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EFFECT OF IODINATION ON THE pK OF SCHIFF BASE DEPROTONATION AND M_{412} PRODUCTION IN PURPLE MEMBRANE

G. Gogel* and Aaron Lewis†

*Department of Chemistry, Colgate University, Hamilton, New York 13346, and School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853

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Summary

Previously, kinetic resonance Raman measurements as a function of pH have been used to demonstrate that, microseconds after light absorption, the pK of Schiff base deprotonation during the bacteriorhodopsin photocycle is 10.2 ± 0.3, whereas before the light event, the pK is > 12 (2). In this investigation, we have iodinated purple membrane suspensions and have found that the pK of Schiff base deprotonation in the photocycle has been lowered to between 7 and 8 for iodinated bacteriorhodopsin. These results, together with our previous data on the pK of Schiff base deprotonation, suggest that the amino acid tyrosine could be a critical component in the deprotonation mechanism.

Over the past several years, kinetic resonance Raman spectroscopy has been a very useful tool in the investigation of the molecular mechanism of the membrane bound proton pump bacteriorhodopsin from Halobacterium halobium (1,2,3, 4,5,6,7). The center of molecular activity in this proton pump is a retinylidene chromophore linked by a protonated Schiff base to the ε-amino group of a lysine residue (8). One of the first applications of resonance Raman spectroscopy to this protein demonstrated that light deprotonates this Schiff base linkage (8). In an initial application of kinetic resonance Raman spectroscopy, we were able to determine that this deprotonation occurred at pH 7 on a timescale of microseconds (1). In this investigation, we were also able to use the structurally selective nature of kinetic resonance Raman spectroscopy to relate the Schiff base deprotonation to the appearance, within 40 μs after light absorption, of the M_{412} kinetic intermediate, which is thought to be associated with the establishment of the proton gradient across the bacterial cell membrane. Subse-

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quently, we investigated the nature of the pH dependence of Schiff base deprotonation and M_{412} evolution. We discovered that the rate of Schiff base deprotonation microseconds after light absorption and M_{412} evolution were strongly pH dependent and had a pK of 10.2 ± 0.3 (2) even though, in the dark, the pK of the Schiff base was apparently > 12.

Two possible explanations could account for this pH dependence microseconds after light absorption. Firstly, our observations could be the result of alterations in the Schiff base pK microseconds after light absorption to 10.2 ± 0.3. This explanation cannot a priori be ruled out, since Schiff base deprotonations are facilitated by nucleophiles and the OH^- of the medium had tens of minutes to exchange into the site of the Schiff base linkage before the experiment was performed. In addition, this Schiff base is deprotonated in microseconds after light alters its pK. However, a second option is also capable of explaining this apparent pK alteration. In the alternate scenario, the Schiff base pK is lowered to $< 10.2 \pm 0.3$ microseconds after light absorption and then deprotonation is controlled by a group in the protein of pK $\sim$10.2 rather than the OH^- of the medium. This protein group could be deprotonated by the high pH medium and catalyze Schiff base deprotonation microseconds after light absorption by the chromophore. The experiments described in this paper were aimed at discriminating between these two options and resolving further our understanding of the molecular mechanism of proton gradient formation in this membrane protein.

A likely candidate for nucleophilic facilitation of Schiff base deprotonation with a pK of $\sim$10 is the amino acid tyrosine (9). One would expect that if a tyrosine was responsible for the catalytic effect, on Schiff base deprotonation, then mono-iodination of this tyrosine would shift the pK of this deprotonation from $\sim$10.2 to $\sim$8.2. As demonstrated in this paper, our data strongly implicate tyrosine in the deprotonation of the Schiff base and suggest that iodination of the purple membrane mono-iodinates the tyrosine/tyrosines responsible for catalyzing Schiff base deprotonation.

Materials and Methods

Halobacterium halobium S9 was cultered by standard procedures (10,11) and purple membrane fragments containing bacteriorhodopsin were isolated and purified by the method of Kanner and Racker (11). Purple membrane fragments were iodinated by the procedure of Hunter and Greenwood (12) using a forty to one molar ratio of iodine to bacteriorhodopsin. Amino acid analyses were performed on a Beckman 119 Cℓ amino acid analyzer following sample hydrolysis with either 5.7 M HCℓ or mercaptoethanesulfonic acid. Prior to HCℓ hydrolysis, samples were treated with performic acid by the method of Hermans and Lu (13). The amount of iodinated tyrosine was determined by the difference between iodinated and non-iodinated purple membrane samples, and these analyses indicated that ∿4-1/2 of the 11 tyrosines in bacteriorhodopsin had been iodinated.

Kinetic resonance Raman measurements were made on buffered purple membrane suspensions in a Spex spinning cell. The laser line used was the 457.9 nm line of an argon ion laser. A laser beam of known diameter was focused on one side of the spinning cell so that the average time a molecule spent in the illuminated area could be calculated from the rate of spin of the cell, which could be varied. Raman scattering was collected through a spectrometer previously described (1). The pH of the purple membrane samples was controlled by suspending the membranes in several 0.05 M buffers varying in pH from 7 to 10.6 (phosphate, tris, and bicarbonate buffers). The pH of the samples after the experiments had changed by less than 0.1 pH unit.

Results and Discussion

Iodination of the purple membrane produces a change in the absorption maximum from 570 nm to 550 nm, and this is essentially independent of pH in the region we have considered (pH 7 to 11). The resonance Raman results demonstrated no alterations in any vibrational frequencies. This can be seen clearly in Figure 1A which compares a KiRRS of native (A) and iodinated purple membrane (B). Notice the unaltered frequency of the bR_{570} (C=C stretch) at 1526 cm^{-1} and even the C=C stretch of the kinetic intermediate M_{412} at 1564 cm^{-1}. This is also the case for the important C=N-H⊕ and C=N vibrations at 1640 cm^{-1} and 1619 cm^{-1}, respectively, and other vibrational modes displayed in this spectrum and in other regions of the resonance Raman spectra of these membranes. This is most significant for two reasons. Firstly, this highly sensitive structural data indicates that the center of photochemical and molecular activity in this proton pump is unaltered by iodination. Secondly, in view of the altered absorption spectrum, the unaltered Raman data indicate that the absorption changes are the result of a through space effect rather than the effect of some group directly bonded to the chromophore. Furthermore, the lack of any change in the unusually low frequency C=N-H⊕ stretch at 1640 cm^{-1} indicates that iodination does not directly effect the group or groups responsible for this lowered frequency.

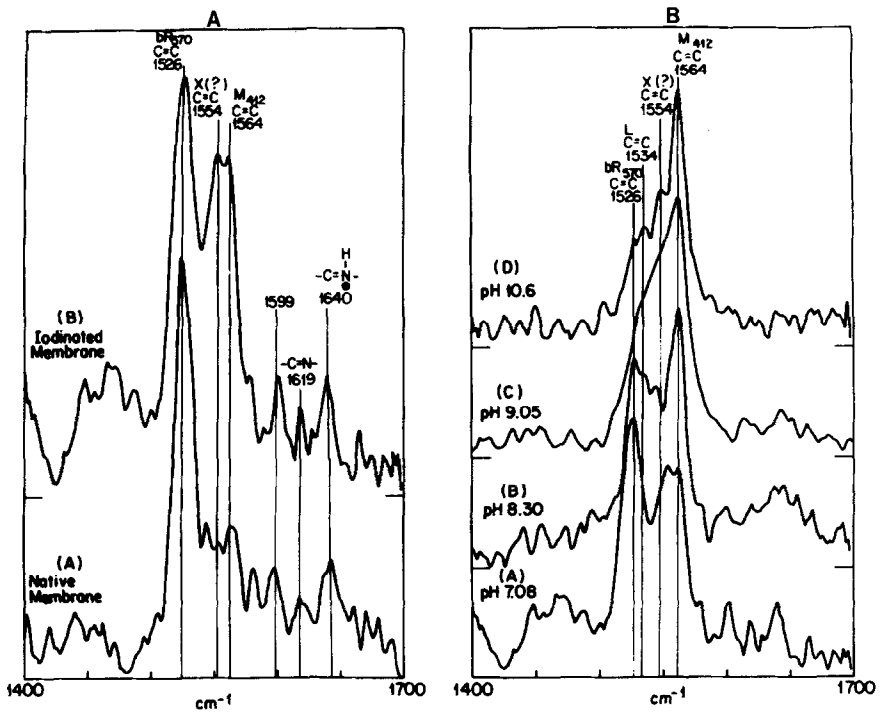

Figure 1A Kinetic resonance Raman spectra of native (A) and iodinated (B) purple membrane obtained with a 10 μs transit time at pH 7.

Figure 1B Kinetic resonance Raman spectra of iodinated purple membrane at pH = 7.08 (A), 8.30 (B), 9.05 (C), 10.6 (D) with a 10 μs transit time.

In spite of the above, there is one significant alteration in the spectra compared in Figure 1A. The contribution at short times (∿10 μs) of the M_{412} C=C stretch is significantly increased in the iodinated membranes. The C=C stretch of other intermediate species which precede M_{412} production are also present with considerably enhanced intensity, e.g., the C=C stretch of X(?) at 1554 cm^{-1}. In addition, the increased intensity of the C=C stretching frequencies of the L and X(?) intermediates can clearly be seen even at longer times when spectra of iodinated membranes are compared to the spectra of the native species. This persistence of contributions from L and X(?) to the spectra of iodinated membranes is also quite apparent when the spectra at short times are compared as a function of pH in Figure 1B. In fact the spectrum at pH 10.6 shows very little contribution from bR_{570} suggesting essentially complete de-

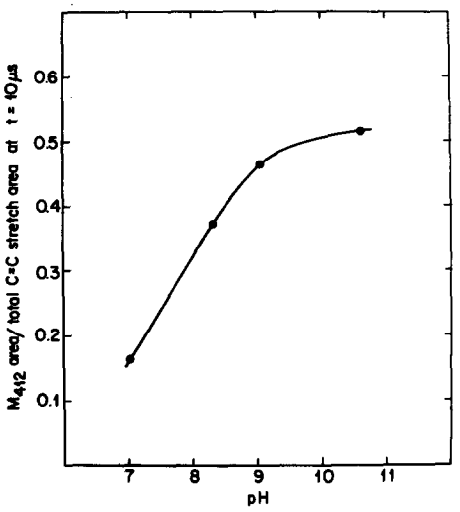

Figure 2 A plot of M_{412} area/total C=C stretch area at 10 μs versus pH.

pletion of this species. This is certainly not the case for high pH KiRRs spectra of native membrane previously published from this laboratory (2). On the other hand, in the KiRRS spectra of iodinated membranes at pH 10.6 we detect the most prominent peaks observed to date for L and X(?). Using a peak fitting program we were able to resolve four peaks in the C=C stretching region in all our spectra. An analysis of the peak areas of each species indicated the interesting observation that the L, 1534 cm^{-1} band, and the X(?), 1554 cm^{-1} band, exhibited different intensity profiles as a function of residence time and pH. This supports the assumption that these two bands may be arising from different species.

These peak areas were also used to determine the percentage of the 1564 cm^{-1} C=C stretch area to the total C=C stretch area. A plot of this percentage as a function of pH for the initial time of 10 μs was used as an approximation of the initial slope of M_{412} formation. The results are seen in Figure 2, and these results indicate that the previously observed increase in M_{412} production between pH 9 and 10 is now observed between pH 7 and 9. This is close to the pK of mono-iodinated tyrosine, which occurs at 8.2.

In conclusion, this data strongly implicate tyrosine as being the pK 10.2 group controlling Schiff base deprotonation (2). In addition, the data suggest that these tyrosine/tyrosines are not initially interacting directly with the chromophore or its Schiff base. Thus other groups must be responsible for the altered nature of the C=N-H⊕ stretching frequency in bacteriorhodopsin and its high pK before light absorption (14). In view of these results, the following additional comments can now be made about the molecular mechanism of this proton pump. The high pK of the group controlling Schiff base deprotonation (2) and the function of this proton pump to pHs down to ∿3 indicate a process with a significant amount of energy must occur which directly or indirectly deprotonates this high pK protein group. The most likely event to cause this effect is certainly light absorption by the chromophore. This could cause deprotonation of a high pK protein residue (e.g., arginine) (14,15), as previously suggested, which could subsequently deprotonate the tyrosine microseconds after light absorption. The production of deprotonated tyrosine in microseconds before M_{412} production, which is essential for the catalytic effect we postulate in this paper, is supported by the results of Bogomolni, et. al. (16), Hess and Kuschmitz (17), and Kalisky et. al. (18). Particularly the work of Hess and Kuschmitz clearly demonstrates the production of a single deprotonated tyrosine microseconds after light absorption but before M_{412} production. This is based on kinetic absorption data in the region of tyrosine's absorption. Furthermore, our large increase in M_{412} production is hard to rationalize in terms of increases in the rate of K or L production, since these rates are picoseconds and 2 μs, respectively. Thus, our large increase probably is the result of the processes that transform L to M, since much smaller increases in these rates could account for the large change in M production. Therefore, the effect of iodinated tyrosine seems to occur with a rate that is comparable to the rate of the tyrosine deprotonation detected by Hess and Kuschmitz (17). In summary, then, our data indicate that, in addition to the well established role of tyrosine in the decay of M_{412} (19), a critical role appears to exist for tyrosine in the framework of the other proton relay amino acid residues we suggested earlier (13).

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