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Primary charge motions and light-energy transduction in bacteriorhodopsin

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The bacteriorhodopsin protein (bR) in the cell membrane of *Halobacterium halobium* is a light-driven proton pump. Many details are known about its structure and the molecular mechanism of proton translocation. The events may be characterized by: (1) the changes in light absorption after photon excitation (the photocycle); (2) the charge motion cycle inside the protein: the steps taken by the proton during translocation; (3) the retinal cycle: changes in isomerization and protonation; and (4) the opsin cycle: alterations of protonation of different amino acids in the apoprotein. From a review of existing data a more or less concise picture of the parallelism of the above four cycles emerges, which may be valuable as a model for understanding other types of molecular pumps.

1. Introduction

An important aim of present day research is to understand the molecular mechanism of the different steps in light-energy transduction. The simplest system is the bacteriorhodopsin protein (bR), which is known as a light-driven proton pump located in the cell membrane of *Halobacterium halobium* [1]. Absorption of light by bR generates an electrochemical potential gradient across the membrane, which is used for ATP production.

The amino acid sequence of bR was determined long ago [2,3]. Recently, the gene of bR has been isolated [4], modified genes have been expressed, and the functioning of genetically modified bR has been studied [5].

The three-dimensional structure of bR has been known since 1975 to approx. 6 Å resolution [6] and from 1986 in projection to 3.5 Å resolution [7]. The data show that bR spans the membrane in seven helices, has a long tail (17 amino acids) at the cytoplasmic side and a shorter (three amino acids) part at the external side. The retinal chromophore is linked by a protonated Schiff base to the Lys 216 residue and makes an angle of 70° with the membrane normal.

These data on the structure of bR are not sufficient for the elucidation of the proton translocation. Therefore, different physical, physicochemical and biochemical methods were applied in order to gain a deeper insight into the mechanism of the bR proton pump. This paper is an attempt to overview the results from light absorption (ultraviolet, visible. infrared), resonance Raman (RR) and solid-state NMR studies, and especially the endeavour of our laboratory to explore the charge motion inside bR. The aim is to construct an acceptable model which may serve as a summary of the previous ones and a starting point for those subsequently proposed.

2. The bR photocycle

2.1. Light absorption changes

The first characterization of the bR proton pump was described is the work of Lozier et al. [8]. They found that bR undergoes characteristic

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changes in the light absorption spectrum after absorbing a photon. The lifetimes showing the intermediates of different absorption spectra in the case of light-adapted bR cover a rather broad range. In fig. 1 the so-called photocycle is shown, based on the work of Lozier et al. [8], over the very short time range for the data in ref. 9, and regarding proton release and uptake from our data [10]. We call this photocycle a restricted photocycle because the intermediate N [8], different branchings [11,12], and the numerous M [13], P [14], R [15] and other intermediates which were already reported are omitted. We feel these restrictions to be necessary in order to reach a more or less concentrated picture.

It is clear that the absorption changes reflect important events in bR during proton translocation. One may look for the proton motion within bR, and changes in the isomeric and protonation states of the light-absorbing retinal and variations in the opsin part of bR. In this way it is possible to construct cycles of charge motion, retinal and opsin.

2.2. Charge motion cycle of bR

Nature made bR to translocate protons which surely have to cross the molecule. The possibility of recording the charge motion in bR arose when researchers succeeded in incorporating purple membranes (pms), containing bRs isolated from halobacteria, into model membrane systems in a particular orientation [16–19]. After a laser flash, a photocurrent and photovoltage were observed, indicating the electric events connected with proton translocation.

It has been shown that pms have a permanent electric dipole moment and by using it they can be oriented in a suspension by weak electric fields (15–20 V/cm [20]). The particular orientation may be immobilized in gels or by drying the sample [21–23]. Approx. 10^{14}–10^{15} bR molecules may be excited in oriented samples by a laser flash, and a macroscopic current is measurable [23]. Detailed analysis of the time and amplitude of the electric signals detected by simple Pt electrodes makes it possible to assign proton displacements in bR to different transitions in the photocycle [24]. This charge motion cycle is shown in fig. 2. The data

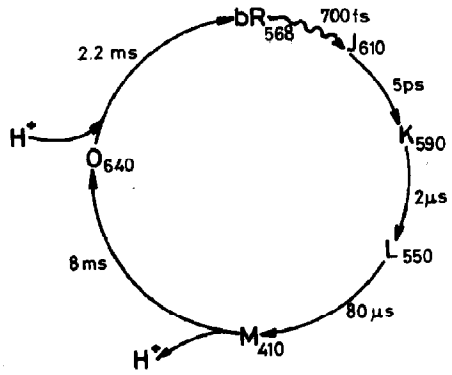

Fig. 1. The photocycle of bR (restricted). Lifetimes relate to room temperature.

on model membrane systems and in suspension are in rather good agreement.

The displacements may be grouped into (a) internal (bR-K, K-L and L-M), and (b) external (M-O and O-bR) transitions.

In the former group, the motions occur deep within the protein: in our experiments with bRs in different environments, with various modifications they always occurred unperturbed [25]. Motions in the latter group lead the protons out to the external and in from the internal compartment. These motions are greatly influenced by modifications [25].

The algebraic sum of the areas (A_i) of all the electric signals normalized to the first negative signal A_1 (corresponding to the bR-K transition considered proportional to the number of photocycles), i.e., $R = -\Sigma A_i/A_1$, is taken as the proton-pumping activity of bR. $R \sim 40$ for native bR at pH 6–7, deviations from this figure indicating variations in the number of translocated protons per photocycle.

2.3. The retinal cycle

The retinal in bR undergoes a cyclic change in isomerization and protonation. Laser Raman, Fourier transform infrared spectroscopy (FTIR) and solid-state NMR results are in agreement in showing that all-*trans*-retinal with protonated Schiff base is the ground state of the proton-pumping bR, the K-intermediate has a distorted 13-*cis* conformation, this distortion being already

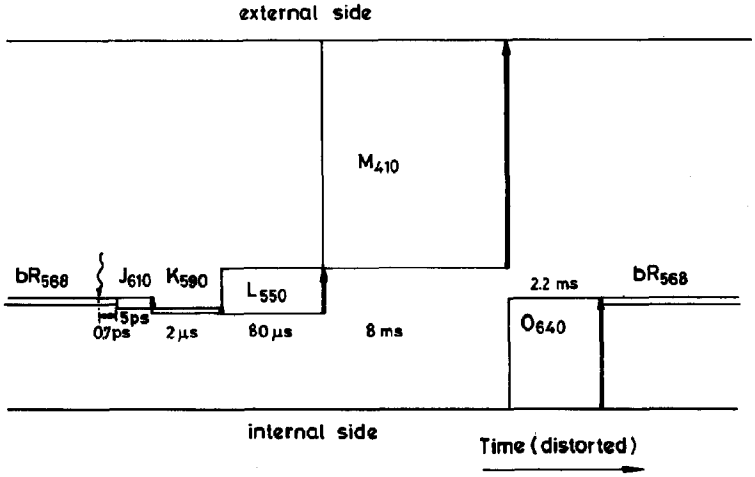

Fig. 2. Charge motion cycle of bR. The length of the arrows is proportional to the calculated displacement of a single proton.

lifted in the L-intermediate, the Schiff-base becomes deprotonated during the L-M transition but the retinal remains in the 13-*cis* state in the M-intermediate, and the retinal in the O-intermediate is already in the all-*trans* state with a protonated Schiff base [26–28]. The retinal cycle (for the restricted photocycle) is shown in fig. 3.

2.4. *The opsin cycle*

Ultraviolet absorption and FTIR data indicate that some amino acids undergo cyclic protonation changes. Protonation changes of tyrosines and carboxylic acids (aspartic acids) [29–33] have been reported. The data, however, are far from com-

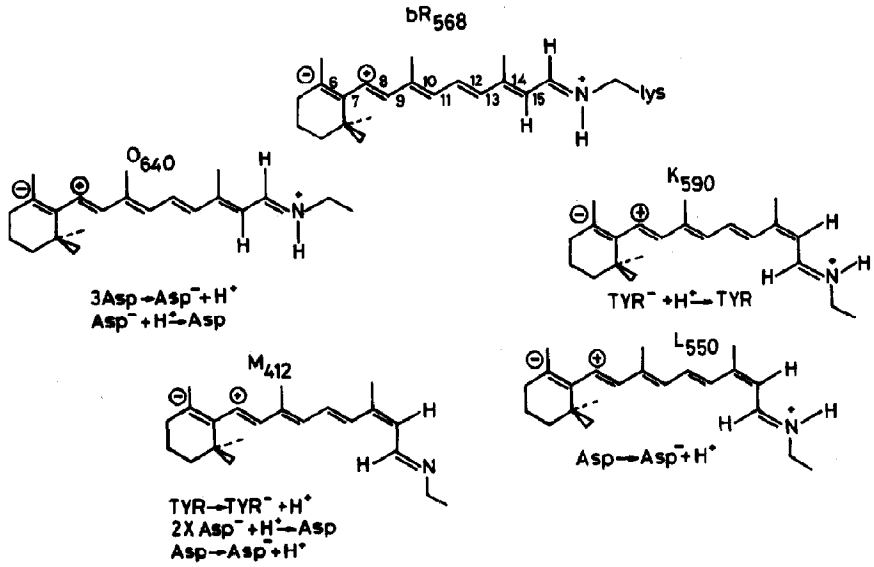

Fig. 3. The retinal cycle of bR. (Taken in modified form from ref. 26.) Protonation changes reported from FTIR and ultraviolet spectroscopy [29–33] are also indicated.

plete, and therefore it is not yet appropriate to construct an 'opsin cycle'.

In section 3 the characteristics of the different intermediates will be discussed and the events during the opsin part of bR photocycle will also be introduced. The recorded changes of protonation of the amino acids are included in fig. 3.

3. Characterization of ground and intermediate states of bR

3.1. Ground state

The ground state of bR in the dark contains a mixture of approx. 40% all-*trans*- and approx. 60% 13-*cis*-retinal (at 20 °C). The protonated Schiff base presumably forms a weakened H-bond with a neighbouring O^- of the carboxylic end of an amino acid residue [34]. NMR measurements also provide information showing that a positive and a negative charge are located near to the β-ionene ring of the retinal [35] (fig. 3). The data were used to interpret theoretically the absorption maximum ($\lambda = 568$ nm) of all-*trans*-bR [36]. According to this interpretation the difference in wavelength between a protonated Schiff base in solution and in bR (the so-called opsin shift $\Delta\lambda = 568 - 440 = 128$ nm) is due to (1) isomerization of the C_6–C_7 bond, (2) the negative and positive charges near to C_6 and C_7 and (3) the weakened hydrogen bond to a counterion A^-.

It is interesting to note that intensive dehydration of bR lowers the absorption maximum of bR to $\lambda = 530$ nm and a different photocycle characterized by absorption changes emerges [37]. Recently, Kovács and Váró [38] succeeded in recording the charge motion cycle of bR_{530} (fig. 4). The R value in this case was zero, demonstrating that charge motions inside the proteins do occur but that the system is unable to translocate protons. Moreover, it was shown that the proton does not escape from the Schiff base region.

This dehydration experiment and chemical experience prompted Sándorfy and Vocelle [39] to suggest that between the Schiff base proton and the counterion A^- a water molecule should be present, with some other water molecules being in the vicinity. There is a double potential well between the Schiff base and the ion A^- with unequal distribution of the proton. Resonance Raman and FTIR spectroscopy senses protons near to the Schiff base only. Further research is needed to decide between this and the previously proposed structure.

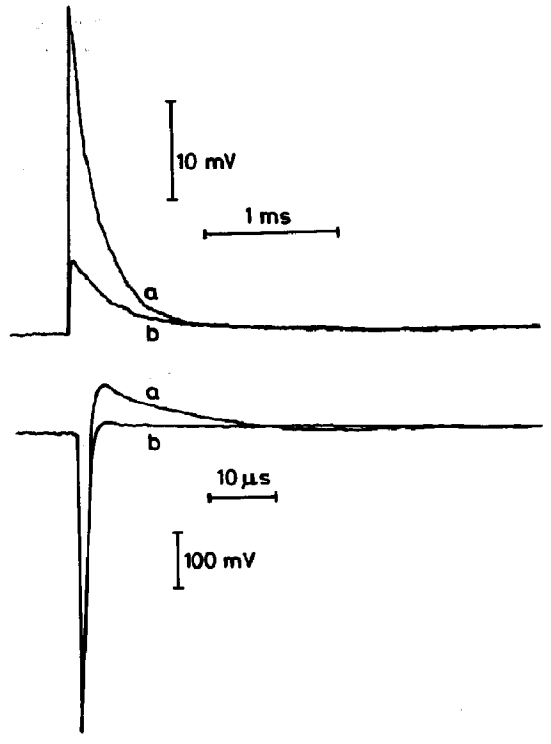

Fig. 4. Comparison of photoelectric signals of (a) dried oriented purple membranes ($p = 568$ nm) and (b) highly dried membranes (maximum 530 nm). The signals are shown in two different time and amplitude ranges. The area of the positive signal in (b) is 1/3 of that in (a) while the large negative components are the same.

60% of the all-*trans*-bR_{568} isomerizes slowly to 13-*cis*-bR_{548} in the dark. Resonance Raman data show that isomerization also occurs at the $C_{15} = N$ double bond which leaves the Schiff base proton in the same orientation as in all-*trans*-bR_{568}.

The charge motion cycle of bR_{548} was determined using our method. The electric signal begins with a fast negative phase, followed by two positives ones (fig. 5 [40]). The value of $R \approx 0$ demonstrates that 13-*cis*-bR_{548} does not pump protons, in accord with previous findings [1].

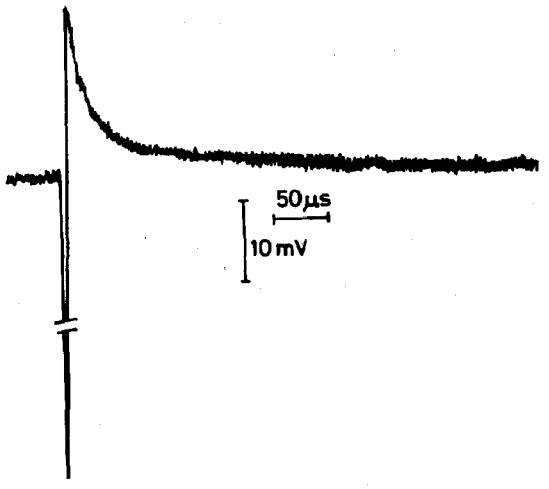

Fig. 5. Electric signal corresponding to the 13-*cis* cycle of bR. The sum of the areas (negative and positive) is zero showing that no proton translocation occurs.

3.2. The J-intermediate

Laser excitation drives the bR within 700 fs into an intermediate with an absorption maximum at $\lambda = 600$ nm [9]. This intermediate was observed only in optical spectroscopy, other data to shed light on its properties not existing.

3.3. The K-intermediate

The K-intermediate is formed from J with a lifetime of 5 ps and $\lambda_{max} = 580$ nm (at room temperature). The intermediate can be trapped at low temperature (below 100 K) and its characteristics can be studied employing different techniques. Resonance Raman and FTIR spectroscopy show that the terminal part of the retinal undergoes a 13-*cis* isomerization. This way the Schiff base proton enters into a protein environment which is different from that in the case of 13-*cis*-bR_{548} in which the C = N double bond is also isomerized [26].

There is disagreement concerning the assignment of the 14–15 C bond. In ref. 26 it is reported to be 14-s-*trans*, but in ref. 41, 14-s-*cis*. The different configurations are compared in fig. 6. The 14-s-*cis* assignment is supported by the theoretical calculation of Tavan and Schulten [42]. Other

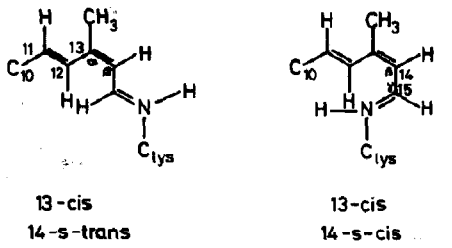

Fig. 6. Configuration of the terminal part of the retinal in the K-intermediate.

vibrations (like hydrogen out-of-plane vibrations) show that the isomerization in the protein packet is not complete: distortions remain in the retinal.

The frequency shift of λ_{max} is explained by considering the greater proton charge of the Schiff base, which is now further from the compensating counterion.

The K-intermediate is assumed to play a key role in energy conversion. By light-driven isomerization the Schiff base proton moves away from counterion A^- thus generating electrostatic energy. Calorimetric measurements show that 16 kcal/mol (35% of the incident photon energy) is conserved in the K-intermediate [43]. This is used later for proton translocation.

The first fast electric signal registered in our measurements corresponds to this charge displacement. From the data $d_1 = -0.13$ nm can be deduced (the negative sign shows that the shift is opposite to the direction of proton translocation). From the geometry of *trans-cis* isomerization (fig. 6) a transmembrane displacement of -0.16 nm can be calculated. The electric signal rises faster than 22 ps [44].

Electric signal measurements at low temperatures (< 100 K), where the K-state may be frozen in, show that after driving bR_{568} into the K-intermediate by means of a pulse of green light the charges can be quantitatively driven back by a red pulse absorbed by the K-intermediate [45].

Taking these data together with the structural data the charge motion related to the *trans-cis* isomerization can be understood. The problem is whether the isomerization and charge motion occur during the formation of J or K, or both, either in parallel or separated in time. The time resolu-

tion of the electric signal and measurements on the stabilized K-intermediate do not allow these questions to be resolved.

In connection with the isomerization it is important to note that in 13-*cis*-bR_{548} the first electric signal is similarly negative (fig. 5). Because isomerization as a cause is excluded we promote the idea of an H-bond potential with an asymmetric double well [39]. In the ground state the proton is nearer to the counterion and jumps across to the well near the Schiff base upon light excitation.

Good evidence exists that changes in the protein part of bR also occur during the bR-K transition. FTIR studies indicate the protonation of a tyrosinate [29].

3.4. The L-intermediate

The L-intermediate is formed with a lifetime of approx. 2 μs (room temperature) from K (λ_{max} = 550 nm). RR and FTIR data agree [26,41] that the retinal remains essentially in the same configuration as in the K-intermediate; only the slight distortion of C–C single bonds is relaxed.

The electric signal indicating a displacement $d_2 = -0.03$ nm of the proton is in agreement with such a relaxation, with small charge motion. A difficulty arises, however, when trying to explain the large blue shift of the absorption maximum (590 → 550 nm). The absorption maxima in general may be understood with the aid of the modified point charge model [36], in which the Schiff base proton and its counterion play an important role. There is seemingly no essential change in these configurations between the K- and L-intermediates, and therefore changes in the charge of other groups in the vicinity of the retinal should occur.

FTIR data show that an aspartic acid group is deprotonated during the K-L transition [30]. Other changes in protonation have not been reported for this transition, therefore the destination of this proton remains unknown. Even this charge motion could be responsible for the displacement d_2.

3.5. The M-intermediate

The M-intermediate is formed with a lifetime of 80 μs from L. $\lambda_{max} = 410$ nm which is explained by the fact that the Schiff base deprotonates during this transition.

Many changes appear in the protonation states of different amino acids. Ultraviolet measurements show that a tyrosine becomes deprotonated during formation of the M-intermediate [31,32], FTIR results indicate protonation of three aspartates and deprotonation of one aspartic acid [30]. Protonation changes of glutamic acids were also reported [33], which were, however, not observed in ref. 30.

Evaluation of the electric signal gives $d_3 = 0.5$ nm. This means a larger forward displacement of the proton from the Schiff base, which should protonate a closely located COO^- group. The protonation of one aspartate, shown by FTIR, fits this hypothesis.

The magnitude of d_3 and its sign were independently confirmed by applying an external electric field to pms [46]. The increased or decreased barrier (against proton transport) increases or decreases the lifetime of the L-M transition. Quantitative evaluation resulted in a value of $d_3 = 0.5 \pm 0.1$ nm.

A negative electric signal appears if the bR in the M-state is illuminated by a blue flash [47]. The area of the signal is equal to that of the previous (positive) signal, demonstrating that the protons return to their original place, i.e., to the Schiff base in all-*trans*-retinal.

These observations imply that the proton released from the Schiff base is still inside the bR, which contradicts findings showing that protons appear in solution more or less concurrently with the formation of the M-intermediate [48]. A possibility for resolving the contradiction is the assumption that the drastically altered charge distribution within the proteins during formation of M is relayed to the surface of the membrane from which bound protons are released. This very small motion is not sensed in the electric signal. The space occupied by the surface proton is later filled by the proton arising from the M-intermediate during its decay.

3.6. The O-intermediate

This species is the most puzzling intermediate. Formed from decay of M in approx. 8 ms, it

decays in approx. 2.5 ms to the bR ground state; $\lambda_{max} = 640$ nm; the retinal is all-*trans* and the Schiff base is protonated [26].

It is problematic to explain the $\Delta\lambda = 640 - 568$ nm = 72 nm shift in the absorption maximum relative to the bR ground state if the retinal is in the same isomeric and protonation state. The presence of other charges in the opsin part is required. Reprotonation of tyrosine and aspartic acids is reported to accompany the decay of M [30–33]; no evidence exists for variations in the charge state of amino acids in the decay after the O-intermediate.

The *cis-trans* isomerization of a retinal with unprotonated Schiff base is rather improbable [49], and therefore an interim protonation should be assumed.

The electric signals indicate a long proton path ($d_4 = 3.1$ nm) during the M-O transition and a shorter one ($d_5 = 1.5$ nm) during O decay. The simple interpretation of d_4 is the translocation of the proton from the M-intermediate (located at a distance of $d_3 + d_1 + d_2 = 0.35$ nm from the Schiff base in the bR ground state) to the external surface of the membrane and of d_5 as the uptake of a proton from the internal side the Schiff base. d_4 and d_5 nicely designate the Schiff base to be 1/3 from the internal surface, in agreement with structural data. If we accept that the O-intermediate is already protonated, it may obtain the proton in the above-mentioned interim process from a proton donor nearby, which is filled during O decay. The negative charge at this proton donor may help to explain the 72 nm shift of the absorption maximum.

4. Mechanism of the proton pump

The process of proton translocation has two phases separated in time and space. The first ends with the formation of the L-intermediate. It has been well established from the above-detailed data that *trans-cis* isomerization and lifting of torsions accumulated during isomerization lead to the L-intermediate. During these steps the proton resides at the Schiff base and moves with it. The surroundings of the Schiff base in the L-intermediate must enable deprotonation, i.e., they should decrease the pK of the Schiff base proton. This moiety has not yet been clearly elucidated. A new charge (an aspartate ion) appears, probably in the vicinity of the Schiff base, during formation of L. It takes only 2 μs to reach the L-state which exists for 70–80 μs.

In the second phase, which involves the L-M-O-bR transitions, the proton leaves the Schiff base during the L-M transition and presumably is anchored by an aspartate ion still deep within the protein. This is supported by the observations that different environments (pH, salt, temperature, humidity) do not essentially change events until formation of M [25]. The photocycle also runs the same way in chemically modified bRs (with, for example, iodinated tyrosines [50], cross-linked carboxyl and lysine groups [50,51] and proteolytically digested protein parts [52]).

The proton must cross large distances to the external surface (3.1 nm) and from the internal surface (1.5 nm). The first model considered events at the retinal as a switch to connect the two proton-conducting pathways [53]. The pathway is constructed from proton-donating and -accepting side chains of amino acids through which the protons are conducted as in ice [54–56]. This theory of proton conduction adequately forecasts the speed of proton transfer [54]. The following experiments, however, are not in favour of this mechanism:

(a) Cross-linking of possible proton donors and acceptors (lysines and carboxyls) varies the lifetimes of the intermediates but does not decrease the number of protons per photocycle [50,51];

(b) Proton translocation ceases below a water content of 0.06 g H_2O/g bR; data show that even for higher water content, protons are pumped only above 0 °C, i.e., in the presence of fluid water [46].

Based on the above data and on the general properties of proteins another simple model was proposed [57]. It is well known from H-^{2}H exchange experiments that different parts of the protein are accessible to water because of the fluctuation in protein structure. This process is rather fast (in the millisecond range). We assume that the proton at the carboxyl group in the M-intermediate is approached by water molecules

through temporarily opened channels, their dipole moment lowers the barrier for proton release and the resultant H_3O^+ is driven out to the external side, and one taken up from the internal side, by the existing internal electric field due to the primary charge separation and surface charges.

Arguments in support of the model are points a and b above and also:

(c) Tryptophan fluorescence data obtained by Plotkin and Sherman [58] demonstrate the existence of intraprotein aqueous regions;

(d) Diamines reverse the direction of the proton flow [59]. In fig. 7 we show that the direction of continuous current generated by continuous illumination of bR oriented and immobilized in gels is reversed by the presence of TEMED.

(e) The model is general and is also applicable for other ions (for example, halorhodopsin, which pumps Cl^- after light absorption [60]).

Data on the de- and reprotonation of aspartic acids [30,33] do not contradict the model. It is highly possible that the conduction of the proton in the water-filled channel may be accelerated by these groups but their elimination, however, is not catastrophic and merely slows down the process, as is observed (b) in refs. 50 and 51.

Recently, genetically modified bRs were prepared and tested for proton translocation [5,61]. Tyrosine, tryptophan, proline, serine and glutamic acid residues were changed by site-specific mutagenesis to other residues. The mutant apoproteins were regenerated by retinals and reconstituted into vesicles. The mutants pumped protons. The experiments point to a general pumping mechanism which is not influenced by many different changes in the protein.

5. Conclusions

It may be seen from the experimental data presented above that the understanding of the bR proton pump is still in the 'hypothesis' state. We believe, nevertheless, that many features of this pump may have a 'model value' in understanding other pumping mechanisms:

(1) The primary energy uptake is of an electrostatic nature: a small motion picks up comparatively large amounts of energy;

(2) Significant changes occur in the charge states of the side chains of some amino acids;

(3) Water is a decisive factor;

(4) Surface charges are involved in pumping;

(5) Many chemical and genetic changes do not abolish pumping, showing its dependence upon general features of the protein structure.

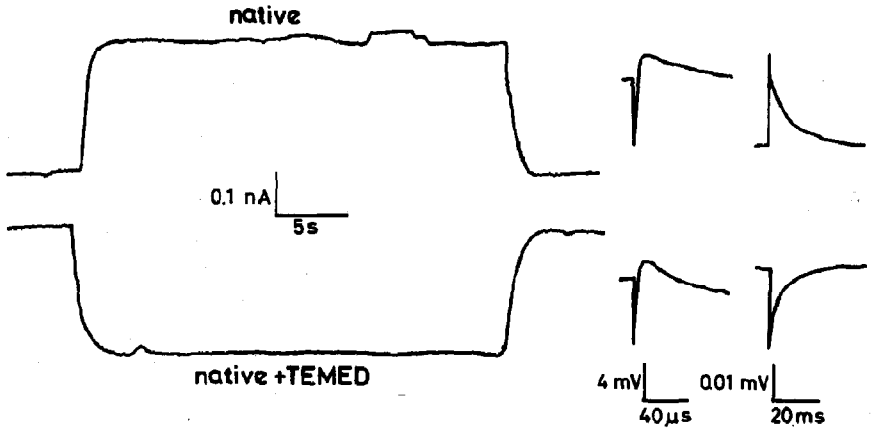

Fig. 7. Continuous current during continuous illumination of pms immobilized in gels. The direction of current is reversed in the presence of TEMED (360 µM bR, 250 µM TEMED, pH 7, room temperature). The transient electric signals are also shown in two different time and amplitude ranges.

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