Cysteine S−H as a Hydrogen-Bonding Probe in Proteins

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The stretching frequency of cysteine S−H is in the 2580−2525 cm^{-1} region, well removed from other vibrations. This is now used for specifically probing changes of H-bonding in the light-driven proton pump of bacteriorhodopsin (BR), with Thr89 substituted for cysteine. The S−H stretch is at 2523 cm^{-1} in BR, at 2477 cm^{-1} upon photoisomerization of the retinal to 13-cis, and at 2576 cm^{-1} after the primary proton transfer. The unusually strong H-bonding with the neighboring Asp85 after isomerization (2477 cm^{-1}) implies steric conflict due to the C_{13}═C_{14} bond rotation. In contrast, lack of such H-bonding after proton transfer (2576 cm^{-1}) presumably correlates with the conformational switching of the protein structure, an essential event in proton pumping.

The S−H group of cysteine (Cys) in proteins is highly reactive toward many reagents and forms a disulfide bond with another S−H. This property has been used extensively for functional studies of proteins by labeling Cys S−H with reagents for absorption or emission probes, photochemical cross-linking, etc.[1] In such cases the Cys residue has to be located near the surface of the protein for the labeling reaction, and no information can be obtained about the interior of the protein. On the other hand, the Cys S−H group is a good indicator of H-bonding inside proteins in infrared spectroscopy.[2] The stretching frequency of the S−H is in the 2580−2525 cm^{-1} region,[3] where other vibrations are absent. In this article, we report a novel approach which utilizes the Cys S−H as a H-bonding probe inside the protein.

We introduced Cys S−H to bacteriorhodopsin (BR), a light-driven proton pump in halobacteria, which contains no Cys. H-bonding networks play a significant role in proton-pumping proteins because the proton is very likely to be transported through a H-bonding network that involves water molecules. BR is the best studied proton pump,[4] whose structure has been recently determined at atomic resolution.[5,6] In BR, light energy is first stored in the protein structure of the K intermediate through the all-trans to 13-cis photoisomerization of the retinal chromophore. As a result of its relaxation, BR translocates a proton from inside the membrane to the extracellular surface. The M intermediate is a product of the primary proton transfer from the protonated Schiff base to Asp85, where conformational switching for vectorial proton transport occurs.[7]

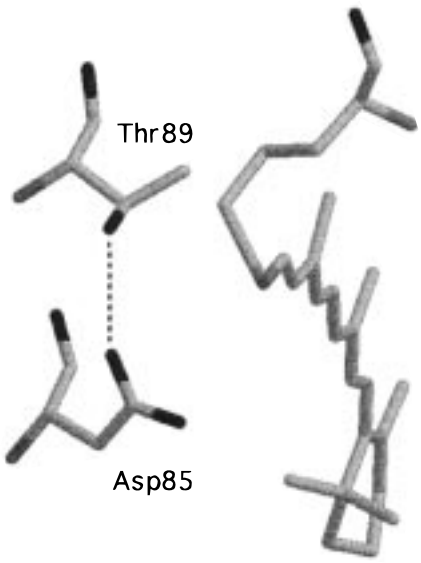

Figure 1. Location of Thr89 shown with the H-bonding acceptor Asp85 and the retinal chromophore bound for Lys216 in the side view of 2BRD. It is similar in the recently determined structure by electron microscopy and X-ray crystallography. The distance between the two oxygens of Thr89 and Asp85 is 3.1 Å in 2BRD[10] and 2.9 Å from the X-ray crystallography.[6]

In the present study, we substitute Thr89 for Cys (T89C). Thr89 is present in the "proton channel" of BR, comprised of helices B, C, and G and involves Asp96, the retinal Schiff base, Asp85, and Glu204 as protonable groups. In addition, Thr89 is located within van der Waals contact of the retinal chromophore near the protonated Schiff base and the O−H group of Thr89 is hydrogen-bonded with COO^- of Asp85 (Figure 1), the counterion of the protonated Schiff base in the unphotolyzed state and the proton acceptor in the M intermediate. Such a structurally important residue is likely to undergo changes in H-bonding upon photoisomerization (K-formation) and proton transfer from the Schiff base to Asp85 (M-formation).

A mutant protein T89C is synthesized as described previously.[8] One of the characteristics of T89C is more rapid dark-adaptation than the wild type. In contrast, low-temperature polarized Fourier transform infrared (FTIR) spectroscopy[9] of a hydrated T89C film at pH 8 (1 mM borate buffer) revealed that both K and M intermediates form normally. Figure 2 shows the infrared spectral change in the S−H stretching region. In both the K minus BR and the M minus BR spectra a pair of positive and negative bands are observed. These bands are absent in the wild type. Therefore, the spectral change originates from the S−H stretching vibration of Cys89. The increased amplitude observed upon tilting the sample film (53.5°) indicates that the dipole moment of the S−H stretch is close to normal to the membrane plane. We attempted

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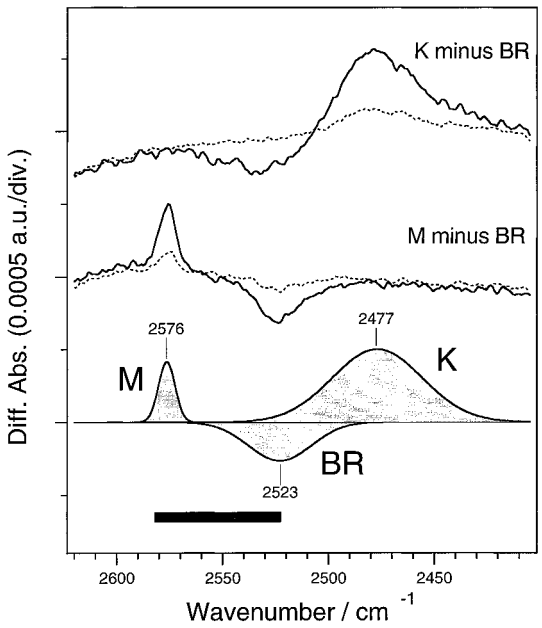

Figure 2. K minus BR (above) and M minus BR (middle) infrared difference spectra of T89C in the 2620−2405 cm^{-1} region. The thick horizontal bar (2580−2525 cm^{-1}) shows the frequency region of S−H stretch in the literature.[3] Solid and dotted lines represent the results of the window tilt at 53.5° and 0°, respectively. The greater change in solid lines indicates that the S−H group is oriented vertical to the membrane. The bottom traces represent the Gaussian fits to the spectra at 53.5°.

Gaussian fitting to the spectra at 53.5°. The fitting yielded the frequencies values of 2477 cm^{-1} for the K intermediate and 2576 cm^{-1} for the M intermediate, whereas the value of BR was determined to be 2523 cm^{-1} from the M minus BR spectrum and 2531 cm^{-1} from the K minus BR spectrum (Figure 2). The difference in BR could be due to the effect of the temperature between 77 and 240 K. Full widths of half-maxima (fwhm) are 35, 50, and 9 cm^{-1} for BR, K, and M, respectively. The area is 2.7 times larger in K and 0.4 times smaller in M than in BR. The angle of the dipole moment to the membrane normal is estimated from the spectra at 0° and 53.5° to be the same (24° ± 5°) among BR, K, and M.

In the literature, the S−H stretching vibration appears in the 2580−2525 cm^{-1} region and its frequency is lower if H-bonding of the S−H group is strong.[3] The frequency of BR at 2523 cm^{-1} thus indicates that the H-bonding is the strongest possible for the S−H group of Cys89. According to the wild-type BR structure,[10] the angle of the H-bonding between oxygen of Thr89 and one of oxygens of Asp85 (Figure 1) is 21° to the membrane normal. Coincidence with the angle of the S−H group (∼24°) strongly suggests that the S−H forms H-bonding with one of oxygens of Asp85 in T89C. The strongest H-bonding of the S−H group does not necessarily indicate that the O−H of Thr89 forms very strong H-bonding because the larger van der Waals radius of sulfur (1.85 Å) than of oxygen (1.40 Å) may force stronger H-bonding in the mutant.

Upon all-trans to 13-cis photoisomerization of the retinal the S−H stretching appears at 2477 cm^{-1} in K. The band has large area with a wide fwhm (50 cm^{-1}) in comparison with those of BR and M. The frequency, which has never been reported as S−H stretch, is about 50 cm^{-1} lower than any in the literature. It implies that the distance between the sulfur of Cys89 and the oxygen of Asp85 becomes much closer, which is probably the result of the chromophore motion in the restricted protein environment. The distance must be in the repulsive region of the H-bonding potential, providing a higher energy state in K. Since the H-bonding structure of the unphotolyzed state is similar in T89C to that of the wild type, it is likely that such structural change occurs between Thr89 and Asp85 in the wild type. A part of light energy storage in K[11] has been explained in terms of the distorted chromophore structure as shown in the strong hydrogen out-of-plane (HOOP) vibrations of resonance Raman spectra.[12] However, nothing is known of structural changes of the protein. The present result provides the first indication of energy storage by the protein.

On the other hand, the S−H stretching appears at 2576 cm^{-1} in the M state upon proton transfer from the protonated Schiff base to Asp85. The band has a much smaller area, with a narrower fwhm (9 cm^{-1}) than those of BR and K. Thus, the H-bonding between Cys89 and Asp85 is very weak in M. Loss of the negative charge at Asp85 presumably affects the weak H-bonding interaction. It is, however, noted that protonation of Asp85 does not necessarily yield loss of H-bonding because protonated Asp85 (COOH) still possesses a possible H-bonding acceptor. The frequency (2576 cm^{-1}) implies that the H-bonding is almost broken. Thus, a certain conformation change is likely to occur for breaking the H-bonding of the S−H group of Cys89 with Asp85. Interestingly, there is almost no difference in the angle between BR and M (∼24°). This may suggest that the atomic movement occurs at the position of Asp85.[13] The reprotonation switch upon M formation will change the accessibility from the extracellular to the cytoplasmic side.[14] The conformation change for the switch is probably composed of various structural elements, one of which is shown in the present result.

Our results show that introduction of Cys inside proteins is a powerful tool to investigate H-bonding network by means of infrared spectroscopy. The similarity of the orientation of the S−H group of T89C to the O−H group of the wild-type BR is shown by determining the similar angles of their dipole moments. The S−H stretch at 2523 cm^{-1} in BR (the strongest H-bonding) shifts to 2477 cm^{-1} upon photoisomerization (unusually strong H-bonding) and to 2576 cm^{-1} upon primary proton transfer (lack of H-bonding). S−H stretching vibrations of intrinsically present Cys have been reported for rhodopsins[15] and heme proteins.[2,16] Thus, introduction of Cys inside proteins is a promising method to probe changes in H-bonding during functional processes of proteins.

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