Interaction of 8-Anilino-1-naphthalenesulfonic Acid with Holo- and Apophosphorylase *b*. Ligand Effects, Resolution, and Reconstitution with Pyridoxal 5′-Phosphate†

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ABSTRACT: When the hydrophobic probe 8-anilino-1-naphthalenesulfonic acid (ANS) is bound to apophosphorylase its fluorescence quantum yield, calculated from uncorrected spectra, is about three times as high as that of ANS bound to phosphorylase *b*. The wavelength of maximum ANS fluorescence was 470 nm with the apoenzyme, and 490 nm with the holoenzyme, also based on uncorrected spectra. Glycerophosphate decreases the fluorescence of mixtures of ANS with either apophosphorylase or phosphorylase, without apparently changing the quantum yields of bound ANS. The half-maximal inhibition of fluorescence by glycerophosphate was similar with both proteins, and approximately 8 mM. AMP has only a small effect on the fluorescence of ANS in the presence of apophosphorylase. When holoenzyme is added to ANS solutions in the fluorimeter cuvet, the change in fluorescence intensity is rapid, and when glycerophosphate or AMP is added to the mixture the decrease in fluorescence is instantaneous. In contrast, mixtures of ANS and the apoenzyme show an initial rapid rise in fluorescence, followed by a slower increase toward a maximal intensity. When glycerophosphate is added to such mixtures, a slow decrease in fluorescence is seen. Addition of phosphorylase *b* to an imidazole–citrate–cysteine mixture at pH 6.2 in the presence of ANS gives an immediate increase in fluorescence, followed by a slower increase toward a maximum. The time required for halfway completion of the slow process was approximately 30 sec, and this time was not influenced by 1 mM AMP, or by a change in temperature from 5 to 15°. Addition of pyridoxal phosphate to an apophosphorylase–ANS mixture in a Tris-cysteine buffer at pH 7.2 was followed by a decrease in fluorescence. The decrease followed first-order kinetics with a half-completion time of 13 min at 22°. In the presence of 1 mM AMP the fluorescence decrease became biphasic, with an initial rapid phase followed by a slower first-order process.

Rabbit muscle phosphorylase *b* contains 1 mol of pyridoxal phosphate per subunit of enzyme. The pyridoxal-5′-P appears to be bound in a hydrophobic pocket on the enzyme molecule, from which it may be removed by means of "deforming agents" and pyridoxal-5′-P binding reagents, imidazole citrate + cysteine (Shaltiel *et al.*, 1966). AMP prevents the removal of pyridoxal-5′-P, but could not be shown to aid the reconstitution of the apoenzyme with pyridoxal-5′-P although AMP was bound to the protein (Hedrick *et al.*, 1966). AMP influences the exchange of apophosphorylase hydrogens with solvent water, and also modifies the effect of pyridoxal-5′-P upon this exchange (Weisshaar and Palm, 1972), but this may be because of overlapping contact areas in the aggregates formed with AMP or by reconstitution.

ANS[1] is bound to phosphorylase with an increase in probe fluorescence (Seery and Anderson, 1972), and other hydrophobic probes are also bound to the enzyme (Radda, 1971).

The removal of the pyridoxal-5′-P from its hydrophobic binding site, with the resulting loosening of the protein structure, might have interesting effects upon the binding and fluorescence of ANS. The present paper describes how resolution affects the fluorescence quantum yield of bound ANS, and how the observed differences may be used for the study of resolution, reconstitution, and of binding of a ligand that has no kinetic consequences, such as glycerophosphate.

Materials and Methods

Rabbit muscle phosphorylase was prepared by the method described by Fischer and Krebs (1962). The crystals were dissolved in 30 mM Tris-acetate buffer at pH 7.2, containing 30 mM mercaptoethanol, or in 30 mM cysteine-HCl (pH 7.0). The enzyme was recrystallized at least three times. Enzyme activity was determined by the method of Hedrick and Fischer (1965) using either maleate or glycerol-P buffers. Protein was determined according to Klungsøyr (1969), or spectrophotometrically, using a value of 12.5 for $A^{1\%}_{278}$. This absorbance represents an average between literature values (Appleman *et al.*, 1963; Kastenschmidt *et al.*, 1968), and gives results identical with those obtained by the modified micro biuret method of Klungsøyr (1969) with bovine serum albumin as a standard. Specific activities between 65 and 75 units/mg of protein were routinely obtained. AMP was removed from the protein by passing it over a short column of activated charcoal (Fischer and Krebs, 1958). The ratio between the absorbances at 260 and 280 nm in treated preparations was less than 0.55.

Apophosphorylase *b* was prepared by the method of Shaltiel *et al.* (1966). In most cases we wanted a product free of glycerophosphate, and for that reason the precipitate with ammonium sulfate after resolution was dissolved in a 30 mM Tris-acetate–30 mM mercaptoethanol–80 mM NaCl buffer (pH 7.2). The same buffer was used to equilibrate and elute the Sephadex G-25 column for separation of the apoenzyme from remaining pyridoxal-5′-P and ammonium sulfate. If NaCl was not added, the protein precipitated during elution. The precipitate could be redissolved by adding NaCl. A minimum of 50 mM NaCl had to be included in the buffer

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[1] Abbreviation used is: ANS, 8-anilino-1-naphthalenesulfonic acid.

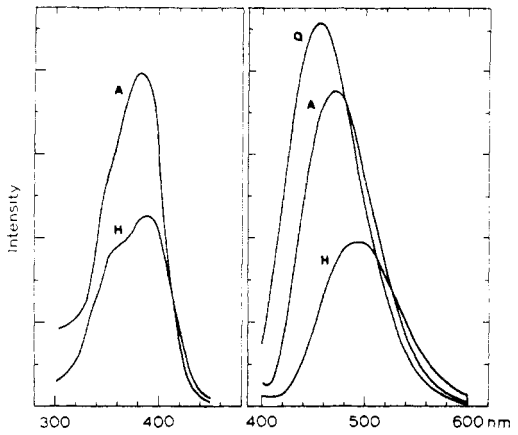

FIGURE 1: Fluorescence spectra of the ANS complexes with phosphorylase *b* (H) and apophosphorylase (A) compared with an emmission spectrum of quinine (Q). Left, exciting wavelength varied; right, emitted light wavelength varied. The protein concentrations were 4 mg/ml, and the ANS concentration was 4 μM in 30 mM Tris-acetate–30 mM mercaptoethanol buffer (pH 7.2). In the experiment with the apoenzyme the buffer also contained 100 mM NaCl.

for fluorescence experiments with apophosphorylase, and this concentration of salt was also used with the holoenzyme when direct quantitative comparisons were made between the two proteins, such as binding of ANS, quantum yields, or effects of ligands on ANS binding.

Resolution experiments were carried out in the solvent described by Shaltiel *et al.* (1966), either undiluted, or diluted with an equal volume of water. The imidazole base (Merck) was not further purified, but the fluorescence from this substance was sufficiently low to permit zero setting of the instrument with the solvent plus 50 μM ANS in the cuvet.

Reconstitution experiments were made in 30 mM cysteine-HCl, adjusted to pH 7.2 with Tris, containing 50 μM ANS and 1 mg of apophosphorylase in 2.5 ml volume. Reconstitution was initiated by adding 2 μl of 10 mM pyridoxal-5′-P on a stirring rod mounted in a specially constructed cuvet housing lid.

Fluorescence was measured on a Farrand spectrofluorimeter, Mark 1, with Heath servo recorder. Thermocontrolled water at the appropriate temperature was circulated through the cuvet housing, and all solutions were preincubated at this temperature before use.

In the case of phosphorylase *b*, titration with ANS or with the ligands AMP and glycerophosphate was carried out as described by Seery and Anderson (1972). When the same technique was used for titration of the apoenzyme with ANS, care was taken to use a sufficiently long interval between each addition of the probe. Mixtures containing apophosphorylase, ANS, and glycerophosphate or AMP required preincubation before reaching a stable fluorescence, and in such experiments separate samples for each ligand concentration were used.

In the fluorimeter the slit for the emitted light is situated halfway along the path of the exciting light, and the light intensity at this point may be easily calculated by the following formula. For a cuvet with a 10-mm light path: $I_{5\text{ mm}}/I_0 = (I_{10\text{ mm}}/I_0)^{1/2} = \text{antilog}\ (-\frac{1}{2}A_{10\text{ mm}})$. We have used this ratio to correct for light absorption in the solution. It gives practically identical results with the correction derived by an integration over the width of the slit (Parker and Barnes, 1957).

ANS was recrystallized as the Mg salt as described by Weber and Young (1964), and further purified on Sephadex G-25 (Klungsoyr, 1971).

Glycerophosphate was the product of Merck, Darmstadt, and AMP, Glc-1-P, and glycogen were obtained from Sigma, St. Louis, Mo.

Results

The ANS complex with apophosphorylase *b* has a more intense fluorescence with maximum at a shorter wavelength than the phosphorylase *b*–ANS complex. This is illustrated in Figure 1 with the fluorescence spectra (uncorrected) of mixtures containing identical concentrations of apoenzyme or phosphorylase and of ANS in both cases. Quinine was used as a standard substance in these experiments, and its emission spectrum is shown for comparison. Maximum fluorescence was seen at 470 nm with apophosphorylase, and at 490 nm with phosphorylase.

The differences in fluorescence observed might be due to differences in the amounts of ANS bound to the two proteins, or to differences in quantum yields of ANS bound to phosphorylase or to the apoenzyme.

To determine the quantum yields we recorded the fluorescence spectra of mixtures containing a low concentration of ANS and three different concentrations of either protein. The spectra were replotted as functions of wave numbers, and the areas under those curves were measured. Double reciprocal plots of areas *vs.* protein concentrations were then used to extrapolate to the areas corresponding to infinite protein concentrations (Figure 2). The fluorescence of a dilute solution of quinine in 0.1 N H_2SO_4 was recorded at the same time. The absorbance of the quinine solution at the exciting wavelength (380 nm) was determined, and also the change in absorbance at 380 nm obtained with 4 μM ANS when added to the protein solutions. Based on a quantum yield for quinine of 0.55 (Pesce *et al.*, 1971) the quantum yield for the ANS–phosphorylase complex was 0.12 and for the ANS–apophosphorylase complex 0.34. It must be pointed out that these calculations were based on uncorrected spectra.

This method for determining the quantum yield is based on the assumption that at infinite protein concentration and low ANS concentration all the probe molecules are bound to the protein. One may further assume that these bound ANS molecules each will give rise to a fluorescence with similar intensity to that of a bound ANS molecule under conditions of limited binding. Rather than recording the complete spectrum, one may use the fluorescence observed at maximum emission intensity and determine this at several protein concentrations and a fixed ANS concentration. Extrapolation to infinite protein concentration by means of double reciprocal plots supplies data to calculate the intensity per μM bound ANS under specified experimental conditions. From this value we then determined the concentration of bound ANS in experiments with low protein concentrations and a series of ANS concentrations. For the two proteins, phosphorylase and apophosphorylase, the fluorescence intensity per μM bound ANS was different, but the number of bound probe molecules per monomer protein was for the concentration range of ANS studied nearly the same for both proteins. This is illustrated in Figure 3, which shows the fluorescence intensity recorded and the calculated number of bound ANS molecules at different ANS concentrations in the presence of phosphorylase or the apoenzyme.

Mixing of solutions of ANS and phosphorylase results in a practically instantaneous increase in fluorescence intensity from that of free ANS. If a ligand is then added which reduces the fluorescence, the decrease in intensity is also rapid

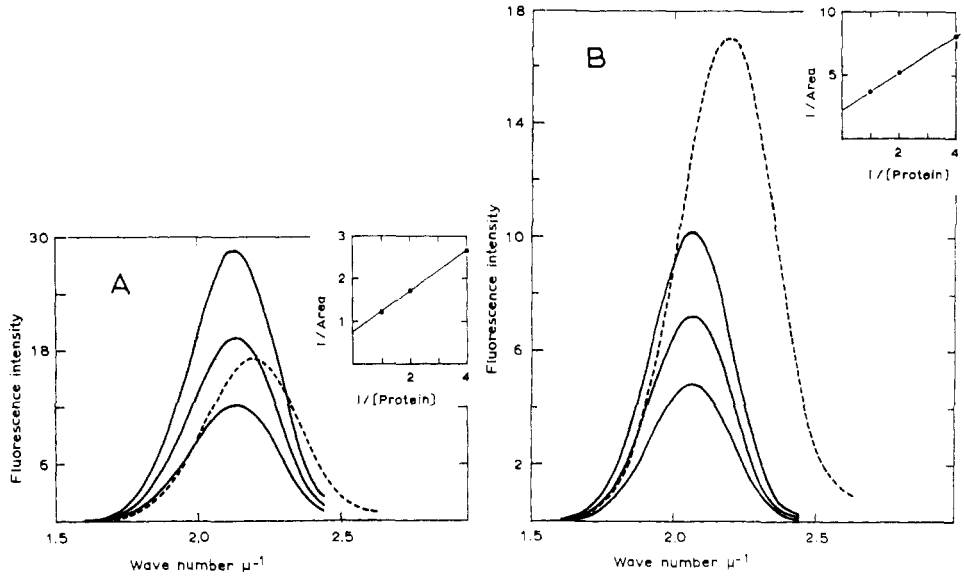

FIGURE 2: Uncorrected emission spectra plotted against wave numbers for the ANS complexes of (A) apophosphorylase, and (B) phosphorylase at three different protein concentrations: 4, 2, and 1 mg/ml. The ANS concentration was 4 μM, in a 30 mM Tris-HCl–30 mM mercaptoethanol–50 mM NaCl buffer (pH 7.2). The wavelength of excitation was 380 nm, and the temperature was 10°. The broken curves represent a quinine standard. The areas under the curves were measured, and by means of reciprocal plots (inserts) extrapolated to infinite protein concentrations. Note the difference in scale between A and B.

(Figure 4). In contrast, addition of ANS to a solution of apophosphorylase results in a rapid rise in fluorescence, followed by a slow phase. Subsequent addition of glycerophosphate caused the fluorescence to decrease, but this was again a slow process (Figure 4). Such slow changes in fluorescence during probe protein interaction have been observed previously in membrane preparations (Freedman and Radda, 1969) and in phosphoribosyladenosine triphosphate synthetase (Klungsøyr, 1971).

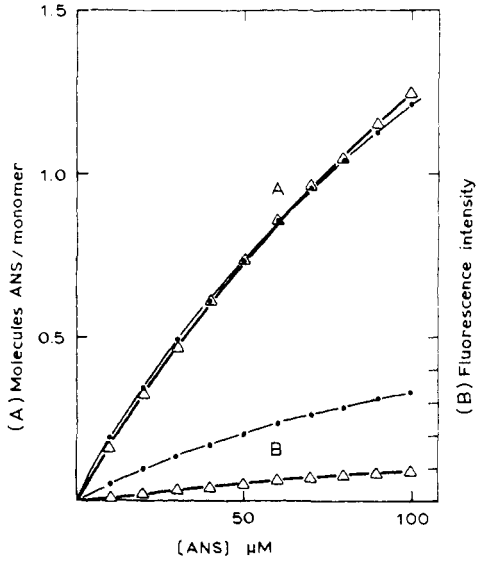

FIGURE 3: ANS binding (A) and fluorescence (B) with apophosphorylase (●) and phosphorylase (△). The protein concentration was 0.5 mg/ml in a 30 mM Tris-HCl–30 mM mercaptoethanol–50 mM NaCl buffer (pH 7.2). A 5 mM solution of ANS was added in 5-μl portions from a microsyringe. For apophosphorylase the additions of ANS were so spaced that a stable fluorescence intensity was obtained before the next addition. To determine the amount of ANS bound the fluorescence intensities were compared to standards, extrapolated to infinite protein concentration. The temperature was 10°, the exciting wavelengths were 380 and 390 nm, and the emitted light wavelengths were 470 and 490 nm for apophosphorylase and phosphorylase, respectively.

Glycerophosphate decreased the fluorescence intensities from mixtures of ANS with phosphorylase and apophosphorylase both. This effect was not due to the increase in ionic strength caused by glycerophosphate. As shown in Figure 5, increase of NaCl from the basal 50 mM to twice that value did not lead to a decrease in fluorescence comparable to that given by 20 mM glycerophosphate with either protein. The affinities for glycerophosphate seem to be similar for both proteins, and close to 8 mM at 10°.

AMP had a powerful effect on ANS–phosphorylase fluorescence, but affected ANS–apophosphorylase fluorescence relatively weakly, as shown in Figure 6.

Seery and Anderson (1972) have shown that AMP decreases the affinity of phosphorylase for ANS, with perhaps

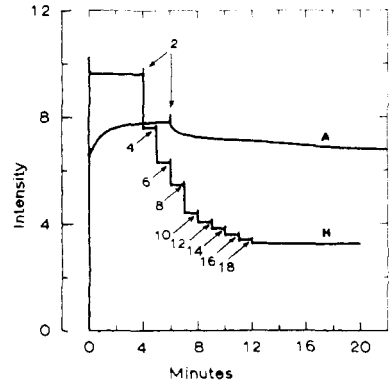

FIGURE 4: Time course of fluorescence change after addition of ANS to phosphorylase (H) or apophosphorylase (A), and after addition of glycerophosphate to the ANS containing mixtures. ANS was added at zero time, and additions of glycerophosphate were made at the points indicated to bring the millimolar concentrations to the levels shown with numbers in each case. The concentrations of protein were 0.4 mg/ml and of ANS 50 μM. The solvent was 30 mM Tris-HCl–30 mM mercaptoethanol buffer (pH 7.2). In the experiments with the apoenzyme the buffer also contained 100 mM NaCl. The temperature was 22°, the exciting wavelengths were 380 and 390 nm, and the emitted light wavelengths were 470 and 490 nm for apophosphorylase and phosphorylase respectively. The scales are arbitrary and not comparable.

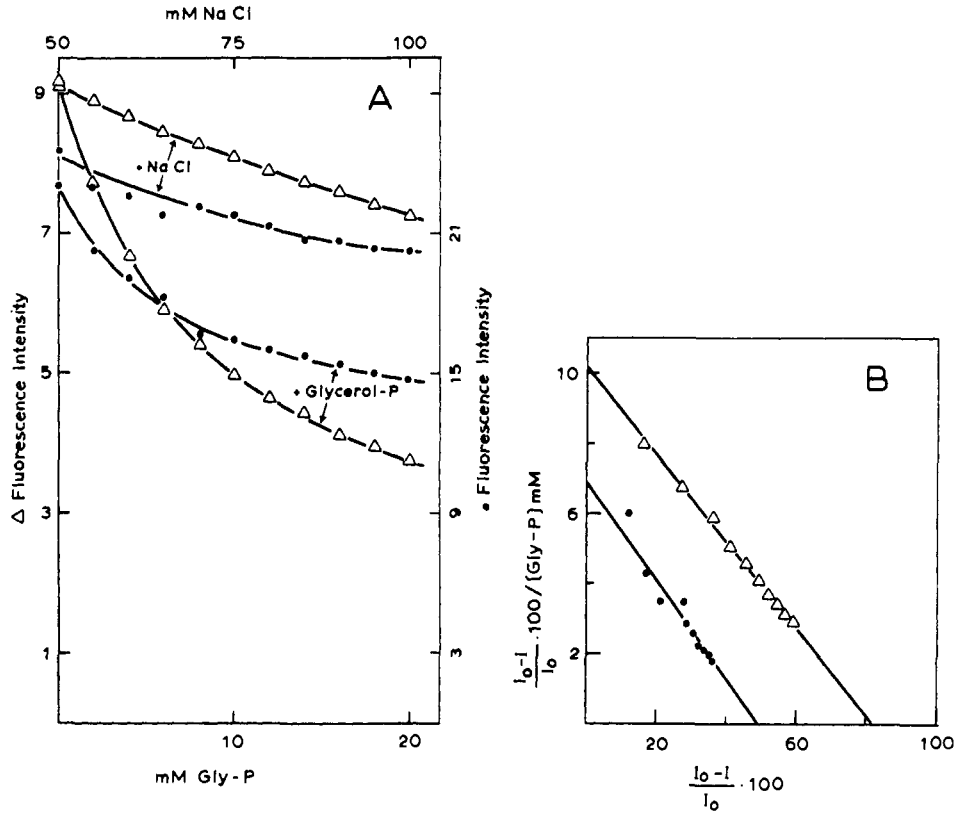

FIGURE 5: (A) Effect of glycerophosphate and NaCl on the fluorescence intensities of mixtures of ANS and apophosphorylase (●) and phosphorylase (△). (B) Scatchard plots of the glycerophosphate effects. The protein concentrations were 0.5 mg/ml, and the ANS concentrations were 50 μM, in a 30 mM Tris-HCl–30 mM mercaptoethanol–50 mM NaCl buffer (pH 7.2). The temperature was 10°. The exciting wavelengths were 380 and 390 nm, and the emitted light wavelengths were 470 and 490 nm for apophosphorylase and phosphorylase, respectively. In the case of apophosphorylase, reaction mixtures containing the appropriate concentrations of glycerophosphate or NaCl were preincubated for 1 hr at 10° before reading the fluorescence intensities. In the experiments with phosphorylase, glycerophosphate or extra NaCl was added stepwise to the ANS–protein reaction mixture from a microsyringe, and the fluorescence was read after each additon.

little change in the quantum yield of the bound probe. We carried out experiments in which we tested the effects of AMP and of glycerophosphate on the μM ANS fluorescence intensities when bound to phosphorylase or apophosphorylase (Figure 7). In both proteins, the reciprocal fluorescence intensities extrapolated to infinite protein concentrations were practically unaffected by the presence of 20 mM glycerophosphate or 200 μM AMP. This indicates that the quantum yields are unaffected by the ligands, but the method cannot determine whether a ligand causes loss in intensity by decreasing the number of ANS sites in the proteins, or by increasing the dissociation constant of the probe protein complex.

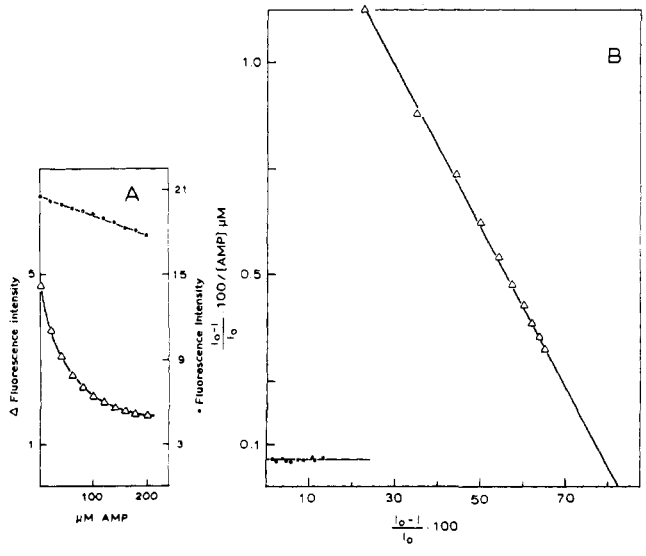

FIGURE 6: (A) Effect of AMP on the fluorescence intensities of mixtures of ANS and apophosphorylase (●) and phosphorylase (△). (B) Scatchard plots of the AMP effects on the two proteins. The experiments were carried out as described in the legend to Figure 5, and in the Experimental Section.

Shaltiel *et al.* (1966, 1969) and Hedrick *et al.* (1969) have described the resolution of phosphorylase *b* from its cofactor pyridoxal-5′-P in the presence of imidazole citrate and cysteine. They postulate a two-step process, with a deformation of the protein conformation preceding the removal of pyridoxal-5′-P. They believed that the deforming process is accompanied by a dissociation, and used this to study the first step in the process. The resolution may be studied by means of the simultaneous loss of enzyme activity, or by the changes in optical properties. The difference in fluorescence quantum yield between ANS bound to phosphorylase and to apophosphorylase may perhaps offer a new tool to study the resolution process of phosphorylase. A typical experiment is presented in Figure 8. In this experiment phosphorylase was added to the resolving mixture plus ANS in the fluorimeter cuvet, and the changes in fluorescence were monitored. The fluorescence intensity immediately rose to an intermediate value, and then continued rising more slowly until a maximum was reached within 5 min. In nondeforming solvents phosphorylase–ANS mixtures immediately reach a stable level of fluorescence, while the fluorescence of ANS–apoenzyme mixtures continues to rise some time after mixing

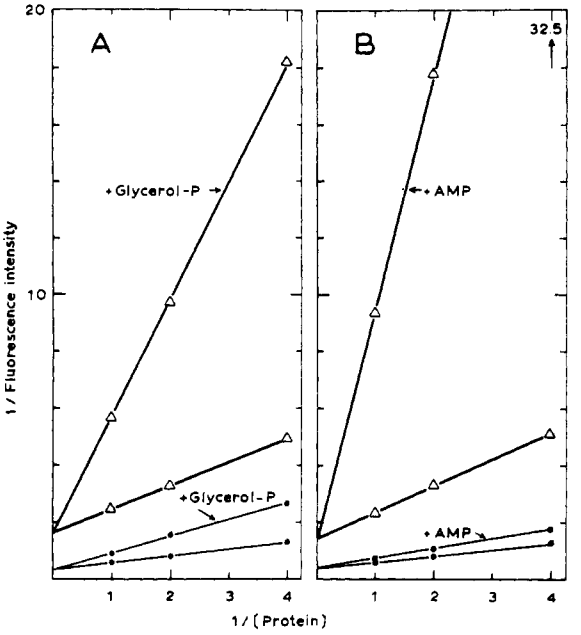

FIGURE 7: Double reciprocal plots of fluorescence intensity *vs.* protein concentration for apophosphorylase (●) and phosphorylase (△). The experiments were carried out as described in the legend to Figure 5, except that the concentration of ANS was 4 μM, and the protein concentrations were 4, 2, or 1 mg/ml: (A) the effect of 20 mM glycerophosphate; (B) the effect of 200 μM AMP.

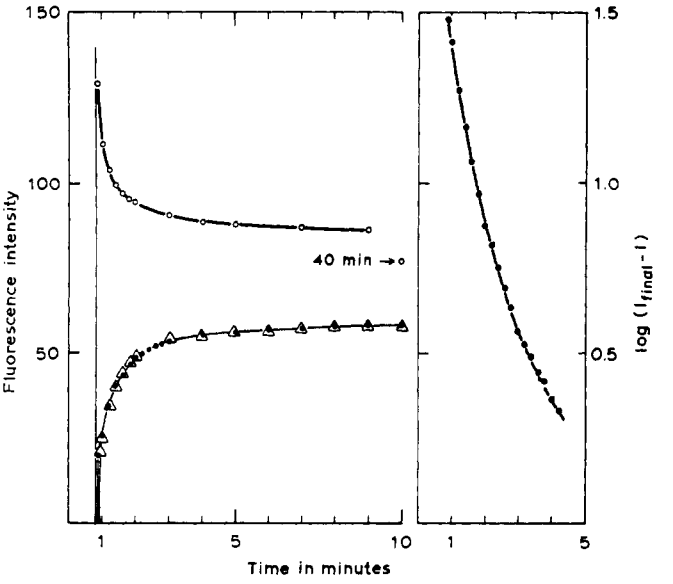

FIGURE 8: The change in fluorescence of the ANS phosphorylase complex under conditions that promote resolution (●). Initially the cuvet contained 0.4 M imidazole, 0.2 M cysteine-HCl, and citric acid to pH 6.2. The ANS concentration was 50 μM, and the temperature was 10°. The recorder reading was adjusted to zero, and then protein was stirred into the solution to a concentraion of 0.5 mg/ml: (△) an identical experiment, but with 200 μM AMP present; (○) a similar experiment with apophosphorylase. In the right-hand part of the graph, the logarithm of the difference between the maximal fluorescence intensity and the intensity at a given time has been plotted against time for the experiment with phosphorylase in the absence of AMP. The exciting wavelength was 380 nm, and the emitted light wavelength was 470 nm.

of the probe with the protein (see Figure 4). Part of the slow rise after adding phosphorylase to the ANS containing resolving mixture might therefore be due to this slow reaction between ANS and the apoenzyme. However, when apoenzyme was added to the resolving reagent in the presence of ANS, the fluorescence intensity immediately rose to a very high level, then slowly decreased toward, but never quite reached, the level of the reaction mixtures with the same initial amount of phosphorylase *b*. It is perhaps justified to consider the slow rise in fluorescence of ANS with phosphorylase in the resolving mixture as a consequence of a step in the deforming process. Remarkably, this process is not influenced by AMP. It is quite rapid, with an initial half-life of 30 sec, and the rate is independent of temperature (Figure 9). The experiments shown in Figure 9 also illustrate another property of the ANS–phosphorylase and ANS–apophosphorylase complexes: the strong dependence of fluorescence on temperature. This temperature effect is considerable also under mild, nondeforming conditions, for example, in Tris-acetate–mercaptoethanol–NaCl buffer (pH 7.2).

Addition of pyridoxal-5′-P to a mixture of apophosphorylase and ANS in a dilute cysteine buffer initiates a slow decrease in fluorescence intensity (Figure 10). The process obeys a first-order law, with a half-life at 22° of approximately 13 min. When AMP is present the process becomes biphasic, with an initial rapid decrease in fluorescence, followed by a slower, first-order process. The AMP effect is not seen when glycerophosphate is present in the reaction mixture.

Discussion

The fluorescence quantum yield of the complex between ANS and phosphorylase is only about one-third of that of the ANS–apoenzyme complex. The emission maximum lies at shorter wavelengths for the latter than for the former, 470 nm for the apoenzyme–ANS complex against 490 nm for the phosphorylase–ANS complex. Even if these data are somewhat inaccurate because of the use of uncorrected spectra, large differences undoubtedly exist.

The binding of ANS to phosphorylase *b* is accompanied by a sharp increase in fluorescence intensity. Additions of ligands that decrease fluorescence lead to rapid drops in intensity. Whatever intermediary states are involved, these must be short-lived on a macroscopic time scale, and probably structurally well defined. The existing or induced fit between protein and ligands is good or easily achieved.

When ANS is bound to the apoenzyme, establishment of an equilibrium situation requires several minutes. Dissociation aggregation processes are probably not the reason for the slow interaction between the apoenzyme and its ligands. AMP, which causes aggregation of the apoenzyme (Hedrick *et al.*, 1966), has little effect upon the fluorescence of the ANS complex of the apoenzyme, while glycerophosphate causes a much greater reduction in the fluorescence of the complex. In the presence of this ligand the apoenzyme is known to be monomeric (Hedrick *et al.*, 1966). Rather, one may speculate that the apoenzyme possesses a larger number of permitted conformational states than the holoenzyme. The induction of fit by perhaps summation of random changes in side chain or backbone conformation apparently is a time-consuming process in the case of ANS binding, and even more so for the accommodation of glycerophosphate.

The difference in fluorescence quantum yield between the apo- and holophosphorylase ANS complexes is a potential tool for the study of enzyme–pyridoxal-5′-P resolution and reconstitution.

Attempts to use this tool in resolution experiments gave results that are difficult to interpret, also in view of existing information about this process. In resolving buffer containing ANS the addition of phosphorylase led to an immediate in-

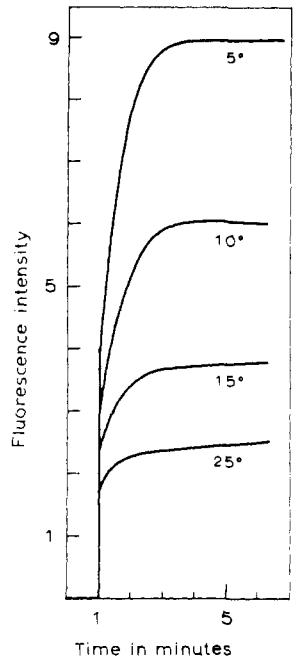

FIGURE 9: Effect of temperature on the rate of increase in fluorescence intensity in a mixture of ANS and phosphorylase in 0.2 M imidazole, 0.1 M cysteine-HCl, and citric acid to pH 6.2. The concentrations of protein and ANS were 0.5 mg/ml and 50 μM, respectively. The fluorescence of the ANS containing solvent was adjusted to zero, and protein was stirred into the mixture as shown by abrupt increases in fluorescence. The exciting wavelength was 380 nm, and the emitted light wavelength was 470 nm.

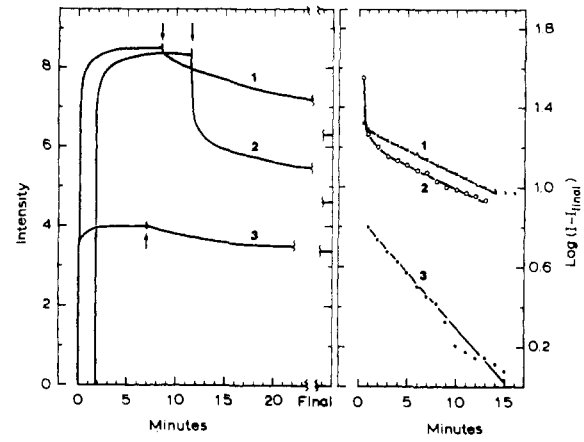

FIGURE 10: Decrease in fluorescence of the ANS–apophosphorylase complex during reconstitution with pyridoxal-5′-P. Curve identification: (1) 0.4 mg/ml of apoprotein in 30 mM cysteine–Tris buffer at pH 7.2 in a total volume of 2.5 ml. ANS was added initially to a concentration of 60 μM. At the point indicated with an arrow 2 μl of a 10 mM pyridoxal-5′-P solution was added. The recorder tracing has been copied in the left-hand part of the graph. In the right-hand part of the graph the logarithm of the difference between the fluorescence intensity at a given time and the final intensity has been plotted *vs.* time after pyridoxal-5′-P addition. The scale has been shifted so that the first minute includes the moment of pyridoxal-5′-P addition. (2) As in 1, except that the reaction mixture contained 1 mM AMP from the beginning. (3) As in 1, except that the reaction mixture contained 30 mM glycerophosphate. The exciting wavelength was 280 nm, and the emitted light wavelength was 470 nm.

crease in fluorescence, then a second phase slower rise in intensity. The first, abrupt increase must obviously be caused by ANS binding to unresolved phosphorylase. It is known (Hedrick *et al.*, 1966) that under the conditions of our experiments, resolution takes place with a half-completion time of approximately 3 min (10°). After 10 min, therefore, a large proportion of the protein must be in the apo form. However, the second phase process that actually takes place has a much higher rate (Figure 8). At 10° it is about six times as rapid as the complete resolution process. Part of the second phase increase in fluorescence might also be due to a slow binding of ANS to the apoenzyme such as that seen under mild conditions at neutral pH. In the resolving buffer, however, preformed apoenzyme binds ANS very rapidly, and actually later slowly undergoes rearrangement to some form that gives less fluorescence. It may therefore be justified to ascribe the fluorescence change after the initial interaction between ANS and the holoenzyme to a process preceding resolution. AMP does not affect the fluorescence change, and it consequently also precedes dissociation of phosphorylase into subunits. This may be a direct demonstration of distortion of subunits within the aggregate, before dissociation.

The finding that the temperature has little effect on the rate with which the fluorescence increases after the first immediate binding phase (Figure 9) is interesting in connection with what kinds of bonds are altered in the initial part of the distortion process. It is possible that breaking of hydrophobic bonds near a bound ANS molecule leads to such rearrangements around the probe that the fluorescence quantum yield increases.

Experiments on reconstitution of apophosphorylase with pyridoxal-5′-P were carried out in a cysteine Tris buffer because glycerophosphate affects the interaction between ANS and the apo- and holoenzyme. The addition of stoichiometric amounts of pyridoxal-5′-P to apophosphorylase led to a decrease in fluorescence intensity. This decrease followed first-order kinetics, and the half-life at 22° was 13 min. As shown by Hedrick *et al.* (1966) the reaction rate was very sensitive to temperature. The reconstituted enzyme reached a specific activity of 80–100% of the mother enzyme.

A biphasic fluorescence change was seen when AMP was present during reconstitution. The initial, rapid, phase was present in experiments with all preparations of the apoenzyme, but the fraction of the total change that took place during the rapid phase varied somewhat from preparation to preparation.

These findings may be interpreted in several ways: AMP may interact with the apoenzyme and an equilibrium situation be established. One state in this equilibrium may reconstitute more rapidly after pyridoxal-5′-P binding than the others. Another possibility is that different populations of apoenzyme conformation exist, and AMP binding affects one or a few of these so that reconstitution after pyridoxal-5′-P binding proceeds rapidly. Above a small excess, the concentration of pyridoxal-5′-P apparently has little effect on the rate of fluorescence change.

Glycerophosphate, itself a ligand of both apo- and holoenzyme, seems to accelerate the change in fluorescence during reconstitution. With this ligand the process remains single phase, first order. AMP has no effect on the pattern of fluorescence change when glycerophosphate is also present. Experiments that indicated the absence of an AMP effect on reconstitution (Hedrick *et al.*, 1966) were made in a glycerophosphate buffer.

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The Protoporphyrin–Apoperoxidase Complex. Photooxidation Studies†

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ABSTRACT: Protoporphyrin IX, a photosensitizer, was found to combine in 1:1 stoichiometry with apoisozyme C of horseradish peroxidase. The complex at pH 9 and 25° was irradiated with visible light. After photooxidation to an uptake of 2 mol of O_2/mol of apoenzyme, the visible absorption spectrum remained unchanged, indicating that the complex was still intact. The porphyrin-free photooxidized apoenzyme was found to be homogeneous on disc gel electrophoresis at pH 4.5, but was less cationic than the native molecule. Spectrophotometric titrations revealed that the ability of the apoenzyme to recombine with hemin was altered markedly after photooxidation. This effect was accompanied by a lowered enzymatic activity, which reached only 25% of the normal value in the presence of 100-fold molar excess of hemin. Amino acid analysis of the 2-O_2 photoproduct indicated that only one histidyl residue had been lost. Tryptic peptide maps of native and photooxidized apoenzyme were prepared. Of the five Pauly-positive peptides observed on maps of the unphotooxidized protein, one (peptide I) was conspicuous by its absence on maps of the 2-O_2 photoproduct. Peptide I was isolated, subjected to amino acid analysis, and found to be a pentadecapeptide containing one histidyl residue. Our results suggest that the selectively photooxidized histidine in protoporphyrin–apoperoxidase is actually located in the heme binding site of peroxidase itself.

The iron-free derivative of heme, protoporphyrin IX, is a potent photosensitizing agent that acts *via* the formation of singlet molecular oxygen (Dalton *et al.*, 1972). We have been exploring the possibility of using proto[1] as a specific photosensitizer for probing the active sites of hemoproteins in which heme can be replaced by proto. The rationale behind this approach is the following: photooxidizable residues lying close to the porphyrin ring should be identifiable on the basis of their greater susceptibility to attack by excited oxygen than residues far removed from the porphyrin binding site. Experiments to test this idea were carried out on the model complex proto–apomyoglobin by Breslow *et al.* (1967), and more recently by Mauk and Girotti (1973b). One particular residue in the myoglobin sequence, His-93, was found to be unusually photoreactive (Mauk and Girotti, 1973b). This observation is consistent with the fact that His-93 is known to coordinate directly with heme iron in crystalline myoglobin (Kendrew, 1962). The high degree of selective modification observed with proto–apomyoglobin indicated that the technique described might be applicable to other hemoproteins.

We have recently undertaken photooxidation studies on the proto–horseradish apoperoxidase complex with the object of probing the enzyme's active site. Although horseradish peroxidase has been the subject of extensive kinetic studies in the past, until recently relatively little work has been done on the amino acid sequence or the conformation of this heme-containing glycoprotein (Welinder *et al.*, 1972; Welinder and

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[1] Abbreviations used are: proto, protoporphyrin IX; apoMb, sperm-whale apomyoglobin; Tos-PheCH$_2$Cl, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; HRP, horseradish peroxidase (isozyme C unless specified otherwise); apoHRP, apoperoxidase; apoHRP(1-O_2), apoHRP(2-O_2), apoperoxidase photooxidized to an uptake of 1 and 2 molar equiv of oxygen, respectively, in the presence of 1 molar equiv of proto; unphotooxidized apoHRP, native apoenzyme that was never in contact with proto.