Lifetime of M Intermediate in the D96N Mutant of Bacteriorhodopsin Determined by a Photoelectrochemical Method

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The recombinant D96N mutant of bacteriorhodopsin (bR) was immobilized on SnO_2 electrode that contacts an aqueous electrolyte. By laser pulse excitation, photocurrent behavior of D96N was analyzed to evaluate the lifetime of the M intermediate involved in proton transfer reactions. The half-life of the M intermediate in the photocycle was about 5 s, which is 10^3-fold longer than that of wild-type and shows the crucial role of D96 in the uptake of protons.

The photocycle of bacteriorhodopsin (bR), the light-driven proton pump in *Halobacterium salinarum*, comprises a series of intermediates that is initiated by all-trans to 13-cis isomerization of retinal. The latter causes deprotonation of the retinal Schiff base, which stimulates proton release to the extracelullar side. The Schiff base is subsequently reprotonated by D96 that is located at the cytoplasmic side.[1]

The importance of D96 in the proton uptake process was first pointed out by Mogi et al.[2] They prepared a site-specific mutant by replacing D96 with Asn (D96N) and showed that D96N has no proton pumping activity due to accumulation of the M intermediate[3] in lack of proton donor. The decay of the M intermediate therefore correlates to the rate of proton uptake and reprotonation of the Schiff base.[4,5]

We have previously demonstrated[6,7] that the photocurrent behavior of bR at the electrode/electrolyte interface is a useful probe for the molecular mechanisms in the proton release and uptake processes that link to the M formation. In this paper, we report a photoelectrochemical approach to determine the lifetime of the M intermediate and the role of D96 in proton uptake reaction by using the mutant D96N.

D96N was expressed in *Halobacterium salinarum* by the procedure as described before.[9] The D96N mutant was suspended in pure water for film preparation. Construction of the electrochemical cell that immobilizes D96N basically follows our previously reported method.[8]

Light source was a 150 W xenon arc lamp and used with an infrared cut-off filter (Toshiba IRA-05) and a band-pass filter (HOYA G550). Photocurrent 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 Gould Model 420 and/or Hewlett Packard Model 54520C digital storage oscilloscope.

Figure 1 shows the profile of photoelectric response obtained for D96N. The transient positive (cathodic) and negative (anodic) signals by turning-on and off of light, respectively, represent capacitive currents induced by a surface potential shift at SnO_2 electrode, caused by the proton release and uptake reactions of the protein. The suppression of the negative response results from the lack of proton uptake activity in D96N. This, as well as, observed slow decay of the positive response compared to the wild-type[8] are assumed to give a long lifetime for the M intermediate (13-cis state) that lasts until the proton uptake recovers the initial state (all-trans state) of the D96N photocycle. However, the lifetime of M in D96N is not clear because lack of kinetic information on the recovery of the initial state.

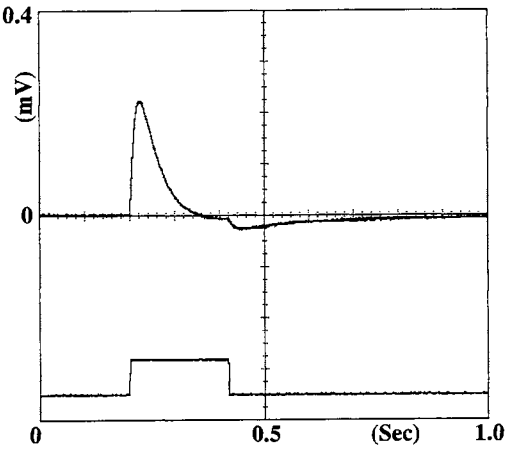

Figure 1. Response profile of D96N immobilized at the SnO_2/electrolyte (0.1 M KCl, pH 8.0) interface in an electrochemical cell. The cell was irradiated 0.25 s with continuous green light supplied by a 150 W xenon arc lamp. Light intensity pattern is given below the response. Photocurrent was converted into dc voltage (ordinate).

To estimate the lifetime of the M intermediate in D96N, we carried out kinetic investigations by laser pulse excitation. The D96N-immobilized electrochemical cell was excited with a 7-ns pulse (<10 mJ/cm^2) of the 532 nm second harmonics supplied by a Nd-YAG laser (Continuum Surelite I). Following the initial pulse excitation that causes substantially total conversion to the M intermediate, the cell was pulse-excited again with different time lags which allows for diminution of the intermediate. Because the M intermediate only absorbs in blue region (<500 nm, maximum at 412 nm), the response amplitude exhibits the population of the initial all-trans state D96N. We have previously confirmed that only all-trans state generates photocurrent.[10]

Figure 2 shows the response data collected with different time intervals (0.1 to 30 s) between two excitations. At a time interval of 0.1 s, a majority of D96N stays at M, which is detected with the 412 nm absorption of M. The response amplitude recovers with an increase in the time interval, i.e.,

with the decay of M and eventually the half-lifetime of M decay is around 5 s and completion of decay to recover the initial D96N takes more than 30 s. In contrast, the wild-type bR takes less than 10 ms to complete the photocycle. This shows that the D96 displacement with N retards the decay of the M intermediate by more than 10^3 times that of wild-type. We confirmed that the addition of azide (up to 100 mM) to electrolyte no longer retards the M decay of D96N and the recovery occurs even at interval of 0.1 s, which coincides with the results reported by Tittor et al.[11] and supports the fact that azide can participate in proton shuttling between the Schiff base and the bulk aqueous phase.[12]

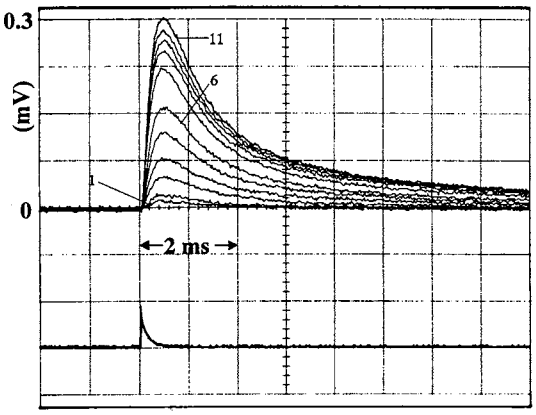

Figure 2. Dependence of the D96N response (second excitation) on the time interval after the first excitation, measured at pH 8.0. The profiles 1 through 10 were obtained at intervals of 0.1, 0.2, 0.5, 1, 3, 5, 10, 15, 20, 30 s after first excitation and 11 is the response of the first excitation. Laser pulse pattern is given below the response.

In Figure 3, it is shown that the M decay in the wild-type undergoes retardation when measured at pH 10. Similar to the case in D96N, wild-type bR was found to undergo retardation of the M decay by raising pH of the electrolyte. Knowing that the pKa of D96[13] is >11 and, at pH 10, D96 stays protonated and functions as proton donor, this phenomenon may not be due to deprotonation of D96. We consider that the low proton concentration in the bulk electrolyte is responsible for the observed retardation, that is, inefficiency in proton pumping.

We determined the lifetime of the M intermediate in D96-

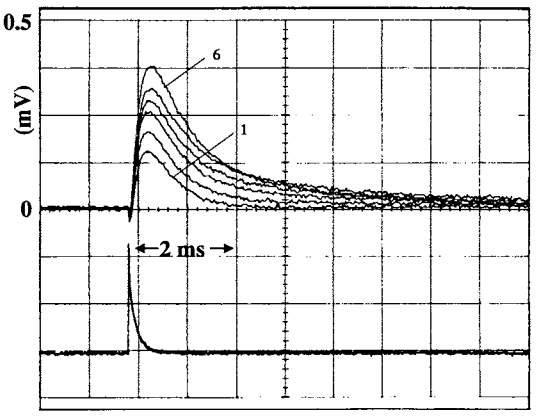

Figure 3. Dependence of wild-type response (second excitation) on the time interval, measured at pH 10. The profiles 1–5 and 6 correspond to 0.1, 0.2, 0.5, 1, 2 s and the first excitation, respectively. Laser pulse pattern is given below the response.

lacking mutant of bR and showed that D96N normally releases protons by illumination but takes much time until the next proton release occurs. Our photoelectrochemical evaluation demonstrates that D96 in the cytoplasmic half of bR is crucial to effect the reprotonation of the retinal Schiff base and play the key role in controlling the lifetime of the M intermediate.

References and Notes

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