Effects of Arginine-82 on the Interactions of Internal Water Molecules in Bacteriorhodopsin†

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ABSTRACT: Arg82, one of the residues near the protonated Schiff base of bacteriorhodopsin, facilitates proton release to the medium during the L-to-M reaction of the photocycle, but retards the rate of proton transfer from the Schiff base to Asp85. In order to understand the role of Arg82 in these processes, the structural changes upon formation of the M intermediate were studied by Fourier transform infrared spectroscopy of the hydrated films of Arg82 mutants at pH 9.5. The negative band at 1700 cm^{-1} in the BR→M spectrum due to the deprotonation of Glu204 was absent when Arg82 was replaced with alanine (R82A), but present with small amplitude when residue 82 was a glutamine (R82Q), or a lysine (R82K), with a shift to 1696 cm^{-1}. The O−H stretch of water at 3643 cm^{-1} is shifted toward a lower frequency in R82Q, R82K, and R82A in the unphotolyzed state. However, R82Q retains a fraction of the unshifted band. Another O−H stretch is prominent in R82Q around 3625 cm^{-1} but absent in R82A and probably in R82K. In parallel, R82Q retains a fraction of the slow component of the formation of the M intermediate, which is almost completely absent in R82K and R82A. These results, along with previous data for the mutants of Glu204, suggest that the guanidium group of Arg82 influences the H-bonding of water molecules located close to Asp85 and Arg82−Glu204 regions, and the rate of proton transfer from the Schiff base to Asp85. The amide group of Gln82 can substitute for it but weakly.

Bacteriorhodopsin is a transmembrane protein in the purple membrane of *Halobacterium salinarium* which contains a retinal chromophore bound covalently to Lys216 through a protonated Schiff base. Light absorbed by the all-trans form of bacteriorhodopsin (BR)[1] results in active proton transport. The chromophore interacts weakly through the Schiff base with the protein, especially with the anionic Asp85 and Asp212, the positively charged Arg82, Tyr57, and water molecules (DeGroot et al., 1989; Henderson et al., 1990; Dér et al., 1991; Humphrey et al., 1994; Brown et al., 1994a; Fischer et al., 1994; Maeda et al., 1994; Govindjee et al., 1995; Kandori et al., 1995). Upon absorption of light, BR goes through a series of intermediates called J, K, L, M, N, and O consecutively (Lozier et al., 1975). Proton transfer from the Schiff base to Asp85 in the L-to-M conversion is followed by proton release from a residue other than Asp85, because Asp85 remains in a protonated state throughout the photocycle (Siebert et al., 1982; Pfefferlé et al., 1991; Bousché et al., 1992; Souvignier & Gerwert, 1992; Hessling et al., 1993). Proton uptake from the cytoplasmic medium follows proton transfer from Asp96 to the Schiff base in the M-to-N reaction (Otto et al., 1989).

The pK_a value of Asp85 increases from 2.5 to about 7 when Arg82 is replaced with neutral residues like glutamine or alanine (Subramaniam et al., 1990; Thorgeirsson et al., 1991; Brown et al., 1993; Balashov et al., 1993), and increases only slightly when Arg82 is replaced with the cationic lysine residue (Balashov et al., 1995). However, there is no effect on the pK_a of Asp85 in the R82Q/D212N double mutant (Brown et al., 1995b).

The single amino acid mutants R82Q and R82A release the proton to the extracellular side only after the uptake from the cytoplasmic side (Otto et al., 1990; Balashov et al., 1993, 1995; Brown et al., 1995a). Proton release from R82K was observed at the normal time in the photocycle, but is greatly reduced in extent (Balashov et al., 1995). These findings led to the idea that the protonated Arg82, or residues close to it, is a source of the released proton. Normal proton release occurs, however, even without Arg82 in the mutant of R82Q/D212N (Brown et al., 1995b) or at high pH in R82Q (Govindjee et al., 1996). Recently Glu204 was identified as the residue that releases the proton (Brown et al., 1995a).

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[1] Abbreviations: BR, *all-trans*-bacteriorhodopsin; FTIR, Fourier transform infared.

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The L-to-M transition in the wild type becomes extremely rapid above pH 9 (Hanamoto et al., 1984). Similar rapid conversion occurs, but independently of pH, in R82A and R82K (Balashov et al., 1993, 1995). It is known that a water molecule is coordinated with Asp85 (Maeda et al., 1994) and weakly to Asp212 (Kandori et al., 1995). This water molecule undergoes stronger H-bonding upon L formation and may be part of the structure for proton transfer from the Schiff base to Asp85 in the L-to-M transition (Brown et al., 1994a). The same water molecule is also affected by the mutation of Glu204, and may be involved in the deprotonation of Glu204 (Brown et al., 1995a).

In this paper, the effects of Arg82 on water, and their influence on the L-to-M reaction as well as the resulting deprotonation of Glu204 in the M state, were studied by Fourier transform infrared (FTIR)[1] and time-resolved visible spectroscopy of the mutants of Arg82. In these mutants, Arg82 is replaced with lysine (R82K), glutamine (R82Q), or alanine (R82A). R82K retains a positive charge and R82Q H-bonding ability. The results suggest that the effects on water molecules of Arg82 in the photocycle are mediated through H-bonding.

MATERIALS AND METHODS

The mutants of R82A, R82K, and R82Q were constructed, and their proteins were isolated as described by Balashov et al. (1993, 1995) and Brown et al. (1993), respectively.

Dried films were prepared from purple membrane suspensions in 0.005 M borate buffer (pH 9.5) on a BaF_2 window in room air. They were hydrated by putting 1 μL of water (H_2O or $H_2{}^{18}O$) in the sample cell before mounting in an Oxford cryostat DN1704. Light-adaptation was done at 274 K by irradiation with >500 nm light for 5 min. The relatively stable N photointermediates of these mutants were depleted by holding at 274 K for 10 min before further cooling. FTIR spectra were measured in a BioRad FTIR FTS60A/896 spectrometer. Temperature was controlled by an Oxford ITC-4 temperature controller. All spectra were drawn after normalizing the negative band at 1202 cm^{-1}. The pure BR→M spectrum was recorded by irradiation with >500 nm light for 1 min at alkaline pH at 230 K (Pfefferlé et al., 1991). The sample was warmed to 273 K to restore the BR state, and then the same procedure was repeated. Four to five recordings were averaged.

Microsecond time-resolved absorbance change at 410 nm was recorded for the suspensions in 0.1 M NaCl and 0.005 M borate buffer (pH 9.5) as described previously (Yamazaki et al., 1995).

RESULTS

Sample Manipulation. Mutants of Arg82 substituted by neutral residues, R82Q and R82A, undergo the blue−purple chromophore transition in the range of neutral pH (Brown et al., 1993; Balashov et al., 1993). All the experiments were conducted at pH 9.5, where the blue forms in R82A and R82Q are absent. This pH is also favorable for R82K, which can be completely light-adapted only at alkaline pH, in spite of the fact that it is in the purple state at pH 7 (Balashov et al., 1995).

BR→M Spectra. The three mutant proteins of Arg82 exhibited spectral features similar to those of the wild type in the 1800−800 cm^{-1} region (Figure 1). The single C═C

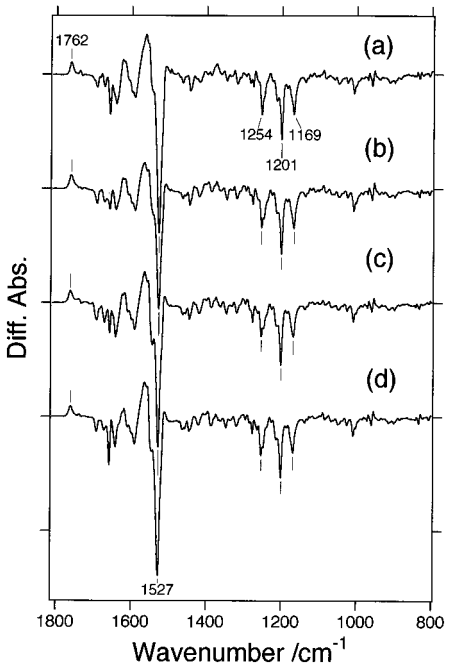

FIGURE 1: BR→M spectra in the 1800−800 cm^{-1} region for wild type (a), R82Q (b), R82K (c), and R82A (d). Vertical solid lines indicate the presence of the band at the same frequency as in (a). One division in the ordinate is 0.025, 0.043, 0.033, and 0.053 absorbance unit for (a), (b), (c), and (d), respectively.

stretch at 1527 cm^{-1} and the absence of the negative band at 1184 cm^{-1} of 13-*cis*-retinal in the difference spectra of all the mutants indicate that the spectra in this study are exclusively due to the photoconversion of the all-trans species of the purple forms. The band characteristic of the protonated Asp85 of the wild type at 1762 cm^{-1} (a) was preserved in R82Q (b), R82K (c), and R82A (d). The result with R82Q (b) is consistent with the data shown previously (Brown et al., 1994b). The absence of any influence from the positive charge of Arg82 on the frequency of this C═O stretching mode is also shown in R82A (d). This is consistent with a notion by Dioumaev and Braiman (1995) that the Asp85 COOH group is in a highly nonpolar environment in the M intermediate. The corresponding band at 1755 cm^{-1} in the N intermediate was also unaffected in R82Q and R82A (not shown).

Figure 2 shows the spectra expanded in the 1710−1680 cm^{-1} region. The wild type exhibits a negative band at 1700 cm^{-1} as a shoulder of a larger band at 1692 cm^{-1} (a). The 1700 cm^{-1} band is ascribed to the deprotonation of Glu204 (Brown et al., 1995a). A similar shoulder, but at 1696 cm^{-1}, appears in R82K (c). R82A (d) exhibits no such shoulder. On the other hand, the peak at 1692 cm^{-1} of R82Q (b) is accompanied by a shoulder at 1700 cm^{-1}, though smaller than that of the wild type. These results indicate that in R82Q, but not in R82A, a small fraction of Glu204 deprotonates. A small proton release in the normal time range with R82Q at pH 8 (Govindjee et al., 1996) is consonant with this. A small negative component in the pyranine signal due to proton release in the L-to-M conversion at pH 7.3 (Brown et al., 1995a; Govindjee et al., 1996) may correspond to the limited deprotonation of Glu204 in R82Q.

The BR→K spectra in the 1800−800 cm^{-1} region, recorded by irradiation at 77 K for all the mutants, were identical with that of the wild type (data not shown). Since the L

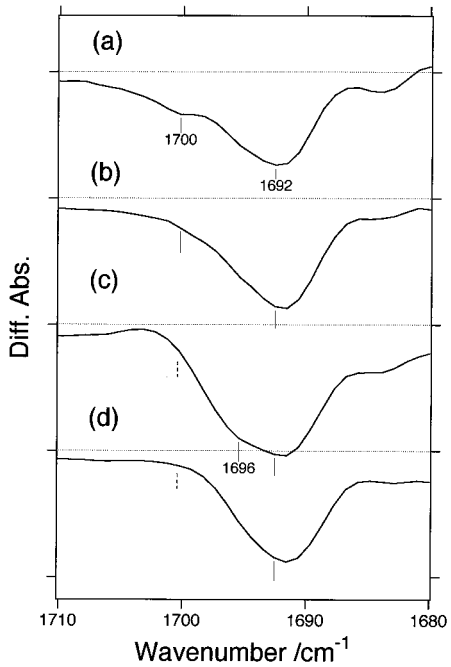

FIGURE 2: BR→M spectra in the 1710−1680 cm^{-1} region for wild type (a), R82Q (b), R82K (c), and R82A (d). Vertical solid lines indicate the presence of the band at the same frequency as in (a) and vertical dashed lines the absence of the band that is present in (a). One division in the ordinate is 0.0038, 0.0065, 0.0050, and 0.0044 absorbance unit for (a), (b), (c), and (d), respectively.

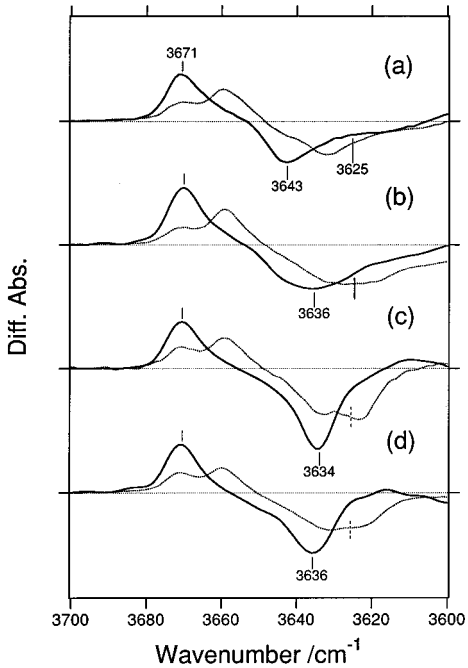

FIGURE 3: BR→M spectra in the 3700−3600 cm^{-1} region in H_2O (solid lines) and $H_2{}^{18}O$ (dotted lines) for wild type (a), R82Q(b), R82K (c), and R82A (d). Vertical solid lines indicate the presence of the band at the same frequency as in (a) and vertical dashed lines the absence of the band that is present in (a). One division in the ordinate is 0.0075, 0.0061, 0.0103, 0.0070, 0.0060, 0.0055, 0.0039, and 0.0050 absorbance unit for solid lines in (a), (b), (c), and (d), and for dotted lines in (a), (b), (c), and (d), respectively.

intermediate is short-lived at alkaline pH, the pure BR→L spectra of the mutants were only obtained after subtraction of the BR→M spectra. Each BR→L spectrum was similar to that of the wild type. Distinct in the mutants were the more intense 1748 cm^{-1} band of Asp96 and the absence of the 1729 cm^{-1} band of Asp115 (data not shown), as observed in D85N and D212N (Kandori et al., 1995).

Water Structural Changes. The BR→M spectra in the 3700−3600 cm^{-1} region are shown in Figure 3. The spectra were also recorded in $H_2{}^{18}O$. As shown previously at neutral pH (Maeda et al., 1992), the wild type at pH 9.5 (a) exhibits negative and positive bands at 3643 and 3671 cm^{-1}, respectively. Remained bands in $H_2{}^{18}O$ at these frequencies, which disappeared completely in the previous experiments at neutral pH (Maeda et al., 1992), might arise from incomplete removal of water in the film in the presence of the buffer salt. The positive band at 3671 cm^{-1} of the M intermediate was not affected at all in these mutant proteins.

The 3643 cm^{-1} band of the wild type in the unphotolyzed state (a), which was attributed to a water molecule located close to Asp85 (Maeda et al., 1994), shifts to 3634 and 3636 cm^{-1} in R82K (c) and in R82A (d), respectively. The corresponding band of R82Q appeared to be broader than the others (b). It is comprised of a band at 3636 cm^{-1} with an accompanying smaller band in higher frequencies, as for the wild type. Also a shoulder around 3625 cm^{-1} is present in R82Q (b) but is absent in R82A (d) and probably in R82K (c). It is present, however, with a smaller intensity in the wild type (a). All these bands showed clear shifts by 8−10 cm^{-1} in $H_2{}^{18}O$, and thus can be attributed to the O−H stretches of water.

Kinetics of the L-to-M Conversion. In Arg82 mutants, the formation of the M intermediate is rapid and independent of pH (Balashov et al., 1993, 1995; Brown et al., 1995a), in contrast to the wild type which displays such a rapid rise of

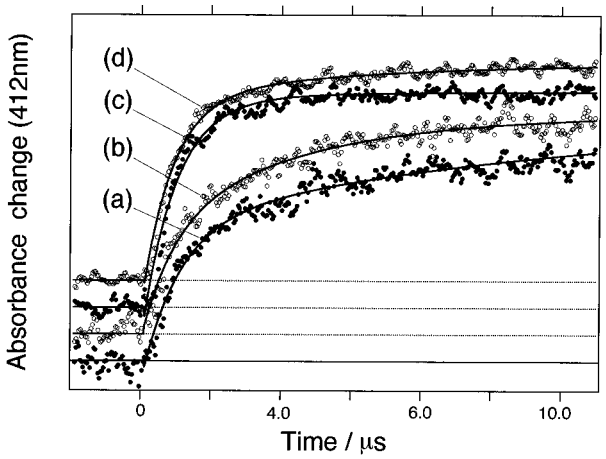

FIGURE 4: Kinetics for the formation of the M intermediate of the wild type (a), R82Q (b), R82K (c), and R82A (d) for the suspensions in 0.1 M NaCl and 0.005 M borate buffer (pH 9.5).

the M intermediate only at alkaline pH. The rate for the M formation was measured for the mutants of Arg82, along with the wild type. Figure 4 shows that M formation of all the species proceeds with almost the same rate at the beginning, and then the rates for the wild type and R82Q become slower. Each curve is composed of two exponential functions. The rapid one with the time constant of about 1 μs comprises about half the amplitude for the wild type (1.1 μs; 59%) and R82Q (0.9 μs; 48%) but more than 80% for R82A (0.8 μs; 84%) and R82K (0.9 μs; 97%). The slower components of the wild type and R82Q exhibit time constants of 12 and 4 μs, respectively. These data for the wild type, R82A, and R82K are nearly coincident with those of Liu (1990) and Balashov et al. (1993, 1995).

DISCUSSION

Arg82 is one of the components of the protein domain that surrounds the Schiff base, and its replacement with either glutamine, lysine, or alanine inhibits the normal proton release during the L-to-M process (Otto et al., 1989; Balashov et al., 1933, 1995; Brown et al., 1995a). In accordance with this, the deprotonation of Glu204 in the proton release (Brown et al., 1995a), manifested as a negative band at 1700 cm^{-1} in the wild type (Figure 2a), was absent in R82A (Figure 2d). However, a small fraction of the band was retained in R82Q (Figure 2b), and, with a shift to 1696 cm^{-1}, also in R82K (Figure 2c). These are in accordance with normal proton release in these mutants, though small in extent (Balashov et al., 1995; Govindjee et al., 1996).

The weakly H-bonding O−H of the water molecule at 3643 cm^{-1} (Figure 3a), coordinated to Asp85 in the unphotolyzed state (Maeda et al., 1994), was shifted to lower frequencies in the mutants of Arg82. However, a part of it remained at 3643 cm^{-1} in R82Q (Figure 3b). An additional negative band present around 3625 cm^{-1} for R82Q, though abolished in R82A and probably in R82K, was detected in the wild type with a lower intensity.

The effects of Arg82 on the rate of proton transfer in the L-to-M reaction were observed even at pH 9.5 (Figure 4) where the L-to-M transition becomes extremely rapid, even for the wild type. The wild type exhibited a biphasic M rise, with a slower component of about 50% amplitude with $\tau_{1/e} = 12\ \mu s$. R82Q also exhibited such a slower component, although its rate was more rapid than for the wild type. R82A and R82K did not show such a slower component with significant amplitude.

Thus, glutamine in place of arginine at position 82 partly preserves the properties of the wild type in (a) the negative band at 1700 cm^{-1} due to the deprotonation of Glu204 in the M intermediate, (b) normal structural changes of water molecule in the Asp85 domain, and (c) slower L-to-M conversion than the other two mutants. The mutations to lysine or alanine, on the other hand, result in the complete abolition of these properties. It appears that the positive charge of arginine is not responsible for at least some of the wild-type-like properties of the mutants because replacement of Arg82 with lysine abolishes them. These properties probably originate instead from a common feature between arginine and glutamine that is absent in alanine and lysine. It is most likely to be the N−H at the ϵ-position, which is able to function as an H-bonding donor.

Internal water distribution in known protein structures was studied by Thanki et al. (1988). They noted a clustering of water around the N−H at the ϵ-position of both glutamine and arginine. On the other hand, water is more scattered around the amino group of lysine. This difference could arise from the free rotation of the amino group of lysine, in contrast to the fixed planar guanidium group of arginine and the amide group of glutamine. Thus, the functional groups of the guanidium group of arginine and the amide group of glutamine resemble each other in their abilities as donors in H-bonding. Alanine does not have any such functional groups.

The shift of the 3643 cm^{-1} band and the abolition of the broad band around 3625 cm^{-1} due to water were also observed in E204Q (Brown et al., 1995), which does not release a proton from the normal proton release group. On the other hand, E204D, which exhibits normmal proton release, displayed the unshifted band at 3643 cm^{-1} and an intense band at 3625 cm^{-1}. These results suggest that the O−H stretching vibration around 3625 cm^{-1} in the mutants of R82Q and E204D arises from a water molecule that is located between Arg82 and Glu204. These water molecules are also related to the proton transfer from the Schiff base to Asp85. A whole structural model based on molecular dynamics calculations by Humphrey et al. (1994) suggests that the ϵN−H of Arg82 is connected to Asp85 through H-bonding of intervening water molecules and Asp212, and another H-bonding chain of water molecules links Arg82 to Glu204.

The faster phase of the rise of M was suggested to reflect the equilibration of L and M_1, followed by a shift to complete proton transfer as the M_1 to M_2 reaction proceeds (Zimányi et al., 1992). According to this model, the absence of a slower phase in R82A and R82K (Figure 4) would indicate that Asp85 is a better proton acceptor without H-bonding to Arg82 through water molecules. The partial shift to the faster rise observed in R82Q is consistent with this, since Glu82 retains similar water-mediated H-bonding to Asp85.

Electron density was not observed by Henderson et al. (1990) for Arg82, and its location could not be assigned. Electrostatic calculations of the pK_a of ionizable groups (Bashford & Gerwert, 1992) suggested that its side chain should be directed toward the Schiff base. It has been suggested that Arg82 changes its position during the photocycle (Balashov et al., 1993; Scharnagl, 1995). The present results indicated that it is quite likely, since the C═O stretch frequencies in the M and N intermediates are unaffected in all of the Arg82 mutants (Figure 2). Further, the O−H stretch of the water molecule presumed to be associated with Asp85 (Maeda et al., 1994) is unaffected in the M intermediate (Figure 3).

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