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Properties of Asp^{212} → Asn Bacteriorhodopsin Suggest That Asp^{212} and Asp^{85} Both Participate in a Counterion and Proton Acceptor Complex near the Schiff Base*

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Richard Needleman‡, Man Chang‡, Baofu Ni‡, György Váró§¶, José Fornés§‖, Stephen H. White§, and Janos K. Lanyi§**

From the §Department of Physiology and Biophysics, University of California, Irvine, California 92717 and the ‡Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201

The gene coding for bacteriorhodopsin was modified *in vitro* to replace Asp^{212} with asparagine and expressed in *Halobacterium halobium*. X-ray diffraction measurements showed that the major lattice dimension of purple membrane containing the mutated bacteriorhodopsin was the same as wild type. At pH > 7, the Asp^{212} → Asn chromophore was blue (absorption maximum at 585 nm) and exhibited a photocycle containing only the intermediates K and L, *i.e.* a reaction sequence very similar to that of wild-type bacteriorhodopsin at pH < 3 and the blue form of the Asp^{85} → Glu protein at pH < 9. Since in the latter cases these effects are attributed to protonation of residue 85, it now appears that removal of the carboxylate of Asp^{212} has similar consequences as removing the carboxylate of Asp^{85}. However, an important difference is that only Asp^{85} affects the pK_a of the Schiff base. At pH < 7, the Asp^{212} → Asn protein was purple (absorption maximum at 569 nm) but photoexcitation produced only 15% of the normal amount of M and the transport activity was partial. The reactions of the blue and purple forms after photoexcitation are both quantitatively accounted for by a proposed scheme, K ↔ L_1 ↔ L_2 → BR, but with the addition of an L_1 ↔ M reaction with unfavorable pK_a for Schiff base deprotonation in the purple form. The latter hinders the transient accumulation of M, and the consequent branching at L_1 allows only partial proton transport activity. The results are consistent with the existence of a complex counterion for the Schiff base proposed earlier (De Groot, H. J. M., Harbison, G. S., Herzfeld, J., and Griffin, R. G. (1989) *Biochemistry* 28, 3346–3353) and suggest that Asp^{85}, Asp^{212}, and at least one other protonable residue participate in it.

Illumination of the light-driven proton pump, bacteriorhodopsin, causes a cyclic sequential reaction via the spectroscopically distinct intermediate states J, K, L, M, N, and O. The two key events in this reaction are the loss of the retinal Schiff base proton to the extracellular side, resulting in the M state, and the subsequent reprotonation of the Schiff base from the cytoplasmic side. At least two aspartate residues participate in this process. Asp^{85} is in a hydrophilic region of the protein which opens to the extracellular medium (Henderson *et al.*, 1990); this is the residue involved in the proton release phase of the transport (Braiman *et al.*, 1988; Gerwert *et al.*, 1989, 1990; Butt *et al.*, 1989; Stern *et al.*, 1989). Asp^{96}, on the other hand, is occluded in a hydrophobic region located nearer the cytoplasmic side (Henderson *et al.*, 1990); this residue functions as the proton donor to the Schiff base (Gerwert *et al.*, 1989, 1990; Butt *et al.*, 1989; Holz *et al.*, 1989; Tittor *et al.*, 1989; Otto *et al.*, 1990; Stern *et al.*, 1989).

The proposed role for Asp^{85} is supported by much self-consistent evidence. Its replacement with asparagine produces a blue chromophore (with a red-shift of 33 nm, Mogi *et al.*, 1988; Subramaniam *et al.*, 1990; Otto *et al.*, 1990) whose Schiff base will not deprotonate effectively during illumination (Stern *et al.*, 1989). As expected, proteins containing any nonprotonable replacement at Asp^{85} will have the same properties as the wild type with Asp^{85} protonated (Subramaniam *et al.*, 1990; Otto *et al.*, 1990). Replacement of Asp^{85} with glutamate on the other hand raised the pK_a for reaching this state.[1] Thus, Asp^{85} and the Schiff base seem to have an intimate and unique relationship: the anionic Asp^{85} not only determines the purple color of the chromophore but functions as a counterion which stabilizes the protonated Schiff base and the residue which accepts the Schiff base proton during the L → M reaction.

Asp^{212} is also located on the extracellular side of the Schiff base, and at about the same distance from the NH$^+$ group as Asp^{85} (Henderson *et al.*, 1990). It may be protonated in the second half of the photocycle (Braiman *et al.*, 1988; Gerwert *et al.*, 1990). Yet little could be said definitively about the involvement of this residue in the chromophore and proton transfer. Its replacement with asparagine resulted in a chromophore with some unexpected properties. 1) Light adaptation, which normally converts an equilibrium mixture of 13-*cis* and all-*trans* bacteriorhodopsins to 100% all-*trans* and thereby causes a 10-nm red-shift in the absorption maximum, caused a blue-shift in the mutant (Mogi *et al.*, 1988; Subramaniam *et al.*, 1990). In the Asp^{212} → Glu mutant at least, this was related to the fact that it was the dark-adapted chromophore which contained nearly entirely all-*trans* retinal, while light adaptation gave a mixture of isomers (Duñach

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¶ Permanent address: Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary.

‖ Fellow of the Brazilian National Research Council (CNPq-Brazil). Permanent address: Instituto de Matemática e Fisica, Universidade Federal de Goiás, Bl.IMF-2-74000-Goiânia, 60, Brazil.

** To whom correspondence should be addressed. Tel.: 714-856-7150; Fax: 714-856-8540.

[1] J. K. Lanyi, J. Tittor, G. Váró, G. Krippahl, and D. Oesterhelt, manuscript in preparation.

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et al., 1990). The Fourier transform infrared ethylenic stretch depletion band of Asp^{212} mutants also suggested a mixture of all-*trans* and 13-*cis* chromophores (Rothschild *et al.*, 1990). 2) Since Asp^{212} carries a negative charge as does Asp^{85}, its replacement might have been expected to have had a similar effect on the chromophore as replacement of Asp^{85}. However, the light-adapted Asp^{212} → Asn protein absorbed to the blue of the wild type rather than to the red (Mogi *et al.*, 1988; Subramaniam *et al.*, 1990; Stern *et al.*, 1989). 3) Unlike the Asp^{85} → Asn mutant whose Schiff base deprotonated in the dark with a pK_a of 7 (Otto *et al.*, 1990), the Asp^{212} → Asn chromophore was unchanged at least up to pH 10 (Subramaniam *et al.*, 1990). 4) Even though the characteristic purple-blue transition at acid pH was attributed to protonation of Asp^{85} rather than Asp^{212}, the Asp^{212} → Asn protein did not show the spectral shift at low pH (Subramaniam *et al.*, 1990). 5) Unlike the wild type, the Asp^{212} → Asn chromophore was destabilized by illumination (Marinetti *et al.*, 1989; Duñach *et al.*, 1990). 6) Light-dependent deprotonation of the Schiff base occurred under some conditions, but the amount of M in the photocycle was much less than in wild type (Otto *et al.*, 1990; Stern *et al.*, 1989; Rothschild *et al.*, 1990), as was transport activity (Mogi *et al.*, 1988; Marinetti *et al.*, 1989). 7) With the Asp^{212} → Asn protein in detergent micelles, the time-course of absorption change at 650 nm resembled that in a mutant with Asp^{85} replacement (Otto *et al.*, 1990), but the same protein in a lipid-reconstituted sample had quite different absorption changes in this wavelength region (Stern *et al.*, 1989). On the basis of these findings, it was suggested (Otto *et al.*, 1990; Subramaniam *et al.*, 1990) that Asp^{212} must play an important role in the chromophore, perhaps as an alternate counterion to the Schiff base, and that its presence is required for efficient proton release in the first half of the photocycle. A structural role for Asp^{212} seemed to be indicated by the instability of the Asp^{212} mutants in general, the changed light-adaptation behavior (Mogi *et al.*, 1988; Duñach *et al.*, 1990), and the greatly slowed rate of retinal reconstitution as compared with wild type (Mogi *et al.*, 1988). In particular, hydrogen bonding between Tyr^{185} and Asp^{212} was suggested to stabilize the relative orientations of helices F and G (Rothschild *et al.*, 1990).

Nevertheless, the specific function of Asp^{212} has remained elusive. Furthermore, these observations were obtained with mutated bacteriorhodopsin expressed in *Escherichia coli*. We have found[1] that when Asp^{85} was replaced with glutamate, but as an *in vivo* mutation in a halobacterial species, the chromophore had different properties than the same protein produced in *E. coli*. The apparent cause for this was that the latter does not readily assemble into two-dimensional crystalline arrays ("purple membranes"). It seems reasonable to assume that there is less gross structural alteration in mutated bacteriorhodopsins when they form these arrays. Since a *Halobacterium halobium* expression system is now available (Ni *et al.*, 1990), we constructed the Asp^{212} → Asn bacteriorhodopsin with this method and examined its properties in purple membrane-like arrays. As much as possible, we had attempted to interpret the data in terms of specific effects on individual steps of the photocycle. The results, given in this report, remove any uncertainties in the earlier data which may be due to the expression system. Our conclusion is that replacing this residue does not greatly perturb protein structure and produces a chromophore with very similar properties to those one finds after Asp^{85} is replaced or protonated. This indicates a close functional interaction between Asp^{212} and either the Schiff base or Asp^{85}, or more likely both, consistent with participation of these residues in the multicomponent counterion complex proposed earlier on other grounds (De Groot *et al.*, 1989, 1990). The involvement of a third protonable amino acid residue is suggested by the appearance of pH dependence in the Asp^{212} → Asn protein, both for chromophore absorption and Schiff base deprotonation.

MATERIALS AND METHODS

Strains, Growth Conditions, Sample Preparation, Transport Assay—The clone which produced Asp^{212} → Asn bacteriorhodopsin was constructed by transformation of *H. halobium* L-33, which lacks carotenoids and gas vacuoles and contains the stable insert ISH2 (DasSarma *et al.*, 1984) in the *bop* gene which encodes bacteriorhodopsin. For controls, *H. halobium* S-9 was used. The cells were grown in a medium containing the following per liter: 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 3 g of citric acid trisodium salt, dihydrate, 2 g of KCl, 0.2 g of $CaCl_2$, 10 g of Oxoid peptone, 50 mM Tris·HCl, and a 10,000 × dilution of a trace metals solution. The latter consisted of 1.3 g of $ZnSO_4 \cdot 7H_2O$, 0.3 g of $MnSO_4 \cdot H_2O$, 0.8 g $Fe(NH)_4SO_4 \cdot 6H_2O$, 0.15 g of $CuSO_4 \cdot 5H_2O$ in 200 ml of 0.1 N HCl. Where required, mevinolin (a generous gift of Merck Sharp and Dohme) was added to the autoclaved medium from an ethanol stock. Bacteriorhodopsin was prepared from cultures of 1–9 liter medium without mevinolin, grown as described before (Oesterhelt and Stoeckenius, 1974), under normal laboratory illumination. It was collected from sucrose gradients at the high buoyant density characteristic of purple membrane, which has a lower lipid/protein ratio than the rest of the cytoplasmic membrane. The circular dichroism band in the visible exhibited the bilobe shape characteristic of wild-type bacteriorhodopsin in purple membrane (*e.g.* Heyn *et al.*, 1975; Ebrey *et al.*, 1977), indicating that the mutation did not abolish the trimeric arrangement within the array. The yield of purified Asp^{212} → Asn bacteriorhodopsin was about 18 mg/liter culture; this is comparable to the highest yields of the wild-type protein achieved. The purple membrane samples were in 100 mM NaCl, 50 mM phosphate, at the pH indicated. Some of the measurements were with samples encased in polyacrylamide gel (Mowery *et al.*, 1979) because at pH near 4 the membranes aggregated.

Cell envelope vesicles were prepared by the sonication method (Lanyi and MacDonald, 1979) and were stored in 4 M NaCl. One hour before the proton transport assays, they were diluted 1:30 into 3 M KCl in order to load them with KCl and thereby eliminate effects from sodium/proton antiport; light-induced pH changes were measured at various light intensities as before (Váró *et al.*, 1990). Interference from halorhodopsin was minimized by performing the assays at pH 5.5. Incident light intensity was determined with a thermopile. The bacteriorhodopsin contents of the vesicles were estimated from light-adapted *minus* dark-adapted difference spectra (as in Bogomolni *et al.*, 1980) in a 2-mm cuvette. It was assumed that the molar extinction of the purple form of the mutated protein is not so different from that of wild-type as to introduce a significant error; the maximal amplitude change upon light adaptation was about 60% of the wild type (*cf.* below).

Mutagenesis—A 2.7-kilobase-long *Bam*H3-*Hin*dIII fragment containing bop (a generous gift of D. Oesterhelt, Max Planck Institute for Biochemistry, Martinsried, Germany) was cloned into M13mp19. Site-directed mutagenesis was according to Nakamaye and Eckstein (1986), using a kit from Amersham Corp. After identification of the mutation, the coding region of the gene was sequenced to confirm that no additional mutations had occurred.

Plasmids—The expression plasmids were derivatives of pWL102 (Lam and Doolittle, 1989). The unique *Kpn*I and *Xba* sites were removed along with the adjacent *Hin*dIII site to form plasmid pRN2357. The mutated M13mp19 replicating form was cut with *Bam*H3 and *Hin*dIII and cloned into pUC18 to form pRN2390. The *Eco*RI-*Hin*dIII fragment containing the Asp^{212} → Asn *bop* gene from pRN2390 was then inserted into pRN2367 which had been cut with *Eco*RI and *Hin*dIII, to form pMC1D212N.

Transformation—The method described earlier (Cline and Doolittle, 1987; Ni *et al.*, 1990) was used, with some modifications. The major change was the use of increased amounts of DNA. L33 cells were grown to a density of about 5×10^9/ml. One ml of culture was centrifuged and suspended in 200 μl of spheroplasting buffer (2 M NaCl, 27 mM KCl, 50 mM Tris·HCl, pH 8.75, 15% sucrose), and 20 μl 0.5 M EDTA in the same solution was added. When microscopic examination indicated that all of the cells were converted to spheroplasts, 200 μl was transferred to a tube containing 1–10 μg of DNA in 20 μl of spheroplasting solution plus 0.5 M EDTA. After incubating

20 min at room temperature, 220 μl of filter-sterilized polyethylene glycol solution (60%, v/v, purified polyethylene glycol 600 in unbuffered spheroplasting solution) was added with gentle but thorough mixing. Standing for 20 min at room temperature was followed by addition of 1 ml of regeneration salt buffer (4.3 M NaCl, 80 mM $MgSO_4$, 27 mM KCl, 1.4 mM $CaCl_2$, 10 mM sodium citrate, 50 mM Tris·HCl, pH 7.2, 15% sucrose) and centrifugation for 15 min at 20% maximum speed in a Sorvall-Du Pont 24S microcentrifuge. The supernatant was removed, 1 ml of growth medium containing 15% sucrose was added, and the culture was incubated overnight, without shaking, at 37 °C. Aliquots were then plated in 3-ml regeneration soft agar overlays (growth medium with 0.8% agar, 15% sucrose) onto plates containing the same medium with sucrose but 1.5% agar. Mevinolin (5 μg/ml) was added to both bottom and top layers. After about 2 weeks of incubation at 37 °C, a stereo microscope was used to pick single colonies; these were transferred to hard agar plates containing growth medium and mevinolin as above.

X-ray Diffraction—The diffraction experiments were carried out at room temperature (23 °C) using nickel-filtered CuK_α radiation produced by a Jarrell-Ash Microfocus x-ray generator operating at 250 watts. Diffraction patterns were recorded on Kodak DEF-5 film using an Elliot (1965) toroidal camera with D-sector optics. Good diffraction patterns were obtained with 12–16-h exposures. Partially oriented samples were prepared by painting small amounts of pelleted membranes on the outer surface of a 1-mm x-ray capillary with a small spatula. The sample was allowed to dry and then equilibrated under argon for 24 h in a sealed chamber containing a saturated aqueous $NaNO_2$ solution to maintain the relative humidity at 66%. The 1-mm capillary was then sealed inside a 1.5-mm capillary containing a drop of the same saturated salt solution and allowed to equilibrate for another 24 h. Membrane pellets were prepared from dispersions of purple membranes suspended in 0.1 M sodium acetate buffer, pH 5.0, by centrifuging 1 h at 200,000 × *g*. Line spacings for calculating interplanar distances were determined directly from the films.

Spectroscopy—Spectra were measured with a Shimadzu UV-250 spectrophotometer, in the dark-adapted state (incubation overnight in the dark at room temperature) and in the light-adapted state (illumination for 5 min with white light from a 150-watt tungsten-halogen lamp). Time-resolved difference spectra were determined on light-adapted samples with a gated optical multichannel analyzer, as before (Zimányi *et al.*, 1989). Single wavelength measurements were with a dual-beam transient spectrophotometer described before (Váró and Lanyi, 1990a). All spectroscopy was at room temperature. Spectral and kinetic analysis was as before (Váró and Lanyi, 1990b), but with a program for numerical integration written in gwbasic.

RESULTS AND DISCUSSION

Crystalline Structure of Purple Membranes Containing Asp^{212} → Asn Bacteriorhodopsin—X-ray diffraction of semi-dry, partially oriented purple membranes from wild type and mutant showed both to be highly ordered and to have P3 hexagonal unit cells. As shown in Table I, the lattice constants for the two types of membranes are identical within experimental error and are in excellent agreement with the results of Henderson (1975) and Blaurock (1975) for wild-type bacteriorhodopsin. Densitometric traces of the films are given in Fig. 1. While not examined in detail, the relative intensities of the reflections are approximately equivalent. Inasmuch as the lattice formed by the Asp^{212} → Asn protein is like wild type, its three-dimensional structure cannot have been greatly perturbed.

Spectrum of the Asp^{212} → Asn Bacteriorhodopsin—Fig. 2*A* shows absorption spectra for a dark-adapted and light-adapted sample in 100 mM NaCl at pH 8. As in wild-type bacteriorhodopsin, the maximum of the chromophore band shifted about 9 nm *in the red direction* upon light adaptation; the extinction also increased as in wild type although only by 50–60% as much. The light-adapted maximum was at 584 nm, which is 16 nm *red-shifted* from wild type. These results are the opposite of what had been expected from results with the protein expressed in *E. coli*, but consistent with earlier arguments (Lanyi *et al.*, 1988) that Asp^{212} should function as at least part of the counterion to the Schiff base; the external point-charge model of rhodopsins (Nakanishi *et al.*, 1980; Spudich *et al.*, 1986) predicts that removal of a negative charge from the immediate vicinity of the Schiff base will cause a shift toward longer wavelengths, as found.

The absorption maximum of the Asp^{212} → Asn chromophore was dependent on pH. Fig. 2*A* contains dark-adapted and light-adapted spectra also at pH 4; both of these are *blue-shifted* relative to the spectra at pH 8. Fig. 2*B* shows that the light-adapted and dark-adapted absorption maxima both exhibit the blue-shift at lowered pH, with an apparent pK_a of 6.7. Above this pH the light-adapted maximum was at 585 nm, and below it was at 569 nm. The phenomenon was observed in 100 mM NaCl and in 200 mM Na_2SO_4 solutions, as well as in 4 M NaCl, but in the latter case the apparent pK_a was 8.0 (not shown). All spectral changes upon illumination and pH shift were reversible.

On the one hand, the pH dependence in Fig. 2 confirms the earlier result (Subramaniam *et al.*, 1990) that the spectrum of the Asp^{212} → Asn bacteriorhodopsin did not change in the alkaline range up to pH 10. This is unlike the behavior of the Asp^{85} → Asn protein in which the Schiff base pK_a is lowered to 7, as detected by shift of the absorption band to 410 nm above this pH (Otto *et al.*, 1990). Thus, even though Asp^{212} and Asp^{85} both appear to have counterion-like effects on the NH^+ group (which implies electrostatic interaction), only Asp^{85} affects its pK_a (which implies hydrogen bonding). On the other hand, in contrast with earlier findings (Subramaniam *et al.*, 1990) we observe a second spectral species at lower pH, whose maximum is similar to wild type (Fig. 2*B*). Indeed, transient absorption changes at 410 nm (Fig. 2*C*) indicated that this species, but not the 585 nm form, produces M upon illumination. However, the amount of M which had accumulated transiently was only about 15% of what is observed under similar conditions in wild-type bacteriorhodopsin (*cf.* also below). At pH 4–5, the decay of M was biphasic, but at pH ≥6 the amplitude of the slower process disappeared (Fig. 2*C*), in contrast with the simple M decay kinetics of the wild-type protein in this pH region.

At still lower pH, the chromophore was unstable: unlike the wild type, the Asp^{212} → Asn protein was destroyed at pH 2, when set with either HCl or H_2SO_4 in the absence of salts (not shown). Presumably, at this low pH Asp^{85} is protonated as in wild type, but lack of an ionized residue at position 212 now leaves the Schiff base charge entirely uncompensated. A requirement for at least 1 ionized carboxyl residue for chromophore stability was suggested by Subramaniam *et al.* (1990) because the chromophore of the Asp^{212,85} double mutant would not reconstitute. Unexpectedly, our attempts to produce monomeric Asp^{212} → Asn protein failed because incubation with 2% Triton X-100 or 1% octylglucoside rapidly bleached the chromophore even in the dark.

Proton Transport by Asp^{212} → Asn Bacteriorhodopsin—The initial rate of light-driven proton extrusion was measured in cell envelope vesicles containing either wild-type bacteriorhodopsin or the mutant. Under the conditions used, the purple form of the mutated protein predominated. At very low light intensities where the response for both systems was in the linear range (*e.g.* at 0.25 milliwatt/cm^2), the rate of transport/min was 2.9 ± 0.2 H^+/bacteriorhodopsin for the wild-type protein and 1.0 ± 0.1 H^+/bacteriorhodopsin for the mutant (not shown). Thus, the Asp^{212} replacement reduced proton transport activity to about one-third.

Photocycles of the Two Spectral Forms of Asp^{212} → Asn Bacteriorhodopsin—Fig. 3, *A* and *B*, shows measured time-resolved difference spectra after flash excitation of the Asp^{212}

TABLE I

Lattice parameters (in angstroms) for purple membranes containing wild-type and Asp^{212} → Asn bacteriorhodopsin

Miller indices	Wild-type		Asp^{212} → Asn	
	Interplanar distance	Lattice parameter	Interplanar distance	Lattice parameter
(1, 0)	53.09	61.30	54.42	62.84
(1, 1)	31.12	62.24	31.12	62.24
(2, 0)	26.73	61.73	26.90	62.12
(2, 1)	20.48	62.57	20.19	61.68
(3, 1)	15.02	62.53	14.81	61.66
(4, 0)	13.50	62.35	13.26	61.25
(4, 1)	11.81	62.49	11.77	62.28
(5, 0)	10.75	62.07	10.75	62.07
(4, 2)	10.07	61.53	10.02	61.22
(5, 1)	9.43	60.63	9.55	61.40
(4, 3)	8.74	61.39	8.80	61.81
(6, 1)	8.23	62.32	8.17	61.86
(7, 0) (5, 3)	7.67	62.00	7.62	61.59
(7, 1)	7.10	61.90	7.09	61.81
Average lattice parameter: (S.D.)		61.9 (0.5)		61.8 (0.4)

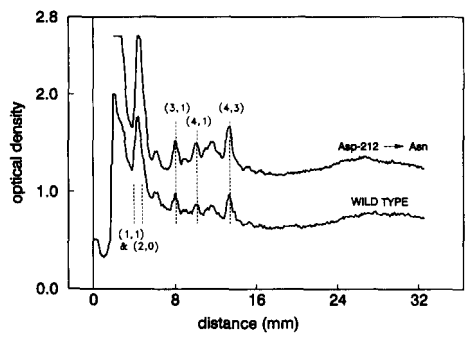

FIG. 1. **Densitometer scans of x-ray films from diffraction experiments on purple membranes from wild-type *H. halobium* and the Asp^{212} → Asn mutant.** The scans are from the second film in a stack of four. The scan of the mutant has been shifted upward by 0.6 optical density units for clarity. The Miller indices for the principal diffraction peaks assuming a P3 hexagonal lattice are indicated. The *(1,1)* and *(2,0)* peaks are not separable in these scans but can be identified visually on the films. The *(1,0)* peak is relatively small and is obscured in these scans by parasitic scatter.

→ Asn protein in 100 mM NaCl at pH 8, *i.e.* in its blue form. They bear some resemblance to difference spectra reported earlier for this mutant (Stern *et al.*, 1989). Up to a few microseconds, they are similar to the difference spectra for the wild-type chromophore (*e.g.* Váró and Lanyi, 1990b), but after this time absorption increase near 410 nm is virtually absent, consistent with the results in Fig. 2*C*, and between 400 μs and 6 ms the spectra hardly change. The quantum yield was approximately the same as for wild-type bacteriorhodopsin. As described before (Váró and Lanyi, 1990b), the difference spectra were used to calculate component absorption spectra, and the measured spectra were then decomposed into sums of these to give the time-resolved concentration changes. The assumptions in this kind of analysis are discussed in the earlier report. The decomposition left residuals without persistent structure and within the noise level (not shown). The measured difference spectra could be described with two spectroscopic species only, with absorption spectra nearly identical to those of the K and L states of wild-type bacteriorhodopsin (Fig. 4*A*). The kinetics of the interconversions of these states contained a time constant for the initial K-to-L transition, a second for the L → BR reaction, and a third between the two (Fig. 4*B*). The additional time constant indicates the existence of an additional kinetic entity. The

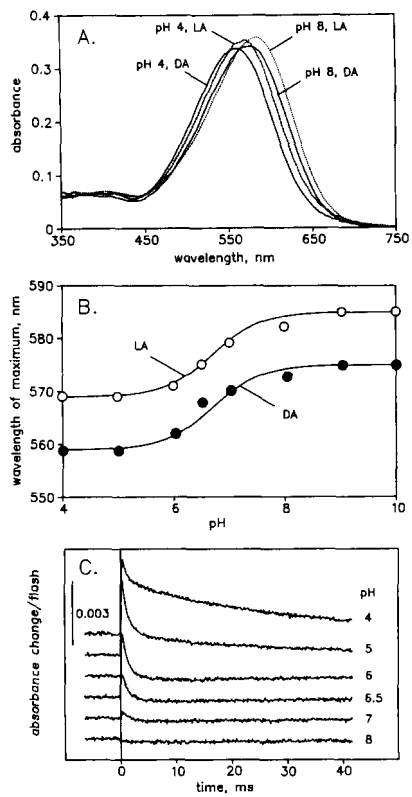

FIG. 2. **Absorption spectra of dark-adapted and light-adapted Asp^{212} → Asn bacteriorhodopsin, and light-induced absorbance changes at 410 nm, at various pH.** Conditions: 100 mM NaCl, 50 mM phosphate at the indicated pH. *DA*, dark-adapted; *LA*, light-adapted protein. *A*, absorption spectra at pH 4 and pH 8. *B*, absorption maxima at various pH. ●, dark-adapted; ○, light-adapted. *C*, time course and amplitudes of absorbance changes after flash excitation at various pH. The flash was at time zero. The amplitudes at pH 4–5 were about 15% of that in a wild-type sample under the same conditions.

simplest model which accounts for the results includes a second L-like state, *i.e.* K ↔ L_1 ↔ L_2 → BR. Fig. 4*B* shows the fit of this model. The second L state is analogous to the second M state in wild-type bacteriorhodopsin. Although the L_2-to-L_1 back-reaction is significant, unlike the M_2-to-M_1 back-reaction, it seems likely that this step reflects a confor-

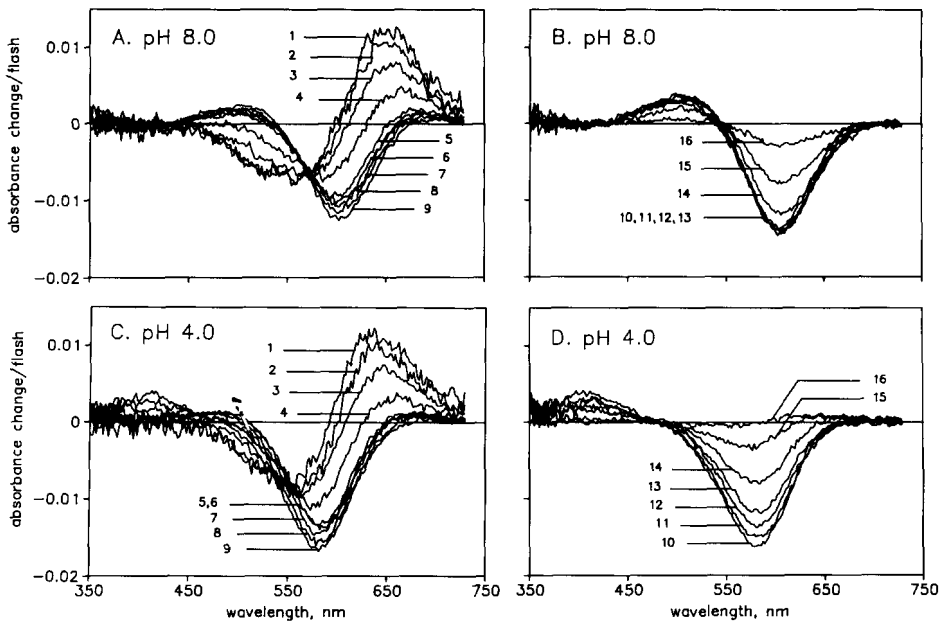

FIG. 3. **Measured time-resolved difference spectra of the blue (*A* and *B*) and purple (*C* and *D*) forms of Asp^{212} → Asn bacteriorhodopsin.** Conditions were as in Fig. 1*A*; the samples were light-adapted. Delay times after photoexcitation are indicated by *numbers*. From *1* through *16*, these were: 100 ns, 250 ns, 600 ns, 1.5 μs, 4 μs, 10 μs, 25 μs, 60 μs, 150 μs, 400 μs, 1 ms, 2.5 ms, 6 ms, 15 ms, 40 ms, 100 ms.

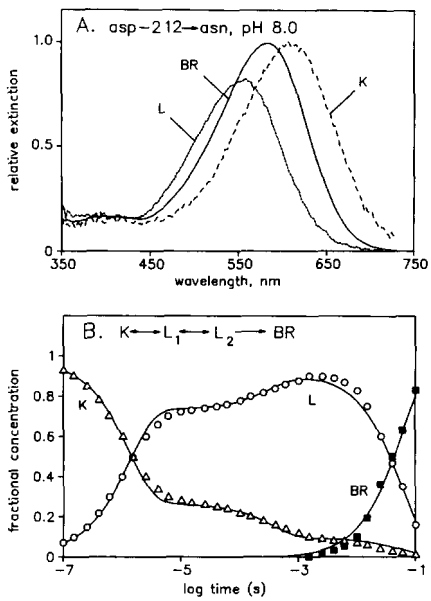

FIG. 4. **Calculated absorption spectra of the photointermediates of Asp^{212} → Asn bacteriorhodopsin and their time-dependent concentrations after photoexcitation at pH 8.** The spectra and kinetics were derived from data such as that in Fig. 2, *A* and *B*. *A*, ——, bacteriorhodopsin (*BR*); -----, L-like intermediate; – – –, K-like intermediate. The spectra were averaged and smoothed. *B*, △, K; ○, L (*i.e* L_1 + L_2); ■, BR. The *line* describes the model shown with the following rate constants: $k_{KL1} = 5.55 \times 10^5\ s^{-1}$; $k_{L1K} = 2.0 \times 10^6\ s^{-1}$; $k_{L1L2} = 2.5 \times 10^3\ s^{-1}$; $k_{L2L1} = 1.0 \times 10^3\ s^{-1}$; $k_{L2BR} = 25\ s^{-1}$.

mational change such as suggested (Fodor *et al.*, 1988; Váró and Lanyi, 1990c)[2] to occur also in wild type when access of the Schiff base is changed from Asp^{85} to Asp^{96} during proton translocation. If this is so, the protein is destined to undergo a conformational transition in the photocycle whether the Schiff base deprotonates or not.

A photocycle which contains only K and L has been described in two other systems: wild-type bacteriorhodopsin at pH < 2 (Mowery *et al.*, 1979; Kobayashi *et al.*, 1983; Váró and Lanyi, 1989) and the blue form of Asp^{85} → Glu bacteriorhodopsin at higher pH.[1] Much evidence indicates (*cf.* also Butt *et al.*, 1989; Subramaniam *et al.*, 1990; Otto *et al.*, 1990) that in both cases residue 85 had been protonated, and this is what prevented the deprotonation of the Schiff base and resulted in the truncated photocycle. Blocking Schiff base deprotonation apparently allows recovery of BR from L. Because the same effect is now observed after replacement of Asp^{212}, we must conclude that in the blue form of Asp^{212} → Asn bacteriorhodopsin proton transfer from the Schiff base to Asp^{85} is as dependent on the carboxylate group of Asp^{212} as on the carboxylate of Asp^{85}.

A similar measurement of time-resolved spectra was performed with the purple form of the Asp^{212} → Asn protein, in 100 mM NaCl at pH 4. Fig. 3, *C* and *D* shows the measured difference spectra. It is evident that the early spectra resemble those of the blue form (Fig. 3, *A* and *B*), but after about 15 μs absorbance increase at 410 nm indicates that a small amount of M is present, and absorbance increase above 600 nm at the end of the photocycle indicates the formation of O. The small amplitude of M, and the intermediate(s) which follow it, distinguish these spectra from those of the wild-type photocycle, however. The derived component spectra (Fig. 5*A*) consist not only of K and L, as for the blue form, but also of M, N, and O (the latter two are not shown as their amplitudes were too small for anything but rough estimates). The existence of a Schiff base deprotonation and reprotonation pathway of the correct vectoriality is indicated by the fact that the purple form of the protein has some proton transport activity (*cf.* above).

The data thus indicate that in this sample about normal amounts of K and L were produced but much less M, N, and O. The calculated kinetics (Fig. 5*B*, *points*) was complicated, particularly for L. Modeling such a complex system is necessarily ambiguous. Nevertheless, it is important to develop a strategy for finding the model for this kind of kinetics because several other mutated bacteriorhodopsins also seem to exhibit high apparent quantum yield for the photoreaction but low amplitude for M (Stern *et al.*, 1989). The most conservative approach would assume a heterogeneous sample and two independent photocycles. However, there is no reason to suspect that at pH 4 the Asp^{212} → Asn protein is heteroge-

[2] G. Váró and J. K. Lanyi, manuscripts in preparation.

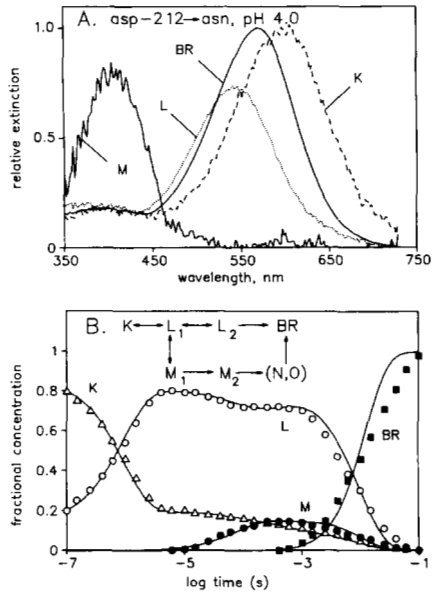

FIG. 5. **Calculated absorption spectra of the photointermediates of Asp^{212} → Asn bacteriorhodopsin and their time-dependent concentrations after photoexcitation at pH 4.** The spectra and kinetics were derived from data such as those in Fig. 2, *C* and *D*. *A*, ——, bacteriorhodopsin (*BR*); -----, L-like intermediate; - - -, K-like intermediate; ——, M-like intermediate. The spectra were averaged and smoothed. Spectra for an N-like and an O-like species are not shown; they accumulated but not in sufficient quantity for critical evaluation. *B*, △, K; ○, L (*i.e.* $L_1 + L_2$); ●, M (*i.e.* $M_1 + M_2$); ■, BR. The *line* describes the model shown with the following rate constants: $k_{KL1} = 8.3 \times 10^5\ s^{-1}$; $k_{L1K} = 2.0 \times 10^5\ s^{-1}$; $k_{L1L2} = 1.0 \times 10^3\ s^{-1}$; $k_{L2L1} = 1 \times 10^3\ s^{-1}$; $k_{L2BR} = 125\ s^{-1}$; $k_{L1M1} = 2.5 \times 10^3\ s^{-1}$; $k_{M1L1} = 1.0 \times 10^4\ s^{-1}$; $k_{M1M2} = 500\ s^{-1}$; $k_{M2N,O} = 667\ s^{-1}$; $k_{N,OBR} = 330\ s^{-1}$. The fit at the end of the photocycle is not as good as the rest because the model for the recovery of BR was simplified as shown; this had its effects mainly after 10 ms.

neous. Instead, we chose to begin with the photocycle of the blue form (Fig. 4*B*) and searched for a self-consistent modification which would explain the behavior of the purple form. Production of M in such a model would have to be from L, as in wild type. However, if the L_1-to-L_2 transition has a conformational basis related to that of the M_1-to-M_2 transition, as we suggested (*cf.* above), access of the Schiff base to its proton acceptor would be in L_1 but probably not in L_2. Consistency with the wild-type photocycle thus suggests a model branched at L_1 (Fig. 5*B*). For the sake of simplicity, we did not resolve the M-to-BR pathway into individual reactions. The data (Fig. 5*B*) fit this model remarkably well. The low amplitudes of M and the intermediates of the subsequent pathway to BR were explained by the fact that the $L_1 \leftrightarrow M_1$ equilibrium, although rapid, is in favor of L_1, and thereby the parallel $L_1 \leftrightarrow L_2$ equilibrium allowed only limited accumulation of M. With the rate constants for these steps in the model, the amplitude of M would have to be as low as actually found (Fig. 5*B*). We suggest therefore that the photocycle proceeds in essentially the same way in the purple and blue forms of the Asp^{212} → Asn protein, but in the purple form deprotonation of the Schiff base will occur. The latter is with different kinetics than in wild type because the deprotonation equilibrium $L \leftrightarrow M$ is poised against loss of the proton from the Schiff base, and thus the relaxation pathway to BR without deprotonation competes effectively. Thus, according to this model the effect of the residue replacement at Asp^{212} is to 1) either lower the Asp^{85} pK_a or raise the Schiff base pK_a in the purple form and 2) prevent Schiff base deprotonation virtually entirely in the blue form. The earlier steps of the photocycle, and probably the M → BR pathway once M is produced, are not significantly affected. If this is so, Asp^{212} plays no significant role in the reprotonation of the Schiff base, contrary to suggestions otherwise (Braiman *et al.*, 1988; Rothschild *et al.*, 1990). While other kinetic models (*e.g.* with two independent photocycles) cannot be ruled out, and the proposed scheme contains some assumptions as discussed above, its conceptual self-consistencies seem appealing.

According to a recent hypothesis based on chemical shifts in Schiff base ^{15}N resonance (De Groot *et al.*, 1989, 1990), there may be a three-dimensional hydrogen-bonded complex near the Schiff base comprising for example a water molecule and three protonable amino acid residues. Redistribution of the protons in response to the positive charge of the NH^+ group would create a diffuse negative charge in the complex which can thus function as the counterion. The near equivalence of replacing Asp^{85} and Asp^{212} on the chromophore absorption and the light-induced Schiff base deprotonation, as reported here, would be consistent with this concept if these residues were part of the complex. The important differences in the effects of Asp^{85} and Asp^{212} seem to be that only Asp^{85} will influence the Schiff base pK_a and accept the Schiff base proton during the photocycle. Thus, the two residues cannot occupy symmetrical positions relative to the NH^+ group, such as implied by the simplest version: a postulated complex with perfect tetrahedral geometry around the water oxygen. The influence of at least one other group at this location is indicated by the pH dependency of the spectrum and the photoreaction of the Asp^{212} → Asn protein. The protonation of this (unidentified) residue, which may be also part of the complex, causes blue-shift and allows measurable deprotonation of the Schiff base. Protonation of Asp^{85} would have the opposite effects. From their nearby location (Henderson *et al.*, 1990), candidates for other groups participating in the counterion complex are Tyr^{185}, Tyr^{57}, and Arg^{82}. As a basic residue, Arg^{82} is a logical choice for inclusion in the proposed counterion complex (De Groot *et al.*, 1990). On the other hand, Rothschild *et al.* (1990) have given evidence for hydrogen bonding between Asp^{212} and Tyr^{185}.

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