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Role of aspartate-96 in proton translocation by bacteriorhodopsin

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ABSTRACT **Proton transfer reactions in bacteriorhodopsin were investigated by Fourier transform infrared spectroscopy, using a mutant protein in which Asp-96 was replaced by Asn-96. By comparison of the BR − K, BR − L, and BR − M difference spectra (BR indicating bacteriorhodopsin ground state and K, L, and M indicating photo-intermediates) of the wild-type protein with the corresponding difference spectra of the mutant protein, detailed insight into the functional role of this residue in the proton pump mechanism is obtained. Asp-96 is protonated in BR, as well as another aspartic residue, which is tentatively assigned to be Asp-115. Asp-96 is not affected in the primary photoreaction. During formation of the L intermediate it is subjected to a change in the H-bonding character of its carboxylic group, but no deprotonation occurs at this reaction step. Also, in the mutant protein a light-induced structural change of the protein interior near the Asn-96 residue is probed. The BR − M difference spectrum of the mutant protein lacks the negative carbonyl band at 1742 cm^{-1} of Asp-96 and in addition a positive band at about 1378 cm^{-1}, which is most likely to be caused by the carboxylate vibration of Asp-96. This argues for a deprotonation of Asp-96 in the time range of the M intermediate during its photostationary accumulation. On the basis of these results, it is suggested that the point mutation does not induce a gross change of the protein structure, but a proton-binding site in the proton pathway from the cytoplasmic side to the Schiff base is lost.**

The mechanism of vectorial proton transfer in proteins is one of the central questions in bioenergetics. To obtain insight into this mechanism at an atomic level, the proton pump of bacteriorhodopsin is a suitable model and Fourier-transform infrared (FTIR), a powerful method.

The retinal-containing protein bacteriorhodopsin—the only protein in the purple membrane of *Halobacterium halobium*—converts light into electrochemical energy (1). After photo-isomerization of the chromophore a photocycle with different intermediates, designated J, K, L, M, N, and O, is initiated and accompanied by vectorial proton transfer reactions (for review see ref. 2). These involve not only deprotonation of the $C{=}NH^+$ double bond between the retinal moiety and the lysine residue 216 (denoted Lys-216) upon formation of the M intermediate (3) but also protonation changes of amino acid side chains. By the FTIR spectroscopy of [4-^{13}C]Asp-labeled purple membrane, evidence was provided that four aspartic residues undergo protonation changes in the hydrophobic region of the protein during the photocycle (4, 14). Uniform labeling, however, prevented assignment to specific aspartic residues of the sequence. Structural models, on the other hand, suggest that of the nine aspartic residues present in the protein, only Asp-85, Asp-96, Asp-115, and Asp-212 are located in the helical intramembrane region of the protein (5). Point mutations of Asp-85, Asp-96, Asp-115, and Asp-212 to asparagine affected the pump activity of bacteriorhodopsin (6). However, these results could not distinguish between an indirect effect, in which neutralization of a charge could influence the protein structure, and a direct effect on the pump mechanism, in which part of the proton pathway is blocked.

Therefore, one of them, the Asn-96 mutant protein, was investigated by FTIR difference spectroscopy. This allows one to compare the light-induced intramolecular reactions of the mutant with those of the wild type on an atomic level. In contrast to ref. 6, the Asn-96 mutant was obtained not by site-specific mutagenesis of bacteriorhodopsin expressed in *Escherichia coli* but by selection of phototrophically negative mutants of *Halobacterium*, allowing isolation of functionally defective bacteriorhodopsin and its analysis in a natural membrane environment, the purple membrane (7, 8). Comparisons of BR − K and BR − L difference spectra, in which BR indicates the bacteriorhodopsin ground state, indicate similar light-induced reactions in the Asn-96 mutant protein and the wild-type protein. In contrast, the BR − M difference spectra show different reactions in the mutant protein. The results point strongly to deprotonation of Asp-96 in the M intermediate, accumulated under photostationary conditions.

MATERIALS AND METHODS

Purple membrane was isolated from *H. halobium*, *Halobacterium* sp. GRB, and derived mutant strains as described (9). About 150 μg of purple membrane was dried on CaF_2 or AgCl windows and rehydrated to 100% humidity. The windows were placed into a homemade sample chamber. Infrared spectra were recorded on a Bruker IFS 88 FTIR spectrophotometer with 2 cm^{-1} spectral resolution as described (4, 17, 20). Fourier transformation was performed by using the Blackman–Harris apodisation function (10). A subtraction of the difference spectra was performed to reveal the effects of the point mutation by minimizing the parameter Δ^2, in which Δ = (difference spectrum of the wild type) − a ×.(difference spectrum of the mutant) + b, with a and b being the variables for minimization.

RESULTS

The infrared absorbance bands of functionally relevant molecular groups were selected from the background absorbance of the whole protein by recording infrared difference spectra between the ground state (BR) and intermediates (K, L, or M) of the photocycle, which were stabilized at low temperatures (for review see ref. 11). Negative bands in the difference spectra then correspond to BR and positive bands to the respective intermediate. Deprotonation of a carboxylic acid is indicated in the difference spectrum by a negative absorbance band in the carbonyl stretching vibration region (1780–1700 cm^{-1}) and a positive absorbance band in the carboxylate symmetric stretching vibration region (1450–1350 cm^{-1}) (12). In the antisymmetric stretching vibration

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Abbreviations: FTIR, Fourier-transform infrared; BR, ground-state bacteriorhodopsin.

region (1540–1650 cm^{-1}), however, the COO$^-$ band is masked by strong C═C chromophore and amide II vibration bands (4). When a carbonyl group is little affected in an intermediate state of the photocycle (e.g., by modified hydrogen bonding), the C═O stretching frequency in the 1700–1780 cm^{-1} region is only slightly shifted. Then one obtains in the difference spectra a "difference band" with a negative peak close to the original position in unphotolyzed BR and, a few cm^{-1} away, a positive peak close to the position in the intermediate state. For such a situation the term "difference band" will be used. BR − K, BR − L, and BR − M infrared difference spectra of the wild type were compared with corresponding difference spectra of the mutant. Infrared absorbance bands missing in the difference spectra of the mutant could be assigned to Asp-96 and bands additionally observed to Asn-96. Further changes could have occurred due to groups which were affected by the amino acid substitution, provided that no drastic change in the overall structure of the mutant protein had taken place.

BR − K. The BR − K difference spectra (data only partially shown in spectra F and G of Fig. 1) of the mutant and wild type are almost identical, indicating similar photoreactions in the two proteins. A difference is observed at about 1560 cm^{-1}, where the intensity of a positive band is decreased in the mutant difference spectrum.

BR − L. The BR − L difference spectra of wild type and mutant (Fig. 1, spectra A and B) again are similar, pointing to local but not general differences of the L state in the two proteins. These differences occur at the bands marked by arrows in Fig. 1 and involve only a few residues. As in the BR − K difference spectra, a positive band at about 1560 cm^{-1} is lacking in the mutant spectrum, causing a loss of intensity at 1563 and 1554 cm^{-1} (Fig. 1, spectrum B). Furthermore, an increase is observed at 1648 cm^{-1} (Fig. 1, spectrum B). In general, difference bands at about 1560 cm^{-1} and 1650 cm^{-1} can be caused by a small conformational change of the protein backbone, which shifts the amide I (about 1660 cm^{-1}) and amide II (about 1545 cm^{-1}) bands. Therefore, the different intensities at 1650 cm^{-1} and 1560 cm^{-1} can indicate a slightly different light-induced conformational change involving one or two groups of the backbone in the mutant protein (13).

In the carbonyl region of the mutant difference spectrum an additional difference band is seen with maxima at 1704 cm^{-1} and 1698 cm^{-1} (compare Fig. 1 spectra C and D/E). This additional absorbance band is not caused by a carboxylic acid, because its frequency is invariant in D_2O (Fig. 1, spectrum E) but is most likely due to the carbonyl vibration of the $CONH_2$ group (12) of Asn-96. The frequency shift of this vibration points towards a change of the microenvironment of Asn-96 in the K-to-L transition. To confirm the interpretation of the difference band at 1704/1698 cm^{-1} a second point mutant, in which Asp-96 is replaced by a glycine residue, was investigated. The lack of the 1704/1698 cm^{-1} difference band in this mutant confirmed our assignment of this band to Asn-96.

The most interesting change is seen in the band pattern at about 1740 cm^{-1}. After comparison of the difference spectra of mutant and wild type it is evident that the band pattern in the wild type consists of two carbonyl difference bands: one shifting from 1737 to 1729 cm^{-1} (Fig. 1, spectrum D) and another shifting from 1740 to 1748 cm^{-1} (Fig. 1, spectrum C). Because the latter difference band is lacking in the difference spectrum of the mutant, it now can be unambiguously assigned to the carbonyl vibration of Asp-96. Earlier interpretations of this band pattern assumed an overlap of a narrower, negative, and a broader, positive, band (4) caused by deprotonation and protonation of two internal aspartic residues in L, called Asp 1 and Asp 1*, therefore must be modified. Also, a possible overlap of a negative band and a difference band with positive peak at 1739 cm^{-1} as described in ref. 14 can no longer be sustained. The data document that Asp-96 occurs protonated in BR as well as another aspartic acid, called Asp 1 according to ref. 4, which absorbs at 1737 cm^{-1}. The frequency shift of Asp-96 from about 1740 cm^{-1} to 1748 cm^{-1} indicates that this residue becomes exposed to a more hydrophobic environment during the K-to-L transition. No positive band is missing in the mutant BR − L difference spectrum in the symmetric COO$^-$ stretching vibration region from 1450 cm^{-1} to 1300 cm^{-1}. Therefore, a deprotonation of Asp-96 is very unlikely for the K-to-L transition.

The second carbonyl difference band 1737/1729 cm^{-1} (Fig. 1, spectrum D) in principle either can be caused by the carbonyl vibration of Asp 1 shifting from 1737 cm^{-1} to 1729 cm^{-1} due to an environmental change or may represent two different Asp residues. In the latter case, the negative band at 1737 cm^{-1} would be due to Asp 1 deprotonating during the K-to-L transition and the positive band at 1729 cm^{-1} would be due to a more water-exposed carboxylate group which becomes protonated during this transition. However, deprotonation of Asp 1 was suggested for the K-to-L transition, because a positive COO$^-$ band of an aspartic residue is observed at 1400 cm^{-1} in the BR − L difference spectrum (4, 14). The difference band 1737/1729 cm^{-1} can be caused by Asp 1 alone, if an environmental change of Asp 1 (for example in K) is followed by its deprotonation (for example in L) and if the BR − L low temperature difference spectra

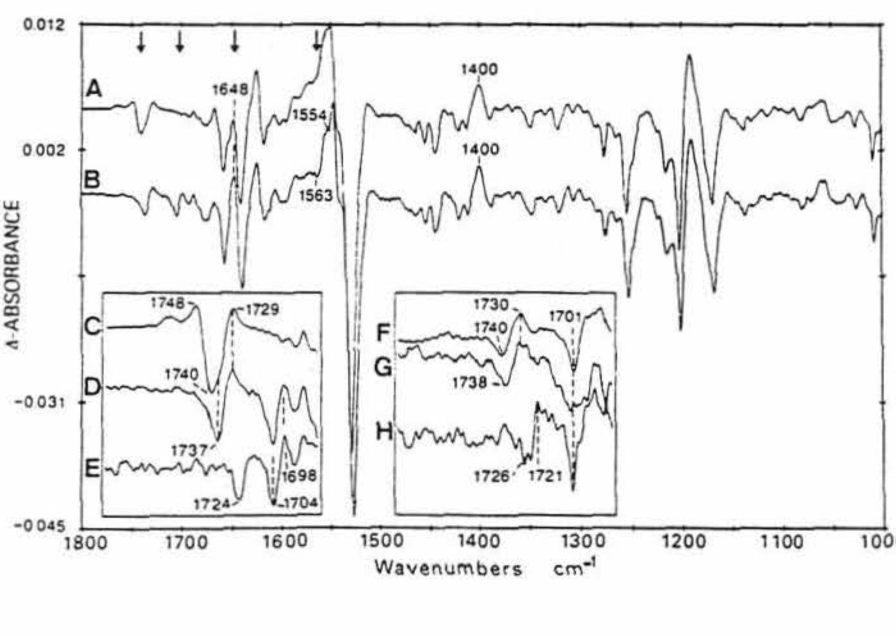

FIG. 1. FTIR difference spectra of bacteriorhodopsin. A, BR − L difference spectrum taken in H_2O at 170 K with spectral resolution of 2 cm^{-1}. B, Corresponding difference spectrum of the mutant in which Asp-96 is changed to Asn-96, recorded under the same conditions as A. C, Expansion of the spectral region 1800–1680 cm^{-1} of A. D, Expansion of the spectral region 1800–1680 cm^{-1} of B. E, BR − L difference spectrum of the mutant in deuterium oxide (D_2O) at 1800–1680 cm^{-1}. F, BR − K difference spectrum of the wild type at 1800–1680 cm^{-1} taken at 70 K in H_2O. G, BR − K difference spectrum of the mutant taken in H_2O at 1800–1680 cm^{-1}. H, BR − K difference spectrum of the mutant taken in D_2O at 1800–1680 cm^{-1}.

consist of a mixture of protein states, one containing protonated Asp 1 exposed to a changed environment and one containing an already deprotonated Asp 1. The mixture could be caused by different substates in which the proteins are trapped at low temperatures (15) or due to different L intermediates (16). In agreement with this interpretation the BR − K difference spectra (Fig. 1, spectra F and G) of both wild type and mutant show a small difference band at 1740/1730 cm^{-1}, which is expected for the preceding environmental change. This small difference band is most likely caused by Asp 1 (14) and not by a glutamic residue (4). On the other hand, the BR − K difference spectra show a small Asp carboxylate vibration at 1400 cm^{-1}, indicating that this vibration might not be caused by Asp 1. This would point to an environmental change of Asp 1 in the BR-to-L transition and not to a deprotonation as suggested previously (4, 14).

Summarizing the results obtained from analysis of the BR − K and BR − L difference spectra, we conclude that the band pattern around 1740 cm^{-1} is caused by the carbonyl vibration of Asp-96, which shifts upon an environmental change in L from 1740 cm^{-1} to 1748 cm^{-1}, and that a second protonated aspartic residue, called Asp 1, absorbs at 1737 cm^{-1} in BR. It seems unlikely that a third aspartic residue, which would become protonated in L, is reflected by the positive band at 1729 cm^{-1}; it is more likely that the carbonyl vibration of Asp 1 is shifted to 1729 cm^{-1} in K and L. Because two carbonyl difference bands, not a negative carbonyl band as suggested previously (4, 14), are seen in the BR − L difference spectra, deprotonation of an aspartic residue in L must be questioned.

BR − M. The M intermediate is defined by deprotonation of the Schiff base. In the deprotonated state the C—C stretching vibrations in the fingerprint region show no significant infrared intensity compared to BR, K, L, and N (17). Therefore, this region would indicate the presence of intermediates other than M in the photostationary state. Only difference spectra of the wild type and the mutant were compared; they agreed highly in the fingerprint region, indicating that the same chromophore state was stabilized.

The BR − M difference spectra of mutant and of wild type are again similar, but they show more deviations from each other than the BR − K and BR − L difference spectra. As already seen in the BR − L difference spectra, the absorbance band at 1649 cm^{-1} is increased and also the additional difference band due to the carbonyl vibration of Asn-96 is seen at 1703/1696 cm^{-1} (see Fig. 2, spectra A, B, C, and D). In contrast to the BR − L difference spectra, the band at 1557 cm^{-1} is increased and an additional negative band is present at 1669 cm^{-1} (Fig. 2, spectra A and B). Both bands can arise from intensity increases of difference bands caused by shifts of the amide I and amide II absorbance bands due to conformational changes of the protein backbone as discussed already for the BR − L difference spectra.

In the carbonyl region a negative carbonyl band at 1743 cm^{-1} and not a difference band as in the BR − L difference spectra is lacking in the difference spectrum of the mutant protein. This negative band can therefore be assigned to the disappearance of the carbonyl vibration of Asp-96 in the M intermediate accumulated under photostationary conditions. Compared to the wild type the carbonyl vibration of an aspartic residue absorbing at 1761 cm^{-1} and called Asp 2 in ref. 4 is decreased. Therefore, the carbonyl vibration of a third aspartic residue absorbing at 1757 cm^{-1} and called Asp 3 in ref. 4 is more pronounced in the difference spectrum of the mutant. In contrast to the BR − L difference spectra, changes also occur in the carboxylate vibration region between 1350 and 1450 cm^{-1} in the difference spectrum of the mutant protein. At about 1378 cm^{-1} part of a positive band and at about 1417 cm^{-1} part of a negative band are missing (Fig. 2, spectra A, B, E, and F).

To quantify the deviations between the difference spectra of mutant and wild-type protein, a subtraction procedure as described in *Materials and Methods* was performed, and the results are shown in Fig. 3 for BR − L difference spectra in A and BR − M difference spectra in B. The subtraction gives evidence that in the carbonyl region at 1742 cm^{-1} only a negative band is lacking in the BR − M difference spectrum of the mutant indicated by the negative peak at 1742 cm^{-1} (Fig. 3, spectrum B). This observation is in contrast to the BR − L difference spectrum, in which a difference band disappears in the mutant, indicated by the negative peak at 1741 cm^{-1} and the positive peak at 1748 cm^{-1} (Fig. 3, spectrum A). Also, the decrease in intensity of the carbonyl band of Asp 2 in the mutant difference spectrum is indicated by the positive peak at 1762 cm^{-1} in Fig. 3, spectrum B. In the carboxylate vibration region a negative peak at 1417 cm^{-1} and a positive peak at 1378 cm^{-1} appear (Fig. 3, spectrum B). These peaks are most likely due to the carboxylate vibrations of Asp 2 and Asp-96, which appear or disappear if the corresponding carbonyl vibrations disappear or appear due to a protonation change. This argues strongly that Asp 2, as suggested by Engelhard *et al.* (4), and also Asp-96 undergo protonation changes during the photocycle. It is unlikely that at the same time another carboxylate vibration is shifted by 39 cm^{-1} from 1417 to 1378 cm^{-1}.

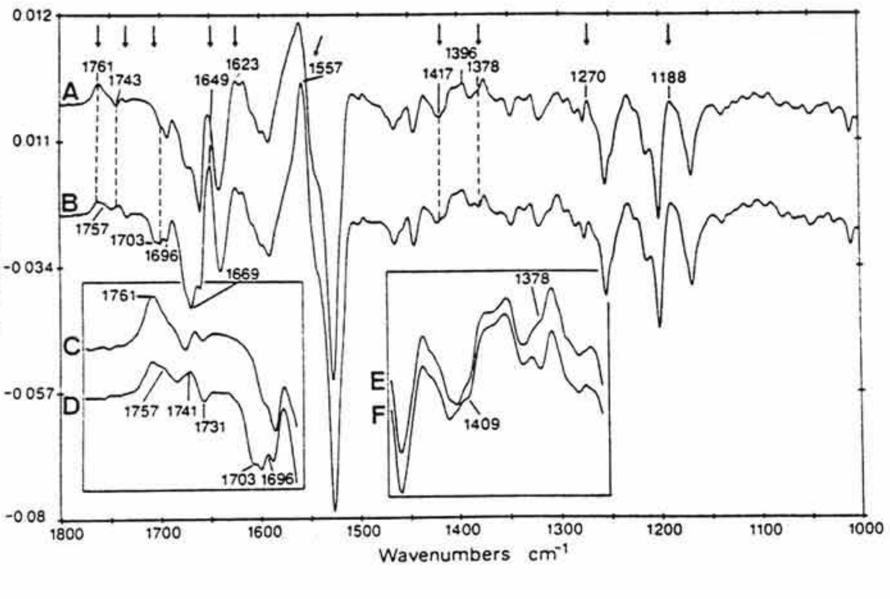

FIG. 2. BR − M difference spectra of bacteriorhodopsin taken at 272 K in H_2O with 2 cm^{-1} spectral resolution. The M intermediate is stabilized under photostationary conditions. Thereby small contributions of the N intermediate are present, but in the same ratio in both samples, as indicated in the fingerprint region. A, wild type; B, mutant; C, expansion of A from 1800 to 1680 cm^{-1}; D, expansion of B from 1800 to 1680 cm^{-1}; E, expansion of A from 1450 to 1350 cm^{-1}; F, expansion of B from 1450 to 1350 cm^{-1}.

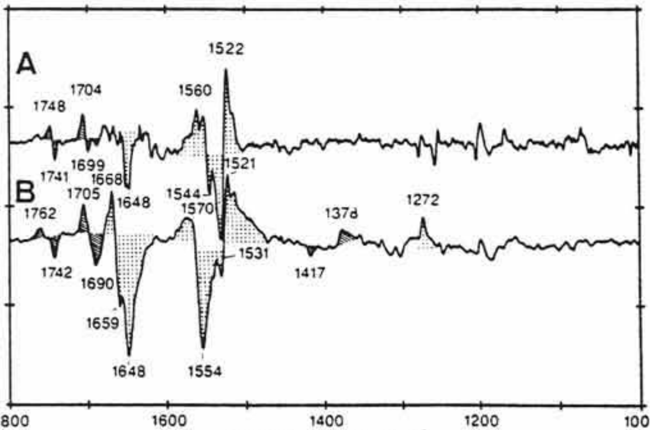

FIG. 3. A, Subtraction of the BR − L difference spectra of the wild type and the mutant; B, corresponding subtraction of the BR − M difference spectra. For details see text.

The subtraction of difference spectra in Fig. 3 shows, furthermore, the shift of the carbonyl vibration of Asn-96 from 1705 cm^{-1} and to about 1690 cm^{-1} in L and M, strong difference bands at the frequency position of the amide I and amide II vibrational modes at about 1650/1670 cm^{-1} and at about 1550/1570 cm^{-1}, respectively. The changes are larger in the BR − M than in the BR − L difference spectra. Interestingly, the vibration of most likely Tyr-185 also shows a change in the difference spectrum of the mutant indicated by the positive peak at 1272 cm^{-1} (18).

The small decrease of the carbonyl band at 1762 cm^{-1} indicates that a slightly different opsin state is stabilized under conditions in which M is photostationarily accumulated in the mutant sample compared to the wild type. This is not surprising, if in the latter case deprotonation of an internal group takes place, which is prevented in the former sample. Control experiments performed at different temperatures (19) and pH values showed differences in the fingerprint region around 1188 cm^{-1}, indicating that, depending on external parameters, different mixtures of N (20) and M intermediates are stabilized. Furthermore, these control experiments showed that the intensity of the band at 1396 cm^{-1} correlates with the increase of the band at 1188 cm^{-1}. In contrast to earlier suggestions (14), this band cannot be assigned to the carboxylate vibration of the group, which absorbs in its protonated form at 1742 cm^{-1}. As the carbonyl band at 1742 cm^{-1} is missing in the difference spectrum of the mutant, the carboxylate vibration should also vanish. Subtraction of the BR − M difference spectra (Fig. 3, spectrum B) shows agreement in the fingerprint region, indicating that similar chromophore states, and, hence, the same photocycle intermediates are stabilized in the two samples. Furthermore, subtractions of the BR − M difference spectra of the mutant and the wild type taken under different external conditions show agreement in the fingerprint region and again the lack of a negative band at 1742 cm^{-1} and a positive band at about 1378 cm^{-1}. Therefore, the bands are most likely due to deprotonation of Asp-96 and are not caused by absorbance bands of different photocycle intermediates.

DISCUSSION

The results obtained are summarized in Table 1, which includes information already published (4, 21, 22).

In the BR and K states two internal aspartic residues, Asp-96 and Asp 1, are protonated. Independently, NMR experiments also gave evidence for two protonated internal aspartic residues in the ground state (23). Because four different carbonyl vibrations of internal aspartic residues are observed in the IR difference spectra it is evident that at least two internal aspartic residues undergo protonation changes during the photocycle. In agreement with ref. 14, the difference band in the BR − K difference spectrum shifting from 1737 to 1729 cm^{-1} and the difference band in the BR − M difference spectrum shifting from 1731 to 1741 cm^{-1} is assigned to an aspartic and not a glutamic residue (4).

The BR − L difference spectrum of the mutant shows that the band pattern around 1740 cm^{-1} consists of two difference bands of two different aspartic residues that are protonated in BR. The results gave evidence that one difference band is caused by a light-induced change of the H-bonding character of the carboxylic acid of Asp-96 in the L intermediate. The other difference band is likely to be caused by the protonated carboxylic acid of Asp-115. This assignment is based on the observation of only two protonated aspartic residues in BR and on chemical modification experiments which point to protonation of Asp-115 (24). In contrast to earlier interpretations (4, 14), the BR − L difference spectrum of the Asn-96 mutant protein shows clearly that not only a negative band is present. Therefore, deprotonation of an aspartic residue in the L intermediate (4, 14) is not likely. Both protonated aspartic residues probe light-induced changes of their microenvironment. This is corroborated by the mutant protein also showing a change in the microenvironment of the exchanged amino acid Asn-96 in the L intermediate.

In the photostationary M intermediate the carbonyl vibration of Asp-96 at 1742 cm^{-1} disappears and a band at about 1378 cm^{-1} appears, which is most likely the carboxylate vibration of Asp-96. These observations point strongly to deprotonation of Asp-96. The apparent negative band at 1742 cm^{-1} is smaller than the carbonyl band observed at 1763 cm^{-1}, because its intensity seems to be masked by the positive carbonyl bands at 1757 and 1741 cm^{-1}.

These results are supported by effects of the point mutation seen on photocycle kinetics: the rise time of M is not affected, but the M-to-BR reaction pathway is slowed down from 5 ms to 450 ms (25). During this reaction the Schiff base is reprotonated from the cytoplasmic side. Because only small structural changes are observed in the mutant compared to the wild type, it can be concluded that the point mutation,

Table 1. Summary of protonation changes of internal aspartic residues

	BR 570	K 590	L 550	M 412 / (N*)
^{115}(*) Asp 1	$-\mathrm{C}\genfrac{}{}{0pt}{}{{=}\mathrm{O}}{-\mathrm{OH}}$	H−bond change	$-\mathrm{C}\genfrac{}{}{0pt}{}{{=}\mathrm{O}}{-\mathrm{OH}}$ / $-\mathrm{C}\genfrac{}{}{0pt}{}{\mathrm{O}}{\mathrm{O}}{}^{-}$ (?)	$-\mathrm{C}\genfrac{}{}{0pt}{}{{=}\mathrm{O}}{-\mathrm{OH}}$
	1737cm^{-1}	1729cm^{-1}	1400cm^{-1} (?)	1740cm^{-1}
^{96} Asp	$-\mathrm{C}\genfrac{}{}{0pt}{}{{=}\mathrm{O}}{-\mathrm{OH}}$	$-\mathrm{C}\genfrac{}{}{0pt}{}{{=}\mathrm{O}}{-\mathrm{OH}}$	H−bond change	$-\mathrm{C}\genfrac{}{}{0pt}{}{\mathrm{O}}{\mathrm{O}}{}^{-}$ (*)
	1742cm^{-1}	1742cm^{-1}	1748cm^{-1}	≈ 1376cm^{-1} (*)
$^{85}_{212}$(*) Asp 2	$-\mathrm{C}\genfrac{}{}{0pt}{}{\mathrm{O}}{\mathrm{O}}{}^{-}$	$-\mathrm{C}\genfrac{}{}{0pt}{}{\mathrm{O}}{\mathrm{O}}{}^{-}$	$-\mathrm{C}\genfrac{}{}{0pt}{}{\mathrm{O}}{\mathrm{O}}{}^{-}$	$-\mathrm{C}\genfrac{}{}{0pt}{}{{=}\mathrm{O}}{-\mathrm{OH}}$
	≈1417cm^{-1} (*)			1762cm^{-1}
$^{85}_{212}$(*) Asp 3	$-\mathrm{C}\genfrac{}{}{0pt}{}{\mathrm{O}}{\mathrm{O}}{}^{-}$	$-\mathrm{C}\genfrac{}{}{0pt}{}{\mathrm{O}}{\mathrm{O}}{}^{-}$	$-\mathrm{C}\genfrac{}{}{0pt}{}{\mathrm{O}}{\mathrm{O}}{}^{-}$	$-\mathrm{C}\genfrac{}{}{0pt}{}{{=}\mathrm{O}}{-\mathrm{OH}}$
				1757cm^{-1}

Notations are in agreement with ref. 4. The assignments marked by (*) are not definitely proven (see text). Asp 1 is tentatively assigned to Asp-115. It is questionable if Asp-115 is deprotonated in L. Interestingly, it is exposed to different environments in K and M. The detailed deprotonation kinetics of Asp-96 and protonation kinetics of Asp 3 should be obtained by time-resolved FTIR spectroscopy (21). It seems to correlate with the M-to-N transition. The assignment of the carboxylate vibration of Asp 2 is tentative. Furthermore, it is suggested (5) that Asp-85 and Asp-212 are in the helical region of the protein; these two residues will have to be assigned to Asp 2 and Asp 3.

which prevents deprotonation of residue 96, strongly inhibits the normal proton pathway from the cytoplasmic side to the Schiff base.

The amplitudes of the carbonyl bands of Asp 3 and Asp-96 increase, corresponding to an increasing contribution of the N intermediate (20), in photostationary mixtures. Therefore, protonation of Asp 3 and deprotonation of Asp-96 seem to correlate with the M-to-N reaction. Thus, Asp-96 is likely to be the proton donor to the Schiff base or to Asp 3. Nevertheless, protonation of Asp 3 and the Schiff base is still observed in the mutant protein. This points to alternative proton pathways with different kinetic efficiency. Furthermore, the results show a small difference in the light-induced conformational change of the protein backbone in the mutant. Such change can also contribute to a less efficient proton pathway.

The finding that a light-induced change of H bonding of an internal protonated carboxylic acid is followed by its deprotonation seems to be of general relevance for other proteins. Internal protonated carboxylic acids, responding to a change in their microenvironment or undergoing protonation changes during photoreactions, have been recorded also in rhodopsin (26) and halorhodopsin (27). Aside from their functional relevance in retinal-containing proteins, protonated carboxylic acids are also involved in proton translocation in ATPases (28).

In this context it is interesting to note the distribution of protonated and deprotonated aspartic residues in the ground state of bacteriorhodopsin. On the basis of the structural model of Engelman *et al.* (5), assigning Asp 2 and Asp 3 tentatively to Asp-85 and Asp-212, we here discover that in the ground state the two protonated aspartic residues seem to be located on the proton uptake side between chromophore and cytoplasmic side, whereas the two deprotonated aspartic residues seem to be located on the proton release side between chromophore and extracellular side. Since Asp 2 is protonated with the same kinetics as the Schiff base is deprotonated (4), this residue (Asp-85 or Asp-212) is most likely the proton acceptor in the proton release pathway.

Current theoretical concepts of proton conduction in proteins assume chains of hydrogen bonds in which amino acid side chains and the protein backbone participate, resulting in a fast continuous proton conduction along a "proton-wire" in the microsecond time range (29, 30). Indeed, no limitation of the photocycle kinetics by protonation reactions has been found (31). On the other hand, removal of Asp-96 slows down the photocycle kinetics about 100-fold and the reprotonation of the Schiff base becomes rate limiting (32). Furthermore, the experimental results point to a mechanism in which the protein adopts discrete intermediate stages. Thus, the appropriate model is to assume that two (or even more) proton-binding sites are involved in the de- and reprotonation of the Schiff base. In such a model a binding site is defined as a group that occurs in a deprotonated and protonated form with an appreciable lifetime. The absence of any of these binding sites—e.g., Asp-96—then slows down the catalytic cycle. The exchange of other residues—e.g., tyrosines—potentially involved in the same "proton-wire" will not lead to an appreciable kinetic effect as long as the proton transfer reactions affected are not slowed below the velocity of the rate-limiting step of the overall catalytic cycle.

Note. After submission of this manuscript a contribution dealing with similar experiments was published by Braiman *et al.* (33). Regarding their publication, the following statements should be made: (*i*) Only the assignment of carbonyl stretching vibrations of aspartic residues are discussed. To determine protonation changes of carboxylic acids one has to demonstrate the corresponding absorbance changes in the carboxylate stretching vibration region to distinguish reliably between changes in the H bonding and protonation changes of a carboxylic acid, as pointed out in our *Results*. On the basis of our results we concluded, in contrast, that Asp-96 is not deprotonated in L, but most likely in the time range of the M intermediate. (*ii*) Our tentative assignment of the carbonyl vibrations of Asp 1 to Asp-115 is confirmed. Asp 2 seems to belong to Asp-85. Therefore, Asp-85 is likely the proton acceptor in the proton release pathway. The carbonyl vibration of Asp 3 (4, 22) is not assigned.

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