Effect of Alterations in the Amphipathic Microenvironment on the Conformational Stability of Bovine Opsin. 2. Rate of Loss of Opsin Regenerability†

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ABSTRACT: The activation free energy for the loss of opsin regenerability (the ability of opsin to react with 11-*cis*-retinal to form a bleachable 500-nm absorption band) was measured at all stages in the solubilization of bovine rod outer segment disk membranes by the nonionic detergent octyl β-D-glucoside. The activation free energy in native disk membranes at 23 °C was determined to be 30 kcal/mol. Exposure of the membranes to subsolubilizing levels of detergent lowered the activation free energy by an amount proportional to the amount of detergent incorporated into the membranes. The total reduction in activation free energy due to incorporation of detergent into the membranes was 7 kcal/mol. The transition from detergent-saturated membranes to soluble protein-lipid-detergent complexes, produced by exposing the membranes to higher detergent levels, was accompanied by a further reduction in activation free energy of 1.0 to 1.5 kcal/mol. Finally, removal of bound phospholipid from the solubilized complexes by the addition of a large excess of detergent resulted in an additional lowering of the activation free energy by 1.8 kcal/mol. The activation free energy for the loss of regenerability of solubilized opsin was directly proportional to the amount of phospholipid bound to the soluble protein-lipid-detergent complexes, each bound phospholipid molecule contributing 38 cal/mol. Thus, 70% of the total reduction seen in the activation free energy occurred under conditions in which the protein was still associated with the disk membrane. An additional 18% of the total change could be attributed to the disruption of protein-lipid interactions in soluble opsin-lipid-detergent complexes. These results suggest that phospholipid may influence the conformational stability of integral membrane proteins by both direct and long-range mechanisms. In the case of opsin, the long-range physical properties of the phospholipid bilayer appear to play the major role in stabilizing the native protein conformation.

This report constitutes the second half of an investigation concerning the changes in membrane protein stability which result from the disruption of the native protein-lipid interactions by a nonionic detergent. The preceding paper (Stubbs and Litman, 1978) described the alterations in the composition of the amphipathic microenvironment of bovine rhodopsin produced by exposure of disk membranes to increasing concentrations of octyl β-D-glucoside. Now we turn to the question of how these changes in the microenvironment affect the rate of denaturation of opsin.

It has long been known that photobleached rhodopsin can be regenerated to a high degree by the addition of 11-*cis*-retinal when the protein is located in a phospholipid bilayer (Wald, 1951; Hong and Hubbell, 1973). Attempts to regenerate detergent-solubilized rhodopsin have generally been unsuccessful due to the irreversible denaturation of opsin which occurs in detergents (Hubbard, 1958; Snodderly, 1967; Johnson and Williams, 1970; Ebrey, 1971; Shichi, 1971; Stubbs et al., 1976). While the rate of denaturation of opsin is substantially lower in digitonin than in other detergents, the stability afforded by the digitonin micelle is still less than that provided by the phospholipid bilayer (Hubbard, 1958). Evidently, detergents are unable to fully substitute for the native membrane environment. The mechanism by which detergent solubilization leads to changes in membrane protein properties is unknown, but may result from the removal of a few specifically bound phospholipid molecules, disruption of a nonspecific boundary layer of phospholipid, or modification of the general physical properties of the phospholipid bilayer. The results presented here provide an estimate of the relative importance of these factors in determining the rate of loss of the native conformation of bovine opsin.

Materials and Methods

Disk Membrane Preparation. Rod outer segment disk membranes were prepared from frozen bovine retinas (Hormel) by the method of Smith et al. (1975). The membranes were washed twice and resuspended in buffer consisting of 50 mM Tris base, 50 mM sodium acetate, and 0.2 M KCl adjusted to pH 7 by addition of HCl. Bleached disk membranes were prepared by diluting unbleached disks 5% with 1 M neutralized hydroxylamine and bleaching the sample by exposure to a fluorescent desk lamp for 15 min at room temperature. The resulting colorless disk membranes were washed twice and resuspended in the original volume of Tris-acetate buffer. The molar extinction coefficient of rhodopsin was taken to be 40 000. All procedures were performed in darkness or under dim red light unless otherwise stated.

Octyl Glucoside Synthesis. Octyl β-D-glucoside was prepared from acetobromoglucose (Sigma) and 1-octanol (Fisher Scientific) by the method of Noller and Rockwell (1938) with modifications described by Baron and Thompson (1975).

Opsin Denaturation[1] Rate Assay. In the presence of 11-*cis*-retinal opsin may either denature, or it may undergo regeneration by binding 11-*cis*-retinal to form rhodopsin. The

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[1] In this paper opsin was considered to be denatured when it could no longer combine with 11-*cis*-retinal to form rhodopsin.

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rate equations governing the simultaneous denaturation and regeneration reactions are:

$$\frac{d[opsin]}{dt} = -k_1[opsin] - k_2[retinal][opsin] \quad (1)$$

and

$$\frac{d[rhodopsin]}{dt} = k_2[retinal][opsin] \quad (2)$$

where k_1 is the first-order denaturation rate constant, k_2 is the second-order regeneration rate constant, [opsin] is the concentration of undenatured opsin, and [retinal] is the concentration of unreacted 11-*cis*-retinal. An approximate solution of these equations gives a value for the rhodopsin concentration at infinite time of

$$[rhodopsin]_\infty = [opsin]_0 \frac{\frac{k_2[retinal]_0}{k_1}}{\frac{k_2[retinal]_0}{k_1} + 1} \exp(-k_1 t) \quad (3)$$

where $[opsin]_0$ is the opsin concentration at the start of the denaturation reaction, $[retinal]_0$ is the concentration of 11-*cis*-retinal added, and t is the time between the start of denaturation and the addition of 11-*cis*-retinal. Since eq 3 has the form of a first-order rate equation, a semilog plot of the final rhodopsin concentration vs. t should yield a straight line with slope k_1. The intercept at $t = 0$ will depend on the ratio of the regeneration and denaturation rates. Computer modeling of the reactions showed that the rate determined from the slope of the semilog plot was within 3% of the true denaturation rate for $k_1 \leq 0.025\ s^{-1}$.

The denaturation reaction was started by adding bleached disk membranes to a solution of octyl glucoside in Tris–acetate buffer in a water bath maintained at 23 °C. Six to eight aliquots of 0.2 to 0.5 mL, depending on the opsin concentration, were removed at various time intervals after the start of denaturation and immediately regenerated by the addition of 11-*cis*-retinal (gift of Hoffman-La Roche, Inc.). Approximately 2 mol of retinal (25 mM in ethanol) were added per mol of opsin in the bleached disk aliquot. The final ethanol concentration was less than 0.25% (v/v). After incubating for 3 h at 23 °C in the dark, the samples were diluted to a final volume of 0.55 mL by addition of octyl glucoside and neutralized hydroxylamine in Tris–acetate buffer. The final hydroxylamine concentration was 90 mM, and the final octyl glucoside concentration was between 30 and 100 mM. The rhodopsin concentration in the regenerated samples was measured by recording the change in A_{500} produced by bleaching each sample for 30 s with a high intensity microscope illuminator. The logarithm of the regenerated rhodopsin concentration was plotted vs. t, and the denaturation rate, k_1, was obtained from the slope of this plot. The maximum denaturation incubation times used ranged from 2 min to 5 h. In order to measure opsin denaturation rates at subsolubilizing detergent concentrations or in detergent-free disk membranes, it was necessary to perform the denaturation incubation in the temperature range of 40 to 50 °C. These samples were returned to 23 °C following the addition of 11-*cis*-retinal, and were solubilized by addition of octyl glucoside and hydroxylamine prior to measuring the ΔA_{500} as described above.

Results

The denaturation of opsin may be initiated either by bleaching solubilized rhodopsin or by solubilizing disk membranes which have previously been bleached. The first method

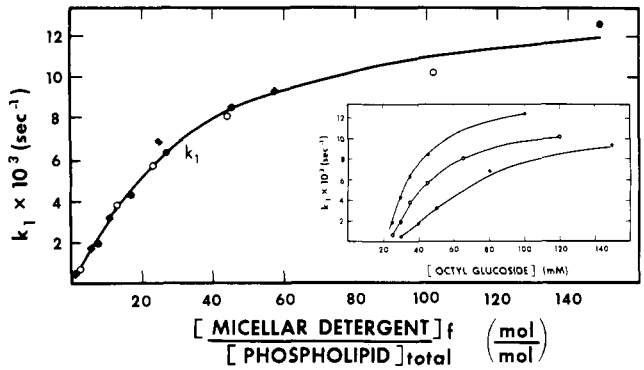

FIGURE 1: (Inset) The opsin denaturation rate (23 °C) is plotted vs. octyl glucoside concentration for three solubilized disk membrane samples with opsin concentrations of (●) 7.5 μM; (○) 15 μM; and (♦) 30 μM. Main figure: the data shown in the inset (same symbols) is plotted vs. the ratio of free micellar octyl glucoside to disk membrane phospholipid in order to normalize the abscissa of each point with respect to the amount of phospholipid bound to opsin. The free micellar octyl glucoside concentration of each curve was calculated by subtracting the octyl glucoside concentration needed to solubilize all of the opsin in the sample (eq 1 of Stubbs and Litman (1978)) from the total octyl glucoside concentration.

(solubilizing and then bleaching) was used in our initial experiments and found to yield denaturation curves displaying complex kinetics due to the slow release of *all-trans*-retinal from the opsin binding site. Consequently, the second method of initiating the denaturation reaction (solubilization of bleached disk membranes) was used in the experiments presented in this study. This method allowed bleaching to be performed in the presence of hydroxylamine, which cleared the retinal binding site on opsin by reacting with *all-trans*-retinal to form retinal oxime. Since the opsin was still situated in the disk membrane, no denaturation occurred, and the excess hydroxylamine could be removed by washing the disks. The denaturation reaction was started by mixing the bleached disks with octyl glucoside to yield the desired final concentrations. The rate of denaturation was measured by removing aliquots at various times and adding 11-*cis*-retinal to trap the undenatured opsin as regenerated rhodopsin.

In the preceding paper (Stubbs and Litman, 1978) the solubilization of disk membranes by octyl glucoside was found to proceed by three distinct stages depending on the relative concentrations of disk membranes and detergent. These three stages were distinguished by the types of protein–lipid structures present in the sample: (1) disk membranes containing varying amounts of incorporated detergent; (2) a mixture of detergent-saturated membrane fragments and soluble protein–lipid–detergent complexes; and (3) soluble protein–lipid–detergent complexes containing varying amounts of phospholipid. The data presented in this paper concerning the effects of changes in the microenvironment on the conformational stability of opsin are discussed with reference to the same three stages of the solubilization process. The results are presented first for fully solubilized disk membrane samples, followed by the results obtained at partially solubilizing and subsolubilizing detergent levels.

Fully Solubilized Disk Membranes. Figure 1 (inset) shows how the denaturation rate of opsin varied as a function of the octyl glucoside concentration in the fully solubilized region for three concentrations of disk membranes. The denaturation rate curves were seen to shift toward higher detergent levels as the concentration of disk membranes was increased. In addition, the curves appeared to intercept the $k_1 = 0$ axis near the critical micelle concentration of octyl glucoside (25 mM, Shinoda et

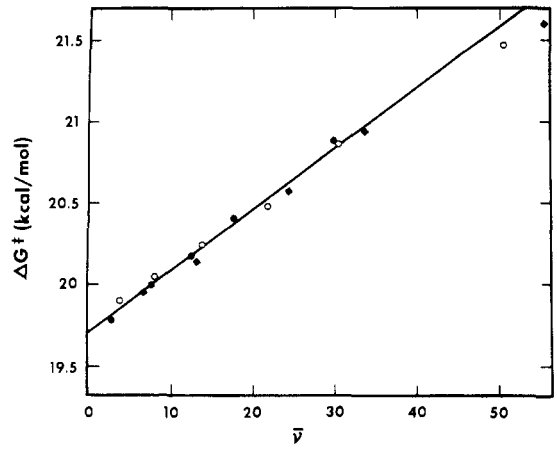

FIGURE 2: The free energy of activation for the loss of opsin regenerability at 23 °C ($\Delta G^{\ddagger}$) calculated from the rate data of Figure 1 (same symbols) is plotted vs. the amount of phospholipid bound to opsin in the octyl glucoside solubilized samples ($\bar{\nu}$, mol of phospholipid/mol of opsin).

al., 1961) and to plateau at high detergent levels. The amount of phospholipid bound[2] to solubilized rhodopsin depends on the ratio of free[3] micellar detergent to membranes (see eq 3 in Stubbs and Litman, 1978). Therefore, the simplest explanation for the observed behavior of the denaturation rate curves is that the amount of bound phospholipid was responsible for modulation of the opsin denaturation rate.

In order to test the above hypothesis, the three sets of denaturation rate data were plotted vs. the ratio of free micellar octyl glucoside to disk membrane phospholipid (Figure 1, main figure). Since the phospholipid in this system partitions between the opsin-lipid-detergent complexes and the lipid-detergent micelles, samples having the same ratio of free micellar detergent to disk membranes will have equivalent levels of phospholipid bound to opsin. As seen in Figure 1 (main figure), the three individual opsin denaturation rate curves obtained with samples of different disk membrane concentrations fell on a single curve when plotted so as to normalize the level of bound phospholipid. Thus, the data shown in Figure 1 quantitatively support the concept that the rate of loss of regenerability of solubilized opsin is governed by the amount of phospholipid bound to the protein.

The relationship between bound phospholipid and opsin stability was further investigated by plotting the activation free energy for the denaturation of opsin vs. the average number of phospholipid molecules bound per rhodopsin molecule. This plot is shown in Figure 2. The activation free energy for each point, $\Delta G^{\ddagger}$, was calculated from the opsin denaturation rate (k_1) as follows:

$$\Delta G^{\ddagger} = \Delta G_0^{\ddagger} - RT \ln (k_1/k_0) \qquad (4)$$

where $\Delta G_0^{\ddagger}$ and k_0 are the activation free energy and denaturation rate, respectively, for an arbitrary reference sample at 23 °C. The reference sample, which was chosen to represent a well-defined point on the denaturation rate curve of Figure 1, had an octyl glucoside concentration of 30 mM and an opsin concentration of 7.5 μM, corresponding to a free micellar detergent to phospholipid molar ratio of 16.2. The denaturation rate of opsin in the reference sample was measured at temperatures of 19, 23, and 27 °C, and the activation parameters calculated from these rates are shown in Table I. The activation free energies determined from the temperature dependence of the denaturation rate for four similar samples with octyl glucoside concentrations of 25, 35, 45, and 100 mM were found to agree with the activation free energies calculated from eq 4.

[2] The term "bound" refers to the phospholipid and detergent contained in the soluble protein-lipid-detergent complexes. No assumptions have been made concerning the structure of these complexes.

[3] The term "free" is used to indicate phospholipid or detergent which is not bound.

TABLE I: Activation Parameters Calculated from the Temperature Dependence of the Opsin Denaturation Rate.

[Octyl glucoside] (mM)	[Opsin] (μM)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal mol^{-1} deg^{-1})	$\Delta G^{\ddagger}$ (kcal/mol)
30	7.5	40 ± 4	66 ± 13	(23 °C) 20.4 ± 0.2
0	5.0	79 ± 7	165 ± 21	(23 °C) 29.9 ± 0.6
				(40 °C) 27.0 ± 0.22

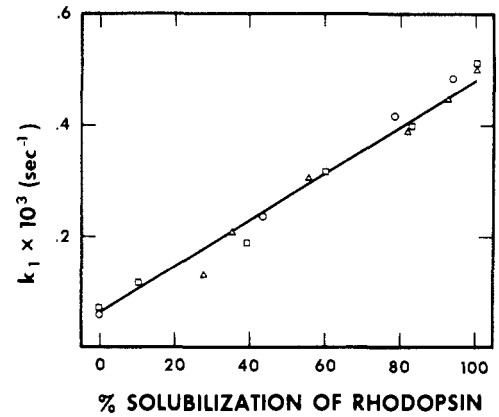

FIGURE 3: The rate of denaturation of opsin (k_1) in partially solubilized bleached disk membrane suspensions vs. percent solubilization of rhodopsin measured in equivalent unbleached samples, 23 °C. Opsin concentrations were (○) 10.6 μM; (□) 24 μM; and (△) 37 μM.

From Figure 2 it can be seen that the denaturation activation free energy increased linearly with the amount of bound phospholipid over the entire fully solubilized range. Assuming that the number of bound phospholipid molecules per opsin molecule was narrowly distributed about the measured mean value, the slope of the line in Figure 2 indicates that each phospholipid molecule bound to the protein-lipid-detergent complex contributed 38 ± 3 cal/mol of free energy toward stabilization of the regenerable opsin conformation. The fact that there was no break in the curve in Figure 2 may be interpreted to indicate that the phospholipid was not functioning as a cooperative unit in stabilizing the opsin structure.

Partially Solubilized Disk Membranes. Opsin denaturation rate measurements were also performed at octyl glucoside concentrations in which the disk membranes were only partially solubilized. Under these conditions, the denaturation rate curves similar to those shown in Figure 1 (inset) were found to have a shoulder in the vicinity of the $k_1 = 0$ intercepts. As was seen under fully solubilized conditions, the curves obtained with higher disk membrane concentrations were displaced toward higher detergent concentrations. When the denaturation rate was plotted vs. the fraction of opsin solubilized (rather than the detergent concentration), the denaturation rate curves obtained with different levels of disk membranes became roughly linear and coincident, Figure 3.

This linear dependence of the denaturation rate on the percent of opsin in the sample solubilized may be explained by assuming the existence of two rapidly exchanging pools of

undenatured opsin in the sample. These two pools consist of detergent-saturated sedimentable membrane fragments and soluble protein–detergent complexes. If the opsin in each pool denatures at a rate characteristic of that pool, and if changes in the detergent concentration only alter the distribution of opsin between the two pools, it can be shown that the observed denaturation rate will depend linearly on the fraction of opsin solubilized. Thus, the left and right end points of the line in Figure 3 may be interpreted as the denaturation rates of opsin in the sedimentable and soluble pools, respectively. From the ratio of these denaturation rates, we estimate that the transfer of opsin from detergent-saturated disk membranes to the soluble phase resulted in a drop of 1.0 to 1.5 kcal/mol in the activation free energy for denaturation.

This simple two-component model for the solubilization process is probably an oversimplification. A wide range of structures may exist in the partially solubilized membrane suspension, leading to a high degree of heterogeneity in the protein's microenvironment and denaturation rate. Nevertheless, the value derived above should still serve as an estimate of the maximum change in denaturation activation free energy experienced by opsin in the transition from the sedimentable to the soluble phase.

Subsolubilizing Detergent Levels. Exposure of disk membranes to octyl glucoside at concentrations below the onset of opsin solubilization also produced dramatic alterations in the opsin denaturation rate. The microenvironment of the protein in this case consisted of a phospholipid bilayer containing varying amounts of incorporated detergent. The rate of loss of opsin regenerability, k_1, and $\ln k_1$ are shown in Figure 4 for a disk membrane sample exposed to subsolubilizing octyl glucoside levels. These measurements were performed at 40 °C in contrast to those previously discussed, which were made at 23 °C. In Figure 4 the opsin denaturation rate is seen to be a highly nonlinear function of the detergent concentration. In contrast, $\ln k_1$, which is proportional to the denaturation activation free energy, appears to be directly proportional to the total detergent concentration. Due to partitioning of the detergent between the aqueous phase and the membranes, the membrane concentration of detergent was directly proportional to the total detergent concentration. Therefore, the reduction in activation free energy for loss of opsin regenerability was linearly related to the amount of detergent incorporated into the disk membranes. The total change in activation free energy over the subsolubilizing range of detergent concentrations was between 6 and 7 kcal/mol.

Measurements of the rate of loss of opsin regenerability were made in native (detergent-free) disk membranes at temperatures between 40 and 50 °C. The activation parameters derived from these measurements are given in Table I. In order to establish the absolute value of the free energy scale in Figure 4, the intercept of the $\ln k_1$ curve was set equal to the free energy of activation for opsin denaturation calculated from these ΔH and ΔS values at 40 °C. Similarly, extrapolation from the high temperature denaturation rate measurements gave a value for the rate of loss of opsin regenerability in native disks at 23 °C of between 1.5×10^{-9} and 1.5×10^{-10} s^{-1}. The ratio of this rate to the denaturation rate in detergent-saturated disks at 23 °C (Figure 3) gave a value of 6.7 ± 0.9 kcal/mol for the total change in denaturation activation free energy due to incorporation of octyl glucoside into the membranes at 23 °C. This value agrees well with the corresponding change in activation energy measured directly at 40 °C (Figure 4), suggesting that the effect of detergent on the stability of opsin in the disk membrane is not strongly temperature dependent.

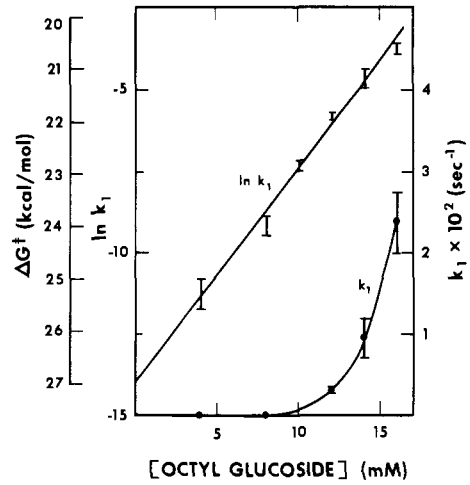

FIGURE 4: The effect of subsolubilizing octyl glucoside concentrations on the denaturation rate of opsin in bleached disk membranes, 40 °C. The scale at left gives the free energy of activation corresponding to the $\ln k_1$ values.

Discussion

The studies described in this and the preceding paper (Stubbs and Litman, 1978) represent an effort to understand the role of phospholipid in stabilizing the native conformation of an integral membrane protein. To this end, detergent solubilization was employed as a means of varying the microenvironment of bovine opsin. The effect of this treatment on the conformational stability of the protein was evaluated by measuring the rate of loss of regenerability.

Hubbard (1958) showed that bleaching rhodopsin in the disk membrane reduced the stability of the protein with respect to thermal denaturation. In the same study, solubilization of the disk membranes by digitonin was also found to lower the thermal stability of both rhodopsin and opsin. These observations demonstrated that the native conformation of rhodopsin is maintained by at least two independent types of interactions: the specific binding of 11-*cis*-retinal, and the association of the protein with a phospholipid bilayer.

The stabilization of rhodopsin by retinal is analogous to the well known ability of substrates to stabilize soluble enzymes, a phenomenon which has also been reported for membrane-bound enzymes solubilized by detergents (Warren et al., 1974; Sussman and Hays, 1977). The ability of a phospholipid bilayer to stabilize integral membrane proteins is evidenced by the fact that membrane-bound enzymatic activities are frequently destroyed by detergent solubilization (Helenius and Simons, 1975).

Bleaching rhodopsin in detergent solutions has been shown to initiate a general denaturation reaction which, in addition to loss of regenerability, results in the exposure of additional sulfhydryl groups (Bonting et al., 1974), loss of approximately one-fifth of the helical secondary structure (Shichi et al., 1969), a reduction in the amount of bound detergent, and aggregation of the protein (Osborne et al., 1974). The loss of regenerability occurs at roughly twice the rate of loss of the helical secondary structure as measured by changes in the ultraviolet circular dichroism (unpublished results). Shichi (1971) also observed that the loss of regenerability and loss of secondary structure are kinetically separate events since the former was found to be more sensitive to detergent than the latter. Hence, the loss of regenerability appears to occur during the initial stage in the denaturation reaction, and likely results from a change in the protein's tertiary structure which destroys the retinal binding site. This conformational change in opsin is likely

TABLE II: Activation Free Energy for Loss of Opsin Regenerability at Different Stages of Disk Membrane Solubilization.

Microenvironment of opsin	$\Delta G^{\ddagger}$ (23 °C) (kcal/mol)	Change in $\Delta G^{\ddagger}$ (kcal/mol)
Detergent-free disk membrane	29.9 ± 0.6	
		6.7 ± 0.9
Disk membranes saturated with detergent	23.2 ± 1.1[a]	
		1.25 ± 0.25
Initially solubilized protein-lipid-detergent complex	22.0 ± 1.1[a]	
		1.8 ± 0.2
Phospholipid-free detergent micelle	20.2 ± 1.2[a]	
	19.7 ± 0.2[a]	

[a] These values were calculated by subtracting the changes in $\Delta G^{\ddagger}$ determined for the preceding stages of solubilization from the value[b] in detergent-free bleached disk membranes. [b] Calculated from the temperature dependence of the denaturation rate of solubilized opsin (Table I) extrapolated to zero bound phospholipid.

analogous to the changes which result in the loss of enzymatic activity upon solubilization of membrane-bound enzymes.

Table II summarizes our results by giving the change in activation free energy for the loss of regenerability for each stage of solubilization. Incorporation of subsolubilizing levels of octyl glucoside into the disk membrane lowered the activation free energy from 30 kcal/mol in native disks to 23 kcal/mol in detergent-saturated disk membranes. The extent of the reduction in activation energy was proportional to the amount of detergent incorporated into the membranes. Increasing the detergent concentration beyond the level of membrane saturation disrupted the disks into soluble protein-lipid-detergent complexes, and produced an additional drop of approximately 1.3 kcal/mol in the activation free energy. Removal of the bound phospholipid from the protein by the addition of a large excess of detergent reduced the activation free energy by another 1.8 kcal/mol to a final value of 20 kcal/mol for opsin in a phospholipid-free octyl glucoside micelle. Essentially the same value was determined from the temperature dependence of the opsin denaturation rate measured in fully solubilized disks, demonstrating the self-consistency of the data.

In these experiments the opsin denaturation rate was sensitive to both the concentration of disk membranes and detergent. In order to interpret the data for solubilized opsin, it was necessary to express the results in terms of the ratio of free micellar detergent to disk membranes (rather than the total detergent concentration). This treatment normalized the data points with respect to the amount of phospholipid bound to the protein. This finding underlines the point made by others (Helenius and Simons, 1975) that the ratio of micellar detergent to membranes should be used as the independent variable in studies concerning the effects of solubilization on the properties of membrane proteins. Furthermore, in order to compare the effects of different detergents, care should be taken to ensure that the amount of phospholipid bound to the solubilized protein is the same in each case. In general, this would require that a phospholipid binding curve be determined for the membrane solubilized in each of the detergents.

Several recent investigations concerning the interaction of integral membrane proteins with phospholipid have focused attention on the first boundary layer of phospholipid in contact with the protein (Warren et al., 1974; Hesketh et al., 1976). These investigators have interpreted the nonlinear dependence of the retention of activity of the sarcoplasmic reticulum Ca^{2+}-ATPase on the amount of bound phospholipid to imply a special importance to the ratio of about 30 phospholipid molecules per enzyme molecule in stabilizing the enzyme's activity. Our own measurements of the rate of loss of the regenerability of opsin showed this rate also to be a nonlinear function of the level of bound phospholipid over the fully solubilized range. However, a calculation of the free energy of activation for the denaturation of opsin as a function of the phospholipid binding (Figure 2) revealed that each molecule of bound phospholipid contributed equally to the stability of the protein. Hence, our results with opsin demonstrate that a nonlinear rate dependence on bound phospholipid is completely consistent with a linear relationship between phospholipid binding and the stabilization of a membrane protein's structure.

The work presented here does not allow us to positively identify the mechanism by which disruption of the native disk membrane structure led to a lowering of the activation barrier to denaturation of opsin. The data suggest, however, that different mechanisms may have been responsible for changes in the stability of opsin located in the phospholipid bilayer of the disk membrane, and opsin located in a lipid-detergent micelle. The incorporation of low levels of detergents into natural and artificial membranes has been shown to have a fluidizing effect on the phospholipid acyl chains (Lenaz et al., 1975), and frequently increases the activity of membrane-bound enzymes (e.g., Ne'eman et al., 1971; Sussman and Hays, 1977). We have observed a similar fluidization of the disk membrane by octyl glucoside as measured by the fluorescence anisotropy of the probe 1,6-diphenyl-1,3,5-hexatriene located in the hydrocarbon region of the membrane (unpublished results). It is tempting to speculate that the enhancement of enzymatic activities as well as the changes in the opsin denaturation rate seen at subsolubilizing detergent levels may have resulted from changes in the amplitude of the conformational fluctuations of the protein caused by detergent-induced alterations in the fluidity of the surrounding phospholipid bilayer.

In contrast to membrane-bound proteins, protein molecules located in lipid-detergent micelles may be expected to be influenced predominantly by direct protein-lipid interactions rather than the long range physical properties of the amphipathic microenvironment. We found that the stability of opsin in these structures was a linear function of the amount of bound phospholipid. Hence, the magnitude of the activation free energy for the loss of regenerability is determined by the sum of the contributions of the individually bound phospholipid molecules, and not through any cooperative interaction among the boundary layer phospholipids. Due to perturbations caused by the presence of the detergent, the effects of bound phospholipid seen on the stability of solubilized opsin must be considered a lower limit to the magnitude of these effects in the absence of detergent. Within the framework of the above interpretation, our data suggests that the major contribution to maintaining the native opsin conformation is provided by the physical properties of the disk membrane phospholipid bilayer, while a lesser but significant contribution may be attributed to the direct interactions between opsin and its boundary layer phospholipid.

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Conversion of Oxyhemoglobin to Methemoglobin by Organic and Inorganic Reductants†

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ABSTRACT: Human oxyhemoglobin is converted to methemoglobin by a wide array of organic and inorganic reductants. Depending upon the concentration and nature of the reductant, varying amounts of deoxyhemoglobin are produced. The general overall sequence is: $FeO_2 \overset{(1)}{\rightarrow} Fe^{III} \overset{(2)}{\rightarrow} Fe^{II}$. The intermediacy of methemoglobin can be demonstrated by direct spectral observation and by cyanide trapping. For organic reductants, the second-order rate constants for (1) vary from >300 (phenylhydroxylamine) to 1.4×10^{-4} M^{-1} s^{-1} (malononitrile). Generally the rates parallel the ease of hydrogen abstraction by iron-bound oxygen from the substrate, and simple hydrocarbons are reactive. Rates for these processes have been ascertained with recrystallized protein, lysed cells, and intact human erythrocytes. At room temperature oxyhemoglobin quantitatively converts benzaldehyde to benzoic acid and hydroquinone to benzoquinone. Rates for inorganic species (process 1) range from $>7 \times 10^3$ (chromous ion) to 0.015 M^{-1} s^{-1} (ferrocyanide). Ferrous ion rapidly deoxygenates oxyhemoglobin by direct attack on the oxy complex but methemoglobin *is not* an intermediate with this reagent. Taken together the results support the theoretical prediction that reductants should oxidize oxyhemoglobin, and they demonstrate at least some degree of radical character to the oxy complex.

A prediction of theory (Castro, 1971) is that oxyhemoglobin should be "oxidized", that is, converted to methemoglobin, by reducing agents. The prediction is a corollary to an explanation of the stability of the iron(II) complex to oxygen. Simply, the G conformation ascribed to the globins sterically prevents the attack of a second iron porphyrin upon the 1:1 oxy complex. These considerations were based primarily on the premise that the mechanism of oxidation of high spin iron(II) porphyrins by oxygen would parallel that of the oxidation of simple metal ions by unsaturated molecules (Castro and Stephens, 1964; Castro et al., 1966) and would entail an intermediate binuclear adduct:

$$M^{n+} + O_2 \rightleftharpoons MO_2^{n}$$
$$MO_2^{n+} + M^{n+} \longrightarrow MOOM^{2n+}$$
$$MOOM^{2n+} \xrightarrow{2H^+} 2M^{n+1+} + H_2O_2$$

While no direct kinetic measurements of the pure high spin systems have been reported, some indirect analysis and qualitative observations lend very strong support to this contention.

Arguments supporting this "axial metal addition" path for the oxidation of high spin iron(II) porphyrins by oxygen have been summarized recently (Castro, 1977), and only the most relevant observations are noted here. Under certain conditions, a term, second order in iron, is found in the rate expression for the oxidation of hemes in pyridine–benzene solutions (Cohen and Caughey, 1968), and solid μ-dioxo adducts were isolated from this reaction (Alben et al., 1968). The extreme sensitivity to oxygen characteristic of the high spin iron(II) complexes is exhibited by pentacoordinate imidazole adducts (the inner coordination sphere of hemoglobin) in amide solvents at room temperature (Castro, 1974). On the other hand, a variety of iron(II) porphyrins reversibly bind oxygen at low temperatures, and 1:1 oxygen complexes have been observed with N-substituted imidazole (Traylor and Chang, 1973; Almog et al., 1974), *N*-butyl mercaptide (Chang and Dolphin, 1976), or no

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