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Influence of Substrates and Effectors on the Binding of 1-Anilino-8-naphthalenesulfonate by Glycogen Phosphorylase*

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ABSTRACT: The binding of 1-anilino-8-naphthalenesulfonate (ANS) to skeletal muscle glycogen phosphorylases *a* and *b* has been studied using a fluorescence method. Phosphorylase *a*, a tetramer, binds ANS more tightly than phosphorylase *b*, a dimer. The titration behavior of both forms of the enzyme at pH 7.1–7.2 under two conditions of ionic strength (0.03 and 0.23 M) indicates the presence of multiple binding sites with overlapping affinity. At low ionic strength (0.03 M), the average dissociation constant for the binding of less than 1 mole of ANS/protomer is approximately 10 μM for phosphorylase *a* and 50–70 μM for phosphorylase *b*. Increasing the ionic strength of the buffer to 0.23 M reduces the affinity of both phosphorylases *a* and *b* for ANS by about tenfold. Substrates and modifiers of enzymatic activity decrease the fluorescence of solutions containing ANS and phosphorylase *b* by as much as 85%. For the phosphorylase *b*–ANS complex, AMP, an activator, and glucose 6-phosphate, an inhibitor, are effective at the lowest concentrations followed by inorganic phosphate and glucose 1-phosphate. Less specific decreases in fluorescence are observed upon addition of salt; 0.4 M NaCl quenches the fluorescence of solutions of phosphorylase *b* and ANS by about 75%. Direct binding measurements were also made using equilibrium dialysis. A good correlation was found between the decrease in the fluorescence intensity and the decrease in the average number of ANS molecules bound either to phosphorylase *a* in the presence of AMP, or to phosphorylase *b* in the presence of AMP or glucose 6-phosphate. All of the effects observed are consistent with a noncompetitive model in which the binding of substrates or modifiers decreases the affinity of the sites which bind ANS.

Rabbit muscle glycogen phosphorylase (EC 2.4.1.1) exists in two states of aggregation: phosphorylase *b*, a dimer inactive in the absence of AMP,[1] and phosphorylase *a*, a tetramer active in the absence of this nucleotide. The monomer has the following well recognized sites: an active site which binds substrates, a site which binds AMP, a specific seryl residue which can be phosphorylated in the *b*-to-*a* reaction, and a site which binds pyridoxal 5′-phosphate by weak covalent bonds. Binding of substrates increases the affinity of phosphorylases *a* and *b* for AMP and *vice versa*. References to the original literature can be found in the review by Cori (1969). Phosphorylase also binds several dyes including 1-anilino-8-naphthalenesulfonate (ANS) (Stryer, 1965), bromothymol blue (BTB) (Ullmann *et al.*, 1964), and 2-methylanilinonaphthalene-6-sulfonate (MNS) (Birkett *et al.*, 1970). This report describes the binding of ANS to phosphorylases *a* and *b* and the interaction which occurs between the ANS sites and the sites which bind substrates and modifiers of enzymatic activity.

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[1] Abbreviations used are: AMP, adenosine 5′-monophosphate; ANS, 1-anilino-8-naphthalenesulfonate; BTB, bromothymol blue; glucose-1-P, glucose 1-phosphate; glucose-6-P, glucose 6-phosphate; K_d, dissociation constant; MNS, 2-methylanilino-6-sulfonate.

Materials

Phosphorylase *b* was prepared from frozen rabbit muscle essentially according to the method of Fischer *et al.* (1958) and recrystallized two to three times. Phosphorylase *a* was prepared from phosphorylase *b* using purified phosphorylase *b* kinase (Krebs *et al.*, 1964). Nucleotides were removed from solutions of the enzyme by treatment with acid-washed Norit. The ratio of absorbances ($A_{260}:A_{280}$) was less than 0.56 for all preparations of phosphorylases *a* and *b*. Enzymatic assays were carried out following the procedure of Hedrick and Fischer (1965) except that 0.05 M sodium glycerophosphate was used instead of the maleate buffer. All preparations had a specific activity of at least 76 μmoles of P_i from glucose-1-P per min per mg when assayed in the presence of AMP. The sedimentation coefficients determined for both forms of the enzyme corresponded to published values (Seery *et al.*, 1967; DeVincenzi and Hedrick, 1967). The protein concentration was determined spectrophotometrically using the absorbancy index at 278 nm (1%, 1 cm) of 11.9 (Appleman *et al.*, 1963). Molar values were calculated using a molecular weight of 92,500 for the phosphorylase monomer (Seery *et al.*, 1967; Ullmann *et al.*, 1968).

The magnesium salt of 1-anilino-8-naphthalenesulfonate (ANS) was prepared according to Weber and Young (1964). Magnesium was removed from solutions of ANS by passage over a column of Dowex 50 in the hydrogen form. The effluent was neutralized with Tris. ANS concentrations were calculated using the value 4.95×10^3 for the molar absorption coefficient at 350 nm (Weber and Young, 1964). Glucose-1-P (Sigma Chemical Co.) was recrystallized from ethanol after precipitation of the contaminating inorganic phosphate with Ba^{2+}. AMP and glucose-6-P (Calbiochem) were used without further treatment. All other chemicals were obtained from commercial sources and were reagent grade. Glass-distilled water was used to prepare all solutions.

Methods

Most of the fluorescence measurements were made using a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation wavelength of 366 nm corresponded to the isosbestic point found when the spectrum of free ANS was compared to that of ANS in the presence of phosphorylase *b*. The wavelength of emission was 520 nm. The slit widths of excitation and emission were 2 and 5 nm, respectively. The observed fluorescence intensities were first corrected for the emission of the enzyme and of free ANS. The correction for protein fluorescence was less than 3% at concentrations of ANS greater than 52 μM, while that for free ANS was never greater than 2%. The intensities were then corrected for the absorption of incident light by the enzyme using the relation

$$I_{cor} = I_{obsd} \frac{[1 - 10^{-OD_1}]}{[1 - 10^{-(OD_1 + OD_2)}]} \frac{(OD_1 + OD_2)}{OD_1}$$

where the subscripts 1 and 2 refer to the optical densities of the ANS and of the protein at 366 nm, respectively. The correction factor ranged from 1.03 to 1.09.

Titration Procedure. A stock solution of phosphorylase (2 ml) was placed in a 1-cm cuvet. The ANS solution was added to the cuvet from a microsyringe. The contents were mixed gently with a polyethylene rod and the fluorescence was recorded after each addition. The total dilution never exceeded 10% and was usually $\leq 5\%$. A parallel titration was carried out using 0.1 M H_2SO_4 and a solution of quinine, the fluorescence standard, which had an optical density equal to that of the ANS solution at the exciting wavelength. A 1-cm cell was used for solutions which had a total optical density of less than 0.5. Solutions containing higher concentrations of ANS were examined in a 2-mm cell.

The relationship between the fluorescence intensity of ANS when it is present exclusively in the bound form (I_b) and the intensity of the matching quinine standard (I_q) was determined at a single concentration of ANS. In a typical experiment, the fluorescence of a 14 μM solution of ANS was recorded at four to five protein concentrations ranging from 20 to 50 μM in phosphorylase monomer. The reciprocal values of the corrected fluorescence intensities ($1/I_{cor}$) were graphed as a function of reciprocal protein concentration ($1/P$). The data were fitted to a straight line using the method of least squares. I_b was obtained from the value of $1/I_{cor}$ when $1/P$ was equal to zero (Jonas and Weber, 1970). The ratio $I_b:I_q$ was used to calculate the values of I_b at higher concentrations of ANS.

For each concentration of ANS, the number of moles of ligand bound per mole of phosphorylase monomer ($\bar{n}$) was calculated from the relation: $\bar{n} = (I_{cor}:I_b)(X_0:P_0)$, where I_{cor} is the observed fluorescence, and I_b is the fluorescence under the same experimental settings of an equal concentration of ANS which is completely bound to phosphorylase, and $X_0:P_0$ is the ratio of total ligand to total protein concentration. The concentration of free ligand, X, is then calculated from the relation $X = X_0 - \bar{n}P_0$.

Dialysis Equilibrium Experiments. ANS binding to phosphorylase was studied by dialysis equilibrium using enzyme concentrations of 3.1–4.6 mg/ml and total ANS concentrations of 60–80 μM. All test systems contained 20 mM Tris-HCl and 2 mM EDTA, pH 7.1–7.2 (22°); additional components included 1–3 mM 2-mercaptoethanol in solutions of phosphorylase *b* and 0.2 M KCl in solutions of phosphorylase *a*. The dialysis cells, purchased from LaPine Scientific Co., were made of Lucite and had a capacity of 1 ml/sector. Membranes were cut from cellulose tubing (A. H. Thomas) which was boiled in several changes of 1% HAc followed successively by 1% $NaHCO_3$, dilute EDTA, and a final treatment with water. The filled dialysis cells were wrapped in aluminum foil to exclude light and placed on a wrist action shaker in an air conditioned room (20–22°). Equilibrium was attained within 18 hr. The physical state of phosphorylase *b* appeared to be unchanged after 18 hr of treatment with 80 μM ANS at 22° as judged by the schlieren pattern in sedimentation velocity. The absorbance of the initial protein solution was determined using a Cary 15 spectrophotometer. The small dilution (1–5%) of the protein by the buffer in the dialysis membrane was ignored. Before sampling, the cells were examined with an ultraviolet light to insure that the green fluorescence of bound ANS was observed only in the protein sector. The ANS concentrations in samples from both sides of the membrane were determined from the absorbance at 366 nm. The ratio of absorbances ($A_{366}:A_{350}$) was used to obtain a value of 4.55×10^3 for the molar absorption coefficient at 366 nm.

Results

Binding Curves. The binding of ANS to both phosphorylases *a* and *b* is accompanied by a considerable fluorescence enhancement. The quantum yield of the dye determined by integration of the corrected emission spectrum increases approximately 90-fold and the emission maximum shifts from 515 to 490 nm.

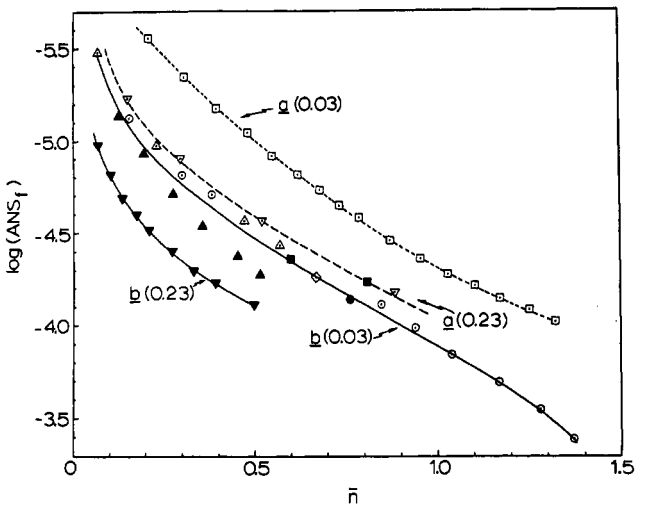

FIGURE 1: Binding of ANS by the monomer of phosphorylases *a* and *b*. Numbers in parentheses indicate the ionic strength of the solvent. Experiments were carried out at 25° in a buffer containing 20 mM Tris-HCl, 30 mM 2-mercaptoethanol, and 2 mM EDTA ± 0.2 M KCl (pH 7.1–7.2), except as noted. The protein concentration ranged from 3.2 to 4.9 mg per ml. The symbols represent different preparations of the enzyme: phosphorylase *b*, titrated at $\Gamma/2 = 0.03$ (○, △, ▲) and $\Gamma/2 = 0.23$ (▼), and phosphorylase *a*, at $\Gamma/2 = 0.03$ (□, no 2-mercaptoethanol in buffer) and $\Gamma/2 = 0.23$ (▽). The curve for phosphorylase *b* ($\Gamma/2 = 0.03$) was drawn using the results from one preparation (⊙); only data points obtained in the 0.2-cm cell are graphed. Dialysis experiments carried out at protein concentrations of 3–5 mg/ml and total ANS concentrations of ~80 μM are indicated by ● for phosphorylase *b* ($\Gamma/2 = 0.03$, 1 mM 2-mercaptoethanol) and ■ for phosphorylase *a* ($\Gamma/2 = 0.23$, no 2-mercaptoethanol in buffer).

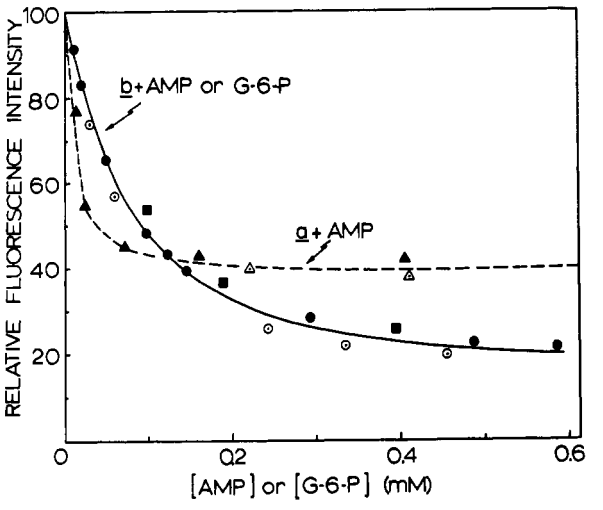

FIGURE 2: Effect of varying concentrations of glucose-6-P and/or AMP on the fluorescence emission of ANS adsorbates of phosphorylases *a* and *b*. The concentration of phosphorylase monomer was 37–50 μM (3.4–4.7 mg/ml), while that of ANS was 40–80 μM. The solvent contained 20 mM Tris-HCl, 30 mM 2-mercaptoethanol, and 2 mM EDTA ± 0.2 M KCl at pH 7.1–7.2 (25°), except as noted. Phosphorylase *a* was titrated with AMP in solvents with $\Gamma/2 = 0.03$ (△, no 2-mercaptoethanol) and $\Gamma/2 = 0.23$ (▲); the initial values of $\bar{n}$ were 0.8 (△) and 0.7 (▲). Phosphorylase *b* was titrated at $\Gamma/2 = 0.03$ with AMP (○, ●) and glucose-6-P (■); initial values of $\bar{n}$ were 0.3 (○) and 0.5 (●, ■). The total dilution by added ligands was less than 2%. G-6-P is glucose-6-phosphate.

The results of fluorimetric titrations of phosphorylases *a* and *b* with ANS in solutions with ionic strengths of 0.03 and 0.23 M are presented in Figure 1. Five different preparations of phosphorylase *b* and three different preparations of phosphorylase *a* were examined. The binding curve for phosphorylase *b* ($\Gamma/2 = 0.03$ M) was drawn using the data obtained from one preparation; representative points from all experiments are included for comparison. The titration behavior of both forms of the enzyme under the two conditions tested clearly indicates the presence of multiple binding sites with overlapping affinity. End points for the titrations cannot be detected at integral values of $\bar{n}$, even though, in the case of phosphorylase *b* ($\Gamma/2 = 0.03$ M), more than 2 log units in free ligand concentration are spanned. Scatchard plots of the data at low ionic strength (0.03 M) reveal that there are at least two classes of sites; the slopes obtained from points below $\bar{n} = 0.8$ correspond to average dissociation constants of approximately 10 μM for phosphorylase *a* and 50–70 μM for phosphorylase *b*. Increasing the ionic strength of the buffer to 0.23 M reduces the affinity of both phosphorylases *a* and *b* by about 10-fold. The results of equilibrium dialysis experiments on phosphorylase *a* ($\Gamma/2 = 0.23$ M) and *b* ($\Gamma/2 = 0.03$ M) at total ANS concentrations of ~80 μM are included in Figure 1. The good agreement between the two types of binding measurements indicates that the relative quantum yield which was obtained at very small $\bar{n}$ is constant at least up to $\bar{n} = 0.8$.

Influence of Substrates and Effectors on the Fluorescence Emission of Phosphorylase b–ANS Complexes. Addition of substrates and modifiers of enzymatic activity to solutions of phosphorylase *b* and ANS results in a decrease in the observed fluorescence (Figure 2) which is not accompanied by a shift in the emission spectrum. The effect of AMP was found to be independent of the initial degree of saturation of the enzyme with ANS from $\bar{n} = 0.3$ to 0.5. In the presence of either the activator, AMP, or the inhibitor, glucose-6-P, the fluorescence could be reduced to 15% of the initial value; concentrations of 70 μM produced 50% of the maximum decrease. Higher concentrations of either inorganic phosphate (4.3 mM) or glucose-1-P (10 mM) were required to yield quenching comparable to 70 μM AMP. In Figure 3 the data at concentrations of ligand which greatly exceed that of the enzyme are analyzed according to the noncompetitive model of McClure and Edelman (1967). The plot of the intensity difference, $I_0 - I$, as a function of $(I_0 - I)/M_0$, where M_0 is the total concentration of the modifier of fluorescence, yields the following apparent dissociation constants (K_{app}): 4 mM for P_i, 9 mM for glucose-1-P, and 0.1 mM for AMP or glucose-6-P.

The apparent dissociation constant has the following relationship to the dissociation constant (K_d) of the binary enzyme–modifier complex (McClure and Edelman, 1967).

$$K_{app} = \frac{\left(\frac{P_0 + X_0}{K_f} + 1\right)K_d}{\frac{P_0 + X_0}{K'_f} + 1}$$

K_f and K'_f are the dissociation constants for the release of ANS from the binary and ternary complexes, respectively. P_0 and X_0 represent the total concentrations of enzyme and ANS, respectively. Thus K_{app} is generally different from K_d. When $K_f < K'_f$, $K_{app} > K_d$; when $K_f = K'_f$, $K_{app} = K_d$; and when $K_f > K'_f$, $K_{app} < K_d$.

Unfortunately direct binding data on the interaction of phosphorylase *b* with substrates and modifiers under our experimental conditions are not available. At 25° in a buffer

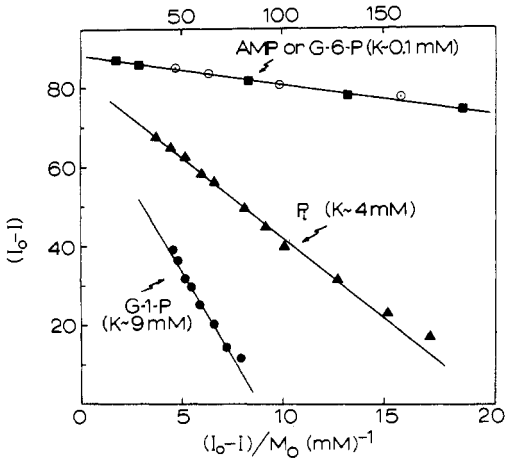

FIGURE 3: Relationship between the fluorescence quenching of phosphorylase *b*–ANS complexes and the binding of substrates and effectors. The test system contained 44 μM phosphorylase *b* monomer and 80 μM ANS in the solvent of Figure 2 without KCl ($\Gamma/2$ = 0.03). The initial value of $\bar{n}$ was 0.5. The initial fluorescence intensity (I_0) was corrected for the decrease (maximum of 2%) upon dilution by glucose-1-P. The addition of other ligands resulted in a decrease in I_0 of less than 2%. Values for the apparent dissociation constants were obtained from the slope of the least-squares line which gave the best fit to the data. The symbols represent titrations with the following ligands: AMP (○), glucose-6-P (■), P_i (▲), and glucose-1-P (●).

TABLE I: Dialysis Equilibrium Experiments: Correlation between the Binding of ANS to Phosphorylases *a* and *b* and the Fluorescence Quenching Induced by Substrates and Effectors.

Enzyme	Treatment[a] (mM)	Expt	$\bar{n}/\bar{n}_0$[b]	Expt	I/I_0[d]
Phosphorylase *b*	AMP (2)	3	0.28 (0.08)[c]	2	0.14 (0.01)[c]
Phosphorylase *b*	G-6-P[e] (4)	3	0.27 (0.07)	1	0.13
Phosphorylase *a*	AMP (0.4)	2	0.37	2	0.40 (0.02)

[a] The buffer contained 20 mM Tris-HCl and 2 mM EDTA at pH 7.1–7.2 (22°); additional components were 1–3 mM 2-mercaptoethanol for solutions of phosphorylase *b* and 0.2 M KCl for solutions of phosphorylase *a*. Total ANS concentration was 40–80 μM. [b] $\bar{n}_0$, the number of ANS molecules bound in the absence of added ligands, was determined from an average of four and five determinations for phosphorylases *a* and *b*, respectively. [c] Root-mean-square deviation of the measurements; the values of $\bar{n}/\bar{n}_0$ for the two experiments using phosphorylase *a* were identical. [d] I_0 is the initial fluorescence intensity before addition of AMP or glucose-6-P. The experiments are those described in Figure 2. [e] Glucose 6-phosphate. Other experimental conditions are given in the Methods.

with an ionic strength approximately four times that used in the present study, Kastenschmidt *et al.* (1968a) reported a K_d of 0.37 mM for AMP. Using the fluorescence increase which is observed when AMP is added to solutions of phosphorylase *b* and MNS, Birkett *et al.* (1970) have estimated the K_{app} for AMP in the presence of Mg^{2+} to be 40 μM. The affinity of the enzyme for low molecular weight substrates has not been determined in the absence of ligands which exhibit heterotropic cooperativity. Apparent K_m values of 2.2 mM for P_i and 3.7 mM for glucose-1-P have been determined from kinetic measurements at 30° in the presence of 0.6% glycogen and 1 mM AMP (Madsen and Schechosky, 1967). In order to fit their kinetic data to an allosteric model, Kastenschmidt *et al.* (1968b) assumed a K_d for glucose-1-P of 5.5 mM at low AMP concentration (0.01 mM). No quantitative data are available for the binding of glucose-6-P to the enzyme.

Influence of Substrates and Modifiers on the Fluorescence Emission of Phosphorylase a–ANS Complexes. AMP decreases the fluorescence intensity of phosphorylase *a*–ANS adsorbates by a maximum of 60–65% (Figure 2). Since increasing the ionic strength of the initial buffer reflects changes in the degree of saturation of the enzyme with ANS, similar end points are reached with or without added salt. At a concentration of 10 mM (total $\Gamma/2$ ~0.03 M), glucose-1-P, glucose-6-P, and P_i each quench the fluorescence of the phosphorylase *a*–ANS mixture by about 30%. The fluorescence intensity of the phosphorylase *b*–ANS mixture is decreased as follows: 42% by glucose-1-P, 60% by P_i, and 85% by glucose-6-P. Since glucose-6-P does not inhibit the activity of phosphorylase *a* (Morgan and Parmeggiani, 1964) and at 1 mM increases the relative mobility of phosphorylase *b* in polyacrylamide gel electrophoresis more than that of phosphorylase *a* (Hedrick *et al.*, 1969), there may be a difference in the affinity of the two forms of the enzyme for the substrate analog. The decrease in fluorescence cannot be explained simply on the basis of ionic strength as the effect of P_i is four to five times that of NaCl at comparable ionic strength. For complexes of fructose 1,6-diphosphatase and ANS, Aoe *et al.* (1970) have reported that the fluorescence emission is quenched 4% by NaCl ($\Gamma/2$ = 0.009 M) and 9% by $MgSO_4$ ($\Gamma/2$ = 0.002 M).

Effect of Salt on Phosphorylases a and b Adsorbates. Nonspecific decreases in fluorescence emission are observed when NaCl is added to the adsorbates. A maximum decrease of 35% for the phosphorylase *a*–ANS complex and 70% for the phosphorylase *b*–ANS complex is observed as $\Gamma/2$ is increased from 0.03 to 0.43 M. As discussed above, salt appeared to decrease the degree of saturation of both forms of the enzyme with ANS.

Correlation between Binding of ANS to Phosphorylases a and b and the Fluorescence Quenching Induced by Substrates and Effectors. When the concentrations of the enzyme and ANS are held constant, the fluorescence decrease can result either from a decrease in the quantum yield of bound ANS or from a decrease in $\bar{n}$. In order to distinguish between these two possibilities, direct binding measurements were carried out. Table I presents the results of equilibrium dialysis experiments on phosphorylase *b* ($\Gamma/2$ = 0.04 M) and phosphorylase *a* ($\Gamma/2$ = 0.24 M) in the presence of glucose-6-P and/or AMP. High salt concentration was not used to damp out charge effects for the phosphorylase *b* system since, at $\Gamma/2$ = 0.2 M, $\bar{n}$ is too low to be measured accurately. There is clearly a direct relationship between the decrease in the fluorescence and the decrease in the average number of ANS molecules bound.

Since the concentrations of AMP used in the dialysis experiments are approximately 70× and 5× the K_d for the phosphorylase *a* (*cf.* Helmreich *et al.*, 1967) and the phosphorylase *b* complex (Kastenschmidt *et al.*, 1968a,b), the decreased affinity for ANS appears to be the result of the binding of

AMP to the activator site. Most likely, the decreased binding of the fluorophore in the presence of glucose-6-P also reflects the binding of the inhibitor to phosphorylase *b*.

Discussion and Conclusions

These data show that the dissociation of bound ANS is the *major* cause of the decrease in fluorescence obtained on the addition of modifiers to solutions of ANS and either phosphorylase *a* or *b*. With phosphorylase *a*, the 60% decrease in fluorescence accompanies a 63% decrease in $\bar{n}$. In the case of phosphorylase *b*, the 86% decrease in fluorescence accompanies a 72 ± 8% decrease in $\bar{n}$. The existence of multiple binding sites and the difficulty in accurately measuring the small values of $\bar{n}$ obtained on the addition of modifiers to solutions of phosphorylase *b* and ANS prevent determination of the quantum yield of the ternary complex in the noncompetitive model. Our results are in conflict with the recent report of Schneider *et al.* (1971) who concluded that metabolites quench the fluorescence of the phosphorylase *b*–ANS mixtures primarily by lowering the quantum yield of bound ANS.

The observation that phosphorylase *a* binds ANS more strongly than phosphorylase *b* is similar to the results which have been obtained with other lyophilic dyes such as bromothymol blue (Ullmann *et al.*, 1964) and 2-methylanilinonaphthalene-6-sulfonate (Birkett *et al.*, 1970). The quenching of the fluorescence of phosphorylase *b*–ANS mixtures by AMP is in direct contrast to the enhanced fluorescence which accompanies the addition of the activator to solutions of phosphorylase *b* and MNS. While AMP decreases the average number of ANS molecules bound to both phosphorylases *a* and *b*, it increases the affinity of *b* for BTB and is without effect on *a*. Neither the binding of BTB nor fluorescence of MNS in the presence of phosphorylase *b* and AMP exceeded that of phosphorylase *a* under comparable conditions; in the case of MNS, the fluorescence increases could be reversed by glucose, an inhibitor. Birkett *et al.* (1970) demonstrated a direct relationship between the increase in fluorescence emission at constant MNS concentration and the per cent tetramer in solutions of either phosphorylase *b*, Mg^{2+}, and AMP, or mixtures of phosphorylases *b* and *a*. In the absence of tetramer formation, either AMP or a divalent metal ion alone had little effect on the fluorescence of the phosphorylase *b*–MNS complex (Birkett *et al.*, 1971). The higher affinity of phosphorylase *a* for ANS may be a consequence of the dimer to tetramer conversion; however, the fluorescence decreases which we report can not be correlated with the state of aggregation of the enzyme. At the concentration of protein, AMP, and other effectors used in this study, phosphorylase *a* exists mainly as a tetramer (Helmreich *et al.*, 1967; Seery *et al.*, 1967). Although phosphorylase *b* is partially associated by AMP at 25° (Kastenschmidt *et al.*, 1968a; Wang *et al.*, 1970), the dimer is essentially the only species present under the experimental conditions in glucose-6-P (Seery *et al.*, 1967; Fischer *et al.*, 1967), glucose-1-P (Fischer *et al.*, 1967), and P_i (Assaf and Graves, 1969). Thus, the phosphorylase *b* dimer, the *a* tetramer, and, possibly, the *b* tetramer have similar binding capacity for ANS in the presence of AMP. Other observations which appear to be unrelated to quaternary structure include both the increase and the decrease in the proton relaxation of Mn^{2+} bound to phosphorylase *b* induced by AMP and glucose-1-P, respectively, as well as the partial quenching (10%) by AMP of the fluorescence of phosphorylase *b* modified with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (Birkett *et al.*, 1971).

There are at least two explanations which can be offered to account for the results obtained in this study. A model could be constructed assuming several hypothetical conformational states which have a low affinity for ANS and which are stabilized by the various ligands. Indeed, there is evidence which suggests that AMP and glucose-1-P favor different structural forms of phosphorylase *b* (Jokay *et al.*, 1965; Damjanovich and Kleppe, 1966; Birkett *et al.*, 1971). On the other hand, without additional data to support a conformational change for each substrate and effector used in this study, a simpler alternative is to propose that the hydrophobic sites which bind ANS cooperate directly with the sites which bind other ligands on phosphorylase *b* and that the interactions between ANS sites and either P_i or glucose-1-P sites are weakened or lost upon conversion to the *a* form of the enzyme.

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Phosphoenolpyruvate Carboxytransphosphorylase. Study of the Catalytic and Physical Structures†

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ABSTRACT: Carboxytransphosphorylase catalyzes the conversion of phosphoenolpyruvate, orthophosphate, and CO_2 to oxaloacetate and inorganic pyrophosphate (oxaloacetate reaction), and when CO_2 is excluded, the irreversible conversion of phosphoenolpyruvate and phosphate to pyruvate and pyrophosphate (pyruvate reaction). The enzyme occurs in three enzymatically active forms: tetramer, dimer, and monomer. The tetramer (crystalline) has an apparent molecular weight of $\sim 4.0 \times 10^5$ g/mole and an $s^0_{20,w}$ of 15.2 S. The dimer is formed from the tetramer in the presence of substrates of the oxalacetate reaction, *i.e.*, phosphoenolpyruvate, orthophosphate, and CO_2. It has a molecular weight of $\sim 2 \times 10^5$ g/mole and an $s_{20,w}$ of ~ 10 S. In the absence of CO_2 the tetramer–dimer transformation does not occur. The monomer is obtained from the tetramer by dialysis against low ionic strength buffer and has a molecular weight of $\sim 1 \times 10^5$ g/mole and $s^0_{20,w} = 7.1$ S. The monomer likewise is converted to the dimer (10 S) in the presence of substrates of the oxaloacetate reaction. The monomer may be a modified form of the enzyme since it is not activated by thiols in the oxalacetate reaction and thus has a lower specific activity than does the tetramer. In the absence of thiols, tetramer and monomer have the same activity in both the oxaloacetate and pyruvate reactions. All three forms, tetramer, dimer, and monomer, were shown to be active by sedimentation through substrates of the oxaloacetate reaction in the presence of malate dehydrogenase and by measurement of the oxidation of NADH with a photoelectric scanner. Electron micrographs show that the tetramer has a rhomboid structure with twofold symmetry, the dimer is rod-like, and the monomer is roughly spherical. In 6 M guanidine and thiol a single component is obtained with a molecular weight of $\sim 0.9 \times 10^5$ g/mole, indicating that the monomer is probably composed of a single peptide chain. At the normal levels of use in an assay, the active species of carboxytransphosphorylase appear to be dimeric in the oxalacetate reaction, and either monomeric or tetrameric in the pyruvate reaction. The catalytically discrete species of different molecular weights of carboxytransphosphorylase may be important in the regulation of the activity of the enzyme, but it is not yet apparent in what manner.

Phosphoenolpyruvate carboxytransphosphorylase (pyrophosphate:oxaloacetate carboxylase (phosphorylating), EC 4.1.1.38) catalyzes reactions 1 and 2. The purification and

$$\text{P-enolpyruvate}^1 + P_i + CO_2 \rightleftharpoons \text{oxaloacetate} + PP_i \quad (1)$$

$$\text{P-enolpyruvate} + P_i \longrightarrow \text{pyruvate} + PP_i \quad (2)$$

crystallization of the enzyme from propionic acid bacteria have been described by Lochmüller *et al.* (1966) and Wood *et al.* (1969a). Its occurrence has been reported in only one other organism, *Entamoeba histolytica* (Reeves, 1970).

It has been determined in previous studies by Lochmüller *et al.* (1966), Wood *et al.* (1966; 1969b), Davis *et al.* (1969), and Willard *et al.* (1969) that the optimal activity of the enzyme in reaction 1 is attained by incubating carboxytransphosphorylase in 1 mM mercaptoethanol and including 0.1 mM Co^{2+} in the assay. Optimal activity of reaction 1 in the direction of formation of oxaloacetate is ~ 24 μmoles/min per mg of protein. Optimal activity in reaction 2 is achieved in the absence of thiol and is ~ 3 μmoles of pyruvate formed/min per mg of protein. When thiol is included in the pyruvate reaction, the enzyme is inhibited 50% (Davis *et al.*, 1969). In the absence of both thiol and cobalt, the rates of the two reactions are about the same.

A mechanism of reaction has been proposed by Wood *et al.* (1969b) and the stereochemistry of reaction 1 has been determined by Rose *et al.* (1969) showing that CO_2 adds to the *si*

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[1] P-enolpyruvate represents phosphoenolpyruvate.