Photoactive yellow protein from the purple phototrophic bacterium, *Ectothiorhodospira halophila*

Quantum yield of photobleaching and effects of temperature, alcohols, glycerol, and sucrose on kinetics of photobleaching and recovery

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ABSTRACT A water-soluble yellow protein from *E. halophila* was previously shown to be photoactive (Meyer, T. E., E. Yakali, M. A. Cusanovich, and G. Tollin. 1987. *Biochemistry.* 26:418–423). Pulsed laser excitation in the protein visible absorption band (maximum at 445 nm) causes a rapid bleach of color ($k = 7.5 \times 10^3$ s^{-1}) followed by a slower dark recovery ($k = 2.6$ s^{-1}). This is analogous to the photocycle of sensory rhodopsin II from *Halobacterium* (which also has $k = 2.6$ s^{-1} for recovery). We have now determined the quantum yield of the photobleaching process to be 0.64, which is comparable with that of bacteriorhodopsin (0.25), and is thus large enough to be biologically significant. Although the photoreactions of yellow protein were previously shown to be relatively insensitive to pH, ionic strength and the osmoregulator betaine, the present experiments demonstrate that temperature, glycerol, sucrose, and various alcohol-water mixtures strongly influence the kinetics of photobleaching and recovery. The effect of temperature follows normal Arrhenius behavior for the bleach reaction ($E_a = 15.5$ kcal/mol). The rate constant for the recovery reaction increases with temperature between 5°C and 35°C, but decreases above 35°C indicating alternate conformations with differing kinetics. There is an order of magnitude decrease in the rate constant for photobleaching in both glycerol and sucrose solutions that can be correlated with the changes in viscosity. We conclude from this that the protein undergoes a conformational change as a consequence of the photoinduced bleach. Recovery kinetics are affected by glycerol and sucrose to a much smaller extent and in a more complicated manner. Aliphatic, monofunctional alcohol-water solutions increase the rate constant for the bleach reaction and decrease the rate constant for the recovery reaction, each by an order of magnitude. These effects do not correlate with dielectric constant, indicating that the photocycle probably does not involve separation or recombination of charge accessible to the protein surface. However, the effects on both bleaching and recovery correlate well with the relative hydrophobicity (as measured by partition coefficients in detergent/water mixtures), in the order of increasing effectiveness: methanol < ethanol < iso-propanol < *n*-propanol < *n*-butanol. We conclude that the change in conformation of the protein induced by light exposes a hydrophobic site to the solvent. This suggests the possibility that light exerts its effect *in vivo* by exposing a region of the protein for binding to a hydrophobic receptor site in the cell, perhaps to a protein analogous to the chemotactic transducers in the cytoplasmic membranes of enteric bacteria.

INTRODUCTION

Photoactive yellow protein (PYP), which has heretofore been found only in the extremely halophilic purple phototrophic bacterium, *Ectothiorhodospira halophila* (Meyer, 1985), is completely water-soluble, and has a molecular weight of 14,000 with a visible absorption maximum at 446 nm. The as yet unidentified chromophore undergoes a series of phototransformations induced by a pulse of laser light (Meyer et al., 1987) that are remarkably similar to those of the sensory rhodopsins from *Halobacterium halobium* (Bogomolni and Spudich, 1982). Recently, we have isolated a similar photoactive yellow protein from a species in a different family from the *Ectothiorhodospiraceae, Rhodospirillum salexigens,* which is a member of the *Rhodospirillacea* (Meyer, Fitch and Cusanovich, unpublished results). Thus, PYP appears to be more widely distributed among the photosynthetic bacteria. This observation, along with the fact that the quantum yield reported here (see below) for the production of the photobleached product is at least as large for PYP as it is for bacteriorhodopsin, argues strongly in favor of the biological significance of the photoactivity of PYP. Unlike the photoactive yellow proteins, sensory rhodopsins from *Halobacterium* are membrane-bound and have molecular weights of 23–25,000 (Spudich and Bogomolni, 1988), which suggests that they are structurally unrelated. A preliminary three-dimensional structure of PYP has been determined (McRee et al., 1989), which shows that it is primarily composed of beta sheet, whereas bacteriorhodopsin is predominantly helical.

It has been previously shown (Meyer et al., 1987) that PYP is unusually resistant to denaturation and has a

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half-life of ~10 min in boiling water. The color is reversibly bleached in the dark during short exposure to high temperatures but recovers completely on cooling. The color is progressively bleached in the dark in urea solutions >5 M and the remaining color can still be photobleached, but with very different kinetics. The recovery reaction in particular is slowed by at least four orders of magnitude. These effects are completely reversible on dilution. The phototransformations are relatively insensitive to pH, ionic strength, or betaine, the principal osmoregulator in *E. halophila*. In the present studies pronounced effects of temperature, aliphatic alcohols, glycerol, and sucrose on *E. halophila* PYP kinetics are demonstrated, which allow some conclusions to be drawn regarding possible structural features of the photobleaching and recovery processes.

Although PYP is structurally distinct from the sensory rhodopsins, and we do not yet know either the physiological function or the identity of its chromophore (these are currently under investigation), the importance of studying this material lies in the fact that it undergoes a reversible photocycle, it has a high quantum yield, and its chromophore has a strongly red-shifted absorption spectrum (~100 nm compared with denatured protein). These are the key features of the rhodopsin/bacteriorhodopsin family of proteins. Furthermore, there is no high resolution 3-D structure available for any photoactive protein. Thus, characterization of the above phenomena in PYP, for which we have strong prospects for a high resolution structure, will be extremely helpful in understanding the analogous properties of visual pigments and related molecules.

MATERIALS AND METHODS

Photoactive yellow protein was prepared from *E. halophila* as previously described (Meyer, 1985). Laser-induced phototransformations were performed as reported by Meyer et al. (1987). All solutions were prepared from reagent grade chemicals and made just before use. Fresh protein was added to each solution immediately before laser flash photolysis, and the absorbance was monitored before and during the experiment to ensure that there was no denaturation. Dielectric constants of alcohol/water mixtures have been reported by Akerlof (1932). Viscosities of glycerol/water and sucrose solutions were obtained from Lange's Handbook of Chemistry (1956) and the Iscotables, Handbook of Data for Biological and Physical Scientists (1982).

The quantum yield for photobleaching of PYP was measured by comparison with triplet state formation in a chlorophyll *a* standard solution. PYP in water was adjusted to the same absorbance (0.42) at 446 nm (the laser excitation wavelength) as the chlorophyll solution in pyridine, which had been made anaerobic by bubbling with deoxygenated argon gas. Calculations were based on a difference extinction coefficient of 25 mM^{-1} cm^{-1} for chlorophyll *a* triplet state at 465 nm (the measuring wavelength), as reported by Linschitz and Sarkanen (1958). The difference extinction for PYP at the measuring wavelength of 445 nm (45.5 mM^{-1} cm^{-1}) was recalculated from Meyer (1985) using the corrected molecular weight based on the amino acid sequence (126 residues; 13,976 daltons, excluding the chromophore). A value of 0.60 was used for the quantum yield of triplet formation for chlorophyll *a* (Bowers and Porter, 1967; Avarmaa, 1979). The quantum yield for photobleaching of PYP was calculated by comparing the absorbance changes produced by the laser flash in the two samples at 465 nm and 445 nm, respectively. For PYP, the calculation was based on the final bleached product, rather than the intermediate species.

RESULTS AND DISCUSSION

It was previously shown that laser flash excitation in the 445 nm absorption band induces a series of transformations in PYP resulting in the formation of a bleached product, which slowly returns to the original state (Meyer et al., 1987). Formation of the first intermediate, which is red-shifted relative to starting material, was previously estimated to occur in <1 μs, but with more recent work we now estimate that it appears in <10 ns (unpublished observations carried out at the Center for Fast Kinetics, University of Texas, Austin). A slower dark reaction, which we previously reported to be slightly biphasic, results in a bleached form of the chromophore. With the present preparation of the protein, this reaction now appears to be monophasic and to have an appreciably larger rate constant in water than was reported in the original study (k = 7,500 s^{-1}; we earlier measured a rate constant of 4,800 s^{-1} for 85% of the reaction and 220 s^{-1} for the remaining 15% of the absorbance change). The final reaction, in which the original absorbance of the protein is restored, is also faster in this present preparation (k = 2.6 s^{-1} vs. 1.6 s^{-1}). We have no explanation at this time for the altered kinetics. These observations require further study.

By comparing PYP transient photobleaching with the formation of the triplet state of chlorophyll by laser flash excitation, the quantum yield for the formation of the final bleached photoproduct is measured as 0.64. This value assumes that the protein is completely bleached by the laser flash. If this is not the case, i.e., if the photoproduct still retains significant absorption at 445 nm, then the quantum yield is even larger. For comparison, the quantum yield for bacteriorhodopsin has been measured to be 0.25 for the bR → M transformation at room temperature (Stoeckenius et al., 1979). We conclude therefore that the light reaction of PYP is efficient enough to be biologically significant.

One parameter that had not been examined in the previous work was the effect of temperature. There is more than a 30-fold increase in the rate constant for the bleach reaction in going from 5°C to 42°C (the rate constant became too large to measure at higher temperatures). The kinetics follow normal Arrhenius behavior as shown in Fig. 1 *a*. If extrapolated to the boiling point of

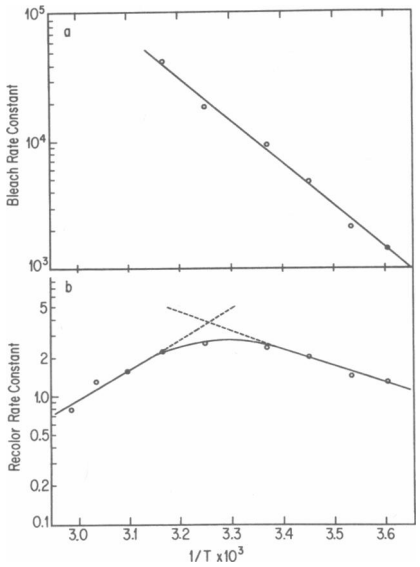

FIGURE 1 Arrhenius plot of temperature effects on kinetics of laser-induced bleach (*a*) and recovery (*b*) of PYP.

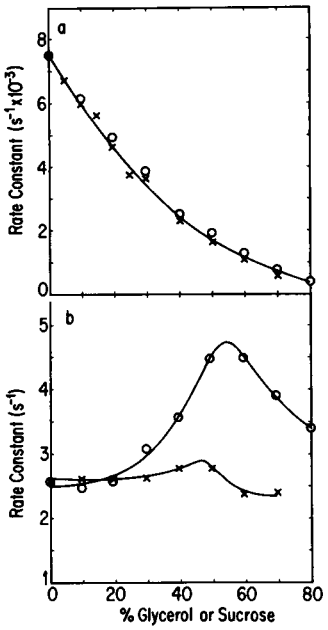

FIGURE 2 Effect of glycerol/water and sucrose/water mixtures on kinetics of laser-induced bleach (*a*) and recovery (*b*) of PYP. O – Glycerol X – Sucrose.

water, the rate constant changes by over three orders of magnitude (the protein has a half-life of 10 min at 98°C in the dark). The calculated activation energy is 15.5 kcal/mol and is similar to values for normal enzyme-catalyzed reactions, as well as to that measured for bacteriorhodopsin (Beece et al., 1981). The kinetic behavior of the recovery reaction on the other hand is more complicated and is less sensitive to temperature. This reaction becomes only twice as fast between 5°C and 35°C (E_a = 5.9 kcal/mol), and then becomes threefold slower up to 62°C (E_a = −11 kcal/mol), the highest temperature examined. The change in slope in the temperature effect for recovery probably reflects conversion of the bleached form to a new conformation that has an altered kinetic behavior. In comparison, all phases of the bacteriorhodopsin photocycle show normal Arrhenius behavior (Beece et al., 1981). Additional evidence for other conformational intermediates arises from observations of the effects of urea on kinetics of the PYP photocycle. Thus, the kinetics for recovery in 5.5 M urea are an order of magnitude faster after illumination by monochromatic blue light than they are when such a solution is irradiated with white light from a fluorescent lamp. Furthermore, the protein bleached by monochromatic blue light in 5.5 M urea appears to have an absorption maximum near 360 nm, whereas the sample bleached by white light does not have a 360 nm peak. These results suggest that the 360 nm form of the protein is also photoactive and can be converted to another form which recovers more slowly. Evidence for additional states of the bleached protein may also be seen from the effects of glycerol and sucrose on recovery kinetics, as documented below.

The principal effect of glycerol addition is to decrease the rate constant for the bleach reaction by an order of magnitude in solutions up to 80% glycerol/water as shown in Fig. 2 *a*. Virtually identical results are obtained with up to 70% sucrose/water solutions. Rate constants for photobleaching are shown in Fig. 3 plotted against the inverse of viscosity, according to the Debye-Smoluchowski equation (Caldin, 1964) for diffusion-limited

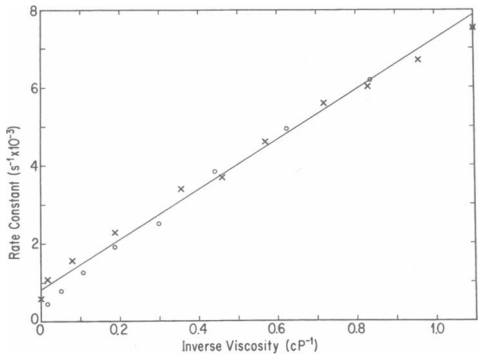

FIGURE 3 Inverse correlation of bleach kinetics in glycerol and sucrose (Fig. 2 *a*) with viscosity. O – Glycerol X – Sucrose.

kinetics. A straight line can be drawn through the data points for both glycerol and sucrose, which indicates that the effect is due primarily to viscosity and does not involve specific solvent perturbation. Based on the results with monofunctional alcohols described below, a small correction to the glycerol kinetics due to a hydrophobic effect may be appropriate, but this cannot change the primary correlation with viscosity. It is concluded from these data that the light-induced bleach of the chromophore results in a conformational change of the protein. This will be discussed further below.

The effect of glycerol on the recovery reaction is more complicated than on the bleach reaction, as shown in Fig. 2 *b*. The reaction becomes somewhat faster in the low viscosity range but eventually slows at the highest viscosity. This suggests that the recovery process proceeds through several different conformational states. At low viscosities, a conformational change may be slowed, which in turn decreases the contribution of a slower relaxing species to the recovery kinetics. At higher viscosities, the protein may become trapped in the more slowly recovering state that is slowed still further by increasing viscosity. In addition, a contribution of the hydrophobic effect may also be present at high concentrations, as described below. These results with PYP are very different from those with bacteriorhodopsin (Beece et al., 1981; Rayfield, 1986), for which the slower steps of the photocycle were much more sensitive to viscosity than were the earlier steps.

The effect of viscosity on recovery kinetics is less pronounced in sucrose than it is in glycerol (Fig. 2 *b*), and may not in fact be significant because the observed variation is almost within experimental error (±10%).

Because the effects of glycerol and sucrose on recovery kinetics are small (less than a factor of 2), it is difficult to draw any specific structural conclusions, except that the rate-limiting step in the recovery process does not appear to involve any large-scale movements of the polypeptide chain that would be especially sensitive to viscosity. Perhaps a nucleation step is required for recovery, which utilizes only a relatively small region of the protein structure. This deserves further study.

The effects of monofunctional aliphatic alcohols on the kinetics of the bleach and recovery reactions are quite large and considerably different from those of glycerol and sucrose. First, it should be noted that the color of the protein is bleached in the dark at high alcohol-water concentrations, but not at low. Furthermore, near the threshold of the dark bleach, the protein is slowly bleached by the measuring beam of the laser flash spectrometer. The effectiveness of the alcohols for inducing dark bleach increases in the order methanol < ethanol < isopropanol < *n*-propanol < *n*-butanol. Other organic solvent/water mixtures such as acetone or dioxane are even more effective in bleaching the yellow protein in the dark.

With all monofunctional alcohols tested, the rate constant for the bleach reaction is increased by an order of magnitude, and the recovery reaction is slowed by an approximately equal amount, over the effective concentration range of that alcohol (cf. Fig. 4). As stated above, these effects are opposite in direction to those in glycerol or sucrose. The effectiveness of the alcohols in altering kinetics parallels their ability to cause dark bleach, i.e., methanol < ethanol < isopropanol < *n*-propanol < *n*-butanol.

These observations with small monofunctional alcohols were also extended to related compounds, which were anticipated to give similar effects. For example, octylglucoside solutions behave like the small alcohols in that the rate constant for the bleach reaction increases to $1.3 \times 10^4\ s^{-1}$ and that for recovery drops to $0.4\ s^{-1}$ in 1% (34 mM) detergent, which is just above the critical micelle concentration. To obtain a comparable change in kinetics in *n*-butanol solutions would require an order of magnitude higher concentration.

The possibility that the alcohols exert their effect through alteration of the dielectric constant must be considered. Although a plot of log rate constant versus inverse of the dielectric constant (Frost and Pearson, 1961) (not shown) approximately correlates the effects of methanol, ethanol, and iso-propanol, the effects of *n*-propanol and *n*-butanol clearly do not fit into this analysis. This is because the dielectric constants for all of the alcohol/water solutions used here are similar, whereas the rate constants are increasingly more sensitive to propanol and butanol than to the other alcohols. Similar effects were reported for binding of ethidium bromide to DNA (Baldini and Varani, 1985), although the deviations of *n*-propanol and *n*-butanol were smaller resulting in a better overall correlation with dielectric constant. It can be concluded from this that the effect of alcohols on PYP kinetics cannot be solely or even principally due to alteration of the dielectric constant of the solution, which implies there is no appreciable charge separation or recombination occurring in a region of the protein that is in contact with the solvent during the photocycle. In contrast, the effects of aliphatic alcohols on the kinetics of the photocycle do correlate quite well with the hydrophobic nature of the alcohol. Thus, the slopes of the plots of log rate constant versus alcohol concentration in Fig. 4 are strongly correlated with the partition coefficient of the alcohols between detergent and water, as shown in Fig. 5. This correlation is also consistent with the large effect of octylglucoside on the kinetics. The partition coefficient of glycerol is about one quarter of that of methanol, and thus the hydrophobic effect of glycerol is expected to be about one-tenth that of methanol on a

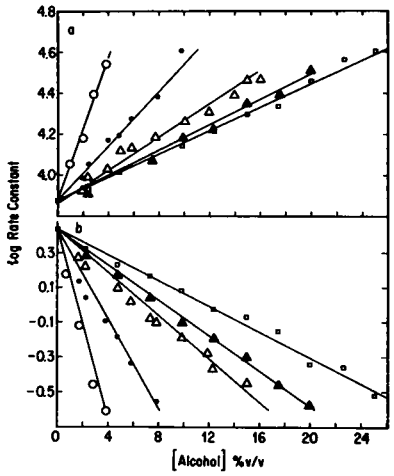

FIGURE 4 Effect of aliphatic monofunctional alcohol/water mixtures on kinetics of laser-induced bleach (*a*) and recovery (*b*) reactions of PYP. □ = methanol ▲ = ethanol △ = isopropanol ● = *n*-propanol ○ = *n*-butanol.

molar basis. This indicates that the correction to the viscosity effect will be small. For sucrose, the effect would be even smaller. Similar hydrophobic effects have been reported for binding of alkyl isocyanides to a model heme in detergent solutions (Olson et al., 1983), for binding of phenols to bovine serum albumin, and for the uncoupling of phosphorylation by organic solvents (Hansch et al., 1965). We conclude from our results with PYP that the conformational change that accompanies the bleach reaction exposes a hydrophobic site on the protein. Such an exposed hydrophobic patch could conceivably interact with a corresponding site on a hypothetical receptor protein. We postulate that this receptor mediates a phototactic response. *E. halophila* is motile and phototactic (McRee et al., 1989). Purple phototrophic bacteria in general show positive phototactic responses at moderate light intensity and negative responses at high light intensity (Clayton, 1964; Armitage, 1988). Furthermore, *E. coli* shows only negative phototaxis at high light intensity and the effect is most pronounced between the wavelengths of 390 and 530 nm. (Taylor and Koshland, 1975). The action spectrum for positive phototaxis in purple bacteria is dominated by the chlorophyll and carotenoid photosynthetic pigments, presumably because of the energy requirement for motility (Clayton, 1964; Armitage, 1988). However, no action spectrum has been measured for negative phototaxis in these organisms, although one would expect it to be in the blue region of the spectrum by analogy to that of *H. halobium* and *E. coli*. Thus, *E. halophila* PYP could conceivably fulfill such a role as a negative phototactic receptor, mediating a negative photophobic response of the cell to high intensity blue light. It is not known how such a phototactic response might be mediated, but it could be due to indirect interaction with the flagella through membrane bound chemotactic transducers (Koshland, 1981; Boyd and Simon, 1982; MacNab, 1987; Furlong, 1987) by analogy with soluble periplasmic receptor proteins. The three-dimensional structures of several of these receptors have been determined e.g., the Leu/Ile/Val-binding protein (Sack et al., 1989) and show no obvious similarity to PYP. Therefore, we have also considered indirect inhibitory effects of photobleached PYP on electron transfer, on ion transport, or on an ATPase. These possibilities are being tested.

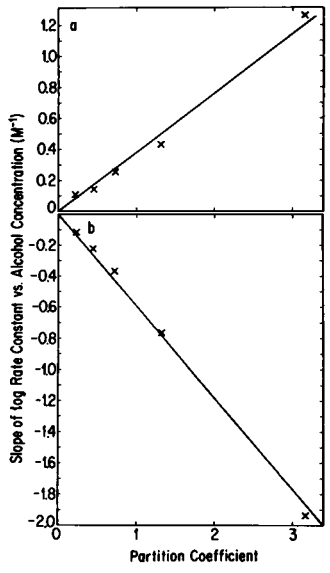

FIGURE 5 Correlation between kinetics of bleach (*a*) and recovery (*b*) in alcohol/water mixtures (slopes of the lines in Fig. 4) and the partition coefficient of alcohols between dimyristoyl lecithin and water (Katz and Diamond, 1974).

In conclusion, organic additives affect the kinetics of PYP bleach and recovery much more than do inorganic salts. Glycerol and sucrose slow the bleach reaction due to changes in viscosity. This is interpreted in terms of a conformational change of the protein that is induced by light. Aliphatic alcohols and octylglucoside increase the rate constant for bleach and decrease the rate of recovery. We consider this to be due to the exposure of a hydrophobic site on the protein to solvent as a consequence of the phototransformation. The effects of alcohols on the kinetics of the photocycle provide the strongest clue to date on the possible biological function of the yellow protein, which may be to bind to a hydrophobic membrane receptor only in the bleached state.

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