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Biochemistry

Aspartic acid substitutions affect proton translocation by bacteriorhodopsin*

(synthetic gene/site-specific mutagenesis/restriction fragment replacement/proteoliposomes)

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ABSTRACT **We have substituted each of the aspartic acid residues in bacteriorhodopsin to determine their possible role in proton translocation by this protein. The aspartic acid residues were replaced by asparagines; in addition, Asp-85, -96, -115, and -212 were changed to glutamic acid and Asp-212 was also replaced by alanine. The mutant bacteriorhodopsin genes were expressed in *Escherichia coli* and the proteins were purified. The mutant proteins all regenerated bacteriorhodopsin-like chromophores when treated with a detergent–phospholipid mixture and retinal. However, the rates of regeneration of the chromophores and their λ_{max} varied widely. No support was obtained for the external point charge model for the opsin shift. The Asp-85 → Asn mutant showed no detectable proton pumping, the Asp-96 → Asn and Asp-212 → Glu mutants showed <10% and the Asp-115 → Glu mutant showed ≈30% of the normal proton pumping. The implications of these findings for possible mechanisms of proton translocation by bacteriorhodopsin are discussed.**

Bacteriorhodopsin (bR), an integral membrane protein, functions as a light-driven proton pump in *Halobacterium halobium*. The protein traverses the cytoplasmic membrane seven times and contains one molecule of all-*trans*-retinal linked as a Schiff base to Lys-216 as the chromophore (Fig. 1). In structure–function studies of this protein, we are investigating the following questions. (*i*) What is the mechanism of vectorial proton translocation? Does it involve proton conduction through the functional groups of certain specific amino acids in the membrane-embedded regions? (*ii*) What is the nature of the interactions between retinal and the protein and how do these interactions change during different stages of the photochemical cycle? (*iii*) What is the nature of the interactions between the membrane-embedded segments that lead to a specific folding pathway?

By recombinant DNA methods we have carried out a variety of amino acid substitutions that were designed to remove specific functional groups (1–4). All of the mutants bound retinal to regenerate bR-like chromophores, and most showed unchanged light-dependent proton pumping. The mutants could be divided into two groups on the basis of their spectral properties. One group showed essentially the native bR absorption spectrum, whereas the second group showed varying but significant spectral shifts from the native bR spectrum. The only two mutants that showed altered proton pumping were Pro-186 → Leu and Tyr-185 → Phe.

With the aim of identifying more mutations that affect proton pumping, we have now carried out single substitutions of all the aspartic acid residues (Fig. 1) except for Asp-242. The latter amino acid was shown (5) not to affect proton pumping in bR. All the aspartic acid residues were replaced one at a time by asparagines; in addition, Asp-85, -96, -115, and -212 were replaced by glutamic acids and Asp-212 was replaced by alanine. The mutant proteins folded correctly as judged by the binding of all-*trans*-retinal and regeneration of bR-like chromophores. Studies of their proton-pumping activity in reconstituted liposomes showed: (*i*) the mutations Asp-85 → Asn, Asp-96 → Asn, and Asp-212 → Glu abolished completely or almost completely proton translocation; (*ii*) the mutants Asp-115 → Glu and Asp-212 → Asn showed reduced (35 and 15%, respectively) pumping whereas Asp-212 → Ala was unstable to light and showed no pumping; and (*iii*) substitution of Asp-36, -38, -102, -104, and -115 by asparagine residues did not affect proton pumping.

We document these findings and discuss their relevance to the mechanism of proton translocation as well as to the current proposals regarding "opsin shift."

MATERIALS AND METHODS

Materials. The plasmid pSBO2, which carries the synthetic bacterioopsin (bO) gene, and the bO expression plasmid pPL-1 have been described (4). L-α-Dimyristoyl phosphatidylcholine ([Myr_2]PtdCho) was obtained from Avanti Polar Lipids, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] (CHAPS) was from Boehringer Mannheim, and all-*trans*-retinal was from Kodak.

Deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (650 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. Restriction endonucleases were from Boehringer Mannheim or New England Biolabs and mung bean nuclease and DNA polymerase (large fragment) were from Pharmacia.

Acetone-washed soybean lipid was a gift from Debra Thompson of this laboratory, and the *Escherichia coli* strain GM119 (*dam3, dam6, supE44, rk$^+$mk$^+$*) was a gift from Harold Drabkin (Massachusetts Institute of Technology).

Purification of Plasmids. Plasmid DNA was isolated by the alkaline extraction method (6) from *E. coli* DH1/pSBO2 cells grown in Luria–Bertani medium overnight. Plasmids were further purified by either CsCl density gradient fractionation followed by A-50m (Bio-Rad) gel filtration, or by phenol extraction, RNase A treatment, and CsCl flotation (7). For digestion with *Bcl* I, pSBO2 DNA was isolated from the *E. coli* strain GM119.

Construction, Cloning, and Expression of the Mutant bO Genes Containing Specific Asp → Asn, Asp → Glu, or Asp → Ala Mutations. The general method for mutagenesis by restriction fragment replacement has been described (1–3). Pairs of appropriate oligonucleotides (Table 1) were synthesized, purified, and annealed to form the appropriate restriction fragments containing the desired codon changes (1). The

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Abbreviations: bR, bacteriorhodopsin; bO, bacterioopsin; [Myr_2]-PtdCho, L-α-dimyristoyl phosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DA, dark adapted; LA, light adapted.

*This is paper VII in the series "Structure–Function Studies on Bacteriorhodopsin." Paper VI is ref. 1.

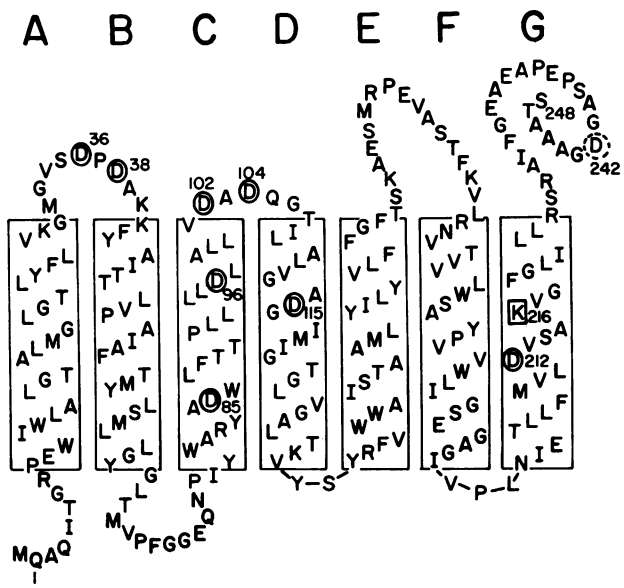

FIG. 1. A secondary structure model for bO with the location of aspartic acid residues (encircled) that were mutated in the present work. Asp-242 (broken circle) was not mutated (see text). Lys-216 that carries the Schiff base is boxed. The N-terminal sequence, Met-Gln-Ala-, shown is that encoded by the synthetic gene. The N-terminal sequence in the natural bO (from purple membrane) is <Glu-Ala-Gln-. A–G are α-helical membrane segments.

three-fragment ligation procedure (1) was used to insert synthetic fragments in place of their counterparts in the wild-type synthetic bO gene in the plasmid pSBO2. The codon for Asp-36 is on the 5′ overhang of a *Bam*HI site. To construct Asp-36 → Asn mutant, oligonucleotides that contain the altered codon were synthesized complementary through the *Bam*HI site. These were annealed and ligated to the large *Bam*HI–*Bsm* I fragment of pSBO2, which was blunt-ended at the *Bam*HI site with mung bean nuclease, in a two-part ligation. The sequences of the mutant segments in the bO genes were confirmed by direct plasmid sequencing with the dideoxy method (8, 9). Mutant bO genes were excised as the small *Hin*dIII–*Eco*RI fragments from pSBO2 and cloned into corresponding sites of the λ P_L expression vector pPL1 (1, 4).

The mutant pPL-SBO2 plasmids were introduced into *E. coli* strains C600/pC_I857 or W3110/pC_I857 as described (1, 3). The expression of the mutant bO genes and the purification of mutant apoproteins by solvent extraction and ion-exchange chromatography have been described (10).

Chromophore Regeneration. bR-like chromophores were generated from bO in a solution of 1% [Myr_2]PtdCho, 1% CHAPS, 0.2% $NaDodSO_4$, and 50 mM sodium phosphate (pH 6.0) by the addition of all-*trans*-retinal in the dark at 20°C (1, 3). Dark-adapted (DA) and light-adapted (LA) λ_{max} refer to absorption spectra taken after overnight dark adaptation and after 5 min of irradiation from a 300-W projector lamp equipped with an appropriate long-pass filter (510–550 nm) at 4°C, respectively.

Proton Pumping. The mutant bR proteins were reconstituted into asolectin liposomes by a modification of the octylglucoside dilution method (1, 3) in which the concentrations of asolectin and octylglucoside in the micellar solution before a 1:25 dilution were 1.9% and 1.6% (wt/vol), respectively. Vesicles were irradiated by light from a 300-W projector lamp, which had been filtered through a 495-nm long-pass filter and focused onto a sample vessel thermostated at 30°C. Light-dependent alkalinization of the medium was monitored by a pH electrode as described (1, 3).

Table 1. Aspartic acid residues and the substituting amino acids in bO

Asp residue replaced		Substituting			
No.	Codon	amino acid	Codon	Restriction fragment*	
36	GAT	Asn	AAC	*Bam*HI†–*Bsm* I	(106–157)
38	GAT	Asn	AAC	*Bam*HI–*Bsm* I	(106–157)
85	GAC	Asn	AAC	*Apa* I–*Bgl* II	(240–289)
		Glu	GAA	*Apa* I–*Bgl* II	(240–289)
96	GAT	Asn	AAC	*Apa* I–*Bcl* I	(240–313)
		Glu	GAA	*Apa* I–*Bcl* I	(240–313)
102	GAC	Asn	AAC	*Bgl* II–*Nar* I	(286–340)
104	GAT	Asn	AAC	*Bgl* II–*Nar* I	(286–340)
115	GAC	Asn	AAC	*Nar* I–*Bss*HII	(339–378)
		Glu	GAA	*Nar* I–*Bss*HII	(339–378)
212	GAC	Asn	AAC	*Ssp* I–*Xho* I	(607–681)
		Glu	GAA	*Xba* I–*Xho* I	(631–681)
		Ala	GCT	*Xba* I–*Xho* I	(631–681)

*Numbering of nucleotides starts as −3 at ATG of the N-terminal Met codon of the synthetic bO gene. Numbers in parentheses are positions of first and last base in the fragment.
†Synthetic duplex was complementary through to the *Bam*HI site to produce a blunt-end fragment.

RESULTS

Construction and Expression of bO Mutants. Table 1 shows the eight aspartic acid residues and their codons that were replaced, the substituting amino acids, and their codons. All of the mutations were introduced by replacement of restriction fragments in the wild-type bO gene with synthetic counterparts containing the changed codons. The restriction fragments involved and their nucleotide positions in the gene are also shown in Table 1. DNA sequences of the synthetic segments cloned into pSBO2 were confirmed by direct plasmid sequencing with the dideoxy method by using appropriate synthetic primers. In selected cases where particularly interesting phenotypes were observed, the entire mutant bO genes were sequenced. For this purpose, the expression vectors pPL-SBO2 containing Asp-85 → Asn, Asp-96 → Asn, Asp-212 → Asn, and Asp-212 → Glu mutations were isolated from the fermentor cultures from which the mutant proteins had been purified. All of the mutant bO proteins could be purified to apparent homogeneity as described (10).

Asp → Asn Mutants at Positions 36, 38, 102, and 104. These four mutants regenerated bR-like chromophores with λ_{max} values close to that of the wild type (Table 2). They all showed normal λ_{max} shifts upon light–dark adaptation (Table 2) and normal proton pumping activities (Table 3).

Table 2. Chromophore regeneration and spectral characteristics of the aspartic acid mutants

Mutant	$t_{1/2}$ of chromophore regeneration, min	Regeneration, %	λ_{max}, nm DA	LA
bR (wild type)	1.0	80	551	561
Asp-36 → Asn	1.3	60	550	560
Asp-38 → Asn	1.3	72	553	563
Asp-85 → Asn	20	34	587	594
Asp-85 → Glu	<0.1	57	556	560
Asp-96 → Asn	2.3	73	553	560
Asp-96 → Glu	1.2	72	553	561
Asp-102 → Asn	1.1	72	553	563
Asp-104 → Asn	2.1	76	550	560
Asp-115 → Asn	6.0	42	545	544
Asp-115 → Glu	4.6	34	541	543
Asp-212 → Asn	31	64	560	548
Asp-212 → Glu	38	57	584	581
Asp-212 → Ala	50	51	540	—*

*Light unstable.

Table 3. Proton-pumping activity of mutants containing aspartic acid substitutions in bR

Mutant	Initial rate, H^+ per bR per sec	Steady state, H^+ per bR
bR	3.4	51
Asp-36 → Asn	3.9	56
Asp-38 → Asn	3.6	58
Asp-85 → Asn	ND	ND
Asp-85 → Glu	1.1	20
Asp-96 → Asn	0.1	3
Asp-96 → Glu	2.8	47
Asp-102 → Asn	3.3	47
Asp-104 → Asn	3.5	48
Asp-115 → Asn	1.9	40
Asp-115 → Glu	1.1	20
Asp-212 → Asn	0.5	6
Asp-212 → Glu	0.2	2
Asp-212 → Ala*	ND	ND

The values for each mutant are the average of at least six independently reconstituted preparations. ND, not detected.
*Light unstable.

Asp-85 → Asn and Asp-85 → Glu Mutants. Asp-85 → Asn regenerated a bR-like chromophore at a rate that was ≈20 times slower than that of the wild type. The extent of chromophore regeneration was only ≈30% and the λ_{max} showed a large red shift in the LA and DA forms (594 nm and 587 nm, respectively) (Table 2 and Fig. 2). The mutant Asp-85 → Asn was completely devoid of proton pumping activity when tested by the standard method after reconstitution into liposomes (Table 3 and Fig. 2).

In contrast to all other bR mutants so far studied, the mutant Asp-85 → Glu regenerated the bR-like chromophore at a rate faster than that of the wild type (Table 2 and Fig. 2). The λ_{max} (LA, 560 nm; DA, 556 nm) was slightly red-shifted with a minor component at 610 nm. In lauryl maltoside solution, the mutant showed a largely red-shifted chromophore (DA, 609 nm) with an increased ε value of 89,500. The mutant showed proton pumping activity about one-third of that observed with the wild type (Fig. 2 and Table 3).

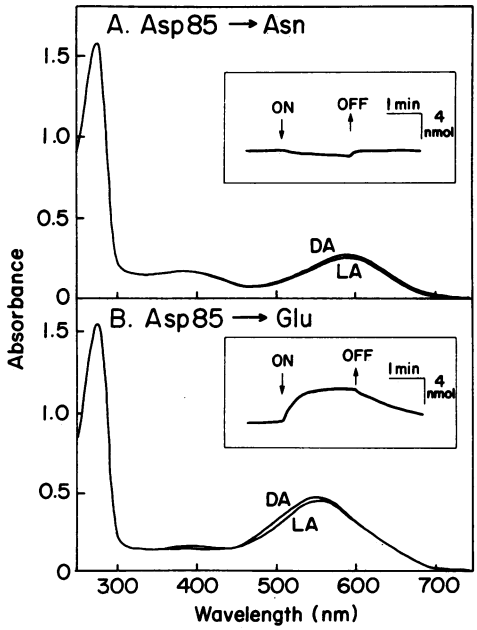

FIG. 2. (*A* and *B*) Absorption spectra and proton pumping behavior of the regenerated mutants Asp-85 → Asn and Asp-85 → Glu, respectively. The pumping experiments were done by using 300 pmol of Asp-85 → Asn (*A Inset*) and 100 pmol of Asp-85 → Glu (*B Inset*). The start and termination of illumination are shown by arrows pointing down or up, respectively. Apparent proton release seen in Asp-85 → Asn mutant is an artifact due to electrical noise.

Asp-96 → Asn and Asp-96 → Glu Mutants. Both mutants regenerated the wild-type chromophore with rates comparable to that of the wild type (Table 2 and Fig. 3). Both mutants showed normal light adaptation. When reconstituted into liposomes the Asp-96 → Asn mutant showed <3% proton pumping relative to the wild type whereas the Asp-96 → Glu mutant was normal (Table 3 and Fig. 3).

Asp-115 → Asn and Asp-115 → Glu Mutants. Both mutants regenerated slightly blue-shifted chromophores (Table 2) at rates ≈5 times slower than the wild type. The extent of regeneration was relatively low in both cases. The Asp-115 → Asn and Asp-115 → Glu mutants showed somewhat reduced pumping (60 and 30%, respectively) (Table 3).

Asp-212 Mutants Containing Asn, Glu, and Ala Replacements. All the three mutants regenerated chromophores in the extents of 50–65% but the rates were very low (30–50 times slower than the wild type, Table 2). The Asp-212 → Asn mutant showed a wild-type λ_{max} whereas the Asp-212 → Glu mutant showed a highly red-shifted chromophore (DA λ_{max}, 584 nm) with an unusually high ε value of 88,000. Both mutants (Asp-212 → Asn and -Glu) exhibited reversed λ_{max} shifts upon light adaptation (DA λ_{max} > LA λ_{max}). The Asp-212 → Ala mutant showed a slightly blue-shifted chromophore (λ_{max} at 540 nm) that was unstable to light (Fig. 4). The Asp-212 → Asn and Asp-212 → Glu mutants showed severely reduced proton pumping (≈15% and ≈7%, respectively, of the wild type). No pumping was detected with the Asp-212 → Ala mutant presumably because of its instability to light.

DISCUSSION

In continuing structure–function studies of bR, we have examined the effects of the substitution of aspartic acid residues. Previously, Fourier transform infrared spectroscopy (FTIR) studies have indicated the involvement of aspartic acid residues in proton pumping (11–14) and a

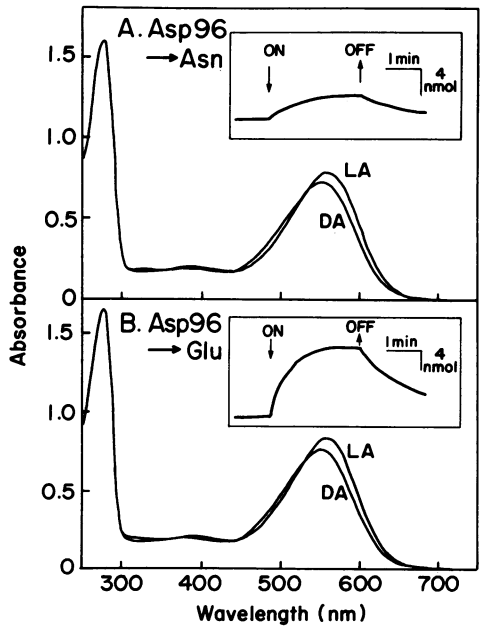

FIG. 3. (*A* and *B*) Absorption spectra and proton pumping behavior of the regenerated mutants Asp-96 → Asn and Asp-96 → Glu, respectively. The following amounts were used in pumping experiments: 300 pmol of Asp-96 → Asn (*A Inset*) and 100 pmol of Asp-96 → Glu (*B Inset*).

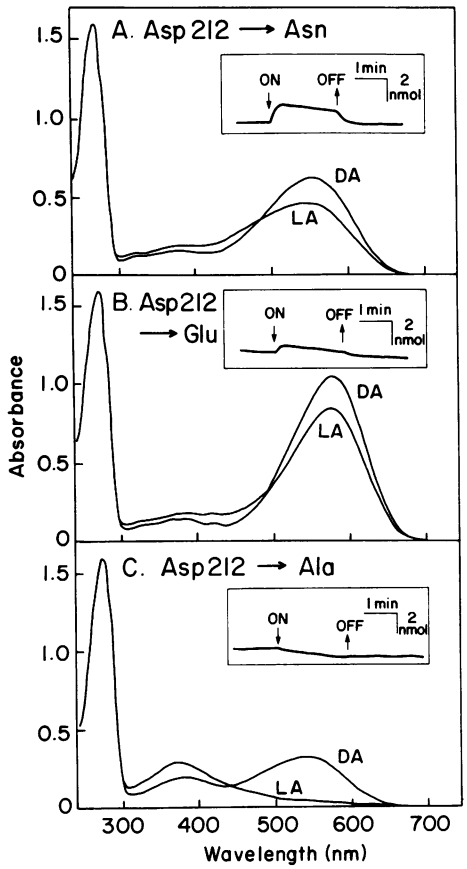

FIG. 4. (*A–C*) Absorption spectra and proton pumping behavior of the regenerated mutants Asp-212 → Asn, Asp-212 → Glu, and Asp-212 → Ala, respectively. The amount of the reconstituted bR used was 300 pmol in all cases (*A–C Insets*).

chemical modification experiment has suggested that Asp-115 residue may be involved (15, 16). The results reported herein show that, whereas substitution of the four residues Asp-36, -38, -102, and -104 (Fig. 1) does not have any significant effect, individual substitutions of Asp-85, -96, and -212 (Fig. 1) exert profound effects on the properties of bR and the substitution of Asp-115 also has significant effects. We discuss, first, the effects on chromophore regeneration and spectral properties and, next, the effects on proton pumping.

The Opsin Shift and the External Point Charge Model. Protonated Schiff base formed from all-*trans*-retinal and an alkylamine shows a λ_{max} between 400 and 440 nm (17). However, an essentially identical chromophore present in bO and visual pigments shows λ_{max} in the range of 500–600 nm. To account for this general large red shift (designated the opsin shift) (18, 19), it has been proposed that negative charges, presumably from carboxylate ions of aspartate or glutamate, are present within a certain distance (≈4 Å) and that these anions interact with the protonated Schiff base and with the β-ionone part of the polyene chain. Specifically, in the case of bR, the two interactions proposed are (*i*) between Asp-212 and the protonated Schiff base at Lys-216 and (*ii*) between Asp-115 and the polyene backbone. Additional smaller contributions to the red shift in bR have also been suggested (20).

If the opsin shift is due, in large part, to interactions of the retinylidene Schiff base with nearby charged amino acids, substitution of these charged amino acids by neutral ones should eliminate or greatly reduce the opsin shift. Replacement of Glu-194, expected to be near the retinal β-ionone ring (21), by glutamine showed only insignificant effect on the bR spectrum (3). In the present work, none of the aspartic acid mutations resulted in a significant reduction of the opsin shift. In fact, the replacement Asp-212 → Glu gave a very large red shift and a large increase in molar extinction coefficient. The Asp-212 → Asn mutant gave a minor shift (Table 2) and the replacement Asp-212 → Ala caused only a minor blue shift. Therefore, our results do not support the current postulates for the opsin shift. We believe, instead, that interactions between the polyene chain and the side chains of certain amino acids, particularly tryptophans, make important contributions to the red shift (3).

However, our results with the Asp-212 mutants do suggest the requirement of an interaction between the Schiff base and the amino acid residue at position 212 for stability and photocycling of bR. Thus, the three Asp-212 mutants regenerate the chromophores 30–50 times slower than the wild type, and the light–dark adaptation behavior is reversed. Further, Asp-212 → Ala mutant is light-unstable.

Proton Translocation. The present work shows that at least three aspartic acid residues—Asp-85, Asp-96, and Asp-212, and, possibly, Asp-115—are candidates for involvement in the proton translocation process. Thus, the Asp-85 → Asn mutant was devoid of proton pumping, Asp-96 → Asn mutant showed <5%, and Asp-115 → Glu mutant showed ≈30% of normal proton pumping. Further, the three Asp-212 mutants showed striking effects on proton pumping. The mutants Asp-212 → Glu and Asp-212 → Asn showed ≈10% and ≈20% proton pumping, respectively, of the normal whereas the Asp-212 → Ala mutant showed no detectable pumping, presumably because it was unstable to light. Therefore, we conclude that Asp-85 and Asp-96 (presumed helix C, Figs. 1 and 5) are probably important in proton translocation, and Asp-115 (presumed helix D) could possibly be involved. Asp-212 may either be a participant in proton translocation or may regulate protonation–deprotonation of the Schiff base.

The most significant conclusion from the present results is that proton translocation involves several amino acids and, therefore, a proton conductance mechanism (22) is likely. Further, the present findings provide a clearer focus for further investigation of the overall proton translocation pro-

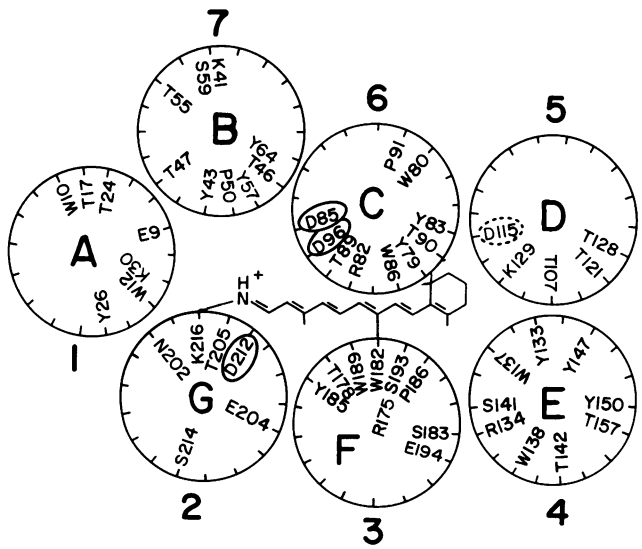

FIG. 5. A helical wheel projection map for bO. Seven α-helical membrane segments A, G, F, E, D, C, and B were assigned locations 1–7, respectively, in the projection map. All the residues of the following amino acids are shown: tyrosine, tryptophan, proline, aspartic acid, glutamic acid, arginine, lysine, serine, threonine, and asparagine. Asp-85, -96, and -212, in which proton pumping is strongly affected on substitution, are shown in solid ovals. Asp-115 mutation that shows reduced H^+ pumping is shown in dotted oval.

cess. There are several questions that must now be addressed. How many components does the process involve? Where does proton translocation begin and where does it end? From the present work, one possible starting point for proton translocation is Asp-96. A second possibility is the protonated Schiff base (Figs. 1 and 5). The Schiff base proton is known to undergo very rapid exchange with water in the dark (23, 24), and it has also been suggested that the protonated Schiff base may be in tight association with a water molecule (25). If proton translocation begins at the Schiff base, how does the proton arrive at this site? Is there a water pore (a channel) that makes water accessible to the Schiff base from the cytoplasmic side? Two reactions of bR show that, at least on illumination, water is accessible to the Schiff base. The protonated Schiff base in purple membrane can be bleached with NH_2OH and it can also be reduced with $NaBH_4$. These reactions are promoted by light. Therefore, at least when bR is photocycling, aqueous solutions of these reagents establish contact with the Schiff base. Alternatively, the water pore could exist even in the dark and light would isomerize the C_{13}-C_{14} double bond to the cis form that would undergo stereo-specific reaction with the reagents.

To visualize the above effects of mutations in bR we have developed a helical wheel model (Fig. 5) of the protein, positioning the helices in the contours of the electron diffraction map of bR_1 (26) on the basis of diffraction (26–32), fluorescence energy transfer (33), and mutagenesis (1, 3, 34) studies. The rotational orientation of the helices was selected on the basis (*i*) of the hydrophobic moment of the helices and (*ii*) the results of site-specific mutagenesis. No interhelical loops are shown, since their position in the map is unclear and since no effect has been observed for mutations in these regions. Mutations that alter the chromophore λ_{max} and regeneration rate (1, 3, 34) all fall on the faces of helices C, D, F, and G, which are able to arrange to form a retinal binding pocket. The residues Asp-85, Asp-96, and Asp-212, whose mutagenesis affects proton pumping, fall in a region near the Schiff base and may define the region through which the protons being translocated pass.

If proton translocation begins with the Schiff base, is Asp-212 the first acceptor of the proton or does the interaction between the Schiff base and Asp-212 simply perform a control function in photocycle? According to the present information, the proton should then translocate to helix C (Asp-96 and then to Asp-85). It is not clear at present if the distances involved would allow such proton transfers directly. The immediate question is: Are there additional amino acids that participate? Extension of the mutagenesis approach to the remainder of the hydrogen-bond forming amino acids, glutamic acids, arginines, threonines, and serines, may be revealing.

An integral part of the present work with the aspartic acid mutants is the determination of their photocycle characteristics so as to learn if or where a block is occurring in each mutant. Biophysical studies of the mutants now described and of additional ones are required to help delineate the pathway of proton translocation.

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