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A defective proton pump, point-mutated bacteriorhodopsin Asp96 → Asn is fully reactivated by azide

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Addition of azide fully restored the proton pump activity of defective bacteriorhodopsin (BR) mutant protein Asp96 → Asn. The decay time of M of BR Asp96 → Asn, the longest living intermediate, was decreased from 500 ms at pH 7.0 to ~1 ms under conditions of saturating azide concentrations. This decay was faster than the decay of M in the wild-type, where no such azide effect was detectable. Stationary photocurrents, measured with purple membranes immobilized and oriented in a polyacrylamide gel, increased upon addition of azide up to the level of the wild-type. Different small anions of weak acids restored the pump activity with decreasing affinity in the order: cyanate > azide > nitrite > formiate > acetate. The activation energy of the M decay in the mutant was higher in the presence (48 kJ/mol) than in the absence (27 kJ/mol) of 100 mM azide even though the absolute rate was dramatically increased by azide. This effect of azide is due to the substitution of a carboxamido group for a carboxylic group at position 96 which removes the internal proton donor and causes an increase in the entropy change of activation for proton transfer which is reversed by azide.

Key words: azide/bacteriorhodopsin/proton pump activity

Introduction

Archaebacterial photosynthesis depends on the action of bacteriorhodopsin (BR) as a light-driven proton pump. The catalytic cycle of this retinal protein is determined by a thermoreversible *trans* to 13-*cis* isomerization of its chromophoric group (for reviews, see Stoeckenius and Bogomolni, 1982; Kouyama *et al.*, 1988). A second conspicuous reversible change of the molecule upon photoexcitation is the reversible protonation of the Schiff base by which retinal is linked to the ϵ-amino group of the lysine residue 216 in the polypeptide chain. This reaction involving the so-called M-intermediate occurring in the 13-*cis* configuration (Pettei *et al.*, 1977; Tsuda *et al.*, 1980) has been repeatedly suggested to be essentially involved in vectorial proton transfer and thus the chromophore is considered to act as the light-activated switch in the proton pump (Oesterhelt and Tittor, 1989). Besides the *cis/trans* configuration and the protonated/deprotonated state of the Schiff base, at least one additional conformational state must exist to guarantee the vectorial nature of the translocating process. This state has been postulated to be a 14 s-*cis* conformation of the retinal moiety (Schulten and Tavan, 1978) or a different protein conformation (Fodor *et al.*, 1988). In addition to the reactions localized around and coupled to the chemical changes of the Schiff base, proton transfer pathways to and from the proton binding sites close to the active switch must exist which allow conduction of protons through the protein in the ms time range (Merz and Zundel, 1981; Nagle and Tristram-Nagle, 1983). Indeed protonation changes of aspartic acids (Engelhardt *et al.*, 1985; Eisenstein *et al.*, 1987; Braiman *et al.*, 1988a) and tyrosine residues (Bogomolni *et al.*, 1978; Hess and Kuschmitz, 1979; Roepe *et al.*, 1987; Braiman *et al.*, 1988b) during the catalytic cycle of BR have been demonstrated by Fourier transform infra-red spectroscopy (FTIR) and UV spectroscopy. In particular the four aspartic acids Asp85, Asp96, Asp115 and Asp212 have been tentatively assigned to be internal to the protein structure and two of them were shown to occur protonated (Asp96 and Asp115), the others unprotonated (Braiman *et al.*, 1988a; Gerwert *et al.*, 1989).

The ion translocating pathway has been suggested to have the intrahelical pore space of the circularly arranged seven α-helices of the protein as a structural basis in both the proton pump BR and in its chloride-translocating counterpart halorhodopsin (HR). HR also undergoes *trans* to *cis*-isomerization but no deprotonation of its Schiff base in the ms time range of the catalytic cycle was found (Oesterhelt *et al.*, 1985). Instead changes of chloride affinity occur (Lanyi and Vodyanoy, 1986). Occasionally, however, a proton is lost to the aqueous phase and the molecule is trapped in a blue-light-absorbing intermediate state before it is protonated in the time range of seconds and returns to its initial state. If azide is added in such an experiment, the Schiff base becomes catalytically de- and reprotonated and these processes can be accelerated up to 1 ms (Hegemann *et al.*, 1985b; Lanyi, 1986). Thus it seems that in both molecules ion transfer pathways exist inside the helical arrangement which are specific for protons and anions respectively. Azide short-circuits the ion pathway of protons from and to the Schiff base in HR and thus can be called a protein internal protonophore.

Random mutagenesis and subsequent selection for halobacterial cells carrying inactive BR (Soppa and Oesterhelt, 1989) as well as site-specific mutagenesis of BR expressed in *Escherichia coli* (Mogi *et al.*, 1988) led to the identification of aspartic acid residues 85 and 96 as causally involved in the regulation of the velocity of proton transfer from and to the Schiff base (Butt *et al.*, 1989; Holz *et al.*, 1989; Marinetti *et al.*, 1989). One specific mutation, the change of the carboxylic group of aspartic acid 96 into a carboxamido group of asparagine or the complete removal of this side chain by replacement with a glycine residue, caused a dramatic retardation of reprotonation in BR, thereby slowing the pump. In this report we show that azide is able to compensate the mutational defect BR Asp96 → Asn, Gly restoring efficient catalysis of proton translocation.

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Results

Azide-accelerated reprotonation of the Schiff base

Time-resolved absorption measurements after flash photolysis revealed that in wild-type BR the M decay could be described by two time constants of 4 ms and 10 ms (Groma and Dancshazy, 1986), whereas in both mutants the decay of M was monophasic and slower than in wild-type as shown in Figure 1A–D respectively. The exchange of Asp96 → Asn resulted in a time constant of 540 ms while the M state of the mutant protein Asp96 → Gly decayed with 120 ms. The retardation of the M decay explains the inactivity of both proteins in the halobacterial cell. Addition of azide accelerated the M decay in both cases as shown for 1 mM azide (Figure 1A) BR Asp96 → Asn and 10 mM azide BR Asp96 → Gly (Figure 1B) concentrations. The increase of the rate constant of decay as a function of the concentrations of azide is plotted in Figure 2. The affinity constant K_a and the maximal velocity V_{max} of the azide-accelerated reprotonation were revealed as 50 mM and 800 s^{-1} by Michaelis–Menten analysis for the Asp96 → Asn mutant protein. The affinity constant K_a reflects the concentration where the half maximal acceleration of the reaction was reached. Replacement of Asp96 by glycine instead of asparagine leads to lower affinity for azide and a lower maximal velocity of reprotonation. This mutant showed no clear saturation behaviour, thus for most experiments the mutant protein Asp96 → Asn was further used.

As shown in Figure 3 the M decay of the mutation Asp96 → Asn depends on proton activities of the medium. By plotting the logarithm of the decay of the M intermediate versus pH, we obtained a straight line up to pH 9, with a slope of 0.3, indicating that on the basis of a bimolecular reaction the effective proton activity for reprotonation of M is different from the proton activity in the bulk phase. At higher pH values (>9) no further increase of the decay time was observed. It is noteworthy that on measuring the acceleration of M decay by 100 mM azide at pH 8.5 we again found the same decay constant as at pH 5.2. Three protonation pathways can be distinguished: the one in the wild-type via Asp96, the one in the mutant BR without azide and finally the one in the mutant with azide.

Both mutant proteins reach the level of the M decay rate of the wild-type upon saturation of the system with azide, thus indicating that azide as a basic anion with a pK of 4.7 is able to replace Asp96 as a catalyst for reprotonation of the Schiff base. The retinylidene chromophore is positioned within the intrahelical pore with its angle of the C=C transition moment tilted 21° from the plane of the membrane. The Schiff base group on the G helix has been assumed to be ~17 Å away from the C-terminal surface and ~15 Å from the N-terminal surface (Hauß et al., 1989). Thus it is conceivable that both de- and reprotonation involve catalytic groups which are responsible for the appropriate reaction speed. Asp85 is suspected to fulfil the role for

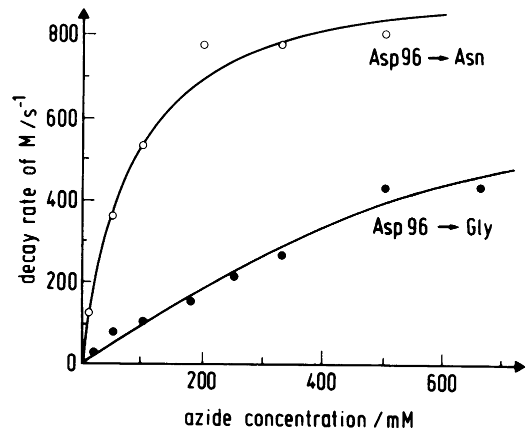

Fig. 2. Dependence of M decay on azide concentration. The decay rate of the M intermediate was measured at 410 nm for BR mutants Asp96 → Asn and Asp96 → Gly. Experimental conditions were as described in the legend to Figure 1 except that pH was adjusted to 5.2.

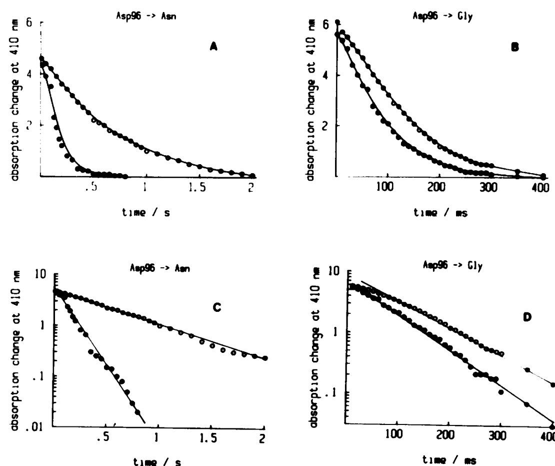

Fig. 1. Effect of azide on the M decay of mutated BR. Time-dependent absorption change at 410 nm of BR mutant Asp96 → Asn (**A** and **C**) and Asp96 → Gly (**B** and **D**) after excitation with a laser flash at 590 nm (3 mJ) without azide (open symbols) and with addition of 1 mM (**A**) and 10 mM (**B**) azide (closed symbols). PMs were suspended at 0.7 OD in 100 mM NaP_i pH 7.0, temperature was kept constant at 19°C. In **C** and **D** the semilogarithmic plots of data from **A** and **B** respectively are shown to demonstrate the monotonic decrease and evaluation of decay constants.

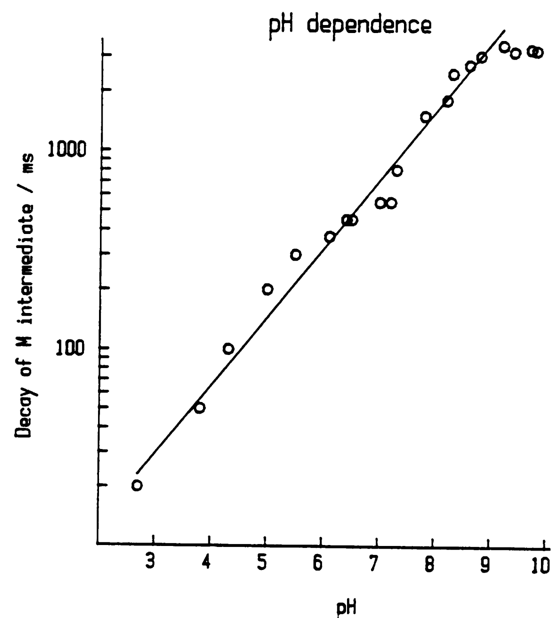

Fig. 3. Dependence of M decay on H^+ activities in the external medium. PM was suspended at 0.1 in 20 mM phosphate or Tris buffer and pH was adjusted with concentrated NaOH or HCl. Excitation wavelength was 590 nm and the wavelength of observation was 410 nm.

deprotonation (Butt *et al.*, 1989) and Asp96 for reprotonation. The replacement of Asp96 by azide can be explained if one assumes that azide (N_3^-) and hydrogen azide (HN_3) diffuse faster through the mutated protein structure than the proton. Alternatively, azide could occupy a site close to the Gly96 or Asn96 replacing the carboxylic function of Asp96 in the wild-type.

Regeneration of efficient proton pumping by azide

Evidence that the reprotonation of the Schiff base accelerated by azide in the ms time range is a vectorial reaction is provided by the experiments shown in Figures 4 and 5. Photocurrents of mutant purple membranes (Asp96 → Asn) attached to a black lipid membrane (BLM) were measured and the conductivity of the BLM increased by adding 3 μM FCCP and 10 μM monensin to allow stationary photocurrents (Bamberg *et al.*, 1979). Only a small stationary current was detectable (Figure 4A) without azide. With azide the stationary current increased (Figure 4B) and became comparable to that of wild-type purple membranes under the same conditions (data not shown). Control experiments showed that this current increase was not due to an azide-induced conductivity increase of the BLM (data not shown). To demonstrate that azide restored the activity of the mutant proton pump to that of the wild-type the following experiment was devised. Wild-type and mutant purple membranes were mixed in a ratio of 1:1 and the sample oriented and immobilized in a polyacrylamide gel. In this system the wild-type purple membranes provided an internal standard. If azide catalyses vectorial reprotonation from the cytoplasmic side, this agent should increase the proton pump activity of the sample by a factor of two. Indeed, this expected increase of stationary current was found (Figure 5) and thus azide restores the pump efficiency of the mutant to that of the wild-type. Thus a proton-specific transfer pathway from the cytoplasm to the Schiff base exists and it is proved by reactivation of the pump that this pathway is of mechanistic importance.

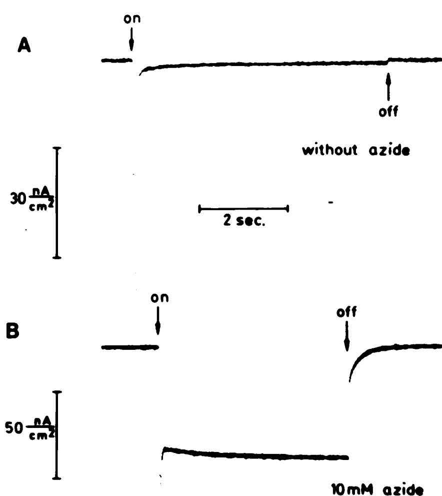

Fig. 4. Light-induced currents from PMs of mutant Asp96 → Asn attached to a BLM. 20 μM of BR in 100 mM NaCl and 10 mM Na citrate pH 6.0 were added from one compartment after doping the membrane with 3 μM FCCP and 10 μM monensin. Stationary photocurrents upon illumination (indicated by arrows) with yellow light (λ >530, Schott, Mainz, FRG, intensity 100 μW cm^{-2}) were measured (**A**) without azide and (**B**) with 10 mM azide. Stationary photocurrents from the wild-type under identical conditions are of the same size (not shown) as those of the mutant with azide.

Anion specificity

The specificity of the mutant BR Asp96 → Asn with respect to the anion which allows reactivation of proton transport was tested for several anions known to catalyse Schiff base deprotonation in HR also (Lanyi, 1986). The dependence of stationary currents in BLM-attached purple membranes on the concentration of several anions was measured. Table I shows the affinity constants K_a of the anion together with the p*K*s of the corresponding acids.

The most efficient catalysts were found to be the anions azide and cyanate which also act best in the reversible deprotonation reaction of HR (Hegemann *et al.*, 1985b). Size and p*K* seem to determine the efficiency to regenerate the pump activity. A detailed analysis revealed a general acid–base catalytic mechanism for HR (Lanyi, 1986).

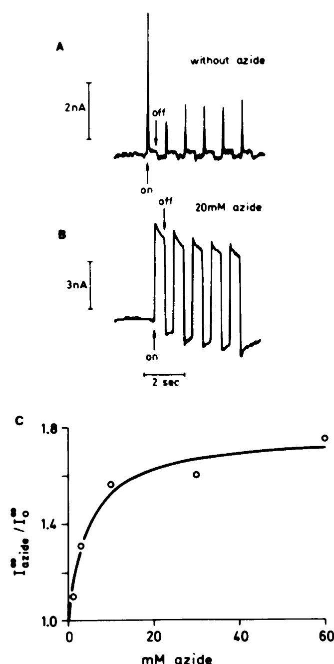

Fig. 5. Photocurrents of BR Asp96 → Asn oriented and immobilized in a polyacrylamide gel (**A**) before and (**B**) after addition of 20 mM azide. (**C**) PMs of wild-type BR and mutant Asp96 → Asn mixed in a ratio of 1:1 oriented and immobilized in a polyacrylamide gel 10 mM MOPS pH 6.4 and 30 mM NaCl. Stationary photocurrents under different azide concentrations 0–60 mM were determined with increasing light intensities (100% light = 2 W/cm², λ >495 nm). The ratio of the saturation current I^∞ (azide) in different azide concentrations to that without azide I^∞ (0) is plotted against the azide concentration.

Table I. Affinity constant K_a of different anions for BR Asp96 → Asn suspended in 100 mM NaCl, 10 mM MOPS, pH 6.4 in the reactivation of transport activity

		p*K*s	K_a (mM)
Azide	N_3^-	4.5	3–4
Cyanate	OCN^-	3.7	0.9
Nitrite	NO_2^-	3.3	10
Formate	$HCOO^-$	3.8	61
Acetate	CH_3COO^-	4.8	139
Thiocyanate	SCN^-	0.85	No effect

The difference between the K_a value for azide found in photochemical experiments and the value given in this table is mainly due to the different types of experiment.

Demonstration of such a mechanism for the mutant BR will require extended studies.

Activation energy of the reprotonation reaction

The red-shift of the BR chromophore accompanying the M decay is indicative of the protonation of the Schiff base independent of later steps in the photocycle. Thus determination of the temperature dependence of M decay in the mutant allows calculation of the activation energy barrier for the proton to reach the Schiff base. A value of 27 kJ/mol was found (Figure 6). This is half the value of 54 kJ/mol found for the monophasic decay of the M state in wild-type BR in salt/ether suspension (Oesterhelt and Hess, 1973). In aqueous suspension the corresponding activation energy is similar (Sherman and Caplan, 1975) but complicated by the fact that M decay is at least biphasic. The activation energies for these reactions are in the range from 71 to 79 kJ/mol (data not shown; Maurer *et al.*, 1987). Although in BR Asp96 → Asn the rate of M decay is drastically reduced the activation energy is not increased as expected but lowered compared to the wild-type. From the Eyring equation:

$$k = k_B T/h \times \exp(\Delta S^{\#}/R) \times \exp(-\Delta H^{\#}/RT)$$

where the constants k_B, T, h, R, $\Delta S^{\#}$ and $\Delta H^{\#}$ reflect the Boltzmann constant, absolute temperature, Planck's constant, the common gas constant, the entropy of activation and the enthalpy of activation according to the transition state theory. The entropy of activation $\Delta S^{\#}$ for the proton transfer in mutant and wild-type is different and the values are calculated as −140 J/mol degree and +50 J/mol degree at 20°C respectively. These values indicate that the proton transfer reaction in the mutated protein structure lacking the internal proton donating site Asp96 has a higher entropy change to reach the transition state than the proton transfer reaction in the wild-type.

Addition of 100 mM azide increases both the entropy of activation to −26 J/mol degree and the activation barrier of reprotonation to 48 kJ/mol and restores the free energy of activation found in the wild type. These findings are inconsistent with a model for the reprotonation step in the Asp96 → Asn mutant protein, in which a higher activation barrier was postulated to explain the slow regeneration of the BR initial state (Holz *et al.*, 1989).

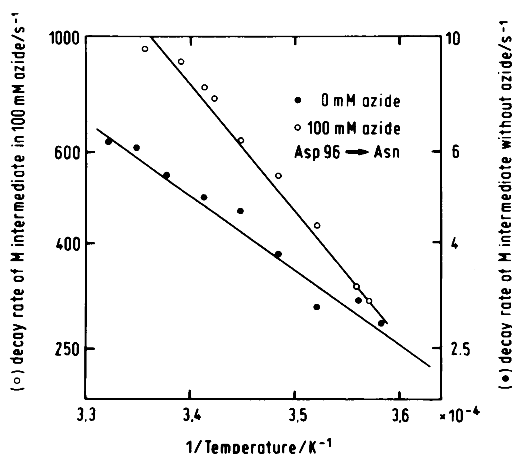

Fig. 6. Arrhenius plots of M decay of mutant Asp96 → Asn before (closed circles) and after (open circles) addition of 100 mM azide. 25 μM PMs were suspended in 100 mM KP_i pH 5.2 optical conditions as described in Figure 1.

Discussion

Comparison of protonation pathways in HR and BR

The chloride pump HR has some striking structural similarities with BR (Blanck and Oesterhelt, 1987; Lanyi *et al.*, 1988; Oesterhelt and Tittor, 1989) which are expected on the basis of the three function similarities: binding of all-*trans* retinal, thermoreversible *trans* to 13-*cis* photoisomerization and ion transport. A conspicuous difference, however, exists between primary structures of the two bacterial rhodopsins: the two residues in HR which correspond to those thought to be responsible for de- and reprotonation in BR, Asp85 and Asp96 in helix C are a threonine and an alanine respectively. Consequently no protons are exchanged in HR in the ms time range between HR and the aqueous phases. Deprotonation does occur but on a slower time scale resulting in inactivation of the chloride pump because it takes minutes for thermal regeneration of the initial state of HR (Hegemann *et al.*, 1985a). Addition of azide to HR leads to catalytic exchange of protons between the aqueous phase and the Schiff base (Hegemann *et al.*, 1985b) and it was shown that the Schiff base is accessible to protons only from the cytoplasmic side (Lanyi, 1986). The same pathway of access for protons in mutant BR was demonstrated here. In both molecules reprotonation depends on external parameters, especially the pH (Hegemann *et al.*, 1985b; Butt *et al.*, 1989). Nevertheless under comparable conditions, i.e. the absence of azide, a remarkable difference in the rate constants is found. Both mutated BRs are regenerated faster by a factor of at least 100 compared to HR indicating that the structure of the latter protein is a more efficient proton insulator.

There is another interesting difference in reprotonation of the two pumps. As in all known retinal proteins, the intermediates of BR and HR are photochemically active. Blue light absorbed by the M intermediate of BR or HRL_{410} photochemically regenerates the respective initial states. Blue light induced reprotonation in HR generates a transient current of the same sign as chloride transport (Hegemann *et al.*, 1985a) thus proving the identical sidedness of photochemically induced proton uptake and azide catalysed

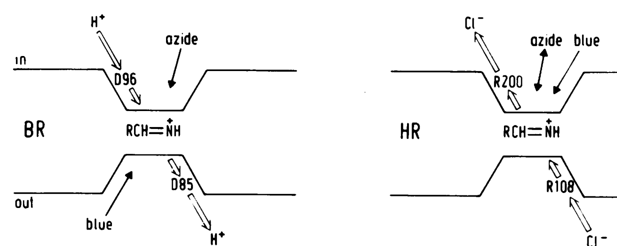

Fig. 7. Sidedness of the blue light effect in BR and HR. Selected amino acids are shown in a possible arrangement relative to the insulated retinal, i.e. the Schiff base within the membrane. The thin arrows indicate the direction of blue-light or azide-induced proton movement, whereas the double arrows indicate the movement of protons (**left**) or chloride (**right**) across the membrane including the amino acids which serve as donors and acceptors. In the case of hR the assignment of Arg108 and Arg200 to this function is hypothetical.

proton uptake in HR, i.e. from the cytoplasmic side where Asp96 is located in BR. Photochemical regeneration of BR from its M state, on the other hand, is linked to proton uptake from the same side to which it was released and consequently blue light inhibits BR (Ormos *et al.*, 1978). Thus blue light induced proton uptake in BR and HR are from opposite sides. A diagramatic representation of the various ion translocation pathways in BR and HR is presented in Figure 7.

Insulation of retinal within the protein

The described reactivation of a defective point mutated BR by application of azide provides insight into several features of proton translocation by BR. First it proves that the protonated Schiff base is sufficiently insulated within the membrane and not freely accessible from the bulk phase. Further evidence for this statement comes from the fact that in the wild-type BR the rate constants are unaffected by pH over a wide range. In BR the protonated residue Asp96 bridges the insulation barrier. In contrast HR lacks this residue and has no need for efficient reprotonation due to its function as a chloride pump. Consequently Asp96 can be regarded as a specific internal binding site for protons serving to reprotonate the Schiff base in the ms time range. This situation favours the view that the insulated retinal within the membrane serves as a proton switch during its isomerization cycle, whereas the vectorality of the transport is governed by the protein moiety. While Asp85 occurring deprotonated in the protein is involved in proton release from the Schiff base to the external side, Asp96 due to its chemical environment occurs protonated even at pH values >7 and thus is an effective proton donor for the Schiff base.

Mode of action of azide

The M decay involves transfer of a proton to the Schiff base and is seen as a spectroscopic red-shift. Apparently, wild-type and mutant protein structures have two different pathways for this reaction. Asp96 causes a higher activation barrier in the wild-type protein but a smaller change in entropy of activation resulting in a kinetic optimization. Azide functions to replace Asp96 in the mutant and might do so by one of three different mechanisms: (i) equal diffusion of azide and hydrogen azide to and from the Schiff base which is seen for HR and is best described as an internal protonophore. Since we do not have experimental evidence that azide catalytically enhances the proton transfer, i.e. also accelerates proton release to the cytoplasmic side, this mechanism is not yet proven; (ii) Hydrogen azide diffuses to the Schiff base, donates a proton and the resulting ion pair is stable until *cis* to *trans* isomerization of the retinylidene moiety has taken place and azide can be released; (iii) azide occupies a binding site, e.g. close to that where Asp96 is located in the wild-type, and accepts a proton from the outside and transfers it to the Schiff base. The raising of the entropy of activation by azide to the value found in wild-type points to the third possibility but none of these mechanisms is yet proven. More detailed investigations of the kinetic parameters will eventually distinguish between alternatives but care must be taken since alternative pathways of protonation might function at different pH values.

In summary the mutationally defective BRs and the restoration of their function by azide presents a unique opportunity for studying the details of ion movement within the structure of a proteinaceous energy converter.

Materials and methods

Purple membranes (PMs) containing mutated BR were isolated as described (Soppa and Oesterhelt, 1989; Soppa *et al.*, 1989).

All chemicals were of analytical grade and purchased from Merck, Darmstadt, FRG. FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone) was kindly provided by Dr P.Heytler (DuPont de Nemours & Co.) Monensin was a kind gift from Dr G.Szabo (University of Texas, Galveston, USA).

Flash photolytic experiments were performed with a polychromatic flash apparatus described previously (Uhl *et al.*, 1985) and a dye laser as actinic light source (FL 3001, Lambda Physics, Göttingen, FRG) tuned to 590 nm, (Rhodamine 6G).

Electrical transport studies were performed with PMs adsorbed on one side of the planar lipid membrane and with PMs oriented in an electrical field and subsequently immobilized in a polyacrylamide gel (Eisenbach *et al.*, 1977; Der *et al.*, 1985).

Optically BLMs with an area of $\sim 10^{-2}$ cm^2 were formed in a Teflon cell filled with an appropriate electrolyte solution (1.5 ml in each compartment). The membrane-forming solutions contained 1.5% (w/v) diphytanoyllecithin (Avanti Biochemicals, Birmingham, AL) and 0.025% (w/v) octadecylamine (Riedel-de-Haen, Hannover, FRG) in *n*-decane to obtain a positively charged surface (Dancshazy and Karvalhy, 1976). PM suspensions ($OD_{578} = 10$) were sonified for 1 min in a sonication bath and aliquots of 20 μl were added under stirring to the rear compartment. Photosensitivity of the samples developed in time and reached the maximal value after $\sim$40 min. The membrane was illuminated with a 250 W halogen lamp. Light reached the sample after passing through the front compartment. The irradiance of continuous light source was up to 2 W/cm^2 when filtered through a heat protecting glass and a 496 nm cut-off filter.

The membrane cell was connected to an external measuring circuit via Ag/AgCl electrodes which were separated from the Teflon cell by salt bridges. The current was measured with a current amplifier (Keithly, model 427). Stationary currents were obtained after addition of monensin and FCCP. Further details of this system are described in Bamberg *et al.* (1979).

The PMs from wild-type and mutant cells were oriented in an electric field and immobilized by polymerization of acrylamide contained in the sample according to Eisenbach *et al.* (1977) and Der *et al.* (1985). Gel pieces of $1.3 \times 8 \times 7$ mm were cut and bathed for 3 days in electrolyte solution. Platinized platinum electrodes protected from incident light were immersed on both sides of the gel into the bathing solution and connected with a current amplifier. The measured stationary current I^{∞} in this system is correlated with the light-induced proton translocation by BR. The cuvette was shielded in a thermostatted Faraday cage with a 7×7 mm window for the actinic light. The irradiance of continuous light was up to 2 W/cm^2 filtered by two heat protecting glasses and a 495 nm cut-off filter.

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