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REVIEW

Infrared Methods for Monitoring the Protonation State of Carboxylic Amino Acids in the Photocycle of Bacteriorhodopsin

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Abstract—This review deals with the role of carboxylic amino acids in the proton-transport activity of bacteriorhodopsin. The main focus is on the infrared data, which allow direct monitoring of the protonation/deprotonation of specific residues during the proton movement in the course of the photocycle. Additional attention is paid to the potential use of carboxylic acids in proteins as internal sensors, based on the sensitivity of their IR frequencies to the immediate environment.

Key words: retinal proteins, FTIR, time-resolved, aspartic acid, glutamic acid, COOH stretch, symmetric and antisymmetric stretching vibrations, carbonyl, carboxyl, carboxylate

PHOTOCYCLE AND PROTON TRANSLOCATION IN BACTERIORHODOPSIN

Bacteriorhodopsin (bR), a natural photochromic protein from *Halobacterium salinarum* (formerly *H. halobium*), acts as an active proton pump driven by light quanta of solar radiation [1, 2]. Photoinduced proton translocation creates a transmembrane proton gradient, which can be used as the energy source to drive physiologically important reactions. In the elementary act of a single turnover, a single proton is transported across the membrane. This process is called the photocycle. Under ambient quasi-physiological conditions the photocycle is finished within ~30 msec, with the protein returning back to its initial state. The photocycle is characterized by a sequence of spectrally distinct transient states, intermediates, with the corresponding lifetimes covering the range from femto- to milliseconds. The photocycle rates are both temperature- and pH-dependent. In oversimplified form of the photocycle can be represented as a linear sequence of intermediates: bR→J↔K↔L↔M↔N↔O→bR [3-8] (for recent reviews see, for instance, [9, 10]). Kinetically distinct and functionally important substates were found for all intermediates: for K [11-14], for L [15-17], for M [8, 18-20], for N [21-24], and for O [21, 25].

From the point of view of mechanisms of light energy utilization, the photocycle can be subdivided into two main stages. During the "early" portion of the photocycle, bR→J↔K↔L, the energy of the light quantum is absorbed by the retinal chromophore, and then transferred from the chromophore to the protein. By the end of this stage, in the L intermediate, the energy is stored in the strained conformation of the protein and in an unstable configuration of the protonated Schiff base (i.e., proton donor) with expected [26] pK_a well below its initial value, pK_a ~ 13, before excitation [27]. While no protons are changing their binding sites during this part of the photocycle, the "later" part, L↔M↔N↔O→bR, is directly coupled to proton translocation across the membrane through a "proton transport chain". This chain is actually a transiently changing network of donor–acceptor interactions, and it is subdivided into two "half-channels", with the chromophore strategically placed between them. Both half-channels include specific proton-binding sites whose proton-affinity is transiently modulated during the photocycle by processes both in the chromophore (e.g., charge redistribution along the polyene chain [26, 28, 29], isomerization [2, 30, 31]) and in the protein (e.g., conformation and/or access changes [32, 33], and pK_a coupling [34-36]).

While only one proton is effectively transported across the membrane during a single turnover of the photocycle, this translocation involves shifts of at least four protons, and the proton ejected on extracellular surface is not the same as the one picked up on the cytoplasmic side. Prior to excitation these four protons are residing in three binding sites inside the protein plus the "fourth" proton is picked up from the bulk on the cytoplasmic side during the photo-induced pumping. The three initially occupied internal binding sites are: 1) the protonated Schiff base (SBH^+); 2) the "proton releasing complex"

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(PRC$^+$), and 3) the Asp96 (D96H$^+$)[1]. These three binding sites are initially stable protonated residues. In the course of the photocycle they are at first transiently transformed into proton donors, and then back into proton acceptors. That is, those groups have to undergo a transient change of their pK_a (or rather proton affinity) from relatively high to relatively low and then back to high. Besides these initially protonated binding sites, for the photocycle to proceed, there should exist at least one initially unprotonated potential binding site, i.e., proton acceptor (a group with relatively low pK_a) which has to undergo pK_a (i.e., proton affinity) shifts in the reverse order.

While different amino acids (for instance, tyrosine, lysine, cysteine, histidine, and arginine) have potentially exchangeable protons, the wide range of pH, in which bacteriorhodopsin preserves its basic functionality severely limits the choices for the possible residues to act as transient/reversible donor/acceptor binding sites. Carboxylic amino acids are especially suited for this role due to specific ability of their pK_a to be readily modulated by local electrostatic interactions in an extremely wide range from 2 to 11 [35-43].

Two techniques—site-specific mutagenesis [44-47] and IR spectroscopy [48-50], especially FTIR in time-resolved mode [13, 14, 20, 51, 52]—provided the possibility to both modify and to monitor the changes at truly molecular level during the photocycle. Other techniques, notably NMR, produced additional evidence [53-55] mostly in accord with IR. As a result, a semi-consensus qualitative picture exists of the main molecular events during the photocycle, and the following review will focus on these consensus features, sometimes at the expense of the historical time-line.

INFRARED FEATURES OF CARBOXYLIC AMINO ACIDS

From an IR point of view aspartic and glutamic amino acids possess a combination of features which make them unique for monitoring protein activity. A protonated carboxyl group, R-COOH, is characterized by three main IR bands: O–H stretching, ν_{OH}, at approximately 3500 cm^{-1}, C=O stretching, ν_{COOH}, at 1700-1780 cm^{-1}, and O–H bending, δ_{OH}, at 1200-1300 cm^{-1} [56-61]. The corresponding deprotonated carboxylate form, R-COO$^-$, displays two main IR bands due to stretching vibrations: antisymmetric, ν_{as}, at 1550-1610 cm^{-1}, and symmetric, ν_s, at 1300-1420 cm^{-1} [57-59, 61, 62]. In retinal proteins δ_{OH} and ν_{as} are hidden under strong IR absorption bands of the fingerprint, ν_{C-C}, and amide II, ν_{CONH}, regions, correspondingly, and they are not very useful for practical purposes [25, 50, 61]. The ν_s falls in the region of numerous bands due to CH_3 bending vibrations and might be difficult to detect. Thus, for diagnostic purposes the most valuable band is that of protonated C=O stretch, ν_{COOH}. First of all, its position at 1700-1780 cm^{-1} is unique for IR [61]. No other bands could be seen in this region in proteins, and any induced feature in this region of differential spectra unambiguously signals either protonation or environmental change involving carboxylic amino acid residues. Furthermore, this vibration produces an IR band with an extinction coefficient of 250-1200 M^{-1}·cm^{-1} [57, 58, 61]. This value is among the highest in the mid-IR[1]. For bacteriorhodopsin-containing thin films, this high extinction coefficient of ν_{COOH} vibration gives rise to transient signals of up to 0.001 OD under moderate flash excitation [20, 63]. Conversely, a 1000 M^{-1}·cm^{-1} extinction coefficient of the ν_{COOH} band could allow detection of a change in protonation of a single aspartic/glutamic acid residue in bacteriorhodopsin even in a monolayer film produced by the Langmuir–Blodgett technique[2].

Both the position and the shape of the ν_{COOH} band are very sensitive to the local environment. In the vapor phase (i.e., monomers free of any interaction) this band could be as high as 1780 cm^{-1} [56], and its shape could be quite accurately described by a Gaussian with FWHM of 8-13 cm^{-1}. On the other hand, in cyclic dimers in concentrated solutions (i.e., under extremely strong H$^+$-bonding) this band is at 1710-1715 cm^{-1} [57, 58, 64, 65]. In dimeric form the band normally is both broadened (FWHM ~ 25 cm^{-1}) and is predominantly Lorentzian, rather than pure Gaussian. Less extensive/specific external influence, for instance in weakly hydrogen-bonding solvents, produces C=O stretching bands at around 1725 cm^{-1} [66, 67].

In the absence of specific interactions, the position of ν_{COOH} is correlated with the so-called Onsager's parameter, $2(\varepsilon - 1)/(2\varepsilon - 1)$ [67, 68], which reflects the intensity of local electrical fields stabilizing the COOH electric dipole moment. This correlation is usually valid for ν_{COOH} ~ 1730 cm^{-1}, and could be used to monitor environmental changes of carboxylic amino acids inside proteins [67]. In molecular terms, this phenomenological correlation means that (in the absence of specific interactions like hydrogen bonding) the ν_{COOH} is a function of the dielectric constant, ε, of the environment. On the other hand, changing the dielectric constant shifts equilibrium between the charged/deprotonated and neutral/protonated forms of the carboxylic group, which is

[1] Both here and below the (+) sign will be used to stress that this particular group is protonated in the particular stage of the photocycle; the PRC$^+$ is not a single residue.

[1] Compare this to the corresponding extinction of H_2O: 20.8 M^{-1}·cm^{-1} at 1640 cm^{-1} [141], which produces a factor of 10 attenuation for each 8.7 μm thickness.

[2] Assuming a 0.00002 OD residual noise level and saturating flash excitation (~30% cycling in time-resolved FTIR measurements).

effectively the shift of the pK_a [69]. Thus, for many practical cases (ν_{COOH} ~ 1730 cm^{-1}), the upshift in the position of the protonated carboxyl stretching frequency, ν_{COOH}, reflects an increase in the pK_a of the COOH group (~5 cm^{-1} per pK_a unit). That is, the higher ν_{COOH} values correspond to the elevated pK_a, and the opposite correlation [65] obtained for covalently bonded substituents at the alpha-carbon atom is inapplicable in this case.

CARBOXYLIC AMINO ACIDS IN BACTERIORHODOPSIN

The primary sequence of bacteriorhodopsin from *Halobacterium salinarum* contains 18 carboxylic amino acids: 9 aspartic and 9 glutamic. Out of these eighteen, four are buried inside the protein: Asp85, Asp96, Asp115, and Asp212. Another six are on/near the membrane surfaces: four (Asp36, Asp38, Asp102, Asp104) are on the cytoplasmic surface and two (Glu194, Glu204) are near the extracellular one. Still another three are on the loops: Glu74 on the extracellular and two (Glu161, Glu166) on the cytoplasmic side. The remaining five are on the tails: four (Glu232, Glu234, Glu237, and Asp242) are on the COOH- and Glu9 on the N-tail.

The eight latter residues on loops and tails (Glu9, Glu74, Glu161, Glu166, Glu232, Glu234, Glu237, and Asp242) are usually believed to be too far to play any active role. Besides, some of them are not conserved [70, 71].

Out of the remaining ten carboxylic acids, the roles of the four internal aspartic acids (Asp85, -96, -115, -212) are unambiguously established. These four aspartates are both highly conserved, and when absent (e.g., Asp85 and Asp96 in halorodopsins or in corresponding mutants) impede the proton translocation. Out of these four Asp85 and Asp212 are normally unprotonated [54, 55, 72] (pK_a < 2.5 [35, 36, 40-43]), while the other two (Asp96 and Asp115) have initially high pK_a [53, 55, 72]. The two glutamic acids near the extracellular surface (Glu194 and Glu204) are a part of the proton-releasing complex [73-75]. The roles of the remaining four aspartic acids on the cytoplasmic surface (Asp36, -38, -102, and -104) are still a somewhat controversial topic. Originally these residues were proposed to play crucial/specific role in proton translocation either as a cluster [76-78], or individually (e.g., Asp38 [79]). These conclusions seem to be highly controversial, since mutation (single or in combinations) of all those aspartates failed to produce [80] the expected pronounced effect.

INFRARED DATA ON INTERMEDIATES OF THE PHOTOCYCLE OF BACTERIORHODOPSIN

Participation of carboxylic acids in the photocycle of BR was already noted in the early publications involving IR spectroscopy [48, 49]. The strongest bands in the carboxyl region belong to Asp85, -96, and -115. Protonated (positive) band of Asp85 is found at 1761-1762 cm^{-1} in M [48, 49, 72, 81, 82]. It is downshifted to 1752-1756 cm^{-1} in both N [52, 63, 83, 84] and O [25, 52, 63, 85]. Negative (depletion) band of Asp96 is at 1740-1742 cm^{-1} [51, 72, 81, 83, 86, 87], while that of Asp115 is at 1733-1735 cm^{-1} [72, 81, 87]. Both Asp96 and Asp115 are subjected to environmental shifts—Asp96 in L and M, Asp115 in L, M, and N—giving rise to differential bands in IR spectra. The corresponding positive bands of Asp115 are at 1727-1729 cm^{-1} in L [72, 81, 86, 88], at 1740-1742 cm^{-1} in M [86, 87], and at 1738-1740 cm^{-1} in N [86, 87]. Likewise, the positive bands arising from perturbed C=O stretch of Asp96 in intermediates are at 1748 cm^{-1} in L [72, 81, 86, 88] and at either 1736 cm^{-1} [87] or 1742 cm^{-1} [89] or 1748 cm^{-1} [72] in M. Asp96 is deprotonated in N [90] and, therefore, only a negative depletion band is present in N [84, 85, 87, 91]. Out of all the intermediates, the least is known about carboxylic bands in O state [25, 52, 63, 85]. Environment-induced shifts differ not only for different intermediates, but even for one and the same transient state when measured in different mutants. For instance, the shift in ν_{COOH} of Asp115 in N state varies between 2 cm^{-1} (in wild type at high pH) and 8 cm^{-1} (e.g., in E194Q/D96N mutant) creating differential spectra that look (qualitatively) misleadingly different.

Symmetric COO^- stretching vibrations are more difficult to assign and, consequently, reports on these are not always consistent. The ν_s were reported for initially deprotonated aspartates in bR state (i.e., measured as depletion/negative band in the differential spectra): for Asp85 at 1417 cm^{-1} [86], 1391 cm^{-1} [81], 1385 cm^{-1} [82, 92], 1384 cm^{-1} [93], or 1381 cm^{-1} [83], and for Asp212 at 1417 cm^{-1} [86], 1387 cm^{-1} [25] or 1393 cm^{-1} [92] (see also [93] for other ν_s unassigned COO^- bands). Likewise, ν_s was reported for the initially protonated Asp96 as a new positive band in N state at 1376 cm^{-1} [86], 1387 cm^{-1} [94], 1399 cm^{-1} [83], and 1402 cm^{-1} [82]. Asp115 does not become deprotonated during the photocycle, and its ν_s was never assigned.

Unlike the data on aspartic acid residues, IR evidence on the involvement of native glutamic acids is quite controversial [73, 81, 93, 95, 96]. For instance, there are still no reliable assignments of IR bands to the COOH group of either Glu194 or Glu204 [73, 96], the two residues whose active participation in proton release is evident from their phenotypes [73, 74]. On the other hand, mutagenic substitution of an aspartic acid with a glutamic one was shown to downshift the ν_{COOH} stretching band by 22-36 cm^{-1} for both Asp85 and Asp96 (to Glu substitution) [51, 72, 97, 98]. If a similar downshift could be expected between the COOH band of Asp194, which is at 1720 cm^{-1} [75] in the M state of the E194D mutant, and Glu194 in wild type, than the corresponding IR line

will be hidden under the strong negative feature (well known but still never unambiguously assigned either) below 1695 cm^{-1}.

Isotope substitution directly affects the position of IR bands and the easily exchangeable proton of the carboxyl group is routinely used to test for the deuterium-induced shift of ν_{COOH} of approximately 9-14 cm^{-1} for non-hydrogen bonding case, expected from both theory and model compounds studies [88, 95, 99]. In bR (and its mutants) H/D exchange was reported to produce a 8-15 cm^{-1} downshift in ν_{COOH} of Asp85 in M [25, 49, 50, 75, 88, 100], N [25, 88], and O [25, 63]; Asp96 in L [50, 88] and N [25, 86]; Asp115 in L [88], N [25], and O [25, 63]; Asp194 in M (in the E194D mutant); and the surface carboxylic acids in blue (cation-depleted) bR_{605} [101]. As was proposed from the model compounds studies [88], the presence of strong hydrogen bonding, especially an H-bond to the OH group, decreases the magnitude of the deuterium-induced shift of ν_{COOH} to ~1-5 cm^{-1}. Such "unusually" small deuterium shifts were reported in bacteriorhodopsin for Asp96 in L (~1 cm^{-1} [88]) and for Asp212 in O (~4 cm^{-1} [25]).

^{13}C-labeling could be specifically applied to aspartates without affecting glutamates [50, 95]; unfortunately, it is more difficult to label glutamates without affecting aspartates [95], and only one such attempt was reported [93]. Based on simple calculations, ^{13}C-labeling of the carbonyl group is estimated to induce a downshift of its stretch frequencies by ~38-40 cm^{-1} for both carboxyl (ν_{COOH}) and antisymmetric carboxylate (ν_{as}) stretching vibration, while the position of the carboxylate symmetric stretch (ν_s) is downshifted by ~10-15 cm^{-1} [25, 50, 102, 103]. These estimates are in accord with measurements on model compounds in which ^{13}C-labeling leads to ~40 cm^{-1} shift in carboxyl stretch, ν_{COOH}, and 18 ± 8 cm^{-1} for symmetric carboxylate, ν_s, vibration frequency (see for instance [25, 50]). In bacteriorhodopsin 4-^{13}C-labeling of the aspartic acids produces a 15 ± 4 cm^{-1} downshift for ν_s of Asp85 [92, 93], Asp96 [25, 88, 92], Asp115, and Asp212 [25, 92] (see also [93]) and 40 ± 3 cm^{-1} downshift for ν_{COOH} of Asp85 [25, 50, 92, 93, 95], Asp96 [25, 50], Asp115 [25], and Asp212 [25].

All the above assignments allowed to correlate specific molecular events involving carboxylic acid to particular steps in the photocycle.

THE SEQUENCE OF PROTONATION CHANGES DURING THE PHOTOCYCLE

The earliest proton-transporting step in the photocycle takes place during the L-to-M transition when the Schiff base proton (the "first" one out of the four "moveable" ones) leaves its initial binding site being transferred to the primary donor, Asp85 [51, 72, 98]. Earlier, conflicting reports were published both pro and contra Asp96 deprotonation in the previous step (K-to-L transition) [72, 86, 100]. However, most of the recent schemes of the photocycle do not include any protonation changes prior to the L-to-M transition, since a protonation change during K-to-L transition seems to be somewhat difficult to rationalize. The next step in the photocycle at physiological (and high pH) is a shift of another ("second") proton during the M_1-to-M_2 transition, which is released from proton releasing complex into the extracellular medium [22, 104-107] (see also [108-111]). This is the so-called "normal" or "early" proton release. At lower pH, i.e., below the pK_a of the proton-releasing site (in the wild-type BR at ~5, which is actually the pK_a of PRC^+ in M [112]), this process is delayed until the end of the photocycle [112], and is called, therefore, "late release". During the next stage, the M_2-to-N transition, a "third" proton is shifted from the primary donor, Asp96, to reprotonate the Schiff base [51, 84, 90, 91, 94, 97, 113, 114]. The N-to-O transition is associated with reprotonation of Asp96 from the cytoplasmic side [84, 85, 105, 115], which is the movement of the "fourth" proton involved in the pumping. The O-to-bR transition is marked by a second (during a single turnover) shift of the "first" proton from Asp85 to reprotonate the PRC^- [25, 51, 85]. Note that this so-called "first" proton is the only one, which participates in two shifts during a single turnover of the photocycle in wild-type bR.

Thus, all photocycle steps starting from the L-to-M_1 transition are associated with proton transfers, and all those proton transfers include participation of carboxylic amino acids. Historically, these five steps of the proton transfers were first detected in the time-resolved photoelectric measurements [108, 109, 116-121] but their molecular interpretation become possible only with proliferation of site-specific mutagenesis and progress in FTIR spectroscopy.

The above outlined scheme is somewhat oversimplified. Both N and O states might form kinetically distinct sub-states (actually separate intermediates), formation and decay of which in turn involve proton shifts, mostly between carboxylic amino acids. For instance, in the mutants involving E194Q and/or E204Q substitution, the proton-releasing complex is severely disturbed specifically blocking the normal ("early") proton release [73, 74]. In these mutants a slow red-shifted O′ is formed after normal O state, different from O in respect of protonation state of Asp85, the proton from which is shifted to Asp212 [25] (see also [122]). Alternatively, in mutants with strongly inhibited N decay (e.g., T46V) a proton (the "fourth" one) disappears from the bulk but fails to immediately appear on its "target", Asp96. During this "delay" it could be observed (at pH ~ 5) being bound to surface carboxylic residues, as is evident from the correspondent transient IR signals [24, 123].

MODULATION OF pK_a OF FUNCTIONALLY IMPORTANT CARBOXYLIC AMINO ACIDS DURING THE PHOTOCYCLE

Prior to excitation Asp85, the primary proton acceptor [72], is characterized by extremely low pK_a (~2.2-2.6) [35, 36, 40-43], which is elevated to pK_a > 10.5 in the late M (presumably M_2) state [40]. In IR this ultra high pK_a is reflected in extremely high position of the ν_{COOH} at 1761 cm^{-1}. At least part of this pK_a increase takes place during the M_1-to-M_2 transition when Asp85 is already protonated, and in terms of energy balance is equivalent to lowering the energy level for the proton of the COOH group of Asp85. This downshift in the energy level (of the particular proton) is only partly dissipative in the photocycle: to the extent of irreversibility of the M_1-to-M_2 transition. In native bacteriorhodopsin the energy flow is optimized, dissipation is minimal, and, therefore, the major part of this energy is transferred to the proton releasing complex through a mechanism of coupling of pK_a values of the Asp85 and that of the PRC^+ [34-36]. Recent X-ray studies proposed a molecular mechanism for this effect on atomic level: H-bonding rearrangements and positively charged Arg82 side chain movement in M [124-126].

In energetic terms pK_a coupling mechanism is similar to the old-fashioned kid's seesaw on which lowering of (the energy level of the proton on the Asp85 on) one side obligatorily leads to the raising of the correspondent (energy) level on the other. That is, instead of dissipating, this energy is used to raise the potential energy of the proton in PRC^+, which is equivalent of lowering its pK_a (~5 units according to [34, 35]). The most probable mechanism for this long-range coupling is a chain of H-bonds[1] directly connecting Asp85 and PRC^+. Besides a chain of interconnected H-bonds, the coupling mechanism could involve some direct Coulombic interaction, aided in part by very low effective dielectric constant (ε ~ 2) environment around Asp85 in M(s), N, and O intermediates [40].

It might be interesting to note here that in the non-transporting sensory rhodopsins such seesaw-like mechanism cannot function due to insufficient "clearance" for seesaw swipes (change in energy/pK_a) when starting from initially much higher pK_a. The static pK_a for the primary proton acceptor Asp75 in pSR-II[2], a homolog of Asp85 in BR, is 5.6 [127], while in SR-I[3] such homolog, Asp76, has an even higher pK_a (~7.2-7.4) [128, 129] and is initially protonated under physiological conditions.

The proton-releasing complex is composed of two glutamic acids (Glu194 and Glu204) [73-75] and a net of hydrogen bonded water molecules [96, 130] with possible participation of other amino acids [96, 131]. In wild type BR, PRC^+ has a pK_a of 9.4-9.7 [35, 36, 43], which is decreased in M to ~5.8, as measured on D96N mutant [112]. Its pK_a should be expected to be restored in O while that of Asp85 has to decrease to below ~9.5 to become the donor for reprotonating the PRC^- during the O-to-bR transition. This latter process is reflected in the IR when the stretch frequency of Asp85 in O is downshifted by ~6-9 cm^{-1} from its position in M. This high frequency of the carboxylic IR band would be consistent (see above in the section "Infrared features of carboxylic amino acids") with pK_a around 9.5 for PRC^+ in O state but would be difficult to reconcile with a proposed much lower estimate of 4.3 [43].

Altering pK_a of the groups in PRC^+ by site-specific mutagenesis opens an opportunity to monitor the timing of the proton transfer shifts within PRC^+: a characteristic IR band at 1720 cm^{-1} coming from transiently protonated Asp194 (in the E194D mutant) was time-resolved in M [75].

The primary proton donor, Asp96 [90, 132], has an initially very high pK_a > 11 [38]. By the time when the Schiff base is reprotonated in the $M_2 \leftrightarrow N$ reaction, pK_a of Asp96 is decreased to ~7-7.5 [43, 82, 104].

In non-excited BR the Schiff base has a pK_a of ~13 [27]. Its pK_a transiently drops in M to facilitate proton transfer to Asp85, and (in D96N mutant) was shown to be ~8.2 in the "late" M [133]. Most probably this nearly 5 pK_a units decrease takes place gradually during L-to-M_1-to-M_2 transitions. A drop in the pK_a of the Schiff base in L (i.e., prior to SBH^+ deprotonation) would bring closer to one another the energy levels of the donor (SBH^+) and the acceptor (Asp85), and, therefore, minimizing dissipation.

When recalculated in pK_a units, the energy stored in the primary act is ~8.7 [134]—apparently enough for the proposed modulation of the pK_a of the Schiff base, Asp85, Asp96, and PRC^+.

CONCLUDING REMARKS

Bacteriorhodopsin is by far the best-studied photochromic protein. Few, if any other system is known to that extent on both functional and structural levels. Two main reasons have powered continuous interest in BR in the past. First, it is probably the simplest natural system that is able to convert sun light into chemical energy. Second, BR is actually a very good "model system", and its study was always a good testing ground for emerging techniques from time-resolved FTIR to crystallization of membrane proteins.

Until very recently archaeal rhodopsins—bacteriorhodopsin, halorhodopsin, and sensory rhodopsins I and II—were thought to constitute an isolated family with quite limited importance for humanity. However, during the last two years closely related opsins were found in fungi (i.e., in

[1] The X-ray structure of M [125] contains an H-bond to Asp85, and IR data [67] indicate that this bond should involve the –OH but not the C=O group of Asp85.

[2] pSR-II is the sensory rhodopsin II from *Natronobacterium pharaonis*.

[3] SR-I is the sensory rhodopsin I from *Halobacterium salinarum*.

Eucarya) (for review see [135]) and a "bacteriorhodopsin-like" protein, proteorhodopsin (PR), was found in marine eubacteria (i.e., in Bacteria) [136]. Both the opsins from fungi (upon retinal binding) [137, 138] and rhodopsin from bacterioplankton [136] exhibit photocycles similar to the archaeal rhodopsins—with a sensory function in the former [135], and as a proton pump in the latter [136]. These findings indicate that bacteriorhodopsin-like pigments could serve as energy sources or photoreceptors among species of various taxa. Proliferation of genomic projects could be expected to extend this "family" even further.

Its now seems likely that PR, rhodopsin from bacterioplankton, provides a previously unknown pathway of light-energy conversion in the oceans worldwide [136, 139]. Thus, a previously semi-obscure family of bacterial rhodopsins—until recently known and interesting mostly to a small group of academics—might be an important component of the Earth's biosphere, being responsible for a non-negligible portion of solar energy conversion.

Sequence comparison of those closely related proteins (which have very high homology) raises interesting questions on the role of particular conserved or semi-conserved residues, including the role of carboxylic acids. For instance, in the so-called "neurospora rhodopsin" (NR) from *Neurospora crassa* homologs of the two key carboxylic residues in bacteriorhodopsin, Asp85 and Asp96, are present. The former, Asp131, acts exactly as in BR, being the primary proton acceptor [140], while the latter, Glu142, does not participate in the Schiff base reprotonation [138].

Just ten or even five years ago the field of "bacteriorhodopsin-related" studies seemed to be on the verge of "recession". Now it is definitely entering a new dynamic phase. The advance of knowledge is known to follow spiral-like cycles. We have entered a "new turn", and the methods, developed and refined on the "model system" of bacteriorhodopsin during the previous decade, are ready to meet new challenges.

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