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Bacteriorhodopsin mutants D85N, D85T and D85,96N as proton pumps

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Abstract

Proton translocation in the BR mutants D85N, D85T and D85,96N was studied by attachment of purple membranes to planar lipid bilayers. Pump currents in these mutants were measured via capacitive coupling and by use of the appropriate ionophores. All mutants have a reduced pK of their Schiff bases around 8–8.5 in common. At physiological pH, a mixture of chromophores absorbing at 410 nm (deprotonated form) and around 600 nm (protonated form) coexists. Excitation with continuous blue light induces in all three mutants an outwardly directed stationary pump current. These currents are enhanced upon addition of azide in D85N and D85,96N by a factor of 50, but no azide enhancement is observed in D85T. Yellow light alone induces transient inwardly directed currents in the mutants but additional blue light leads to a stationary current with the same direction. All the observed currents are carried by protons, so that the consecutive absorption of a yellow and a blue photon leads to inverted stationary photocurrents by the mutants, as observed with halorhodopsin (HR).

A mechanistic model describing the inversion of proton pumping is discussed by the *cis–trans*, *trans–cis* isomerization of the retinal and the different proton accessibility of the Schiff base from the extracellular or the cytoplasmic side of the membrane.

Keywords: BLM; Retinal; Vectorial catalysis; Ion pump; Schiff base

1. Introduction

The channel of a bacteriorhodopsin proton is divided by the Schiff base in a cytoplasmic (CP) and an extracellular (EC) part. For vectorial transport by the protein, D85 in the EC channel serves as an acceptor of the proton from the Schiff base, whereas D96 serves as the internal proton donor to complete the transport process. Between these two essential amino acids retinal is switched by light from *cis* to *trans*, altering the accessibility of the Schiff base for protons from the cytoplasmic to the extracellular side, respectively (for reviews see [1–5]).

Interestingly, in the closely related anion pump halorhodopsin the amino acids equivalent to D85 and D96 are replaced by threonine and alanine, respectively. Halorhodopsin can act as an inwardly directed proton pump after sequential absorption of a yellow and a blue photon, respectively [6]. This is due to a side reaction of the photocycle. With a certain probability the Schiff base deprotonates after absorption of a yellow photon by forming HR_{410}. Concomitant with this reaction the proton is released via the CP

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channel. Blue light reprotonates the Schiff base via the EC channel, so that the proton translocation is completed. This proton translocation in halorhodopsin is amplified by azide, which increases the rate of formation of HR_{410} upon illumination with yellow light. In analogy to HR, D85 was altered in bacteriorhodopsin to D85 → N, D85 → T and D85,96 → N. Then proton translocation in these mutants was studied. Similarly to HR these mutants have a lowered pK of the Schiff base around 8.0. Therefore in the dark at physiological pH a fraction of the molecules exists with the deprotonated Schiff base with retinal in the all-*trans* and 13-*cis* configuration [7]. We report here on the direction of the proton translocation in the mutants. Blue light alone induces stationary currents in the same direction as in wild type, whereas after sequential absorption of a yellow and a blue photon these mutants produce inwardly directed proton transfer similarly as it was seen for HR.

2. Results and discussion

Photocurrents were determined via the capacitive coupling of purple membranes with a planar lipid film (for details see [8]). The appropriate application of ionophores permeabilizes the underlying lipid bilayer for protons, so that stationary photocurrents by the mutant bacteriorhodopsins can be observed. All experiments were carried out at pH 6.7, where a significant amount of the point-mutated bacteriorhodopsins have a deprotonated Schiff base. According to retinal extraction experiments the chromophore has partially an all-*trans* configuration in the respective mutants [7].

When purple membranes are added under stirring to one side of the planar lipid membrane, photosensitivity develops within 20–40 min and only transient photocurrents can be observed (not shown). Upon addition of the light insensitive protonophore 1799, the underlying lipid film becomes selectively conductive for protons. Only after excitation with blue light or white light (blue and yellow) stationary photocurrents with all three mutants can be observed (Fig. 1). For a definition of 'yellow' and 'blue' light, see legend of Fig. 1.

As can be seen in the same figure, yellow light elicited only transient photocurrents. In mutants D85,96N and D85N, azide increases the stationary photocurrent drastically, whereas mutant D85T is unaffected by azide. The main interesting point from these results is that the direction of the photocurrent is dependent on the condition of illumination. Blue light drives a pump current which is in the same direction as that driven by yellow light in wild type.

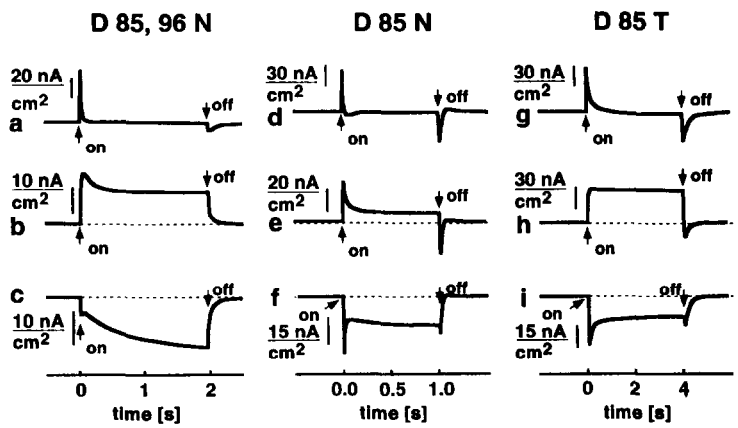

Fig. 1. Examples of stationary photocurrents in yellow, white and blue light. The conductance of the lipid bilayer was between 20 and 50 nS in the presence of 16 mM 1799. The protein was illuminated by yellow ($\lambda > 515$ nm, 2 W/cm^2) light (traces a, d and g), white ($\lambda > 360$ nm, 2.8 W/cm^2) light (b, e and h) or blue ($360 < \lambda < 420$ nm, 0.7 mW/cm^2) light (c, f and i). The vertical bars indicate the measured currents in nA, the horizontal one indicates the time which is the same scale in all experiments shown. A similar figure was shown in a earlier publication [7].

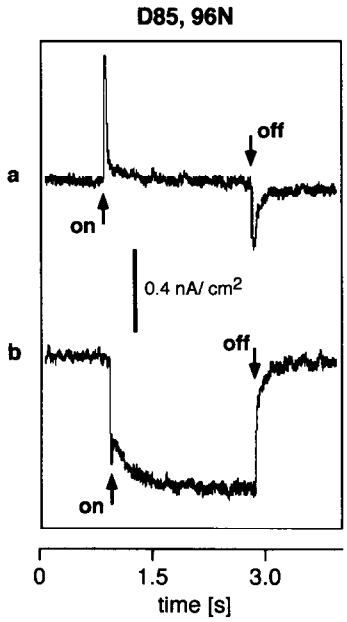

Fig. 2. Stationary pump current of mutant D85,96N induced by cell envelope vesicles adsorbed to planar lipid membranes (a) yellow light, (b) blue light. The electrolyte was composed with 2 M NaCl, 10 mM azide, 5 mM 1799, 20 mM Tris/Hepes, pH 6.8.

White light drives oppositely directed stationary currents. Yellow light alone drives in all three mutants a transient current which is inwardly directed. The determination of the direction of the current was made under the assumption that wild type purple membranes and purple membranes of the mutants adsorb preferentially to the planar lipid film facing the lipid bilayer with the extracellular side. This was determined under the knowledge of the proton pumping in the whole cell and the determination of the direction of the pump current induced by the wild type purple membrane. The assumption that also the mutants adsorb in the same way to the lipid bilayer is strongly supported by experiments with cell envelope vesicles with the mutant D85,96N, where the molecules are oriented in the cell membrane in the same direction as in the whole cell. Fig. 2a,b shows under yellow light and blue light conditions the same direction of current as it has been observed for the three mutants, as described in Fig. 1. This proves that the purple membranes of the mutants adsorb also with the extracellular side to the planar lipid film. It is important to note that in case of the stationary currents the amount of transported charge exceeds by far a current arising from a single turnover. This statement is justified by the following consideration: the current ($I = \Delta Q/\Delta t$) observed e.g. by mutant D85T under white light conditions is about 60 nA/cm^2. This stationary current (Fig. 1h) can be measured under continuous light for at least 20 min (not shown). If a continuous monolayer of purple membrane (D85T) would cover completely the planar film the total charge ΔQ to 1 H^+ per D85T mutant and photocycle would be around 1 μC/cm^2. If a current density of 60 nA/cm^2 exists for several minutes, the transferred charge is far in excess of 1 μC/cm^2. Therefore, many cycles are responsible for the current, showing the continuous function of the mutant as an ion pump. Therefore, the stationary currents reflect directly the transport activity of these mutants.

2.1. The effect of azide

The stationary photocurrents demonstrated in Fig. 1 were obtained in the presence of 50 mM azide. In D85N as well as in D85,96N stationary photocurrents are drastically reduced in absence of azide, but still present [7]. Therefore, it is concluded that the reversal of the stationary currents is not due to azide. In yellow light a transient current is not influenced by addition of azide. A drastic increase of the photostationary current by azide is observed in blue light. In white light a stationary current of opposite sign is observed but also drastically enhanced by azide. This finding clearly excludes that the inversion of the currents is caused by azide.

In order to elucidate the reaction which is accelerated upon addition of azide spectroscopical experiments were carried out. Continuous blue-light illumination of D85N membranes produces a state which absorbs maximally at 610 nm and decays after switching off the light to the initial form absorbing maximally at 410 nm. This decay can be accelerated from 15 s to 500 ms after addition of 300 mM azide at pH 6.5 (Fig. 3). This finding clearly demonstrates that azide accelerates the deprotonation reaction in that mutant. It should be stated that the action of azide in mutant D85N or D85N,96N is an additional one to that described for mutant D96N where azide accelerates the reprotonation of the Schiff base [7,9].

Mutant D85T showed no increased current after addition of azide under any condition of illumination. In fact, the rate of the deprotonation reaction in D85T is 500 ms at pH 6.5 and is accelerated to 300 ms upon addition of azide. In general azide effects on the photostationary current can only be expected if rate-limiting steps in the catalytic cycle are drastically affected. This is obviously not the case in D85T.

The results shown in Fig. 1 are discussed in a model where the bacteriorhodopsin proton channel is separated into two halves by the Schiff base. The uptake and release of the proton is dependent on the accessibility of the Schiff base either from the cytoplasmic or the extracellular side, respectively. From the direction of the current the proton uptake and release can be assigned to the respective half-channel (CP, EC). Proton uptake in blue light occurs via the CP channel as does the release of protons after excitation with yellow light. Two observations support these results. First, in wild type bacteriorhodopsin a deprotonated Schiff base in 13-*cis* (M intermediate) is converted by blue light to all-*trans* and picks up the proton through the EC channel [10]. It seems unlikely that the opposite reaction, i.e. photoisomerization of the deprotonated Schiff base from *trans* to *cis* would cause the same direction of proton uptake in the mutants. The second observation was made during FTIR experiments. After excitation of BR mutants with yellow light a deprotonation of D96 occurs without the Schiff base being

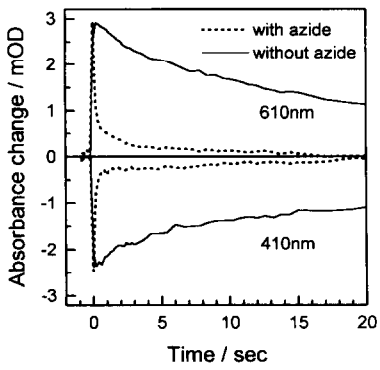

Fig. 3. Photostationary state and its decay of BRD85N upon blue light illumination in absence (solid line) and presence (dashed) of 300 mM azide. Purple membranes of BRD85N were suspended in 6 mM MES/Tris pH 6.5 and 1 M NaCl.

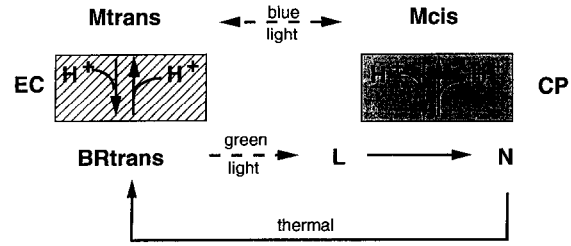

Fig. 4. Reaction scheme for the mutants lacking the proton acceptor D85 accounting for the observed photocurrents dependent on the light quality used. A similar figure was shown in an earlier publication [7].

deprotonated [11]. This strongly argues that proton release in yellow light as well as proton uptake in blue light is through the CP channel.

The possibility of addition of ion-selective ionophores to obtain stationary photocurrents is one of the main advantages of the BLM system. Therefore, beyond doubt all stationary currents shown are exclusively due to proton movements because just the protonophore 1799 is required.

Fig. 4 shows the tentative reaction scheme which explains the different directions of the proton translocation by the same protein molecule. All three mutants have in common that a substantial amount of protonated and deprotonated Schiff base with retinal in all-*trans* and *cis* state coexist at pH 6.8. In both forms light can induce a *trans* to *cis* isomerization. Blue light starts (single photon per cycle) the reaction from the deprotonated state (M_{trans}) and under steady state illumination the outwardly directed current can be observed. This is the first example where the temporal order of release and uptake of the Schiff base proton during the proton transport is inverted compared to the wild type. It demonstrates directly that the Schiff base in the all-*trans* and 13-*cis* form have different affinities and accessibilities for the proton. While the 13-*cis* form is connected preferentially with the CP channel the *trans* form releases and accepts protons via the EC channel.

The proposal for this mechanism in Fig. 4 is as follows: the deprotonated form of the retinal is in the all-*trans* configuration. Blue light causes isomerisation to 13-*cis* with a reprotonation of the Schiff base, yielding an N intermediate, which decays to BR. BR_{trans} releases the proton through the EC channel

under the formation of M_{trans} due to the lowered pK of the Schiff base in these mutants.

The two photon process, where the proton translocation is inwardly directed can be described by the second mechanism: BR_{trans} is excited by a yellow photon forming a stable M_{cis}. The proton is released into the CP channel, since in the *cis* form the Schiff base is connected to CP. Further excitation with a 'blue' photon forms a M_{trans} which is connected to the EC channel, where the proton is taken up to complete the translocation process.

If only yellow light is present, BR_{trans} is transformed to L, which decays thermally via N back to BR_{trans}. If M_{cis} is formed transiently from N, no net transport under these conditions occurs either, since the proton is released and taken up by the same half-channel (CP). Therefore, only transient photocurrents under these conditions were observed.

In summary we have shown that proton translocation in mutant D85X occur in a one photon process in blue light and has the same direction as the proton translocation in wild type upon yellow light illumination. The direction of blue light driven translocation is inverted in white light. This two-photon driven process is similar to the one observed in halorhodopsin. Obviously the key element for the vectorial proton translocation is the light-triggered switch between all-*trans* and 13-*cis* or 13-*cis* to all-*trans* linked to protein conformational changes which have been first demonstrated for BR D96N [12]. Protein relaxation also occurs in the D85X mutants upon pH jump [13,14] but, should, different from wild type, also occur in the protonated state of the chromophore (L → N in Fig. 4). This has not yet been shown. In addition it can be ruled out that the vectoriality of proton translocation is determined by Asp85, because in all three mutants the same direction as in wild type is observed in blue light. The change of vectoriality from blue light to white light indicates that the thermal relaxation to the initial state can compete with a second photochemical reaction (M_{cis} to M_{trans}).

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