On the Azide Effect Regenerating the Proton Channel of Mutated Bacteriorhodpsins

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Effect of azide on proton transfer of bacteriorhodopsin (bR) mutants that lack the proton donor Asp-96 and/or proton acceptor Asp-85 was investigated by a photoelectrochemical method.In the presence of azide, D96N completely recovered the response profile of the wild type and D96N/D85N gave a very small current with the same amplitude as in D85N. On the contrary, D85N showed no azide effect. These results indicate that azide affects the process of shuttling protons from D96 to the Schiff base rather than does the proton transfer from the Schiff base to D85.

In the light-driven proton pump, bacteriorhodopsin (bR), proton is vectorially transferred from the cytoplasmic to the extracellular side. The active sites, located near the center of the protein, are the positively charged retinal Schiff base and its counterion that contains the anionic residue Asp-85 (D85).[1] The proton transfer in the protein begins with deprotonation of the Schiff base and protonation of D85. The latter simultaneously causes release of protons to the extracellular side via Glu-204 (E204)[2] and Glu-194 (E194).[3] The Schiff base is then reprotonated by the initially protonated Asp-96 (D96) located at the cytoplasmic region.[4] Reprotonation of D96 from cytoplasm[5] and reisomerization of the retinal follow at the end of the photocycle. Thus, D96 and D85 act as proton donor and acceptor, respectively, and are indispensable for the light-driven proton transfer.

We previously showed[6,7] that the bR-induced photocurrent at the electrode-aqueous electrolyte interface serves as a direct probe to elucidate the molecular mechanisms of proton pumping, of which the proton release and uptake ultimately effect the cathodic and anodic responses, respectively.[8]

Site specific mutagenesis revealed that replacement of D96 with Asn (D96N) extremely slows the rate of the photocycle (less than 1/1000 that of wild-type) due to inhibition of reprotonation from D96 to the Schiff base.[9] To cope this, use of azide has been found to directly aid the reprotonation of the Schiff base in the D96N mutant in which the photocycle is arrested in the M state[1] due to the absence of D96.[10] More interestingly, with halorhodopsin, a closely related light-driven chloride pump, azide can change its function into proton pump.[11,12] This made us consider thatthe azide addition may accelerate the rate of proton transfer of a specific process that is inhibited in some mutant, thus making it possible to locate the reaction site that azide affects.

In this communication, we report the effect of azide on the proton transfer processes within the mutants D96N, D85N, and D96N/D85N as elucidated photoelecrochemically.

The electrochemical cell was constructed by the procedure as reported previously.[8] The mutant proteins D96N, D85N, and D96N/D85N were received from Profs. R. Needleman and J. K. Lanyi. An aqueous suspension (OD ~ 1 at 5 60 nm) of each mutant was deposited on a conductive SnO_2 electrode to form a thin transparent layer. The aqueous supporting electrolyte was 0.1 M KCl that filled the cell compartment between the SnO_2 electrode and counterelectrode. Azide was added at a concentration up to 100 mM to the electrolyte. With buffer solutions such as Na phosphate and borate, pH of the electrolyte was adjusted to be pH 8.0 at which the maximum activity of proton pumping holds for the wild-type bR. A 300 W xenon arc lamp was used as a light source to excite the bR film. Photocurrent output was measured with a circuit comprising an operational amplifier that converts a small transient current into a dc voltage; the signal was recorded on a Hewlett Packard Model 54520C digital storage oscilloscope. All measurements were done at room temperature.

Figure 1 shows profiles of photoelectric response in the absence and presence of azide for D96N, D85N, and D96N/D85N, where either or both of the proton donor and acceptor are absent. The transient cathodic (positive) and anodic (negative) signals correspond to proton release and uptake, respectively, which are to be capacitive currents induced by a rapid surface potential shift at SnO_2 in response to a pH change in electrolyte.[13]

In the absence of azide, D96N gave a normal cathodic response but the anodic one was significantly reduced in amplitude. In D85N, the response amplitudes were totally suppressed to be about 50 times smaller than that in wild-type. The double mutant D96N/D85N did not give any response. This is because D96N drastically slows the reprotonation of the Schiff base to prolong the lifetime of M the intermediate to the order of seconds, whereas no proton transport occurs in D85N because the proton on the Schiff base is not removed during excitation.

In the presence of azide, the D96N mutant was found to rise a large anodic response and closely reproduce the amplitude of wild-type while the response of wild-type itself remained unchanged with the same azide concentration. The result agreed with those reported by Tittor et al.[10] However, totally distinct behavior from this was observed for the other D85 mutants.D85N did not cause any change in the response profile. The D96N/D85N double mutant produced a small photocurrent, which is comparable with the response amplitude of the D85N single mutant as shown in Figure 1. These results indicate that azide facilitates the proton transfer between the Schiff base and the cytoplasmic proton doner (D96) rather than the transfer between the Schiff base and the proton acceptor (D85). In this regard, Le Coutre et al. suggested that azide binds in the extracellular channel near D85 and accelerates thereprotonation of the Schiff base in D96N by restoring the hydrogen-bonded water structure between the Schiff base and D96.[14]

By comparison of the photoresponse behavior of D85N and D96N/D85N, it appears that the small response of D85N is

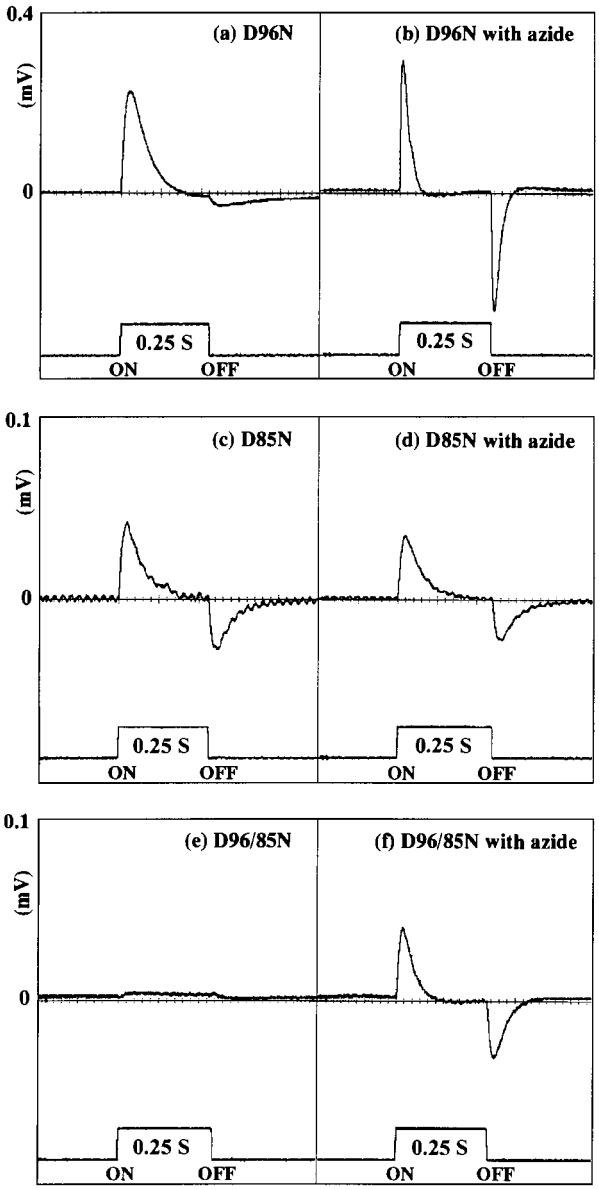

Figure 1. Response profiles of D96N, D85N and D96N/D85N immobilized at the interface of SnO_2 and electrolyte (0.1 M KCl, pH 8.0) without (a), (c), (e) and with azide (100 mM) (b), (d), (f) in an electrochemical cell. Responses are compared which arose from an equivalent amount of bR immobilized on SnO_2. The cell was irradiated 0.25 S with continuous green light supplied by a 300-W xenon arc lamp. Light intensity pattern of irradiation is given below the time course of response. The small transient photocurrent was converted into dc voltage using an operational amplifier.

possibly associated with D96 since the response disappears in D96N/D85N and recovers in the presence of azide. Despite many details still missing for description of the molecular mechanism,our result suggests that the azide-sensitive region is located at the proton transfer route through the cytoplasmic half channel.

On the basis of evidence from our electrochemical method using single and double mutants of D85 and D96, we propose that azide does not catalyze a proton transfer at the extracellular side but, instead of D96, acts as an intermediary in a proton transfer at the cytoplasmic side in the protein.

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