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Properties of a Water-Soluble, Yellow Protein Isolated from a Halophilic Phototrophic Bacterium That Has Photochemical Activity Analogous to Sensory Rhodopsin†

T. E. Meyer,‡ E. Yakali,§ M. A. Cusanovich,*,‡ and G. Tollin‡

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721, and Department of Chemistry, Simmons College, Boston, Massachusetts 02115

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ABSTRACT: A water-soluble yellow protein, previously discovered in the purple photosynthetic bacterium *Ectothiorhodospira halophila*, contains a chromophore which has an absorbance maximum at 446 nm. The protein is now shown to be photoactive. A pulse of 445-nm laser light caused the 446-nm peak to be partially bleached and red-shifted in a time less than 1 μs. The intermediate thus formed was subsequently further bleached in the dark in a biphasic process occurring in approximately 20 ms. Finally, the absorbance of native protein was restored in a first-order process occurring over several seconds. These kinetic processes are remarkably similar to those of sensory rhodopsin from *Halobacterium*, and to a lesser extent bacteriorhodopsin and halorhodopsin; although these proteins are membrane-bound, they have absorbance maxima at about 570 nm, and they cycle more rapidly. In attempts to remove the chromophore for identification, it was found that a variety of methods of denaturation of the protein caused transient or permanent conversion to a form which has an absorbance maximum near 340 nm. Thus, by analogy to the rhodopsins, the absorption at 446 nm in the native protein appears to result from a 106-nm red shift of the chromophore induced by the protein. Acid denaturation followed by extraction with organic solvents established that the chromophore could be removed from the protein. It is not identical with *all-trans*-retinal and remains to be identified, although it could still be a related pigment. The *E. halophila* yellow protein has a circular dichroism spectrum which indicates little α-helical secondary structure (19%). Although the yellow protein is unique in its properties, it has characteristics of both bacterial rhodopsins (in terms of photochemistry) and the animal retinol binding proteins (in terms of solubility).

A water-soluble, yellow protein was previously isolated from the extremely halophilic purple phototrophic bacterium *Ectothiorhodospira halophila* (Meyer, 1985). This protein was found to have a relatively low molecular weight (15000), and the chromophore has a strong absorbance peak at 446 nm (ϵ = 48 mM^{-1} cm^{-1}). The chromophore was not released by a variety of methods of denaturation. This combination of properties is unlike those of any previously characterized protein. Although the spectral properties of the chromophore were reported to have a superficial resemblance to flavins with O, N, or S substituents at the 8-position of the aromatic ring, we now present evidence that the yellow protein has photo-

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‡ University of Arizona.
§ Simmons College.

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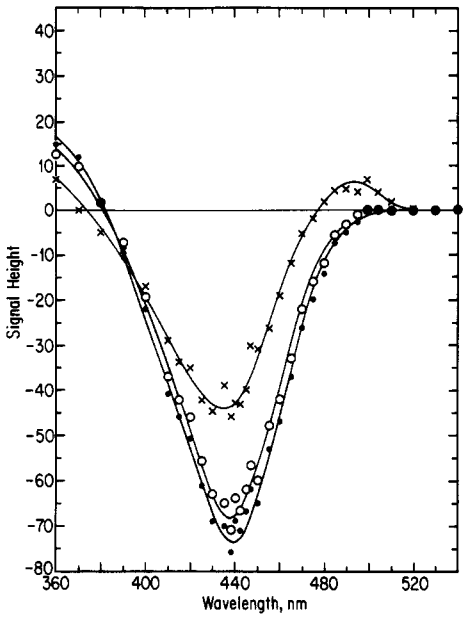

FIGURE 1: Time-resolved difference spectra for the photochemical reaction of yellow protein. Sweep time at each wavelength was 15 ms. The initial phase (×) was obtained by extrapolating the kinetic transient back to the beginning of the second phase. Similarly, the third phase was extrapolated back to obtain the second phase (○). The third phase (●) corresponds to the final absorbance reached prior to decay back to the initial state.

chemical properties which are remarkably similar to those of the retinal-containing sensory rhodopsin from *Halobacterium* (Bogomolni & Spudich, 1982).

Materials and Methods

Yellow protein was extracted from *E. halophila* strain BN9626 and purified according to Meyer (1985). Photochemical kinetics were analyzed by using the laser flash photolysis apparatus described by Simondsen and Tollin (1983). For the laser photolysis experiments (excitation wavelength 445 nm), protein concentrations were typically 10 μM. Under these conditions, approximately 10% of the protein in the illuminated volume of the 1-cm path-length cuvette (~0.1 mL) was photoconverted by a single flash. For kinetic measurements, traces obtained from three to six flashes were usually signal averaged. In multiple flash experiments, approximately 30 flashes spaced 0.25 s apart were sufficient to cause complete conversion to the photobleached form.

Circular dichroism spectra in the region 200–550 nm were recorded with a Jobin Yvon Mark V spectrophotometer, courtesy of Dr. G. Fasman (Brandeis University), and data were analyzed by using the CONTIN computer software program generously supplied by Dr. Stephen Provencher (Provencher & Glockner, 1981; Provencher, 1982a,b). Fluorescence emission and excitation spectra were measured with a Perkin-Elmer Model MPF-2A fluorescence spectrophotometer.

Analyses of denaturation data were carried out as described by Pace (1975), using a two-state model. The free energy term, $\Delta G_D = -RT \ln K_D$, was calculated by using $K_D = f/(1 - f)$, where f, the fraction unfolded, is equal to $(Y_N - Y_{obsd})/(Y_N - Y_D)$. $\Delta G_D^{H_2O}$, the free energy of denaturation in the absence of denaturant, was estimated by linear extrapolation ($\Delta G_D = \Delta G_D^{H_2O} + m$[urea]).

Results and Discussion

Photochemistry. The yellow protein was found to undergo photochemical bleaching when irradiated by a single laser flash (wavelength 445 nm). The kinetics of the initial light-induced reaction were too rapid to be measured by our methodology, but we could establish the existence of a complicated series of dark reactions summarized in reaction Scheme I. Time-resolved difference spectra, shown in Figure 1, indicate that the first observed intermediate, I_1, has much less absorbance at 446 nm and is red-shifted relative to the starting material. Our ability to measure the rise kinetics of I_1 was limited by scattering artifacts and instrument response time, but we could determine that k_1 must be larger than 10^6 s^{-1}. Intermediate I_1 decays to intermediate I_2 on a millisecond time scale,

Scheme I

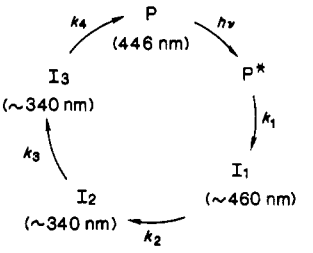

Table I: Rate Constants for Yellow Protein Bleaching and Recoloring upon Laser Flash Photolysis or Steady-State Illumination[a]

condition	k_2 (s^{-1})	k_3 (s^{-1})	k_4 (s^{-1})
H_2O	4781	221	1.6
5 mM phosphate, pH 7.0	3673 ± 339	360 ± 35	3.3 ± 0.2
500 mM phosphate, pH 7.0	2463	346	3.4
5 mM phosphate, 1 M NaCl, pH 7.0	2094	315	1.6
5 mM phosphate, 4 M NaCl, pH 7.0	1850	212	1.1
3 M betaine, pH 7.0	4330	325	5.2
5 mM phosphate, 3 M betaine, pH 7.0	4430	325	6.6
50 mM phosphate, 3 M betaine, pH 7.0	4754	358	6.8
100 mM Tris, 10 mM NaCl, pH 7.5	3090	375	3.8
100 mM Tris, 10 mM $CaCl_2$, pH 7.5	3182	420	3.5
100 mM Tris, 10 mM $MgCl_2$, pH 7.5	3532	419	3.9
100 mM Tris, 10 mM $ZnCl_2$, pH 7.5	3265	379	3.5
100 mM Tris, 100 mM $ZnCl_2$, pH 7.5	3818	296	1.2
15 mM acetate, pH 5.0	6904	210	0.9
14 mM glycine, pH 10.0	3944	239	2.0
2 M urea	4428	277	1.8
3 M urea	4553	212	1.2
4 M urea	4736	244	0.8
5 M urea	3692	186	0.6
5.5 M urea	3670	231	0.4/0.03[b]
6.0 M urea	4419	ND[d]	0.01[c]
6.5 M urea	ND	ND	0.004[c]
7.0 M urea	4627	ND	0.001[c]
7.5 M urea	ND	ND	0.0004[c]
8.0 M urea	ND	ND	0.0001[c]
renatured (5 mM phosphate, pH 7.0)	4350	369	4.4

[a] Unless otherwise stated, k values determined by laser flash photolysis. Bleaching kinetics typically consisted of 85–90% of the k_2 step and 10–15% of the k_3 step. The k_4 values refer to recoloring kinetics. The error limits in the rate constant values obtained in 5 mM phosphate buffer are representative of all the data. [b] The first value is for a flash experiment, and the second value was measured after steady-state illumination. [c] Rate constant measured after steady-state illumination. [d] ND = not determined. At high urea concentrations, a substantial portion of the sample is bleached in the dark, and the transient kinetics following a laser flash are difficult to measure with the remaining native protein.

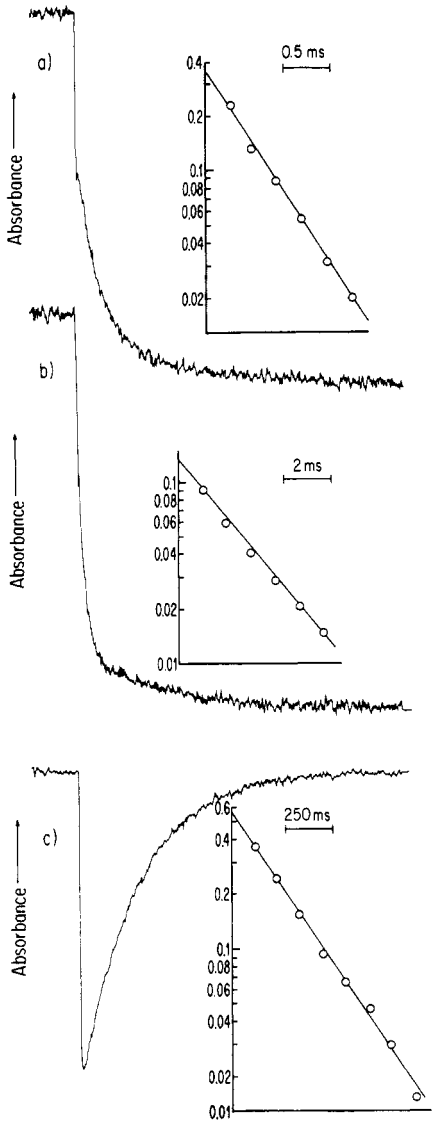

FIGURE 2: (a) Kinetics of the laser-induced photochemical bleaching of the yellow protein on a 4-ms time scale measured at 446 nm. Buffer was 5 mM phosphate. (b) Kinetics on a 15-ms time scale. (c) Recoloring kinetics determined on a 2-s time scale. Inserts are semi-log plots of the kinetic data.

measured either at 505 nm or at 446 nm. This is shown in Figure 2a, with the rate constant (k_2) given in Table I. I_2 is a bleached form of the starting material, as is shown in Figure 1. Analysis of the transient kinetics at 446 nm shows that the dark bleaching reaction is actually biphasic and that a third intermediate (I_3), with approximately the same absorption minimum as I_2 (Figure 1), is formed on a slower time scale (Figure 2b). The rate constant for this reaction, k_3, is given in Table I. This species contributes only 10–15% of the absorbance change at 446 nm. It is possible that I_3 is formed in a parallel rather than a sequential reaction path. However, the kinetics appeared to be monophasic at 505 nm (which monitors mainly I_1 disappearance), thus favoring the sequential pathway shown in Scheme I. It should be noted, however, that the absorbance change was much smaller and noisier at this wavelength and it is difficult to be certain that there was not a small amount of a slower phase.

The more stable intermediates, I_2 and I_3, have absorption maxima below 370 nm, with at least one-third as much absorbance as the original peak at 446 nm, and with an isosbestic point at about 380 nm (with the present optical system, we were unable to make reliable measurements below 360 nm).

Table II: Yellow Protein Denaturation Parameters[a]

condition	$[urea]_{1/2}$ (M)	$\Delta G_D^{H_2O}$ (kcal/mol)	m (kcal mol^{-1} M^{-1})
flash photolysis	4.3	2.2	0.52
steady-state illumination	3.8	5.1	1.36
absorption spectrum	7.4	10.8	1.45

[a] Parameters calculated as described under Materials and Methods by using the data given in Figure 4.

By analogy with urea-denatured protein (see below), we assume the absorbance maximum is near 340 nm. If one also assumes that I_3 is a completly bleached form of the starting material, then the spectrum of I_1 can be approximated by subtracting the difference spectra in Figure 1. In this way, we estimate that I_1 absorption is shifted about 15 nm to the red, i.e., has a maximum at about 460 nm. At very long times (on the order of seconds), the intermediates recolor in a simple first-order reaction to a substance which is spectrally and photochemically identical with the starting material. This reaction is shown in Figure 2c, and the rate constant, k_4, is given in Table I. Recoloring kinetics were the same whether measured with one flash or with multiple flashes where the sample was completely bleached.

E. halophila is an extreme halophile, and the yellow protein would either be located in the periplasmic space and presumably experience a high ionic strength environment or be located in the cytoplasm, where there is a high concentration of betaine, the principal osmoregulator (Galinski & Truper, 1982). Rate constants for the generation of intermediates and recoloring are dependent on ionic strength (Table I). In the absence of buffer, k_2 was increased relative to low ionic strength conditions (5 mM potassium phosphate), and k_3 and k_4 were decreased. However, as the ionic strength was further increased, all three rate constants decreased. In contrast, betaine does not have much of an effect on k_2 and k_3 (Table I) but substantially increased k_4 in both H_2O and buffer (2–3-fold, Table I). Low concentrations of specific cations (Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}) had no significant effect on the observed kinetics (Table I). The photochemical kinetics of the yellow protein were somewhat sensitive to pH when ionic strength was held constant, with k_2 increased at pH 5 and k_3 and k_4 decreased at both pH 5 and pH 10. In general, the recoloring kinetics (k_4) are quite sensitive to the composition of the media while k_2 and K_3 are much less so.

The overall photochemical reaction is similar to that observed with sensory rhodopsin in *Halobacterium* (Bogomolni & Spudich, 1982), in that there is a rapid photoconversion via several intermediates to a form which absorbs at shorter wavelength, followed by a relatively slow recovery to starting material. The photocycle also resembles those of bacteriorhodopsin and halorhodopsin except that these proteins cycle much more rapidly.

Protein Stability. The spectral properties of the yellow protein are relatively unaffected by up to 5 M urea. However, at higher concentrations of urea, there is a progressive loss of 446-nm absorption in the dark and the appearance of a new absorption peak at 342 nm, with an isosbestic point at 381 nm, as shown in Figure 3. Denaturation in urea, as defined by these spectral changes, is consistent with a two-state model, with half-completion at 7.4 M urea (Figure 4, triangles; the calculated thermodynamic parameters are given in Table II). The equilibrium for dark bleaching is reached in less than 10 min at all urea concentrations, and the remaining color can still be photochemically bleached. The kinetics of formation of the intermediates of the photochemical reaction are only

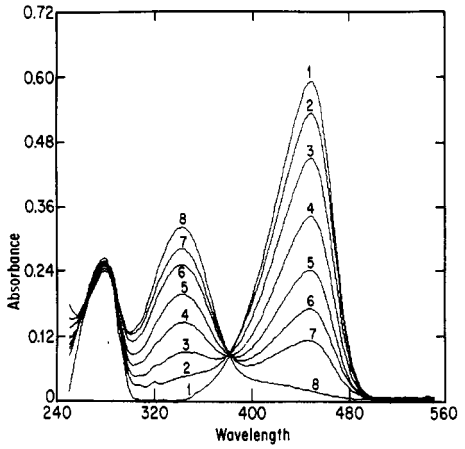

FIGURE 3: Equilibrium urea titration of *E. halophila* yellow protein in the dark. The spectra stabilized in less than 10 min after mixing at all urea concentrations. Traces 1–8 were for 5, 6.5, 7.0, 7.25, 7.5, 7.75, 8.0, and 9 M urea, respectively.

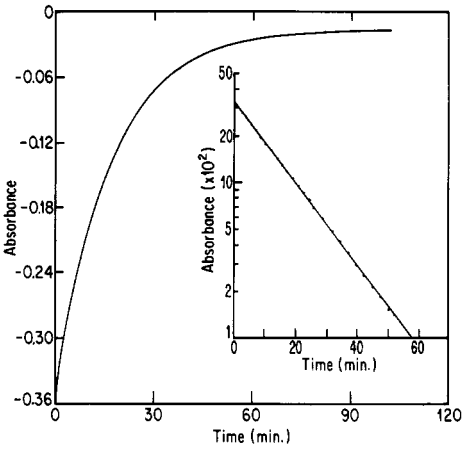

FIGURE 5: Kinetics of recoloring of the yellow protein in 7.0 M urea followed on a 2-h time scale. The yellow color remaining after adding urea in the dark was bleached by exposure to a 40-W fluorescent lamp for 1 min. The insert is a semi-log plot of the data.

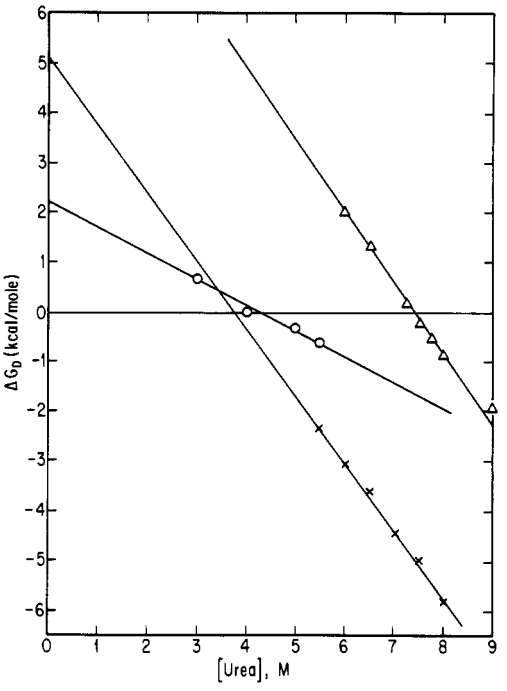

FIGURE 4: ΔG_D for the denaturation of yellow protein as a function of [urea]. ΔG_D was calculated as given under Materials and Methods. (O) Flash recoloring kinetics; (×) recoloring kinetics after steady-state illumination; (Δ) effect of [urea] on the absorption spectrum in the dark. For the calculation of the fraction denatured, Y_N was taken as the kinetic value in H_2O (Table I); Y_D was taken as zero.

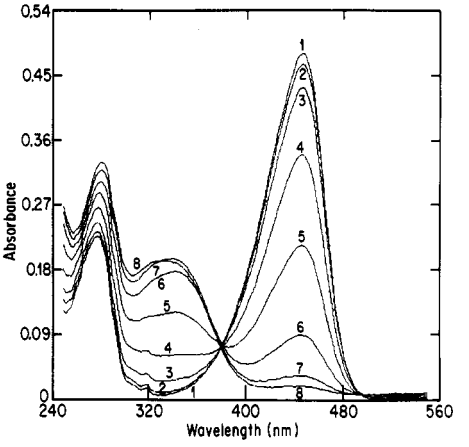

FIGURE 6: Irreversible heat denaturation of the yellow protein as a function of time in 5 mM phosphate buffer, pH 7.0. The samples 1–8 were boiled for 1, 3, 6, 10, 18, and 24 min, respectively.

slightly affected by urea denaturation of the protein, with k_2 increased and k_3 decreased (Table I). Subsequent to steady-state or flash-induced photochemical bleaching of the 446-nm absorption which remains after dissolution of the protein in urea, there is still a dark recoloring phase back to the equilibrium level, but the kinetics are dramatically slowed (by up to 4 orders of magnitude) as compared to the native protein, as is shown in Table I (cf. also Figures 4 and 5). Plots of ΔG_D vs. [urea] for the recoloring kinetics measured in flash experiments (open circles) as well as following steady-state illumination (×) are shown in Figure 4, and the relevant parameters are summarized in Table II. Clearly, the effect of urea on the recoloring kinetics is different from the spectral effect described above, in that the kinetic effects are half-completed at much lower urea concentrations (3.8 and 4.3 M) than required to cause the dark bleach (7.4 M). The slope (m), $\Delta G_D^{H_2O}$, and the urea concentration for half-maximal response are also clearly dependent on the bleaching method, with steady-state illumination resulting in a species having substantially slower recoloring kinetics than is formed upon flash photolysis (Table I, Figure 4). However, in flash experiments in 5.5 M urea, the recoloring kinetics were an order of magnitude slower if the sample had first been exposed to steady-state illumination and allowed to recolor before the flash. This indicates that the protein which is photochemically bleached under steady-state conditions in urea is converted to another form which has slower recoloring kinetics. We have also observed that when such a sample was left in the dark for several hours, the rapid kinetics observed under flash conditions were restored. Urea denaturation is completely reversible by dilution followed by dialysis, since all the color is recovered and the original kinetics are restored (Table I).

The decrease in the rate constant for the recoloring kinetics observed (Table I) for the protein in 1 or 4 M NaCl as compared with 0.5 M potassium phosphate suggests a destabilizing influence of NaCl, which is also reflected by a more rapid denaturation of the protein at high temperature under these conditions (see below). A similar effect is seen for $ZnCl_2$ at lower concentrations, and the pH 5 kinetics also suggest destabilization (Table I).

The yellow protein is remarkably stable to heating. It can be reversibly boiled (97.6 °C) for about 1 min in 5 mM phosphate, during which time the color is completely bleached

in the dark, but immediately returns on rapid cooling. Prolonged boiling causes irreversible conversion to a material having an absorption maximum initially at about 340 nm, which shifts to 330 nm and eventually to even shorter wavelengths (Figure 6). When heated at 90 °C in water, no permanent loss of absorbance was noted in 10 min, and only 50% of the absorbance was lost after 100 min. In 5 and 500 mM phosphate, pH 7.0, only 11% of the absorbance was lost after 10 min at 90 °C. In contrast, in 4 M NaCl, or at pH 5 or pH 10, there was substantial loss of absorbance at 446 nm after 10 min at 90 °C, 100%, 65%, and 84%, respectively. These heat denaturation results provide further evidence that the protein is destabilized in high NaCl and at the pH extremes.

Heat denaturation of the protein in 3 M betaine was approximately the same as in 5 mM phosphate. The protein eventually coagulates on prolonged heating in all buffers, and the 330–340-nm chromophore precipitates with the protein. This indicates that the 340-nm material cannot be free in solution and is still associated with the protein.

Since the similarity in photochemical properties to sensory rhodopsin suggested a protein-bound retinal, we considered the possibility that the chromophore could be bound to the protein via a protonated Schiff base and might be removed by light plus hydroxylamine (Oesterhelt & Schuhmann, 1973; Oesterhelt et al., 1974). Therefore, we illuminated the yellow protein in the presence of 50 mM hydroxylamine. Light was provided by a projector lamp which had the infrared component removed by a 6-cm water filter cooled by ice and which was fitted with a narrow band-pass interference filter (wavelength 438 nm). The yellow color was irreversibly bleached after about 6 h, giving a new absorbance peak at about 340 nm. At the end of the experiment, the protein had coagulated, and the 340-nm chromophore precipitated with the protein. The protein was also incubated in 9 M urea plus 50 mM hydroxylamine overnight and diluted with water. The control sample without hydroxylamine recolored on dilution, but hydroxylamine caused irreversible denaturation to the 340-nm absorbing material, which was insoluble in the absence of urea.

Using procedures which had been applied to bacteriorhodopsin (Oesterhelt et al., 1973), we found that acid treatment followed by petroleum ether extraction yielded a pale yellow colored material. However, this substance had a different R_f value (0.24) from authentic *all-trans*-retinal (0.19) on thin-layer chromatography (85% hexane, 15% diethyl ether), indicating that it is less polar, and stained bright yellow with antimony trichloride, as compared to dark blue-green for *all-trans*-retinal. Thus, we have to conclude that the chromophore of the yellow protein is not identical with retinal but may be a structural analogue. Further studies are under way to complete the chemical identification.

Circular Dichroism. The UV circular dichroism spectrum of the yellow protein as shown in Figure 7 indicated 19% helix and 30% β-sheet secondary structure. These values are very different from bacteriorhodopsin, which contains approximately 50% helix and 18% β-sheet (Jap et al., 1983). The secondary structure is more like the water-soluble retinal binding proteins, which lack photochemical activity. Human retinol binding protein, for example, contains about 8% helix and 57% β-sheet structure (Newcomer et al., 1984). β-Lactoglobulin is composed of about 8% helix and 55% β-sheet structure (Sawyer et al., 1985). These two proteins are apparently homologous to one another (Godovac-Zimmerman et al., 1985). It should be emphasized that secondary structure

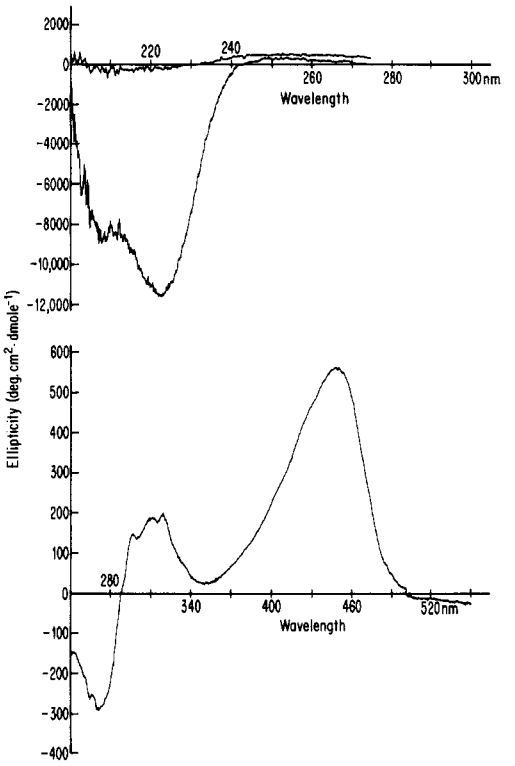

FIGURE 7: UV–visible circular dichroism spectra of the yellow protein in phosphate buffer, pH 7.0.

is the only similarity to the animal proteins, which do not appreciably alter the spectral properties of vitamin A analogues on binding to protein (Horwitz & Heller, 1974).

The visible circular dichroism spectrum of the yellow protein has major positive ellipticity centered at 450 nm, which correlates with the principal peak in the UV–visible absorption spectrum (446 nm). Major ellipticity in visual rhodopsin also correlates with the visible absorption band, but in bacteriorhodopsin, there appears to be exciton splitting in the visible CD bands (Becher & Cassin, 1976). The retinol–retinol binding protein complex has a CD spectrum with positive ellipticity at the chromophore absorption peak (Horwitz & Heller, 1974).

Fluorescence. The yellow protein was weakly fluorescent when excited in the chromophore absorption band. The emission peak was centered near 495 nm (uncorrected), and the excitation spectrum was consistent with the absorption spectrum of the yellow chromophore. Neither free retinal nor the rhodopsins are very fluorescent (Stoeckenius et al., 1979), which is consistent with, but is not indicative of, the presence of a pigment related to retinal in the yellow protein. Weak fluorescence is also compatible with the observed photochemical activity of the yellow protein. On the other hand, weak fluorescence would argue against a role in energy transfer for the yellow protein; i.e., it probably does not function in vivo as an accessory light-harvesting pigment in photosynthesis.

Conclusions

The experiments described above provide evidence that the yellow protein from *E. halophila* strongly resembles sensory rhodopsin from *Halobacterium* in its photochemical properties. It also resembles bacteriorhopsin and halorhodopsin, but to a lesser extent. For sensory rhodopsin, the native form absorbing at about 570 nm is converted to a species absorbing at 680 nm within 1 μs of illumination (yellow protein also generates a red-shifted form in <1 μs), followed by decay to a 373-nm species in 20 μs ($t_{1/2}$ for the yellow protein decay

to form I_2 is $\sim$200 μs), and the return to the starting material occurs with a rate constant of 0.9 s^{-1} [the corresponding rate constant for the yellow protein (k_4) is 3.3 s^{-1}]. The primary differences in these proteins reside in the native absorption spectral maxima (446 nm vs. 570 nm), in their water solubility, and in secondary structure. This does not imply that the photoactive yellow protein is a water-soluble form of either bacterial or animal rhodopsins, but the evidence suggests convergent evolution of unrelated proteins toward similar photochemical properties.

Spectrally monitored denaturation of the yellow protein by urea yields a $\Delta G_D^{H_2O}$ of 10.8 kcal/mol, a value typical of globular proteins (Pace, 1975). However, lower concentrations of urea affect the recoloring kinetics and yield $\Delta G_D^{H_2O}$ values of 2.2 and 5.1 kcal/mol for the flash photolysis and steady-state illumination experiments, respectively. These values are substantially less than those observed for spectral denaturation of the protein and suggest the presence of a more labile domain whose integrity is required for photochemical activity. It appears that steady-state illumination results in the formation of a metastable state which can only recolor slowly. On the basis of the thermodynamic parameters obtained from the denaturation studies, this state is distinct from that observed in laser photolysis. However, we have no chemical or structural information at this time which provides an understanding of the differences between the two kinetic states. The small $\Delta G_D^{H_2O}$ (2.2 kcal/mol) observed for recoloring after flash photolysis coupled with the sensitivity of this reaction to betaine, NaCl, and pH suggests photochemical activity resides in a conformationally labile region of the molecule.

The water solubility of the yellow protein raises interesting questions concerning biological function. One would not expect a role in proton or chloride translocation such as found for bacteriorhodopsin and halorhodopsin because these are membrane phenomena. However, the phototransformed yellow protein could specifically bind to a membrane receptor and thereby cause a physiological response, and thus serve a sensory role. Alternatively, the bleached form could act at the enzyme or gene levels to control a metabolic system. In any event, it is quite clear that this protein provides a unique system for the elucidation of the relationships between protein structure and photochemical transformations. Work is presently under way to solve the crystal structure of the photoactive yellow protein. The crystalline protein diffracts to at least 1.3-Å resolution, and a preliminary analysis has been accomplished (McRee et al., 1986).

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