Fourier transform infrared spectroscopic analysis of altered reaction pathways in site-directed mutants: the D212N mutant of bacteriorhodopsin expressed in *Halobacterium halobium*

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INTRODUCTION

The D212N mutant of bacteriorhodopsin (bR), when expressed in *H. halobium* and purified as purple membrane (PM_{D212N}), undergoes a pH-dependent shift in its absorption spectrum (1). The pK_a of this transition, which is not observed in wild-type PM, is ~6.7. The identity of the group undergoing titration is a mystery, because the protein has no histidines or cysteines and the shift in the chromophore absorption maximum is too small (~10 nm) to be associated with a retinylidene Schiff base protonation change.

It was shown with time-resolved visible difference spectroscopy (1) that the photochemical cycle of PM_{D212N} differs above and below this pK_a. The M_{412} photoproduct was detected only at lower pH values, where it was still significantly reduced relative to the wild type. At high pH, the only intermediates detected in the millisecond time range were 2 *L*-like species with identical visible absorption spectra, which were therefore distinguishable only on the basis of their kinetic properties. We have used static and time-resolved FTIR difference spectroscopy to examine further the light-induced structural changes of this mutant at various pH values. Analysis of these difference spectra yields a more detailed picture of the structural alterations induced by the D212N mutation in the various photointermediates than is possible with visible absorbance measurements alone.

MATERIALS AND METHODS

PM_{D212N} samples were generously provided by Janos Lanyi. Samples were washed in 20 mM TRIS-MES buffer before pelleting for IR spectroscopy. Spectra of the primary photoproduct were obtained at −193°C as described previously (2, 3), except that 0.25 × 6 mm diamond windows (Dubbeldee Harris Diamond Corp., NJ) were used instead of AgCl. Stroboscopic time-resolved FTIR (TR-FTIR) difference spectra (4) were obtained at 20°C using 0.69 ms temporal resolution and 4- cm^{-1} spectral resolution.

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RESULTS AND DISCUSSION

Primary photoproduct

The primary photoproduct difference spectra of PM_{D212N} (Fig. 1) are somewhat different from those previously obtained for the same mutant expressed in *E. coli* and reconstituted in halobacterial lipids (2, 5). Spectral features associated with the 13-*cis* component of dark-adapted bR (bR_{550}) and its primary photoproduct, which were seen in the earlier spectra (5), are not observed in Fig. 1. Evidently, expressing the D212N mutant in a somewhat rigid PM lattice helps to maintain the protein and chromophore in a normal light-adapted configuration.

The spectra in Fig. 1, *A* and *B*, obtained at pH values respectively below and above the pK_a of the visible transition, are generally quite similar to each other and to previously published spectra of the bR → K transition of wild-type PM (3, 6). The most obvious pH-dependent features are the negative bands at 1,255 and 1,275 cm^{-1}, both of which are smaller in the higher-pH spectrum. The 1,275- cm^{-1} band has been assigned previously to tyr-185 (7) and has been associated with a deprotonated tyrosine (8). Moreover, the 1,255 cm^{-1} band is also at a frequency that could be due to a tyrosine C-O vibration, but for a protonated tyrosine (8). The observation that raising the pH eliminates both of these bands is consistent with the idea that tyr-185 is strongly affected by the group being titrated, but is somewhat inconsistent with the idea that tyr-185 itself could be undergoing a titration. In particular, if the negative band at 1,275 cm^{-1} were due to the ionized form of a titratable tyr-185, it should be present at high pH and absent at low pH, which is exactly the opposite of what is observed. It is more likely that tyr-185 is protonated but strongly hydrogen bonded, possibly directly to the unknown group undergoing titration. This hydrogen bond is affected by the bR → K photoreaction, but only (or most strongly) when the unknown group is in a protonated state. The reason why the hydrogen bonding of tyr-185 would not also be affected by the bR → K reaction when the titratable group is deprotonated are not clear. However, the presence of bands in the wild type bR → K spectrum indicative of these same tyr-185 hydrogen

0006-3495/92/04/56/03 $2.00

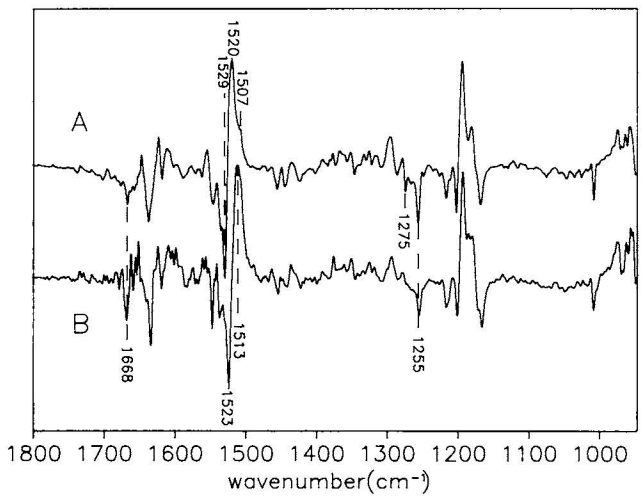

FIGURE 1 Primary photoproduct difference spectra of a PM_{D212N} sample at pH 6.0 (*top*) and at pH 8.0 (*bottom*). The sample temperature was −193°C. The absorbance of the sample measured under red illumination was subtracted from the absorbance of the same sample measured under green illumination as described previously (3). Spectral resolution was 2 cm^{-1}.

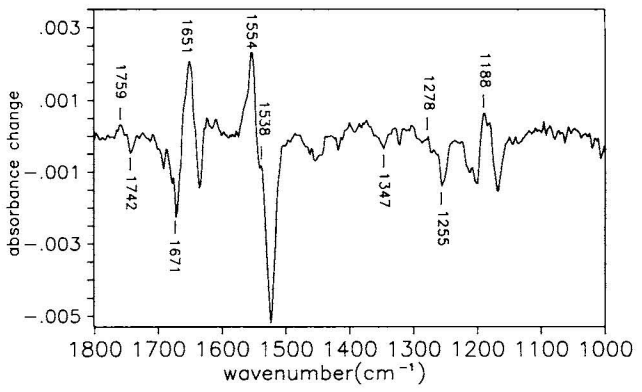

FIGURE 2 Time-resolved difference spectrum of a PM_{D212N} sample, measured at 20°C, pH 8, and ~50% water by weight. Data covering the time range 0.69–2.76 ms were averaged to calculate this spectrum.

bonding changes (5, 7) suggests that the unknown titratable residue is normally protonated in wild type PM in the physiological pH range. Thus its pK_a is higher in the wild type than in the D212N mutant. This is exactly as expected if asp-212 is normally ionized and exerts its effect through a simple electrostatic interaction which raises the relative free energy of the deprotonated state of a nearby ionizable residue.

L_2 photoproduct at high pH

The TR-FTIR data in Fig. 2 demonstrate that the photoproduct of PM_{D212N} that is present in the time range of 0.7–2 ms at pH 8.0 is probably *N*, rather than a second *L* species as suggested previously (1). The difference spectrum in Fig. 2 is nearly identical to that previously published for the bR → *N* reaction of wild-type bR at pH ≥ 9.0 (4, 9), and significantly different from time-resolved (10) and low-temperature (10–12) FTIR difference spectra of the bR → *L* photoreaction. The spectral features that most clearly distinguish Fig. 2 as a bR → *N* rather than bR → *L* spectrum are in the amide I and amide II regions (the negative band at 1,671 cm^{-1} and positive band at 1,554 cm^{-1}) but the absence of a negative band near 1,275 cm^{-1} and the presence of positive bands at 1,538 cm^{-1} and 1,759 cm^{-1} are also important indicators.

We have not obtained spectra of this mutant with sufficiently fast time resolution to analyze the L_1 intermediate detected with TR-visible absorbance measurements (1). However, the observed kinetic behavior of the L_1 and L_2 intermediates, and our current reinterpretation of the latter as *N*, suggests that in this mutant at high pH values there is a direct conversion of *L* to *N* (without any formation of *M*), or else a greatly slowed *L* → *M* decay rate, to a point where it is slower than the *M* → *N* rate, which results in an undetectable *M* concentration at all times.

CONCLUSIONS

At low pH, the bR → K reaction of PM_{D212N} proceeds essentially as with the wild type, and difference bands near 1,275 and 1,255 cm^{-1} are likely associated with changes in the hydrogen-bonding of tyr-185. Although high pH conditions in the PM_{D212N} mutant still allow a fairly normal bR → K reaction, the usual hydrogen bonding changes of tyr-185 are eliminated. Furthermore, at high pH the *L* → *M* decay rate appears to be considerably slowed. Thus the unidentified group with a pK_a of 6.5 may be needed as a proton donor during *M* formation. The group is almost certainly not tyr-185, but it is near to this residue (as well as to the chromophore and asp-212) and is perhaps involved in a hydrogen bond with tyr-185, at least in the D212N mutant.

This work demonstrates that static and time-FTIR difference spectroscopy can provide detailed information about altered reaction pathways in site-directed mutants of a membrane protein.

We are grateful to Janos Lanyi for the PM_{D212N} samples used, as well as for many useful discussions.

M. S. Braiman is a Lucille P. Markey Scholar, and this work was supported by a grant from the Lucille P. Markey Charitable Trust as

well as by National Institutes of Health Biomedical Research Support Grant 5-S07RR05431-30.

REFERENCES

1. Needleman, R., M. Chang, B. Ni, G. Váró, J. Fornés, S. H. White, and J. K. Lanyi. 1991. Properties of $asp^{212} \rightarrow$ asn bacteriorhodopsin suggest that asp^{212} and asp^{85} both participate in a counterion and proton acceptor complex near the Schiff base. *J. Biol. Chem.* 266:11478–11484.
2. Braiman, M. S., T. Mogi, T. Marti, L. J. Stern, H. G. Khorana, and K. J. Rothschild. 1988. Vibrational spectroscopy of bacteriorhodopsin mutants. II. Light-driven proton transport involves protonation changes of aspartic acid residues 85, 96, and 212. *Biochemistry.* 27:8516–8520.
3. Rothschild, K. J., P. Roepe, J. Lugtenburg, and J. A. Pardoen. 1984. Fourier transform infrared evidence for Schiff base alteration in the first step of the bacteriorhodopsin photocycle. *Biochemistry.* 23:6103–6109.
4. Braiman, M. S., O. Bousché, and K. J. Rothschild. 1990. Protein dynamics in the bacteriorhodopsin photocycle: Submillisecond Fourier transform infrared spectra of the L, M, and N photointermediates. *Proc. Natl. Acad. Sci. USA.* 88:2388–2392.
5. Rothschild, K. J., M. S. Braiman, Y.-W. He, T. Marti, and H. G. Khorana. 1990. Vibrational spectroscopy of bacteriorhodopsin mutants: Evidence for the interaction of aspartic acid 212 with tyrosine 185 and possible role in the proton pump mechanism. *J. Biol. Chem.* 265:16985–16991.
6. Siebert, F., and W. Mäntele. 1983. Investigation of the primary photochemistry of bacteriorhodopsin by low-temperature Fourier-transform infrared spectroscopy. *Eur. J. Biochem.* 130:565–73.
7. Braiman, M. S., T. Mogi, L. J. Stern, N. R. Hackett, B. H. Chao, H. G. Khorana, and K. J. Rothschild. 1988. Vibrational spectroscopy of bacteriorhodopsin mutants. I. Tyrosine-185 protonates and deprotonates during the photocycle. *Proteins: Struct. Funct. Genet.* 3:219–229.
8. Rothschild, K. J., P. Roepe, P. L. Ahl, T. N. Earnest, R. A. Bogomolni, S. K. Das Gupta, C. M. Mulliken, and J. Herzfeld. 1986. Evidence for a tyrosine protonation change during the primary phototransition of bacteriorhodopsin at low temperature. *Proc. Natl. Acad. Sci. USA.* 83:347–351.
9. Pfefferlé, J.-M., A. Maeda, J. Sasaki, and T. Yoshizawa. 1991. Fourier transform infrared study of the *N* intermediate of bacteriorhodopsin. *Biochemistry.* 30:6548–6556.
10. Chen, W.-G., and M. S. Braiman. 1991. Kinetic analysis of time-resolved infrared difference spectra of the *L* and *M* intermediates of bacteriorhodopsin. *Photochem. Photobiol.* 53: 905–910.
11. Roepe, P., P. L. Ahl, S. K. Das Gupta, J. Herzfeld, and K. J. Rothschild. 1987. Tyrosine and carboxyl protonation changes in the bacteriorhodopsin photocycle. 1. M_{412} and L_{550} intermediates. *Biochemistry.* 26:6696–6707.
12. Eisenstein, L., S.-L. Lin, G. Dollinger, K. Odashima, J. Termini, K. Konno, W.-D. Ding, and K. Nakanishi. 1987. FTIR difference studies on apoproteins. Protonation states of aspartic and glutamic acid residues during the photocycle of bacteriorhodopsin. *J. Am. Chem. Soc.* 109:6860–6862.