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Review

Reconciling crystallography and mutagenesis: a synthetic approach to the creation of a comprehensive model for proton pumping by bacteriorhodopsin

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Abstract

As a result of the number of new high-resolution structures of the pigment and some of its photointermediates, a realistic model for the functioning of bacteriorhodopsin seems to be finally emerging. However, lack of structural information for some of the key functional states, and contradictions between some published structural models, argue for the use of the synthetic approach, one that includes use of data from both crystallographic and mutagenesis studies. The role of mutagenesis in this synthetic approach falls into two categories. First, to provide additional structural information, and second, to test the predictions of structural models by studying mutant phenotypes. This review urges critical comparisons of the structural and mutagenesis data, as there are problems with their selective and indiscriminate use.

Keywords: Bacteriorhodopsin; Protein structure; Proton pumping; Crystallography; Mutagenesis; Proton pathway

1. Introduction

Bacteriorhodopsin (BR), the halobacterial proton pump, is probably the most studied membrane protein. It consists of seven transmembrane helices and a retinal chromophore covalently linked to the side chain of K216 via a protonated Schiff base. Bacteriorhodopsin is found in halobacterial membranes in a trimeric state, with the trimers forming two-dimensional crystalline patches called the purple membranes. Absorption of a photon by the chromophore starts a cycle of reactions (so-called photocycle) that result in proton translocation across the membrane. The interconversions of the spectrally distinct photocycle intermediates, called J, K, L, M, N, and O, correspond to more or less understood molecular events, including among others photoisomerization and later thermal reisomerization of the retinal, deprotonation and reprotonation of the Schiff base, conformational changes in the protein backbone, proton release from the extracellular surface of the membrane and proton uptake from the cytoplasmic side (for recent reviews see [42,57,89]).

Methodologically, one can divide the research on BR into three periods (for a different historical subdivision of the period of 1970–1995, see [89]). The first period encompassed about 15 years of biochemical and biophysical studies of the wild-type protein. It resulted in a substantial accumulation of basic knowledge but did not yield sufficient insights to create a realistic model for proton transport mechanism. The first breakthrough came with introduction of site-specific mutagenesis of the protein. This started the second period - the period of mutagenesis, which

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included about 10 years of studies of site-specific mutants and lead to the identification of the residues that are the major players in the proton pumping. As a result, a model of the BR functioning began to emerge (for reviews of this period see [30,55,56]). Still, lack of specific structural information at the atomic level left a lot of leeway in interpreting the mutational data.

It appears that what we have been witnessing in the last 3 years constitutes a third period: the period of the high-resolution structures (or crystallography period). There has been an explosive growth in the number of BR structures, with increasing resolutions (Table 1). Although the multitude of these structural models yielded a lot of information toward finalizing the model of the proton transport, to fully accomplish this task one would need to know structures of all the photocycle intermediates. Not all of these structures are available at this time, even though coordinates for the two intermediates (K and M) were recently released ([31,61] and 1CWQ[1]). Furthermore, some of the published structures contain contradictory information about key protein groups and water molecules involved in proton translocations [42,60,93]. Thus, at least at the present stage of BR research, one needs to use a synthesis of the mutational and the structural data in order to make judgements about the conflicts, and to fill in the gaps in the structural information. The two principal questions to be asked now are whether such a synthesis can succeed and what the role of mutagenesis would be in it.

2. Problems of the synthetic approach

The success of the synthetic approach to creation of a comprehensive model for BR would depend on the judicious use of the vast array of mutagenesis data[2] and the novel high-resolution structures of BR and its photointermediates. Unfortunately, neither data pools are free from problems and indiscriminate use of the experimental information may lead to wrong conclusions. Some of the possible pitfalls are presented below.

2.1. Possible problems of using mutagenesis data

2.1.1. Effects of mutations may be overlooked or exaggerated when mutated proteins are expressed heterologously

At the beginning of the period of mutational studies the predominant system for expression of bacterio-opsin was *Escherichia coli* [29,63]. The process of renaturation of the unfolded form of the expressed protein and its reconstitution into lipid vesicles or lipid/detergent micelles often employed an artificial lipid/detergent environment [13]. The properties of some of the mutants obtained this way are quite different from their equivalents expressed homologously in the halobacteria [53,70,86], many of which are assembled in vivo into the trimeric lattice of the native purple membrane. Some mutants are not stable in the reconstituted artificial systems, and partially or fully convert either to the spectrally blue-shifted forms known as P480 or to forms in which the conformation of the retinal is anomalous [28]. Because of these complications, artificially high shifts of the absorption maxima, low pumping activities or abnormal light-adaptation were reported for some of the mutants. For example, the mutants W182F and W189F were reported to have strongly blue-shifted absorption spectra [41], findings not confirmed upon expression of the same mutants in *Halobacterium salinarum* [102]. At the same time, absence of native lipids and disruption of BR timers in these systems led to peculiar photocycles even in a case of the wild-type protein expressed in *E. coli* (eBR). The kinetics of M decay and proton uptake in eBR is about 10 times slower than in the purple membrane, and the M rise is much faster [62], resembling the behavior of purple membrane at pH as high as 10. This sometimes prevented noting important differences that became obvious when the mutants were expressed homologously. For example, a dramatic shift in the Schiff base deprotonation equilibrium in the photocycle of the V49A mutant was not reported in heterologous expression system [39], unlike in the same mutant in the purple membrane [15]. It should be

[1] The structure of the M intermediate, 1CWQ, was recently released by PDB (Table 1) but it has not yet been described in a publication.

[2] Information about mutants of various proteins is available on the web at http://pmd.ddbj.nig.ac.jp/.

Table 1
Structures of BR, its mutants, and photocycle intermediates available at the protein data bank (PDB)

PDB Identification no.	Method	Resolution (Å)	Sample; space group	Ref.	PDB reference
1BRD	Electron diffraction	3.5	WT in purple membrane; p3	[44]	Henderson et al., May 1990
2BRD	Electron diffraction	3.5	WT in purple membrane; p3	[40]	Grigorieff et al., Dec. 1995
1AP9	X-Ray diffraction	2.35	WT in lipidic cubic phase, twinned; $p6_3$	[74]	Pebay-Peyroula et al., July 1997
1AT9	Electron diffraction	3.0	WT in purple membrane; p3	[51]	Kimura et al., Aug. 1997
1BRX	X-Ray diffraction	2.3	WT in lipidic cubic phase, twinned; $p6_3$	[59]	Luecke et al., May 1998
1BRR	X-Ray diffraction	2.9	WT in monoclinic crystals; c2	[32]	Essen et al., July 1998
1BM1	X-Ray diffraction	3.5	WT in honeycomb lattice; p622	[84]	Sato et al., July 1998
2AT9	Electron diffraction	3.0	WT in purple membrane; p3	[65]	Mitsuoka et al., Dec. 1998
1QHJ	X-Ray diffraction	1.9	WT in lipidic cubic phase, non-twinned; $p6_3$	[12]	Belrhali et al., May 1999
1C3W	X-Ray diffraction	1.55	WT in lipidic cubic phase, twinned; p63	[60]	Luecke et al., July 1999
1C8R	X-Ray diffraction	1.8	D96N in lipidic cubic phase, twinned; p63	[61]	Luecke et al., July 1999
1C8S	X-Ray diffraction	2.0	D96N in lipidic cubic phase, M state, twinned; $p6_3$	[61]	Luecke et al., July 1999
1QKO	X-Ray diffraction	2.1	WT in lipidic cubic phase, K state, non-twinned; $p6_3$	[31]	Edman et al., July 1999
1QKP	X-Ray diffraction	2.1	WT in lipidic cubic phase and its K state, non-twinned; $p6_3$	[31]	Edman et al., July 1999
1CWQ	X-Ray diffraction	2.25	WT in lipidic cubic phase and its M state, twinned; $p6_3$	Not available	Sass et al., Aug. 1999
1QM8[a]	X-Ray diffraction	?	WT in honeycomb lattice; p622	Not available	Takeda et al., Sep. 1999

[a]This structure is being processed by the PDB and is not available at the moment.

noted that kinetics of eBR and heterologously expressed BR mutants were much closer to those observed in the purple membrane when high protein to lipid ratios along with halobacterial lipids were used for the liposomes reconstitution [1].

2.1.2. *When BR mutants are used to obtain structural information one should keep in mind that the structure of mutants may differ from that of the wild-type protein*

This could happen as a result of a perturbation of BR folding by a particular mutation, or as a result of instability leading to partial denaturation. A more interesting possibility, though, is that a mutation changes the BR structure so it resembles a photocycle intermediate. Examples of such changes can be found among D85N mutants that appear to mimic the key step in the proton transport, i.e., protonation of the retinal Schiff base counterion. An M-like structure for D85N/D96N [50], as well as N-like structure for F42C/D85N [26], were reported. Also, the triple mutant D96G/F171C/F219L was found to have a structure similar to that of M and N states [92]. These observations present an opportunity for structural insights, but pose a problem for the interpretation of the photocycle intermediates in these mutants.

There may be, in fact, a general problem with

understanding the photointermediates of mutants. It was shown, for example, that structural changes in the M intermediate of D96G mutant are quite different from those of wild type and D96N [92]. In the case of the high-resolution M structure obtained in D96N [61] it may be hard to deduce the pathway connecting the Schiff base with its proton donor in the wild-type BR, because the donor is replaced by an unprotonatable group. Also, the structural information obtained on O intermediate in L93A mutant [91] may be not applicable to this intermediate in the wild type because it has different retinal configuration [24].

2.1.3. Long-range or indirect effects of some mutations may provide misleading information

There are examples where interpreting the phenotypes of mutants in terms of structural changes is made very difficult by long-distance effects. For instance, some mutations in regions far from the retinal can change the spectral properties of the chromophore. This seems to be the case for glutamic acid residues in the extracellular region of the protein [81], which probably affect the chromophore through the hydrogen-bonded network of waters and amino acid side-chains now known to exist from X-ray diffraction [12,60]. The presence of this network, which is responsible for the coupling of the proton affinities of D85 and the proton releasing complex near the extracellular surface [8,78], makes the effects of many mutations in this region look very complicated. In this way, some mutations of the residues close to the extracellular surface (E204, E194) change the kinetics of M formation [25,78], even though these residues are as far as 10 Å from the site of proton equilibration between the Schiff base and D85. Also, there is an evidence that some mutations in this region have unexpected long-range effects via changes in backbone conformation [95]. As for indirect effects on photocycle steps, they occur when a mutation affects a process far from the site of the replacement by influencing a preceding event, which is rate limiting. For example, an observation of slowed proton uptake in a mutant does not necessarily mean that the replaced residue was a part of the proton pathway. It could also happen because one of the steps coupled to the uptake is made slower, such as: (i) the Schiff base reprotonation (as in D38R [79]), (ii) retinal reisomerization (as in W182F [100]), (iii) reversal of the conformational change modulating the pK_a of D96 (as happens upon attaching bulky groups to the cysteine in T170C [17]).

2.1.4. Different replacements of the same residue can give conflicting information

From the results of non-conservative mutations one may misinterpret a role of the replaced residue if a newly introduced residue perturbs the native BR structure or forms new bonds. Replacement of the surface aspartate D38 with arginine produced a rather perturbed phenotype [79], implying an important role in the proton transport for this carboxylic acid. The conservative replacement of the same residue with asparagine did not affect proton pumping [66], and produced much smaller changes in the kinetics of the proton transfers [18] suggesting that D38 is not a proton donor in the photocycle. In the same way, replacement of Y57 with asparagine almost completely eliminated the M intermediate [35,87], while the more conservative, but non-hydrogen-bonding replacement with phenylalanine had a much milder effect [35]. On the other hand, one may use a mutation that is too conservative to reveal a role of the residue replaced. This is probably the case for D115, where a conservative replacement with the hydrogen-bonding asparagine does not significantly perturb the photocycle [98], in contrast to the greatly changed behavior of the D115L mutant (L.S. Brown, A.K. Dioumaev, R. Needleman, J.K. Lanyi, unpublished data), in which there must be a complete loss of hydrogen bonding of the side-chain to T90 [12,32,59].

2.1.5. The effect of a mutation may be masked by the compensatory action of another residue or ion

When looking at the effects of some mutations one should always explore a possibility of a compensatory effect of other residues or ions, which may be able to perform some functions of a lost residue. For example, the negative charge of D212 can be replaced with that of a chloride ion, which restores the functionality of the D212N mutant in a limited pH range [67]. It was suggested that the replacement of a single aspartic acid on cytoplasmic surface would not give usable information about proton pathways, because there are several alternative routes

for the proton conduction and other carboxylic acids will compensate for the loss of the mutated one [51]. In later work [18], results of a simultaneous replacement of the three aspartates closest to the proton channel (D38, D36, D102) with asparagines did not support this idea, showing no significant difference between the single and triple mutants in the kinetics of proton uptake.

2.2. Possible problems of using crystallographic data on BR

2.2.1. Most of the available structural information is about the BR state

It may be unwise to go too far in using structural information about the unphotolyzed state in order to make conclusions about the intermediates, because BR undergoes considerable conformational rearrangements during the photocycle. They include helical tilts, changes in backbone conformation, side-chain rotations, rearrangements of water molecules, and changes in the protonation states of the side-chains [43,61,92].

2.2.2. It is difficult to produce pure intermediate states for structural studies, because the intermediates are usually present as mixtures

Many of the intermediates are separated by very small differences in free energy and thus exist in equilibria, so it is hard to find a temperature range where only one intermediate would be present. When illumination is used to obtain certain intermediates there is always a danger of producing their photoproducts also, making the mixtures even more complex [6]. Some intermediates cannot be accumulated in high concentrations even theoretically, because they can convert back to the unphotolyzed state, thermally or as a result of the illumination. In such cases the composition of the photostationary states will be determined by the light intensity, the decay time-constant, the extinctions at the wavelength of illumination, and the quantum yields of the forward and reverse photoreactions.

2.2.3. The obtained structures may represent just one of a number of possible conformers stable at low temperature

As long as cryogenic temperatures are used for the structural data collection there will be a question if observed structures are the same as at physiological temperatures. This question is important for the mobile extramembrane loops and tails of BR, especially for cytoplasmic E–F loop [68,76], and for side-chains with alternative conformations. It is well known that spectra of many intermediates change at cryogenic temperatures, suggesting that the same may also happen to their structures. There is an indication that both BR and its K intermediate exist as multiple conformers at 90 K [6]. Another example is the attempt to deduce protonation states of carboxylic acids in BR from electron diffraction data taken at 4 K [65]. Theoretically, their proton affinities could change considerably with temperature.

2.2.4. Use of detergents for crystallization may lead to structures different from the native ones

Solubilization of BR with detergents is a usual step in crystallization procedure, and may lead to partial delipidation and, as a result, to a different trimer packing and changed conformation of the monomer. It was shown that even sublytic concentrations of detergents can lead to substantial loss of glycolipids and squalenes, causing changes in the protein conformation and photocycle, some of which are not fully reversible [10,69]. For the BR crystals in lipid cubic phase, Raman and Fourier transform infrared data argue for a functionally intact protein [43], contrary to earlier measurements in a different type of BR crystals where visible spectroscopy revealed a perturbed photocycle [85]. Also, the lattice constant of the hexagonal two-dimensional lattice of the purple membrane is unchanged in the three-dimensional crystals, and there is structural evidence that diphytanyl lipids are retained [12,60], even though the headgroups could not be detected. Mass spectrometry of extracts of these BR crystals detected four out of six major species of lipids present [12].

2.2.5. Different ways of purification, solubilization and crystallization may result in different structures

Various sample preparation techniques may result in crystallographic forms with different lattices or lipid–protein interactions and, consequently, somewhat different BR structures, especially in the loop regions [32,68,84,93]. Some crystal forms in lipid cubic phases demonstrated merohedral twinning,

which, if not taken in account properly, can seriously affect the calculated structural model [103].

2.2.6. Some regions of BR structure may have low reliability

Portions of BR are very labile, have high-temperature factors, and cannot be reliably modeled from X-ray data. This point is not always appreciated by non-crystallographers, and can lead to overinterpretation of the structural models in spite of cautionary advice from their authors. An example is the conformation of the large cytoplasmic E–F loop and the C-terminal part of the protein, especially in the M state [61].

3. Role of BR mutagenesis in the synthetic approach

Taking in account the above-mentioned problems, it is possible to identify two major roles of mutagenesis in the synthetic approach. The first is to help to provide additional structural information, and the second is to ensure functional tests of the predictions derived from structures.

3.1. Helping to provide structural information

Mutants can be used to generate stable and kinetically separated photocycle intermediates for structural studies. Even though the structures of the intermediates in mutants may be not exactly the same as in the wild-type BR (cf. above), they offer some important advantages. The changed kinetics of their photocycles allow accumulating some intermediates to concentrations impossible for the wild type. For example, the structure of the M intermediate obtained with wild-type BR was derived from the samples with 35% occupancy,[1] while the structural data collected with D96N mutant were from the samples with virtually 100% occupancy [61]. Both the high- and low-resolution structural studies took advantage of the ability of the mutants with the Schiff base proton donor D96 replaced (D96G or D96N) to accumulate large quantities of the M intermediate [48,52,82,90,92,101]. In some cases, it was even possible to observe substates of the M intermediate using either D96N or another mutant with slow M decay, D38R [83,92]. Some low-resolution data on conformational changes in the N intermediate were obtained using F219L [92,99], F171C [47], and T46V [92] – the mutants with slow N decay. Published attempts to obtain the structure of the O intermediate are limited to the low-resolution data on L93A, the mutant with very slow O decay [91]. It should be noted, though, that the O intermediate of this mutant is different from that of the wild type, because it has 13-*cis* chromophore [24,49]. Probably, high-resolution structures of the late intermediates (other than K, L, and M) will be obtained soon using other site-specific mutants.

Another important application of mutagenesis is to create sites for labels for providing independent structural information, which can be also used to obtain phase information for X-ray crystallography. One of such methods employs introduction of cysteines into the naturally cysteine-less BR sequence, and derivatization of their thiol groups with labels. Derivatization with mercuric compounds was successfully used in X-ray diffraction studies both for the BR state at positions 103 [54], 27, 100, 170, 171, 222 [71], 101, 130, 160, 231 [11] and for the N and M intermediates at positions 171 and 222, respectively [47,72]. Attachment of nitroxide spin-labels to cysteines allowed gaining structural information about BR loops and helices in unphotolyzed state using positions 72, 90, 92, 169 [2], 125–142 [3], 74, 76, 79, 82, 85, 96, 98, 101, 103, 105, 109 [38], 103, 105, 109, 113, 116, 117, 120, 124, 127, 129 [4], and 154–171 [76]. The kinetics of the structural changes in the photocycle intermediates were followed using spin-labels with single cysteine mutants at positions 72, 101, 105 [88] or at 36, 38, 46, 161 [80]. Use of double cysteine mutants S35C/M163C and A103C/M163C [97] allowed measuring changes in distances between the spin-labels during the photocycle. Finally, chemical modification of introduced cysteines at positions 72, 90, 92, 169 [34] or modification with bulky compounds combined with non-specific crosslinking at positions 27, 35, 100, 156, 163, 170, 222 [17] was used to obtain structural information. In a different approach, cysteine mutants in the vicinity of retinal at positions 118, 121, 138, 141 [33] were used for the mapping of the chromophore pocket by crosslinking to 4-bromoretinal.

3.2. Providing functional information that can test predictions from the structures for the BR state and the photocycle intermediates

The best example of this kind of mutagenesis use is the mechanism of the proton release from the extracellular surface, a still controversial topic. According to majority of structural models, the extracellular region constitutes a network of the hydrogen-bonded side-chains and water molecules. The decrease in the pK_a of the releasing group(s) follows the protonation of D85 [8,78]. The mechanism of this long-range coupling, and the identity of the source of the released proton have been subjects of intense investigation. The several possible structures for the proton release pathway involve various residues, and implicate different mechanisms (Table 2). In this case, mutagenesis studies are quite helpful in determining the residues that are not essential for the normal proton release, even though some of them may affect its kinetics. Replacement of the surface glutamates E9 [25] or E74 (L.S. Brown, R. Needleman, J.K. Lanyi, unpublished data), or both [104], with unprotonatable residues did not affect the kinetics of the proton transfers. These data question validity of the models implying significant role of E9 and E74 in this process [51,74]. The proton release kinetics were normal for the mutant T205V under physiological conditions [59,62], even though the pK_a for the proton release was reported to be much higher than in the wild type [58]. Mutations K129H [37], R134K [64], S193A [58], Y83N [46] and Y83F (L.S. Brown, R. Needleman, J.K. Lanyi, unpublished data) affect pK_a of the releasing group(s) but do not abolish normal (early) release. Replacement of F208 with arginine slows down the proton release without reversing the order of the release and uptake [21], probably by affecting the pK_a of the releasing group. There are only few residues in the extracellular region (except for the immediate members of the Schiff base counterion, such as D85 and D212) that are indispensable for the normal proton release, as judged from the mutagenesis. Replacement of E204 or E194 with unprotonatable residues blocks early proton release and delays it until the end of the photocycle and exchange of these glutamates for aspartates affects the pK_a and kinetics of the proton release [9,16,25]. Also, replacement of R82 with uncharged residues (A, Q) or of its neighbor Y57 with non-hydrogen-bonding residue (F) severely impairs the proton re-

Table 2
Structure-based models of the proton release from extracellular surface of BR

Model	Participating groups	Mechanism of the release and/or source of the released proton
2BRD [40]	E9, Y79, R82, Y83, R134, W189, S193, E194, E204, T205, water[a]	Sequence of transfers between the groups in the network (proton wire)
1AP9 [74]	Water, R82, T205, E9	Delocalized proton transfer in the network
1AT9 [51]	E9, E74, E194, E204, R82, water[a]	Any of the glutamic acids may release a proton
1BRX [59]	E194, E204, R82, water	Water 403 is a source of the proton, which is released via E204 and E194
1BRR [32]	R82, Y83, E194, E204, water[a]	Proton is released from the E194/E204 dyad, which may be associated with water molecule
2AT9 [65]	E194, E204, R82, water	Changed interaction of R82 with the glutamic acids leads to the proton release from E204 or E194 or water molecules associated with them
1QHJ [12]	E194, E204, R82, water	Hydrogen-bonded network releases a proton
1C3W [60]	E194, E204, R82, S193 (backbone and side-chain), Y79 (backbone), water	Three alternative mechanisms are allowed: (i) same as in 1BRX; (ii) same as in 1BRR; (iii) hydrogen-bonded continuum of waters is the proton source
1C8R, 1C8S [61]	E194, E204, R82, Y83, water	Displacement of R82 towards extracellular surface and rearrangement of water molecules stabilizes anionic forms of E204 and E194 and makes them lose the proton they share
1CWQ	E194, E204, S193, R82, Y83, W189, T205, Y79 (backbone), water	Displacement of R82 towards extracellular surface leads to its hydrogen bonding to E204 and E194 and stabilizes their anionic forms making them lose the proton they share[b]

[a]These water molecules were not observed directly, but suggested on the basis of calculated cavities in the structural model.
[b]This mechanism is inferred from the coordinates, as no publication was available at the time this article was written.

lease [5,35,73]. At the same time, there are indications that elimination of the early release is not complete for the mutants R82Q [36] and Y57F (Y. Cao, L.S. Brown, R. Needleman, J.K. Lanyi, unpublished data) that may be consistent with the ability of glutamine to form hydrogen bonds. The properties of the mutants R82K and R82H [7,45] demonstrate altered proton affinities of the proton release complex, and imply an important role for the R82 in the process. This is consistent with the idea that movement of R82 towards extracellular side, following protonation of D85, is the cause of the proton release (Table 2). Recently, such movement was detected in high-resolution structures of the M intermediate ([61] and 1CWQ[1]) and was also suggested by nuclear magnetic resonance investigations of various mutants [75,96].

Summing up the mutagenesis data, one can substantiate deep involvement of at least three residues suggested to be of importance by the most of the structural models, that is, E194, E204, and R82. Still, the problem of the source of the released proton remains unsolved because it was impossible to detect a reliable sign of the transient deprotonation of any glutamic acid residue in the wild-type photocycle [77,104]. At the same time, the transient protonation of D194 in the photocycle of E194D mutant suggested that a carboxylic acid at position 194 might be a part of the proton releasing chain, at least in this mutant [25]. Absence of any carboxyl deprotonation signal in the infrared poses a problem also for the idea that E194/E204 dyad shares the proton that is released [32,61].

The alternative idea, supported by the observation of amplitude changes of a broad infrared continuum, is that the source of the proton is a hydrogen-bonded network of water molecules stabilized by E204, R82 and other residues [77]. Such a network, including 7–8 water molecules, is clearly observed in the unphotolyzed structures with the highest resolution [12,60], but it is not obvious if this rather small hydrogen-bonded cluster can be responsible for the large infrared continuum changes. The idea of an ice-like network was supported by the large deuterium effects of the photocycle steps related to the proton release [23]. New mutagenesis data show, however, that the elimination of the early release by the mutations E194Q or E204Q (or partial elimination by Y57F) does not decrease the kinetic isotope effects of these steps, as expected, but increases them [19], pointing to the independence of the magnitude of the deuterium effects from the occurrence of the early release. However, it is still possible that one of the recently observed water molecules coordinated by some of the three mentioned residues may be the source of the proton.

Unlike the wealth of the functional and structural information available about the hydrogen-bonded network in the proton release pathway, information about the mechanism of the reprotonation of the Schiff base by D96, as well as of the proton uptake from the cytoplasmic surface, is scanty, and these processes are poorly understood. There are very few hydrogen-bonded side-chains and water molecules observable in the unphotolyzed state of BR in this protein region, and a clear proton pathway cannot be identified. This is consistent with an idea that in the unphotolyzed state the retinal Schiff base and D96 are not connected and pK_a of D96 is kept very high, and it is only in the M state that structural changes of the protein allow D96 to become a proton donor [20,94]. One of the two available high-resolution M structures was obtained in D96N mutant [61] that may preclude observing the hypothetical network formed by this aspartate in the M state of the wild-type protein. Nevertheless, this structure gives us a hint on how such pathway may be formed, showing a rotation of the side chains of F219 and L93 that constitute the hydrophobic barrier between the Schiff base and D96. In the other high-resolution M structure[1] a water molecule connects D96 and T46, an arrangement observed in both the BR state and the M intermediate of the D96N mutant [61]. While hydration would probably decrease pK_a of D96 making it a suitable proton donor, one still cannot identify the pathway of the Schiff base reprotonation from this structure. To understand the mechanism of the subsequent reprotonation of D96 one would need to obtain the structure of the N intermediate, which is not yet available. For these reasons, present structure-based models of the pathways of deprotonation/reprotonation of D96 should heavily rely on mutagenesis data.

It is inferred from the structural data that hydrophobic residues surrounding D96 (F42, I45, V49, L93, L99, L100, L219, L223, L224) help to insulate it and keep its pK_a high [32,60,74]. Indeed, direct

titration of the infrared band of the protonated D96 in F42C/D85N and D85N mutants demonstrated that replacement of F42 lowers pK_a of D96 by 1.5 pH units [26]. Also, the phenotypes of F219L [14], L100C [17], and F42C (L.S. Brown, R. Needleman, J.K. Lanyi, unpublished data) demonstrate accelerated deprotonation of D96 along with its slowed reprotonation from the surface that is consistent with a lowered pK_a. Interestingly, a similar phenotype is observed for the T46V mutant [17,62], where the possibility of hydrogen-bonding of T46 and D96 side-chains [32,60] is prevented. This implicates T46 in modulation of the proton affinity of D96, consistent with the 0.6 Å increase in the distance between N96 and T46 in M_N intermediate of D96N mutant (as measured in PDB structures 1C8R and 1C8S). Also, the disruption of the hydrogen bonding of T46 and D96 by the intercalating water molecule was observed in the wild-type M structure.[1] Replacement of several residues close to the cytoplasmic surface such as D38 [18,79], R227 [14,27], and F156 [17] slows down the reprotonation of the Schiff base and accelerates reprotonation of D96, probably by increasing the pK_a of the D96 in a manner opposite to that of the replacements of F42, T46, L100, or F219. The mechanism of such a pK_a increase cannot be completely understood from the available structural data, but possibly relates to the interference of these mutations with the conformational changes in the M state changing the hydration of D96 [83]. As for the residues involved in the reprotonation of the D96, their nature has not been established reliably either from the structure or from mutagenesis. The idea of the involvement of surface carboxylic acids in a 'proton funnel' [22,51] found some support from the mutagenesis [79], but was questioned later upon analysis with more conservative mutations [18] (also cf. above).

It seems that future developments of the synthetic approach will have to include improving the structures of the intermediates K and M, obtaining the structures for the remaining intermediates (L, N, O), as well as producing additional mutants deemed critical by the structural models and systematic studies of their phenotypes. There is a question to be solved regarding substates of the intermediates and their structures, because on logical and in some cases experimental grounds, for each mentioned intermediate at least two functional substates were proposed, and for some as many as four [100]. This will require a lot of sophistication in accumulating and identifying the substate of interest, as many of them are not easily distinguished spectroscopically.

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References

[1] P.L. Ahl, L.J. Stern, T. Mogi, H.G. Khorana, K.J. Rothschild, Biochemistry 28 (1989) 10028–10034.
[2] C. Altenbach, S.L. Flitsch, H.G. Khorana, W.L. Hubbell, Biochemistry 28 (1989) 7806–7812.
[3] C. Altenbach, T. Marti, H.G. Khorana, W.L. Hubbell, Science 248 (1990) 1088–1092.
[4] C. Altenbach, D.A. Greenhalgh, H.G. Khorana, W.L. Hubbell, Proc. Natl. Acad. Sci. USA 91 (1994) 1667–1671.
[5] S.P. Balashov, R. Govindjee, M. Kono, E. Imasheva, E. Lukashev, T.G. Ebrey, R.K. Crouch, D.R. Menick, Y. Feng, Biochemistry 32 (1993) 10331–10343.
[6] S.P. Balashov, Isr. J. Chem. 35 (1995) 415–428.
[7] S.P. Balashov, R. Govindjee, E.S. Imasheva, S. Misra, T.G. Ebrey, Y. Feng, R.K. Crouch, D.R. Menick, Biochemistry 34 (1995) 8820–8834.
[8] S.P. Balashov, E.S. Imasheva, R. Govindjee, T.G. Ebrey, Biophys. J. 70 (1996) 473–481.
[9] S.P. Balashov, E.S. Imasheva, T.G. Ebrey, N. Chen, D.R. Menick, R.K. Crouch, Biochemistry 36 (1997) 8671–8676.
[10] S.M. Barnett, S. Dracheva, R.W. Hendler, I.W. Levin, Biochemistry 35 (1996) 4558–4567.
[11] W. Behrens, U. Alexiev, R. Mollaaghababa, H.G. Khorana, M.P. Heyn, Biochemistry 37 (1998) 10411–10419.
[12] H. Belrhali, P. Nollert, A. Royant, C. Menzel, J.P. Rosenbusch, E.M. Landau, E. Pebay-Peyroula, Structure 7 (1999) 909–917.
[13] M.S. Braiman, L.J. Stern, B.H. Chao, H.G. Khorana, J. Biol. Chem. 262 (1987) 9271–9276.
[14] L.S. Brown, Y. Yamazaki, A. Maeda, L. Sun, R. Needleman, J.K. Lanyi, J. Mol. Biol. 239 (1994) 401–414.
[15] L.S. Brown, Y. Gat, M. Sheves, Y. Yamazaki, A. Maeda, R. Needleman, J.K. Lanyi, Biochemistry 33 (1994) 12001–12011.
[16] L.S. Brown, J. Sasaki, H. Kandori, A. Maeda, R. Needleman, J.K. Lanyi, J. Biol. Chem. 270 (1995) 27122–27126.
[17] L.S. Brown, G. Váró, R. Needleman, J.K. Lanyi, Biophys. J. 69 (1995) 2103–2111.

[18] L.S. Brown, R. Needleman, J.K. Lanyi, Biochemistry 38 (1999) 6855–6861.
[19] L.S. Brown, R. Needleman, J.K. Lanyi, Biochemistry 39 (2000) 938–945.
[20] Y. Cao, G. Váró, A.L. Klinger, D.M. Czajkowsky, M.S. Braiman, R. Needleman, J.K. Lanyi, Biochemistry 32 (1993) 1981–1990.
[21] Y. Cao, L.S. Brown, J. Sasaki, A. Maeda, R. Needleman, J.K. Lanyi, Biophys. J. 68 (1995) 1518–1530.
[22] S. Checover, E. Nachliel, N.A. Dencher, M. Gutman, Biochemistry 36 (1997) 13919–13928.
[23] J. le Coutre, K. Gerwert, FEBS Lett. 398 (1996) 333–336.
[24] J.K. Delaney, U. Schweiger, S. Subramaniam, Proc. Natl. Acad. Sci. USA 92 (1995) 11120–11124.
[25] A.K. Dioumaev, H.-T. Richter, L.S. Brown, M. Tanio, S. Tuzi, H. Saitô, Y. Kimura, R. Needleman, J.K. Lanyi, Biochemistry 37 (1998) 2496–2506.
[26] A.K. Dioumaev, L.S. Brown, R. Needleman, J.K. Lanyi, Biochemistry 37 (1998) 9889–9893.
[27] L.A. Drachev, A.D. Kaulen, H.G. Khorana, T. Mogi, N.V. Postanogova, V.P. Skulachev, L.J. Stern, Photochem. Photobiol. 55 (1992) 741–744.
[28] M. Duñach, T. Marti, H.G. Khorana, K.J. Rothschild, Proc. Natl. Acad. Sci. USA 87 (1990) 9873–9877.
[29] R.J. Dunn, N.R. Hackett, J.M. McCoy, B.H. Chao, K. Kimura, H.G. Khorana, J. Biol. Chem. 262 (1987) 9246–9254.
[30] T.G. Ebrey, in: M. Jackson (Ed.), Thermodynamics of Membrane Receptors and Channels, CRC Press, Boca Raton, FL, 1993, pp. 353–387.
[31] K. Edman, P. Nollert, A. Royant, H. Belrhali, E. Pebay-Peyroula, J. Hajdu, R. Neutze, E.M. Landau, Nature 401 (1999) 822–826.
[32] L.-O. Essen, R. Siegert, W.D. Lehmann, D. Oesterhelt, Proc. Natl. Acad. Sci. USA 95 (1998) 11673–11678.
[33] Y. Feng, D.R. Menick, B.M. Katz, C.J. Beischel, E.S. Hazard, S. Misra, T.G. Ebrey, R.K. Crouch, Biochemistry 33 (1994) 11624–11630.
[34] S.L. Flitsch, H.G. Khorana, Biochemistry 28 (1989) 7800–7805.
[35] R. Govindjee, M. Kono, S.P. Balashov, E. Imasheva, M. Sheves, T.G. Ebrey, Biochemistry 34 (1995) 4828–4838.
[36] R. Govindjee, S. Misra, S.P. Balashov, T.G. Ebrey, R.K. Crouch, D.R. Menick, Biophys. J. 71 (1996) 1011–1023.
[37] R. Govindjee, E.S. Imasheva, S. Misra, S.P. Balashov, T.G. Ebrey, N. Chen, D.R. Menick, R.K. Crouch, Biophys. J. 72 (1997) 886–898.
[38] D.A. Greenhalgh, C. Altenbach, W.L. Hubbell, H.G. Khorana, Proc. Natl. Acad. Sci. USA 88 (1991) 8626–8630.
[39] D.A. Greenhalgh, D.L. Farrens, S. Subramaniam, H.G. Khorana, J. Biol. Chem. 268 (1993) 20305–20311.
[40] N. Grigorieff, T.A. Ceska, K.H. Downing, J.M. Baldwin, R. Henderson, J. Mol. Biol. 259 (1996) 393–421.
[41] N.R. Hackett, L.J. Stern, B.H. Chao, K.A. Kronis, H.G. Khorana, J. Biol. Chem. 262 (1987) 9277–9284.
[42] U. Haupts, J. Tittor, D. Oesterhelt, Annu. Rev. Biophys. Biomol. Struct. 28 (1999) 367–399.
[43] J. Heberle, G. Büldt, E. Koglin, J.P. Rosenbusch, E.M. Landau, J. Mol. Biol. 281 (1998) 587–592.
[44] R. Henderson, J.M. Baldwin, T.A. Ceska, F. Zemlin, E. Beckmann, K.H. Downing, J. Mol. Biol. 213 (1990) 899–929.
[45] E.S. Imasheva, S.P. Balashov, T.G. Ebrey, N. Chen, R.K. Crouch, D.R. Menick, Biophys. J. 77 (1999) 2750–2763.
[46] E.S. Imasheva, S.P. Balashov, T.G. Ebrey, Y. Chen, D.R. Menick, R.K. Crouch, Biophys. J. 78 (2000) 475A.
[47] H. Kamikubo, M. Kataoka, G. Váró, T. Oka, F. Tokunaga, R. Needleman, J.K. Lanyi, Proc. Natl. Acad. Sci. USA 93 (1996) 1386–1390.
[48] H. Kamikubo, T. Oka, Y. Imamoto, F. Tokunaga, J.K. Lanyi, M. Kataoka, Biochemistry 36 (1997) 12282–12287.
[49] H. Kandori, Y. Yamazaki, M. Hatanaka, R. Needleman, L.S. Brown, H.-T. Richter, J.K. Lanyi, A. Maeda, Biochemistry 36 (1997) 5134–5141.
[50] M. Kataoka, H. Kamikubo, F. Tokunaga, L.S. Brown, Y. Yamazaki, A. Maeda, M. Sheves, R. Needleman, J.K. Lanyi, J. Mol. Biol. 243 (1994) 621–638.
[51] Y. Kimura, D.G. Vassylyev, A. Miyazawa, A. Kidera, M. Matsushima, K. Mitsuoka, K. Murata, T. Hirai, Y. Fujiyoshi, Nature 389 (1997) 206–210.
[52] M.H.J. Koch, N.A. Dencher, D. Oesterhelt, H.J. Plöhn, G. Rapp, G. Buldt, EMBO J. 10 (1991) 521–526.
[53] M.P. Krebs, R. Mollaaghababa, H.G. Khorana, Proc. Natl. Acad. Sci. USA 90 (1993) 1987–1991.
[54] M.P. Krebs, W. Behrens, R. Mollaaghababa, H.G. Khorana, M.P. Heyn, Biochemistry 32 (1993) 12830–12834.
[55] M.P. Krebs, H.G. Khorana, J. Bacteriol. 175 (1993) 1555–1560.
[56] J.K. Lanyi, J. Biol. Chem. 272 (1997) 31209–31212.
[57] J.K. Lanyi, J. Struct. Biol. 124 (1998) 164–178.
[58] M. Lu, R. Govindjee, S.P. Balashov, T.G. Ebrey, Y. Chen, D.R. Menick, R.K. Crouch, Biophys. J. 78 (2000) 476A.
[59] H. Luecke, H.-T. Richter, J.K. Lanyi, Science 280 (1998) 1934–1937.
[60] H. Luecke, B. Schobert, H.-T. Richter, J.-P. Cartailler, J.K. Lanyi, J. Mol. Biol. 291 (1999) 899–911.
[61] H. Luecke, B. Schobert, H.-T. Richter, J.-P. Cartailler, J.K. Lanyi, Science 286 (1999) 255–260.
[62] T. Marti, H. Otto, T. Mogi, S.J. Rosselet, M.P. Heyn, H.G. Khorana, J. Biol. Chem. 266 (1991) 6919–6927.
[63] L.J.W. Miercke, M.C. Betlach, A.K. Mitra, R.F. Shand, S.K. Fong, R.M. Stroud, Biochemistry 30 (1991) 3088–3098.
[64] S. Misra, C. Martin, O.-H. Kwon, T.G. Ebrey, N. Chen, R.K. Crouch, D.R. Menick, Photochem. Photobiol. 66 (1997) 774–783.
[65] K. Mitsuoka, T. Hirai, K. Murata, A. Miyazawa, A. Kidera, Y. Kimura, Y. Fujiyoshi, J. Mol. Biol. 286 (1999) 861–882.
[66] T. Mogi, L.J. Stern, T. Marti, B.H. Chao, H.G. Khorana, Proc. Natl. Acad. Sci. USA 85 (1988) 4148–4152.
[67] S. Moltke, M.P. Krebs, R. Mollaaghababa, H.G. Khorana, M.P. Heyn, Biophys. J. 69 (1995) 2074–2083.
[68] D.J. Müller, H.-J. Sass, S.A. Müller, G. Büldt, A. Engel, J. Mol. Biol. 285 (1999) 1903–1909.

[69] A.K. Mukhopadhyay, S. Dracheva, S. Bose, R.W. Hendler, Biochemistry 35 (1996) 9245–9252.
[70] B. Ni, M. Chang, A. Duschl, J. Lanyi, R. Needleman, Gene 90 (1990) 169–172.
[71] T. Oka, H. Kamikubo, F. Tokunaga, J.K. Lanyi, R. Needleman, M. Kataoka, Photochem. Photobiol. 66 (1997) 768–773.
[72] T. Oka, H. Kamikubo, F. Tokunaga, J.K. Lanyi, R. Needleman, M. Kataoka, Biophys. J. 76 (1999) 1018–1023.
[73] H. Otto, T. Marti, M. Holz, T. Mogi, L.J. Stern, F. Engel, H.G. Khorana, M.P. Heyn, Proc. Natl. Acad. Sci. USA 87 (1990) 1018–1022.
[74] E. Pebay-Peyroula, G. Rummel, J.P. Rosenbusch, E.M. Landau, Science 277 (1997) 1676–1681.
[75] A.T. Petkova, J.G. Hu, M. Bizounok, M. Simpson, R.G. Griffin, J. Herzfeld, Biochemistry 38 (1999) 1562–1572.
[76] M. Pfeiffer, T. Rink, K. Gerwert, D. Oesterhelt, H.-J. Steinhoff, J. Mol. Biol. 287 (1999) 163–171.
[77] R. Rammelsberg, G. Huhn, M. Lubben, K. Gerwert, Biochemistry 37 (1998) 5001–5009.
[78] H.-T. Richter, L.S. Brown, R. Needleman, J.K. Lanyi, Biochemistry 35 (1996) 4054–4062.
[79] J. Riesle, D. Oesterhelt, N.A. Dencher, J. Heberle, Biochemistry 35 (1996) 6635–6643.
[80] T. Rink, J. Riesle, D. Oesterhelt, K. Gerwert, H.-J. Steinhoff, Biophys. J. 73 (1997) 983–993.
[81] C. Sanz, T. Lazarova, F. Sepulcre, R. Gonzalez-Moreno, J.-L. Bourdelande, E. Querol, E. Padrós, FEBS Lett. 456 (1999) 191–195.
[82] H.-J. Sass, I.W. Schachowa, G. Rapp, M.H.J. Koch, D. Oesterhelt, N.A. Dencher, G. Büldt, EMBO J. 16 (1997) 1484–1491.
[83] H.-J. Sass, R. Gessenich, M.H.J. Koch, D. Oesterhelt, N.A. Dencher, G. Büldt, G. Rapp, Biophys. J. 75 (1998) 399–405.
[84] H. Sato, K. Takeda, K. Tani, T. Hino, T. Okada, M. Nakasako, N. Kamiya, T. Kouyama, Acta Crystallogr. D: Biol. Crystallogr. 55 (1999) 1251–1256.
[85] G.F.X. Schertler, R. Lozier, H. Michel, D. Oesterhelt, EMBO J. 10 (1991) 2353–2361.
[86] J. Soppa, D. Oesterhelt, J. Biol. Chem. 264 (1989) 13043–13048.
[87] J. Soppa, J. Otomo, J. Straub, J. Tittor, S. Meessen, D. Oesterhelt, J. Biol. Chem. 264 (1989) 13049–13056.
[88] H.J. Steinhoff, R. Mollaaghababa, C. Altenbach, K. Hideg, M. Krebs, H.G. Khorana, W.L. Hubbell, Science 266 (1994) 105–107.
[89] W. Stoeckenius, Protein Sci. 8 (1999) 447–459.
[90] S. Subramaniam, M. Gerstein, D. Oesterhelt, R. Henderson, EMBO J. 12 (1993) 1–8.
[91] S. Subramaniam, A.R. Faruqi, D. Oesterhelt, R. Henderson, Proc. Natl. Acad. Sci. USA 94 (1997) 1767–1772.
[92] S. Subramaniam, M. Lindahl, P. Bullough, A.R. Faruqi, J. Tittor, D. Oesterhelt, L. Brown, J. Lanyi, R. Henderson, J. Mol. Biol. 287 (1999) 145–161.
[93] S. Subramaniam, Curr. Opin. Struct. Biol. 9 (1999) 462–468.
[94] S. Szaraz, D. Oesterhelt, P. Ormos, Biophys. J. 67 (1994) 1706–1712.
[95] M. Tanio, S. Inoue, K. Yokota, T. Seki, S. Tuzi, R. Needleman, J.K. Lanyi, A. Naito, H. Saitô, Biophys. J. 77 (1999) 431–442.
[96] M. Tanio, S. Tuzi, S. Yamaguchi, R. Kawaminami, A. Naito, R. Needleman, J.K. Lanyi, H. Saitô, Biophys. J. 77 (1999) 1577–1584.
[97] T.E. Thorgeirsson, W. Xiao, L.S. Brown, R. Needleman, J.K. Lanyi, Y.-K. Shin, J. Mol. Biol. 273 (1997) 951–957.
[98] G. Váró, L. Zimányi, M. Chang, B. Ni, R. Needleman, J.K. Lanyi, Biophys. J. 61 (1992) 820–826.
[99] J. Vonck, Biochemistry 35 (1996) 5870–5878.
[100] O. Weidlich, B. Schalt, N. Friedman, M. Sheves, J.K. Lanyi, L.S. Brown, F. Siebert, Biochemistry 35 (1996) 10807–10814.
[101] M. Weik, G. Zaccai, N.A. Dencher, D. Oesterhelt, T. Hauss, J. Mol. Biol. 275 (1998) 625–634.
[102] Y. Yamazaki, J. Sasaki, M. Hatanaka, H. Kandori, A. Maeda, R. Needleman, T. Shinada, K. Yoshihara, L.S. Brown, J.K. Lanyi, Biochemistry 34 (1995) 577–582.
[103] T.O. Yeates, B.C. Fam, Structure 7 (1999) R25–R29.
[104] C. Zscherp, R. Schlesinger, J. Tittor, D. Oesterhelt, J. Heberle, Proc. Natl. Acad. Sci. USA 96 (1999) 5498–5503.