground state chemistry, our findings should be of importance for the development of any molecular model.

Our conclusions have been based on earlier MINDO4-CI quantum-chemical calculations involving only a fragment of retinal [14] and on recent results for the complete chromophore employing an improved MNDO method, as shown in Table I. Further unpublished results obtained so far strengthen the point made here. For example, it has been found that certain charge distributions around the protonated Schiff base of retinal can lower the 13-14 double bond rotational barrier to values as low as 6 kcal mol^{-1} and increase the 14-15 single bond rotational barrier to values as high as 20 kcal mol^{-1}. We also determined that the charge environment of the chromophore can actually steer the ground-state stereochemistry of retinal by lowering and increasing barriers for different bonds in selective ways. In conclusion we express our belief that the electrical interactions of the chromophore with its protein environment and the ensuing stereochemical properties will be the key to an understanding of the function of bacteriorhodopsin.

ACKNOWLEDGMENTS

We would like to thank D. Oesterhelt for his support and discussions concerning many aspects of this work. This work has been supported by the Deutsche Forschungsgemeinschaft (SFB-143/C1).

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