# Functional Roles of Aspartic Acid Residues at the Cytoplasmic Surface of Bacteriorhodopsin†

Leonid S. Brown,‡ Richard Needleman,§ and Janos K. Lanyi*,‡

*Department of Physiology and Biophysics, University of California, Irvine, California 92697, and Department of Biochemistry, Wayne State University, Detroit, Michigan 48201*

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ABSTRACT: The functions of the four aspartic acid residues in interhelical loops at the cytoplasmic surface of bacteriorhodopsin, Asp-36, Asp-38, Asp-102, and Asp-104, were investigated by studying single and multiple aspartic acid to asparagine mutants. The same mutants were examined also with the additional D96N residue replacement. The kinetics of the M and N intermediates of the photochemical cycles of these recombinant proteins were affected only in a minor, although self-consistent, way. When residue 38 is an aspartate and anionic, it makes the internal proton exchange between the retinal Schiff base and Asp-96 about 3 times more rapid, and events associated with the reisomerization of retinal to all-trans about 3 times slower. Asp-36 has the opposite effect on these processes, but to a smaller extent. Asp-102 and Asp-104 have even less or none of these effects. Of the four aspartates, only Asp-36 could play a direct role in proton uptake at the cytoplasmic surface. In the 13 bacterioopsin sequences now available, only this surface aspartate is conserved.

In bacteriorhodopsin, the light-driven proton pump of halobacteria, the reaction cycle initiated by photoisomerization of the *all-trans*-retinal to 13-*cis*-retinal includes proton transfers inside the protein and proton exchanges between the surface residues and the bulk (reviewed in *1*−*6*). Together, these add up to the translocation of a proton across the full width of the membrane. The protonation of Asp-85 by the buried retinal Schiff base (the L → M reaction, where in M the retinal Schiff base is deprotonated) and reprotonation of the Schiff base by Asp-96 (the M → N reaction, where in N the Schiff base is reprotonated but the retinal is still 13-cis) have been investigated in great detail (reviewed in *5, 6*). Likewise, the proton release to the extracellular surface upon protonation of Asp-85 early in the photocycle has been studied extensively (*7*−*12*). Much less is known about the proton uptake at the cytoplasmic surface that leads to reprotonation of Asp-96 later in the photocycle.

The capture of a proton from the cytoplasmic side occurs during the photocycle reaction identified as the decay of the N intermediate. It is detected by a transient pH increase at this time (*13*−*15*), and by disappearance of the negative C═O stretch band of Asp-96 upon its reprotonation (*16, 17*). Asp-96 is about 6 Å from the cytoplasmic surface, and surrounded by mostly nonpolar residues (*18*−*22*). Its protonation from the bulk is rather slow, on the millisecond timescale. It is likely that the transfer of a proton from the membrane surface to Asp-96 is through an occluded pathway, similarly to proton transfer from Asp-96 to the Schiff base, and limited therefore by the conformational dynamics of the protein. The large-scale conformational change (*23*−*25*) that involves the cytoplasmic ends of helices F, and to a lesser extent helices B, C, and G, and its reversal, may create the conditions for both of these proton transfers (*26, 27*). Rearrangements of bound water would provide the means to form pathways for transfer of the protons, and to first lower and then raise the p$K_a$ of Asp-96. This idea is consistent with the observed effects of lowered water activity (*28*−*30*) on the respective photocycle reactions, and constitutes a possible mechanism.

Some investigators have maintained that the uptake of protons from the bulk medium, and their entry into the hydrophobic cytoplasmic channel, is aided by the four acidic residues at the surface of the protein, located on the A−B and C−D interhelical loops. Although single replacement of none of these aspartic acid residues (Asp-36, Asp-38, Asp-102, and Asp-104) with asparagine affected proton transport (*31*), the distribution of charges at the surface in the 3 Å resolution electron-diffraction structure had suggested (*19*) that proton uptake could depend on lateral proton exchange within the Asp-36/Asp-38/Asp-102 triad and the Asp-102/Asp-104 pair, and thereby utilize multiple pathways. Quantitative descriptions of the kinetics of proton migration after a proton pulse in model systems, other proteins, and specifically bacteriorhodopsin (*32*−*34*), had demonstrated that a constellation of acidic groups at the surface can serve as an antenna for collecting protons. From such experiments, Asp-36 was identified as the mediator that delivers the proton to the channel leading to Asp-96 (*33*), while the other aspartates at the surface, and perhaps also on the C-terminal tail, would act as proton attractors. In another study (*35*), Asp-38 was proposed to be the essential residue in the proton conduction pathway that connects the Schiff base with the

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* To whom correspondence should be addressed. Telephone: (949) 824-7150. Fax: (949) 824-8540. E-mail: jlanyi@Orion.oac.uci.edu.

‡ University of California.

§ Wayne State University.

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cytoplasmic surface. Direct evidence for these mechanisms could not be obtained, presumably because the p$K_a$ of the surface aspartates would be too low to allow detection of their proposed brief transient protonation. Replacing Asp-38, but not the other aspartates, with cysteine, and particularly arginine, was found to cause slowing of proton transfer from Asp-96 to the Schiff base by up to 20-fold, and for this reason Asp-38 was suggested to have a role in protein conformational changes of importance for proton conduction (*35*). Unlike in the wild type and in the D38C mutant, in D38R FTIR difference spectra did not detect the accumulation of the N intermediate and the amide bands from the protein conformation change associated with this state (*35*). The possibility was raised that lack of N in the photocycle is a profound consequence of the mutation and reveals the essential role for Asp-38 in the transport. Further evidence for this was given from X-ray diffraction, which indicated that conformational changes attributed to the M state (specifically the $M_2$ state) were absent in the photocycle of the D38R mutant (*36*).

In this report we describe an extensive study of the roles of surface aspartates in the events that lead to proton transport. All four aspartates on the cytoplasmic interhelical loops were replaced, one by one and in various combinations, with asparagine, and the phenotypes of these mutants, as well as other mutants also containing the additional D96N replacement, were examined. The results indicate that these surface aspartates affect the various steps of the photocycle of bacteriorhodopsin only in a minor way, and mostly through their negative charges. The four aspartate residues each appear to have different effects on photocycle steps, with none, except possibly Asp-36, implicated in a direct role in proton conduction. This may be because under physiological conditions proton uptake at the cytoplasmic surface does not limit the photocycle reactions, either in the wild-type protein or in the mutants studied. Of the four aspartates, only Asp-36 is a conserved residue. Further, the solar light-flux in natural environments is not high enough to create photostationary states for these proteins in which the turnover of the photocycle would limit the rate of transport. It is not clear, therefore, how evolutionary pressure based on transport function could select for conservation of the aspartates on the cytoplasmic surface. For this reason, the physiological relevance of these residues in proton transport is doubtful.

## MATERIALS AND METHODS

Purple membranes were prepared from *Halobacterium salinarium* by a standard method (*37*). The D36N, D38N, D38N/D104N, D36N/D38N/D102N, D102N, D104N, D102/D104N, D36N/D96N, D38N/D96N, D38R/D96N, D36N/D38N/D102N/D96N, D102N/D96N, and D104N/D96N mutants were constructed as described before (*38*), and isolated after expression in *H. salinarium* as purple membrane patches. Absorption changes were followed after photoexcitation with a Nd:YAG laser pulse (532 nm, 7 ns), as in earlier publications of ours (e.g., *39*). The temperature was 25 °C throughout.

Cell envelope vesicles were prepared by a method described earlier (*40*). Proton transport was determined from the initial rate of pH decrease during illumination through a 520 nm cutoff filter as before (*41*). Although not exactly the same, in the relevant wavelength range the spectral distribution of the light source (*42*) was assumed to be similar to sunlight.

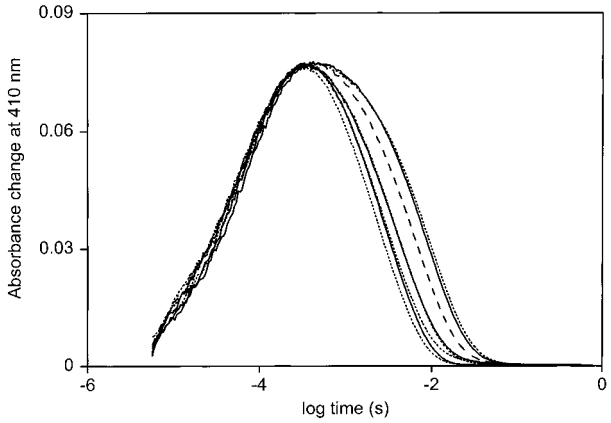


FIGURE 1: Kinetics of the M state at pH 7.0 after flash photoexcitation of wild-type bacteriorhodopsin and various mutants. The traces, measured at 410 nm, refer to the following, in the direction of slower decay: D36N (dotted line), wild type (solid line), D102N (dotted line), D104N (solid line), D102N/D104N (dotted line, nearly coincident with D104N), D38N/D104N (dashed line), D36N/D38N/D102N (solid line), and D38N (dotted line). The amplitudes of the traces are normalized for the sake of comparison. Conditions: 10 mM NaCl, 5 mM sodium succinate, 5 mM Bis-tris-propane, pH 7.0.

Table 1: Decay Time Constants for Wild-Type Bacteriorhodopsin and Various Mutants[a]

| sample | M-decay at pH 7 (ms)[b] | N-decay at pH 10.2 (ms)[c] |
|---|---|---|
| wild type | 3.3 | 316 |
| D36N | 2.8 | 388 |
| D38N | 10.2 | 109 |
| D38N/D104N | 6.9 | 161 |
| D36N/D38N/D102N | 9.3 | 124 |
| D102N | 3.5 | 123 |
| D104N | 4.5 | 255 |
| D102N/D104N | 4.5 | 141 |

[a] Conditions: as in Figures 1 and 2 (second and third columns, respectively). Similar results were obtained in 1 mM NaCl with no buffer added, and with 1 M NaCl (not shown). [b] M-decay was approximated with a single exponential. [c] Last component of decay of absorbance change at 570 nm.

## RESULTS

*Changed Photocycle Time Constants in Mutants of Aspartic Acid Residues at the Cytoplasmic Surface.* At pH ≥7 and in the millisecond time domain upon flash excitation, the transient absorbance changes at 410 and 570 nm reflect mainly the rise and decay of the M and N intermediates. Figure 1 shows traces of the absorbance change at 410 nm for a variety of single and multiple mutants of Asp-36, Asp-38, Asp-102, and Asp-104 at pH 7. Deprotonation of the Schiff base (increase of absorbance, formation of M) is virtually unaffected by these mutations. Reprotonation of the Schiff base (decrease of absorbance, decay of M) is slightly more rapid (by 15%) in the D36N mutant, but slowed to various extents by the other aspartic acid to asparagine replacements (Table 1). The largest effect, about 3×, is in D38N. When residue 38 is an arginine, this inhibition is even greater (*35*). The effects of these mutations are not additive

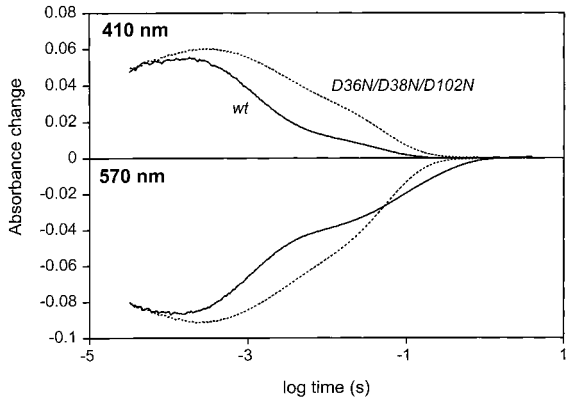


FIGURE 2: Kinetics of the M and N states at pH 10.2 after flash photoexcitation of wild-type bacteriorhodopsin (solid line) and the D36N/D38N/D102N mutant (dotted line). The M state is evident from the transient absorbance increase at 410 nm, the N state from the continued negative absorbance at 570 nm after the M intermediate had decayed. Conditions: 10 mM NaCl, 10 mM CAPS, pH 10.2.

(e.g., compare M-decay in D38N and D104N with that in D38N/D104N). Accumulation of the O intermediate was followed at 660 nm (not shown). Its amplitude at the pH of these measurements was small, but inversely dependent on the time constant of M-decay in the various mutants, as expected since the decay of O was unaffected.

At pH 7, the decay of the N state nearly coincides with the decay of the M state, and the kinetics of N are difficult to dissect (*43, 44*). Figure 2 shows traces at pH 10.2, where the decay of N is much slower than the decay of M, and can be assessed as a separate process.[1] The accumulation of N is evident from the continued negative absorbance at 570 nm after M had completely decayed. Comparison of the D36N/D38N/D102N mutant with wild-type protein indicates that M-decay is slower under these conditions also. However, the mutations cause the decay of N to be faster, i.e., the opposite to its effect on the decay of M. In various mutants, this acceleration relative to the wild type is as much as 3× (Table 1). The D38N and D102N residue replacements have the greatest effect. Again, D36N behaves unlike the others, as it exhibits a slight inhibition (by 20%) of N-decay. The decay of the N state under these conditions is the last event in the photocycle, and thus includes all steps after reprotonation of the Schiff base, i.e., reversal of the protein conformational change, proton uptake that results in reprotonation of Asp-96, reisomerization of the retinal to all-trans, as well as deprotonation of Asp-85 in the extracellular region (*45, 46*). If Asp-38 (or Asp-102) were to participate in proton uptake, one would have expected that this step would be slower in D38N (or D102N) rather than faster, as found. Such inhibition does occur in D36N. Although N-decay is only slightly inhibited, the direction of the effect of the mutation is consistent with the involvement of Asp-36 in proton conduction.

*Effects of the Additional D96N Mutation on the Photocycles of Mutants of Aspartic Acid Residues at the Cytoplasmic Surface*. Replacement of Asp-96 with asparagine removes the internal proton donor to the Schiff base, and the latter is reprotonated directly from the cytoplasmic surface (*29, 47*−*49*). Any effects of the mutations on Asp-96 as a proton donor will be absent. Table 2 shows time constants for the decay of the M state in many of the mutants in Table 1, but with the additional D96N mutation included in each. Because the decay of M in D96N mutants is very slow, the N state does not accumulate. All reactions of the second half of the photocycle, i.e., reprotonation of the Schiff base, reversal of a protein conformational change, uptake of a proton from the cytoplasmic surface, and reisomerization of the retinal to all-trans, merge into a single step. As the time constants in Table 2 indicate, M-decay is not slowed in the D38N/D96N mutant like the decay of M in D38N, but accelerated by about 2×, like the decay of N in D38N (Table 1). In the D102N/D96N and D104N/D96N mutants, M-decay appears unaffected. In the D36N/D96N mutant, however, M-decay exhibits a 1.6× greater time constant. Again, the results suggest that Asp-36, but not the other aspartic acid residues, may be involved with proton uptake.

Table 2: Decay Time Constants for D96N Bacteriorhodopsin and Various Mutants[a]

| sample | M-decay (ms)[b] | N-decay, 90 mM azide (ms)[c] |
|---|---|---|
| D96N | 1425 | 80 |
| D36N/D96N | 2210 | 70 |
| D38N/D96N | 623 | 18 |
| D38R/D96N | 682 | 5 |
| D36N/D38N/D102N/D96N | 988 | 16 |
| D102N/D96N | 1389 | 35 |
| D104N/D96N | 1553 | 69 |

[a] Conditions: for second column, 10 mM NaCl, 10 mM sodium phosphate, pH 7; for third column, as in Figure 3. [b] M-decay was approximated with a single exponential. [c] Last component of decay of absorbance change at 570 nm.

Unlike in the N intermediate of the wild-type protein, in D96N mutants residue 96 cannot become anionic. Nevertheless, an N state does accumulate under some conditions, if defined as an intermediate in which the Schiff base is reprotonated but the retinal is still 13-cis. The decay of this N obviously does not include proton uptake. Its kinetics can be determined in the presence of azide. This weak acid accelerates the proton exchange between the Schiff base and the cytoplasmic surface in D96N mutants (*50*−*52*), but not the steps that follow it (*52*). Thus, as shown in Figure 3, under these conditions the absorbance decrease at 410 nm is more rapid than the absorbance rise at 570 nm, in the same way as occurs in the wild-type protein at high pH (Figure 2). The continued absorbance change at 570 nm after decay of the change at 410 nm (and 660 nm) is evidence for the presence of the N intermediate. In this way, N is detected even in D38R/D96N, although it could not be seen in D38R (*35*). The traces indicate that the decay of N is 5× more rapid in D36N/D38N/D102N/D96N than in D96N (Figure 3A), although much less when D104N/D96N is compared with D96N (Figure 3B). N-decay is particularly rapid in the D38R/D96N mutant (Table 2). The effects of the mutations (Table 2) are remarkably similar to those on N-decay in mutants without the additional D96N mutation (Figure 2, Table 1), with the exception of the D36N/D96N mutant that does not exhibit a slowed N-decay. The acceleration of

[1] Accumulation of N was not observed in D38R, even at the higher pH, consistent with the earlier report on this mutant (*35*). However, N could be observed in D38R/D96N in the presence of azide (cf. below), with a greatly accelerated decay that suggested that its accumulation in D38R could be kinetically hindered.

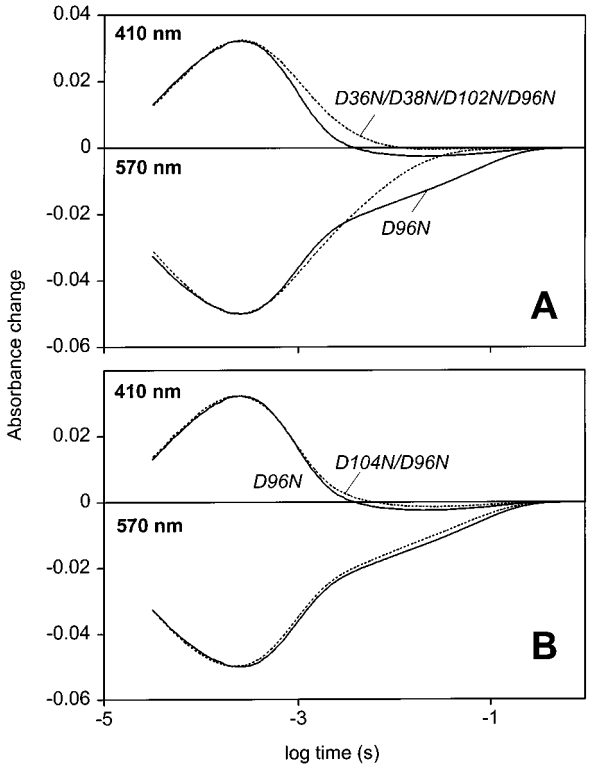


FIGURE 3: Kinetics of the M and N states after flash photoexcitation of D96N bacteriorhodopsin (A and B, solid line) and the D36N/D38N/D102N/D96N mutant (A, dotted line) or the D104N/D96 mutant (B, dotted line). Azide was added at 90 mM to accelerate decay of the M state so that the kinetics of the N state could be followed. Conditions: 9 mM NaCl, 9 mM sodium phosphate, pH 7.0

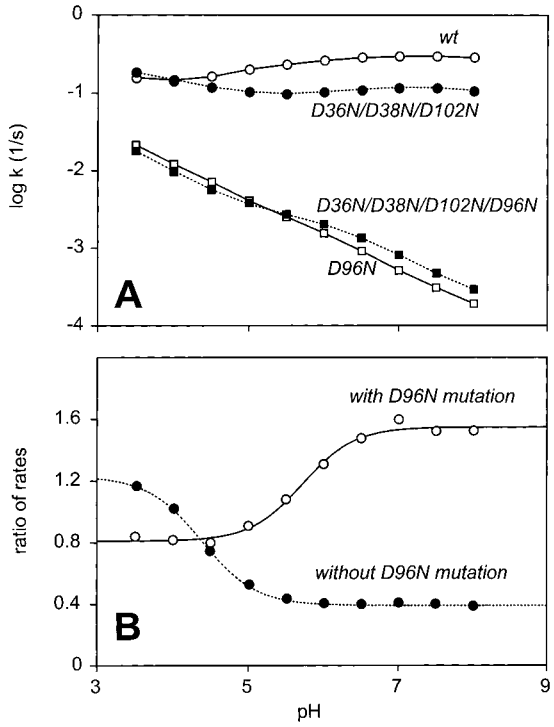


FIGURE 4: pH dependence of the effects of the D36N/D38N/D102N triple mutation on photocycle steps. (A) Rate constants (approximated as a single exponential) vs pH for M-decay in the wild type (open circles, solid line) and D36N/D38N/D102N (closed circles, dotted line), and in D96N (open squares, solid line) and D36N/D38N/D102N/D96N (closed squares, dotted line). (B) Ratios of rate constants in (A) vs pH. Open circles, solid line: rate constant for D36N/D38N/D102N/D96N divided by rate constants for D96N (apparent p$K_a$ = 5.7). Closed circles, dotted line: rate constants for D36N/D38N/D102N divided by rate constants for the wild type (apparent p$K_a$ = 4.4).

N-decay in these mutants of Asp-38 indicates that when an aspartic acid, this residue inhibits a step that is not proton conduction to Asp-96. On the other hand, the absence of slower N-decay in D36N/D96N, where this step does not involve proton uptake, strengthens the conclusion that Asp-36 participates in proton conduction.

*pH Dependence of the Effects of Mutations on the Photocycle Steps*. Figure 4A shows the time constants measured for M-decay in D36N/D38N/D102N (with the wild-type protein as control), and in D36N/D38N/D102N/D96N (with D96N as control), at various pH values between 4 and 8. The pH dependencies in both control samples are complex (*53, 54*), and themselves are so far not well explained. The results in Figure 4A clearly indicate, however, that all effects of the mutations studied are diminished at lower pH. When plotted as ratios, the deviations from the control time constants describe titration curves (Figure 4B), with apparent p$K_a$'s of 4.4 and 5.7 for the two mutants in Figure 4A. If these curves originate from the ionization of aspartic acid residues on the cytoplasmic surface, it would appear that asparagines at positions 36, 38, or 102 produce a phenotype similar to that of protonated aspartic acids. At low pH the ratios of the decay rates approach that of the D36N and D36N/D96N mutants, suggesting again that Asp-36 has a different effect, in this case a pH-independent one, from the other three residues. It would have to be part of this interpretation that the p$K_a$'s of the aspartic acid residues titrated are influenced somewhat by the absence of Asp-96 in D96N.

Although not shown, at low pH the decay of M in D38N and D104N approaches the rate in the wild type, suggesting that Asp-38 and Asp-104, as the collection of the three aspartates in Figure 4, are being titrated. If so, asparagines at these positions are equivalent to protonated aspartic acids. Likewise, the decay of N in several mutants containing the D38N and D96N mutations (Table 2), measured as in Figure 3, also approaches the decay rate in D96N at low pH. The result suggests that the pH dependence of D96N relative to the D36N/D38N/D102N/D96N mutant originates from Asp-38, which contributes most to its effects on the photocycle.

*Conservation of Aspartic Acid Residues at the Cytoplasmic Surface*. Bacteriorhodopsins have been identified in 13 species or strains so far. Figure 5 shows their sequences near Asp-36/Asp-38 and Asp-102/Asp-104, aligned with the program MSA,[2] version 2.1 (*55*).[3] Neighboring segments are included to illustrate the large number of conserved residues that flank these regions. From Figure 5 it is evident that Asp-38 is replaced conservatively by glutamic acid in 2 species, but by arginine or lysine, i.e., with a positively charged residue, in 9 of the 13 species. In five of the cases when residue 38 is an arginine, another acidic residue, glutamic acid, appears at position 34 so as to maintain the pattern of a pair of carboxyl groups two residues apart, but in the other

[2] At http://www.ibc.wustl.edu/ibc/msa.html.

[3] Archaerhodopsin-1 is the same sequence as SG1, reported later in Soppa, et al. (*67*).

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20          30          40                    90          100         110
MGLGTLYFLVKGMGVSDPDAKKFYAITTLV--DWLFTTPLLLLDLALLVDADQGTILALVGADGIMI
salinarium
MFLGMLYFIARGWGETDGRRQKFYIATILI--DWLFTTPLLLYDLGLLAGADRNTIYSLVSLDVLMI
shark
MFLGMLYFIARGWGETDSRRQKFYIATILI--DWLFTTPLLLYDLGLLAGADRNTITSLVSLDVLMI
port
MLIGTFYFIARGWGVTDKKAREYYAITILV--DWLFTTPLLLLDLALLANADRTTIGTLIGVDALMI
mex
MFLGMLYFIARGWGETDSRRQKFYIATILI--DWLFTTPLLLYDLGLLAGADRNTITSLVSLDVLMI
Cruxrhodopsin-1
MFLGMLYFIARGWSVSDQRRQKFYIATIMI--DWLFTTPLLLYDLALLAGADRNTIYSLVGLDVLMI
Cruxrhodopsin-2
MLIGTFYFIVKGWGVTDKEAREYYSITILV--DWLFTTPLLLLDLALLAKVDRVSIGTLVGVDALMI
Archaerhodopsin-1
MLIGTFYFIARGWGVTDKEAREYYAITILV--DWLFTTPLLLLDLALLAKVDRVTIGTLIGVDALMI
Archaerhodopsin-2
MLIGTFYFLVRGWGVTDKDVREYYAVTILV--DWLFTTPLLLLDLALLAKVDRVTIGTLVGVDALMI
Archaerhodopsin-3
MFLGMLYFIARGWGETDSRRQKFYIATILI--DWLFTTPLLLYDLGLLAGADRNTISSLVSLDVLMI
Cruxrhodopsin-3
MFLGMLYFIGRGWGETDSRRQKFYIATILI--DWLFTTPLLLYDLGLLAGADRNTIASLVSLDVLMI
H. japonica
MTLGTLVFVGRGRGVRDRKMQEFYIITIFI--DWLFTTPLLLLDLSLLAGANRNTIATLIGLDVFMI
HT
MTLGTLYFVGRGRGVRDRKMQEFYIITIFI--DWLFTTPLLLLDLSLLAGANRNTIATLIGLDVFMI
Arg-4
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FIGURE 5: Partial sequences from 13 naturally occurring bacteriorhodopsins, aligned with the program MSA, version 2.1 (*55*). Their origins, in the sequence shown, are as follows: *H. salinarium* (*57*), *shark*, *port*, and *mex* (*58*), *cruxrhodopsin*-1 (*59*), *cruxrhodopsin*-2 (*60*), *archaerhodopsin*-1 (*61;* footnote 3), *archaerhodopsin*-2 (*62*), *archaerhodopsin*-3 (*63*), *cruxrhodopsin*-3 (*64*), *H. japonica* (*65*), *HT* (*66*), and *Arg*-4 (*63*). Residues 34, 36, 38, 102, and 104 are shown in boldface type.

four cases residue 34 is a valine. Residue 102 is an aspartic acid only in *H. salinarium*. In the other 12 species, it is either a glycine or a lysine. Residue 104 is an aspartic acid in 11 species, but in 2 cases it is an asparagine. It appears, therefore, that of the four aspartic acid residues at the cytoplasmic surface of the *H. salinarium* protein, only Asp-36 is conserved.

*Light Intensity Dependence of Proton Transport by Wild-Type Bacteriorhodopsin and the D36N/D38N/D102N Mutant*. Replacement of aspartic acids at the cytoplasmic surface, singly or in various combinations, slows the overall turnover of bacteriorhodopsin, although to relatively small extents (Table 1). The requirement for maximizing the rate of proton transport could be an evolutionary pressure for retaining these acidic residues, regardless of whether they are conserved in all known species (Figure 5), but only if the turnover of the transport cycle is slow enough to be limiting. In natural environments of the highest known solar flux, such as Lake Kinneret (Israel) at noon in August, the total energy delivered to the surface between 520 and 650 nm is about 10 mW/cm$^2$ (*56*). Is this a high enough light intensity to drive bacteriorhodopsin to a saturating photostationary state, and thus make the turnover of the cycle be the limiting factor in transport?

Figure 6 shows the light intensity dependence of the rate of proton transport in cell envelope vesicles containing either wild-type bacteriorhodopsin or the D36N/D38N/D102N mutant. As expected, the triple mutant with a 3× slower overall turnover (Table 1) exhibited half-saturation at an approximately 3× lower light intensity. However, at 10 mW/cm$^2$ the rate of transport is limited by light rather than by the turnover rate of the cycle, and the three mutations have no effect on the rate of transport. This will be true for the fourth mutation, from the overall turnover of the photocycle of the D104N mutant (Table 1), as well. According to these results, the reason residues 36, 38, 102, and 104 are aspartates cannot be that they are needed for the rapid turnover of the proton transport cycle.

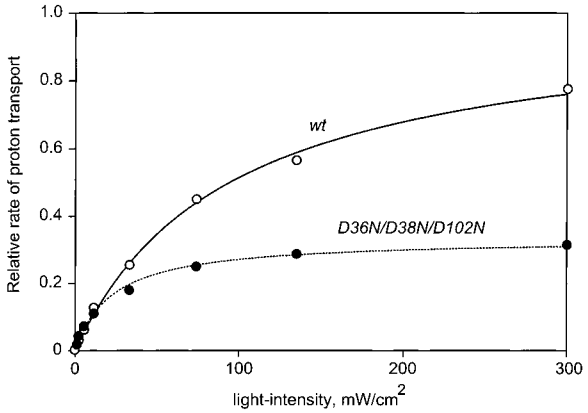


FIGURE 6: Proton transport at various light intensities in cell envelope vesicles containing either wild-type bacteriorhodopsin (open circles, solid line) or the D36N/D38N/D102N mutant (closed circles, dotted line). The measured rates of pH decrease upon illumination of the envelope suspensions with yellow light (>520 nm) were first normalized to the maximal rate calculated at infinite light intensity, and then to a 3× higher rate for the wild type because its photocycle turnover is approximately 3× more rapid (Table 1).

## DISCUSSION

The properties of the numerous aspartic acid to asparagine mutants studied indicate that Asp-36, Asp-38, Asp-102, and Asp-104, located at the cytoplasmic surface of *H. salinarium* bacteriorhodopsin, have small but distinct effects on the photochemical cycle. When residue 36 is an aspartic acid, it slightly inhibits the rate of proton transfer to the retinal Schiff base. Asp-38 and Asp-104 increase this rate by up to 3×, while Asp-102 has no detectable effect (Table 1). Asp-38 and Asp-102 inhibit another photocycle step that is most

likely protein conformational change and/or reisomerization of the retinal to all-trans, by 2−3×, while Asp-104 has negligible effects on this event (Tables 1 and 2). The observed slower M-decay and faster N-decay in Asp-38 mutants could explain why the N state does not accumulate measurably in the photocycle of D38R (*35*, *36*).

The observations that N-decay in D36N (Table 1) and M-decay in D36N/D96N (Table 2) are somewhat slower than in the wild type and D96N, respectively, but N-decay is unchanged in D36N/D96N relative to D96N (Table 2) together suggest that Asp-36 may enhance proton uptake at the cytoplasmic surface. If it does, this enhancement is well below 2-fold. None of the observations suggest that the other aspartic acid residues play any direct role in proton transfer. It was pointed out earlier (19) that the existence of alternative proton-transfer pathways in the Asp-36/Asp-38/Asp-102 triad and Asp-102/Asp-104 pair would result in uninhibited proton uptake in single aspartic acid to asparagine mutants, as found. However, the observed properties of the double and triple mutants (Tables 1 and 2) rule out such alternative pathways. Acceleration of the internal reprotonation of the retinal Schiff base (M-decay) when residues 38 and 104 are aspartic acids appears to be correlated with their negative charges (Figure 4 and Results). The inhibitory effect of residue 38, when it is an aspartic acid, on N-decay appears to be related to charge also, because the time constants for this reaction are in the order D96N > D38N/D96N > D38R/D96N (Table 2), i.e., decreasing time constants in the order of increasing positive charge. Thus, all of the effects of these residues appear to be electrostatic. The lack of clear correlation of the mutations studied with proton uptake is to be expected if the rates of the photocycle steps are limited not by proton transfer but by the much slower conformational changes of the protein.

Of the 4 aspartic acid residues discussed, only Asp-36 is conserved[4] in the 13 species in which amino acid sequences for bacterioopsin are available (Figure 5). However, even when a residue is well-conserved, it may not be essential or important for function. We suggest the following criterion: *if a site-specific mutation makes the turnover rate of the photocycle so slow that at natural solar fluxes light intensity no longer limits proton transport, evolution may have selected and maintained that residue in the wild-type protein.* A very rough estimation from Figure 6 is that natural selection on the basis of transport function becomes a possibility if the overall time constant for the last step of the photocycle is at least a few hundred milliseconds. Replacement of Asp-38 with arginine brings the time constant into this range (*35*). However, Asp-38 is not only not conserved, but often found to be replaced by an arginine or a lysine in natural isolates (Figure 5). Possibly, under these conditions selective pressures could have favored *lower* transport activity. More conservative replacement of Asp-38 and the other three aspartic acid residues at the cytoplasmic surface, i.e., with asparagine, does not change the turnover rate of the photocycle sufficiently to make a difference for natural selection.

[4] Of the two glutamate residues in a cytoplasmic interhelical loop, Glu-166 is conserved either as glutamate or as aspartate, but Glu-161 is not conserved. However, at pH 7 the photocycle of the E166Q mutant is only slightly different from the wild type (our unpublished results).

## REFERENCES


1. Mathies, R. A., Lin, S. W., Ames, J. B., and Pollard, W. T. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 491−518.
2. Oesterhelt, D., Tittor, J., and Bamberg, E. (1992) *J. Bioenerg. Biomembr. 24*, 181−191.
3. Ebrey, T. G. (1993) in *Thermodynamics of membranes, receptors and channels* (Jackson, M., Ed.) pp 353−387, CRC Press, New York.
4. Khorana, H. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1166−1171.
5. Lanyi, J. K. (1993) *Biochim. Biophys. Acta 1183*, 241−261.
6. Lanyi, J. K., and Váró, G. (1995) *Isr. J. Chem. 35*, 365−386.
7. Zimányi, L., Váró, G., Chang, M., Ni, B., Needleman, R., and Lanyi, J. K. (1992) *Biochemistry 31*, 8535−8543.
8. Balashov, S. P., Imasheva, E. S., Govindjee, R., and Ebrey, T. G. (1996) *Biophys. J. 70*, 473−481.
9. Balashov, S. P., Imasheva, E. S., Ebrey, T. G., Chen, N., Menick, D. R., and Crouch, R. K. (1997) *Biochemistry 36*, 8671−8676.
10. Richter, H. T., Brown, L. S., Needleman, R., and Lanyi, J. K. (1996) *Biochemistry 35*, 4054−4062.
11. Richter, H. T., Needleman, R., and Lanyi, J. K. (1996) *Biophys. J. 71*, 3392−3398.
12. Dioumaev, A. K., Richter, H. T., Brown, L. S., Tanio, M., Tuzi, S., Saitô, H., Kimura, Y., Needleman, R., and Lanyi, J. K. (1998) *Biochemistry 37*, 2496−2506.
13. Zimányi, L., Cao, Y., Needleman, R., Ottolenghi, M., and Lanyi, J. K. (1993) *Biochemistry 32*, 7669−7678.
14. Grzesiek, S., and Dencher, N. A. (1986) *FEBS Lett. 208*, 337−342.
15. Heberle, J., and Dencher, N. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5996−6000.
16. Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147−167.
17. Maeda, A. (1995) *Isr. J. Chem. 35*, 387−400.
18. Grigorieff, N., Ceska, T. A., Downing, K. H., Baldwin, J. M., and Henderson, R. (1996) *J. Mol. Biol. 259*, 393−421.
19. Kimura, Y., Vassylyev, D. G., Miyazawa, A., Kidera, A., Matsushima, M., Mitsuoka, K., Murata, K., Hirai, T., and Fujiyoshi, Y. (1997) *Nature 389*, 206−211.
20. Pebay-Peyroula, E., Rummel, G., Rosenbusch, J. P., and Landau, E. M. (1997) *Science 277*, 1676−1681.
21. Luecke, H., Richter, H. T., and Lanyi, J. K. (1998) *Science 280*, 1934−1937.
22. Essen, L. O., Siegert, R., Lehmann, W. D., and Oesterhelt, D. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 11673−11678.
23. Subramaniam, S., Gerstein, M., Oesterhelt, D., and Henderson, R. (1993) *EMBO J. 12*, 1−8.
24. Kamikubo, H., Kataoka, M., Váró, G., Oka, T., Tokunaga, F., Needleman, R., and Lanyi, J. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1386−1390.
25. Vonck, J. (1996) *Biochemistry 35*, 5870−5878.
26. Brown, L. S., Váró, G., Needleman, R., and Lanyi, J. K. (1995) *Biophys. J. 69*, 2103−2111.
27. Váró, G., Needleman, R., and Lanyi, J. K. (1996) *Biophys. J. 70*, 461−467.
28. Váró, G., and Lanyi, J. K. (1991) *Biophys. J. 59*, 313−322.
29. Cao, Y., Váró, G., Chang, M., Ni, B., Needleman, R., and Lanyi, J. K. (1991) *Biochemistry 30*, 10972−10979.
30. Váró, G., and Lanyi, J. K. (1995) *Biochemistry 34*, 12161−12169.
31. Mogi, T., Stern, L. J., Marti, T., Chao, B. H., and Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4148−4152.
32. Nachliel, E., Gutman, M., Kiryati, S., and Dencher, N. A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10747−10752.
33. Checover, S., Nachliel, E., Dencher, N. A., and Gutman, M. (1997) *Biochemistry 36*, 13919−13928.
34. Sacks, V., Marantz, Y., Aagaard, A., Checover, S., Nachliel, E., and Gutman, M. (1998) *Biochim. Biophys. Acta 1365*, 232−240.
35. Riesle, J., Oesterhelt, D., Dencher, N. A., and Heberle, J. (1996) *Biochemistry 35*, 6635−6643.

36. Sass, H. J., Gessenich, R., Koch, M. H. J., Oesterhelt, D., Dencher, N. A., Büldt, G., and Rapp, G. (1998) *Biophys. J. 75*, 399−405.
37. Oesterhelt, D., and Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667−678.
38. Needleman, R., Chang, M., Ni, B., Váró, G., Fornes, J., White, S. H., and Lanyi, J. K. (1991) *J. Biol. Chem. 266*, 11478−11484.
39. Váró, G., Zimányi, L., Fan, X., Sun, L., Needleman, R., and Lanyi, J. K. (1995) *Biophys. J. 68*, 2062−2072.
40. Lanyi, J. K., and MacDonald, R. E. (1979) *Methods Enzymol. 56*, 398−407.
41. Váró, G., Brown, L. S., Needleman, R., and Lanyi, J. K. (1996) *Biochemistry 35*, 6604−6611.
42. Brown, L. S., Dioumaev, A. K., Needleman, R., and Lanyi, J. K. (1998) *Biochemistry 37*, 3982−3993.
43. Váró, G., and Lanyi, J. K. (1990) *Biochemistry 29*, 2241−2250.
44. Váró, G., and Lanyi, J. K. (1991) *Biochemistry 30*, 5016−5022.
45. Richter, H. T., Needleman, R., Kandori, H., Maeda, A., and Lanyi, J. K. (1996) *Biochemistry 35*, 15461−15466.
46. Kandori, H., Yamazaki, Y., Hatanaka, M., Needleman, R., Brown, L. S., Richter, H. T., Lanyi, J. K., and Maeda, A. (1997) *Biochemistry 36*, 5134−5141.
47. Gerwert, K., Hess, B., Soppa, J., and Oesterhelt, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4943−4947.
48. Otto, H., Marti, T., Holz, M., Mogi, T., Lindau, M., Khorana, H. G., and Heyn, M. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9228−9232.
49. Miller, A., and Oesterhelt, D. (1990) *Biochim. Biophys. Acta 1020*, 57−64.
50. Lanyi, J. K. (1986) *Biochemistry 25*, 6706−6711.
51. Tittor, J., Soell, C., Oesterhelt, D., Butt, H.-J., and Bamberg, E. (1989) *EMBO J. 8*, 3477−3482.
52. Brown, L. S., and Lanyi, J. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1731−1734.
53. Eisfeld, W., Pusch, C., Diller, R., Lohrmann, R., and Stockburger, M. (1993) *Biochemistry 32*, 7196−7215.
54. Radionov, A. N., Kalaidzidis, I. V., and Kaulen, A. D. (1996) *FEBS Lett. 399*, 251−254.
55. Lipman, D., Altschul, S. F., and Kececioglu, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4412−4415.
56. Dubinsky, Z., and Berman, T. (1979) *Limnol. Oceanogr. 24*, 652−663.
57. Khorana, H. G., Gerber, G. E., Herlihy, W. C., Gray, C. P., Anderegg, R. J., Nihei, K., and Biemann, K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5046−5050.
58. Otomo, J., Urabe, Y., Tomioka, H., and Sasabe, H. (1992) *J. Gen. Microbiol. 138*, 2389−2396.
59. Tateno, M., Ihara, K., and Mukohata, Y. (1994) *Arch. Biochem. Biophys. 315*, 127−132.
60. Sugiyama, Y., Yamada, N., and Mukohata, Y. (1994) *Biochim. Biophys. Acta 1188*, 287−292.
61. Sugiyama, Y., Maeda, M., Futai, M., and Mukohata, Y. (1989) *J. Biol. Chem. 264*, 20859−20862.
62. Uegaki, K., Sugiyama, Y., and Mukohata, Y. (1991) *Arch. Biochem. Biophys. 286*, 107−110.
63. Ihara, K., Umemura, T., Katagiri, I., Kitajima-Ihara, T., Sugiyama, Y., Kimura, Y., and Mukohata, Y. (1999) *J. Mol. Biol. 285*, 163−174.
64. Kitajima, T., Hirayama, J.-I., Ihara, K., Sugiyama, Y., Kamo, N., and Mukohata, Y. (1996) *Biochem. Biophys. Res. Commun. 220*, 341−345.
65. Yatsunami, R., Kawakami, T., Ohtani, H., and Nakamura, S. (1997) *Nucleic Acids Symp. Ser. No. 37*, 111−112.
66. Kamekura, M., Seno, Y., and Tomioka, H. (1998) *Extremophiles 2*, 33−39.
67. Soppa, J., Duschl, J., and Oesterhelt, D. (1993) *J. Bacteriol. 175*, 2720−2726.

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