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A Calorimetric Study of the Interactions between Phosphorylase *b* and Its Nucleotide Activators†

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ABSTRACT: Interactions of glycogen phosphorylase *b* with AMP and IMP have been studied by calorimetry at two different temperatures: 18 and 25°. Calorimetric titration of the enzyme with AMP shows a biphasic curve suggesting that the enzyme contains two sets of AMP binding sites with different affinity. The high affinity sites are essentially filled at 0.3 mM AMP whereas significant nucleotide binding at the low affinity sites occurs only when AMP concentration exceeds 1 mM. Since phosphorylase *b* is maximally activated at 1 mM AMP, the low affinity sites are probably not directly involved in the AMP activation of the enzyme. The heat of interaction between phosphorylase *b* and 0.5 mM AMP has been measured calorimetrically as a function of the enzyme concentration. This heat per mole of the enzyme increases as the enzyme concentration increases at 18° but not at 25°. Sedimentation velocity experiments have shown that phosphorylase *b*, in the presence of 0.5 mM AMP, undergoes reversible changes between a dimeric and a tetrameric species. These results suggest that the heat of phosphorylase *b*–AMP interaction at 18° consists of the heat of AMP binding and that of the tetramer formation. The enthalpies of the AMP binding and the tetramer formation, as well as the equilibrium constant for the enzyme association calculated from the calorimetric data at 18°, are respectively 2.6 kcal/mol, −85 kcal/mol, and 2.1×10^5 M^{-1}. At 25°, the enthalpy of AMP binding to phosphorylase *b* is found to be −13.2 kcal/mol. In contrast to AMP, IMP does not induce phosphorylase *b* association either at 25 or at 18°. The enthalpy of IMP binding to phosphorylase *b* is about −11 kcal/mol, independent of the temperature. Changes in free energy, enthalpy, and entropy accompanying the AMP induced phosphorylase *b* association at 18° have been calculated from the calorimetric data to be respectively −7.1 kcal/mol, −85 kcal/mol, and 268 eu. These thermodynamic properties indicate that the driving force for the AMP induced enzyme association is enthalpic. Thermodynamic parameters for bindings of AMP and IMP to phosphorylase *b*, both at 18 and 25° have also been calculated. The results indicate that conformational changes in phosphorylase *b* induced by AMP at 18° are different from those induced by IMP and those induced by AMP at 25°.

Skeletal muscle glycogen phosphorylase *b* depends on AMP for catalytic activity (Green and Cori, 1943). Both V_{max} and K_m of the enzyme are functions of the nucleotide concentration. The interactions between the nucleotide activator and phosphorylase *b* have been studied under a variety of conditions by kinetic and equilibrium binding techniques (Madsen and Shechosky, 1967; Buc and Buc, 1967; Kastenschmidt *et al.*, 1968a,b). Using conformational probes, it has been shown that the binding of AMP to phosphorylase *b* is accompanied with conformational changes (Ullman *et al.*, 1964; Buc and Buc, 1967; Birkett *et al.*, 1971), as well as with an enhanced tendency of the enzyme to associate into a tetramer (Appleman, 1962; Sealock and Graves, 1967; Wang *et al.*, 1968). In addition to AMP, IMP and several other nucleotides also activate phosphorylase *b* (Cori *et al.*, 1938; Mott and Bieber, 1968; Okazaki *et al.*, 1968). These activators differ from AMP in that they do not significantly affect the K_m of phosphorylase *b* toward glucose 1-phosphate, nor do they cause the enzyme to associate into a tetramer unless the substrate glucose 1-phosphate or phosphate is also present (Black and Wang, 1968; 1970; Mott and Bieber, 1970). These observations have led to the suggestion that conformational changes of phosphorylase *b* induced by AMP differ from those induced by IMP or other nucleotides (Black and Wang, 1968, 1970).

In spite of extensive studies on nucleotide activation of phosphorylase *b*, information concerning the thermodynamics of the interactions are meager. Kastenschmidt *et al.* (1968a,b) have calculated some thermodynamic parameters for AMP binding to phosphorylase *b* based on kinetic and equilibrium binding data. In our laboratory, microcalorimetric measurements of the heat of interactions between phosphorylase *b* and AMP have been initiated and some preliminary results have been reported (Wang *et al.*, 1970). The present communication concerns itself mainly with the thermochemistry of phosphorylase *b*–AMP interaction and that of the AMP induced enzyme association. The results on calorimetric study of phosphorylase *b*–IMP interaction are also included.

Materials and Methods

Glycogen phosphorylase *b* was prepared according to the procedure of Fischer and Krebs (1962), and was recrystallized four times in 0.04 M glycerophosphate–0.03 M cysteine buffer (pH 6.8) containing 1 mM AMP and 10 mM $Mg(OAc)_2$. For calorimetric and ultracentrifugal studies, phosphorylase *b* preparations were used within 1 week after the fourth crystal-

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lization. The use of freshly prepared enzyme was important since the heat of phosphorylase *b*–AMP interaction as determined by calorimetry and the AMP induced enzyme association were both dependent upon the age of the enzyme preparation.

Cysteine hydrochloride, sodium glycerophosphate, β-mercaptoethanol, shellfish glycogen, adenosine 5′-monophosphate, inosine 5′-monophosphate, and glucose 1-phosphate were obtained from Sigma Chemical Co. Glycogen was treated with Norit A to remove the contaminating nucleotide (Sutherland and Wosilait, 1956). Glucose 1-phosphate was freed of the contaminating inorganic phosphate by $Ba(OAc)_2$ precipitation. Subsequently, Ba^{2+} was removed as $BaSO_4$ by the addition of K_2SO_4 and glucose 1-phosphate was then crystallized as the dipotassium salt in an aqueous ethanol solution.

For calorimetric measurements, the enzyme crystals were centrifuged and dissolved in 0.04 M glycerophosphate–0.03 M β-mercaptoethanol–0.1 M KCl buffer (pH 6.9). The solution was treated with Norit A to remove AMP and then dialyzed against two changes of the same buffer for at least 24 hr. The nucleotide solutions used for calorimetric measurements were prepared immediately prior to the measurements by dissolving the sodium salt of the nucleotides in the last enzyme solution dialysates. The solutions were then titrated to the original pH of the dialysate.

The concentration of phosphorylase *b* was determined spectrophotometrically at 280 nm (Appleman *et al.*, 1963).[1] The enzyme activity was measured in the direction of glycogen synthesis at 30° according to the procedure described by Illingworth and Cori (1953). For routine enzyme assay, the reaction mixture contained 16 mM glucose 1-phosphate and the reaction followed a first-order progress curve with respect to glucose 1-phosphate. For the kinetic study of IMP activation of phosphorylase *b*, a high concentration of glucose 1-phosphate (60 mM) was used and the reaction was essentially zero order with respect to glucose 1-phosphate. Thus, initial velocities for IMP activation of phosphorylase *b* were calculated as OD 660/5 min.

Calorimetric measurements were carried out with a batch type calorimeter, Beckman 190 B. Bi-compartmented vessels were used in all of the experiments reported in the present study. The two compartments of the sample vessel contained separately 7 ml of enzyme solution and 7 ml of the nucleotide solution. In the control vessel, the compartment for the enzyme solution was filled with 7 ml of the dialysate instead so that the heat of nucleotide dilution was automatically corrected for. Separate experiments were made to determine the heat of enzyme dilution. For most experiments corrections for the heat of enzyme dilution were negligible. The instrument and the reaction vessels were calibrated with the heat of reaction between HCl and NaOH. The calibration procedure outlined in the instruction manual for the instrument was closely followed.

Sedimentation velocity experiments were carried out with a Spinco Model E analytical ultracentrifuge using the schlieren optics.

Results

The change in heats accompanying binds of AMP to glycogen phosphorylase *b* was determined by microcalorimetry at 18° as a function of the nucleotide concentration. Figure 1 shows that this calorimetric titration curve of phosphorylase *b* with AMP is biphasic; *i.e.*, it consists of two plateaus. With nucleotide concentrations below 1 mM, the plot has the appearance of a ligand binding curve reaching its ligand saturation at about 0.2 mM AMP. The titration curve appears sigmoidal, therefore suggestive of cooperative bindings of AMP to the enzyme. Since the phosphorylase *b* may undergo reversible association in the presence of AMP, the apparent cooperative AMP binding may have arisen from both changes in enzyme conformations or enzyme associations. The present calorimetric data, however, are not adequate for a detailed analysis of this cooperative ligand binding. With the increase in AMP concentrations above 1 mM, a further rise in the heat of the reaction occurs and the plateau of the second phase of the titration curve is reached at an AMP concentration of approximately 2 mM (Figure 1).

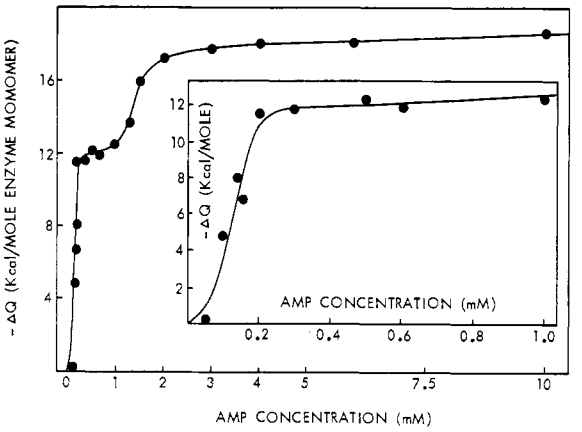

FIGURE 1: Calorimetric titration curve for phosphorylase *b* with AMP at 18°. The enzyme concentration used in calorimetric measurements was 5 mg/ml. Inset: the first phase of the titration curve plotted with an expanded scale.

Phosphorylase *b* has been shown by several groups of workers (Buc and Buc, 1967; Kastenschmidt *et al.*, 1968a; DeVincenzi and Hedrick, 1970; Avramovic-Zikic and Madsen, 1968) to bind two molecules of AMP with dissociation constants of around 0.1 mM. Thus, it suggests that the first phase of the titration curve of Figure 1 represents the binding of these two AMP molecules to the enzyme. The heat of reaction at the plateau of the titration curve, therefore, may be used to calculate the molar enthalpy change of AMP binding at these sites using the value 92,500 as the molecular weight for the monomer of phosphorylase *b* (Seery *et al.*, 1967). The second phase of the titration curve probably arises from bindings of AMP to phosphorylase *b* at additional sites having lower nucleotide affinities. Since this phase of the titration curve appears to occur in a narrow range of the AMP concentration (1–2 mM), binding of nucleotides to these low affinity sites is probably highly cooperative. The calorimetric titration curve, however, cannot be used to calculate the number of the low affinity AMP binding sites on phosphorylase *b*. Since phosphorylase *b* may be fully activated by 1 mM AMP, the low affinity AMP binding sites do not appear to be directly involved in the nucleotide activation of the enzyme. In the present study, only the binding of AMP at the high affinity sites of phosphorylase *b* has been further analyzed.

The calorimetric titration of phosphorylase *b* with AMP was carried out using a constant amount of the enzyme. The use of constant concentration of phosphorylase *b* in the

[1] An absorbance index of 11.7 at 280 nm for a 1% phosphorylase *b* was used in the present work. Other investigators used higher values, around 13 (Buc and Buc, 1967; Huang and Graves, 1970). If the higher value is used, the molar thermodynamic quantities reported in this communication will be increased by 10%.

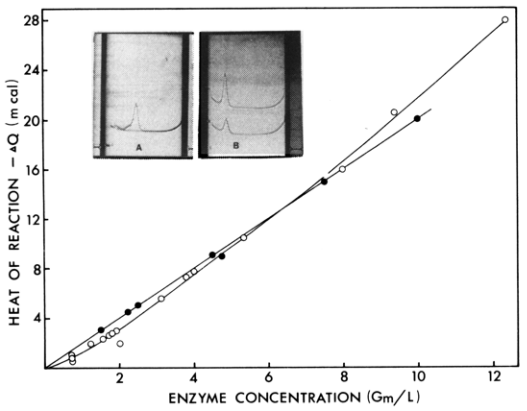

FIGURE 2: The dependence of heats of phosphorylase *b*–AMP interaction upon the enzyme concentration. Total heat of the interaction between phosphorylase *b* and AMP is plotted against the enzyme concentration. The calorimetric measurements were carried out either at 18° with 0.5 mM AMP (○) or at 25° with 1 mM AMP (●). Inset: (A) ultracentrifugal pattern of phosphorylase *b* (6 mg/ml) in the presence of 0.5 mM AMP at 18°; (B) those for phosphorylase *b* at 25° in the presence of 1 mM AMP; upper curve and lower curve are respectively with 5 and 2.5 mg/ml of the enzyme.

calorimetric titration is important because the apparent molar heat of AMP–phosphorylase *b* interaction depends on the enzyme concentration under certain conditions. Figure 2 shows that the increase in the heat of the interaction at 18° with a nucleotide concentration sufficient to saturate the high affinity sites is not proportional to the increase in enzyme concentration, but can be depicted by an upwardly curved line. In contrast, the heat of phosphorylase *b*–AMP interaction at 25° is proportional to the enzyme concentration. Several groups of investigators (Kent *et al.*, 1958; Appleman, 1962; Sealock and Graves, 1967; Kastenschmidt *et al.*, 1968a,b) have shown that phosphorylase *b* may undergo reversible association in the presence of AMP from a dimeric to a tetrameric species, and that this reversible enzyme association is strongly temperature dependent. Thus, one possible explanation for the observed dependence of the apparent molar heat of the phosphorylase *b*–AMP interaction upon the enzyme concentration may be that the enzyme associates at 18°. The measured heat of the interaction, therefore, is the sum of the heat of the nucleotide binding and that of the tetramer formation. Since the relative concentration of the tetramer should increase with an increase in the protein concentration, the apparent molar heat of the reaction is expected to be higher at a higher concentration of the enzyme. This view is supported by the sedimentation velocity experiments. The inset of Figure 2 shows that the schlieren pattern of phosphorylase *b* at 18° in the presence of 0.5 mM AMP contains two diffuse bands indicative of a reversible protein association. In contrast, at 25° phosphorylase *b* in the presence of AMP shows an ultracentrifugal pattern containing one symmetric peak with a sedimentation constant of 8.6 S.

During the course of the present study, it was found that the association property of phosphorylase *b* also depended upon the age of the enzyme preparations. For some enzyme preparations which had been stored under refrigeration over 3 weeks, association into tetramers was indicated by ultracentrifugal examination in the presence of AMP at 25°. Enzymes from these preparations also exhibited a different heat of interaction with AMP as measured by calorimetry. Accordingly, freshly prepared phosphorylase *b* was always used in the present study.

To further analyze the interaction between phosphorylase *b* and AMP and the nucleotide induced enzyme association, it is assumed that the reaction which occurs upon mixing of phosphorylase *b* and AMP may be represented by

$$\text{phosphorylase } b + 2\text{AMP} \rightleftharpoons \text{phosphorylase } b\text{–AMP}_2 \quad (1)$$

$$2\text{phosphorylase } b\text{–AMP}_2 \rightleftharpoons (\text{phosphorylase } b\text{–AMP}_2)_2 \quad (2)$$

Consequently, the calorimetrically determined heat of the reaction between phosphorylase *b* and AMP, Q, would be the sum of the heat of the AMP binding (eq 1), Q_b, and that of the tetramer formation (eq 2), Q_t. Assuming the enthalpy change of the AMP binding and that of the tetramer formation are ΔH_b and ΔH_t, respectively, the following equations may be obtained.

$$Q = [\text{E}]\Delta H_b + [\text{T}]\Delta H_t \quad (3)$$

$$\frac{Q}{[\text{E}]} = \Delta H_b + \frac{[\text{T}]}{[\text{E}]}\Delta H_t \quad (3a)$$

where [E] and [T] are the molar concentration of AMP binding site and that of the tetrameric phosphorylase *b*, respectively. When AMP concentration is sufficiently high so that all of the high affinity AMP binding sites are saturated, eq 2 alone may be sufficient to describe the equilibrium conditions of the various forms of phosphorylase *b*. Accordingly, concentration of the tetrameric phosphorylase *b* may be expressed as a function of the total enzyme concentration.

$$[\text{T}] = \frac{1}{8K}\left(2K[\text{E}] + 1 - \sqrt{4K[\text{E}] + 1}\right) \quad (4)$$

Based on eq 3a and 4, ΔH_b and ΔH_t as well as K, the apparent association constant of tetramerization, may be evaluated from the data in Figure 2. In the present study, an empirical method was used for this purpose. This method consisted of assigning a numerical value for K to calculate [T]/[E] at each protein concentration used in the calorimetric measurement. A set of values for [T]/[E] thus obtained was plotted against Q/[E] according to eq 3a and this plot was then analyzed by a linear regression computer program. The procedure was repeated several times each with a different K value. The computer analyses of the various plots were then compared to obtain a K value giving the best agreement between the experimental data and the straight line plot. In our analyses using this procedure, the K values from 0.5×10^5 M^{-1} with increments of 0.5×10^5 M^{-1} were first analyzed. The result indicated that experimental data agree closest to a straight line when the value for K was 2×10^5 M^{-1}. Subsequently, the analyses were repeated for K values from 1.5×10^5 M^{-1}, with increments of 0.2×10^{-5}–2.5×10^5 M^{-1}. The K value which gave the best fit was found to be 2.1×10^5 M^{-1}. The inset of Figure 3 shows a plot of the experimental results of Figure 2 according to eq 3a using a K value of 2.1×10^5 M^{-1} for the computation. Using this plot, ΔH_b may be computed from the intercept on the vertical axis to be 2.6 kcal/mol and ΔH_t, from the slope, −85 kcal/mol. The deviations of experimental points from linear plots are, however, not pronounced when the value for the equilibrium constant K is changed slightly from 2.1×10^5 M^{-1}. Good agreement to a linear plot may be obtained with the K value set in the range of 1.7–2.5×10^5 M^{-1}. Thus, the values for

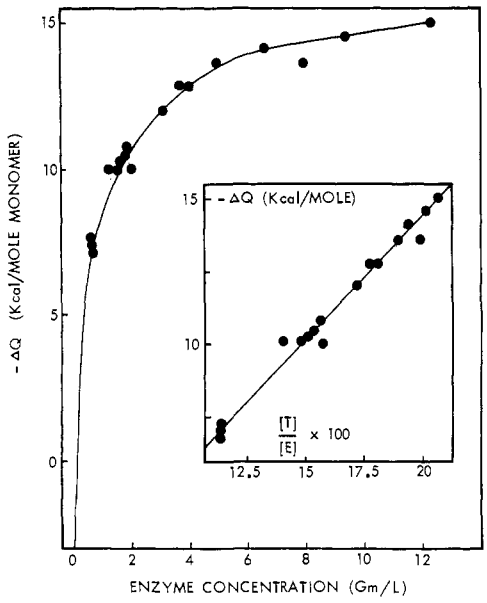

FIGURE 3: The plot of specific heat of phosphorylase *b*–AMP interaction against the enzyme concentration. Solid curve is a theoretical line calculated using values for ΔH_b, ΔH_t and K as respectively 2.6 kcal/mol, −85 kcal/mol, and 2.1×10^5 M^{-1}. Inset: the plot of specific heat of the interaction against the molar ratio of the phosphorylase *b* tetramer to that of the total enzyme binding site. This ratio is calculated assuming a K value of 2.1×10^5 M^{-1}.

ΔH_t and ΔH_b may lie in the ranges of −89 to −83 and 2–3.7 kcal/mol, respectively.

Combining eq 3a and 4 results in the following equations.

$$\frac{Q}{[E]} = \Delta H_b + \Delta H_t \frac{1}{4}\left(1 + \frac{1}{2K[E]} - \sqrt{\frac{1}{K[E]} + \frac{1}{4K^2[E]^2}}\right) \quad (5)$$

$$\frac{Q}{[E]} = \Delta H_b + \Delta H_t \frac{1}{4}\left(1 + \frac{1}{2K[E]} - \sqrt{\left(1 + \frac{1}{2K[E]}\right)^2 - 1}\right) \quad (5a)$$

From these two equations, it is seen that $Q/[E]$ approaches ΔH_b as the concentration of the total enzyme approaches infinite dilution (eq 5a) and that it approaches $\Delta H_b + 1/4\Delta H_t$ when the enzyme concentration becomes very high (eq 5). Figure 3 shows a plot of $Q/[E]$ as a function of enzyme concentration. The solid line passing through most of the experimental points is a theoretical line using the values of 2.6 kcal/mol, −85 kcal/mol, and 2.1×10^5 M^{-1} for ΔH_b, ΔH_t, and K, respectively. It is clear that using these values, eq 5 or 5a gives an adequate representation of the experimental data.

In addition to AMP, IMP activates phosphorylase *b* but the extent of this activation is much lower than that of AMP activation (Cori *et al.*, 1938). The interaction between IMP and phosphorylase *b*, therefore, has also been examined by calorimetry. Figure 4 shows a calorimetric titration curve of phosphorylase *b* with IMP at 25°. It can be seen that IMP concentrations used in the titration far exceeded the concentration of the enzyme. Thus, if IMP binding sites in phosphorylase *b* are equivalent and independent, a linear double reciprocal plot, *e.g.*, reciprocals of the measured heats and IMP concentrations plotted against each other, will be obtained. A double reciprocal plot for the data of Figure 4,

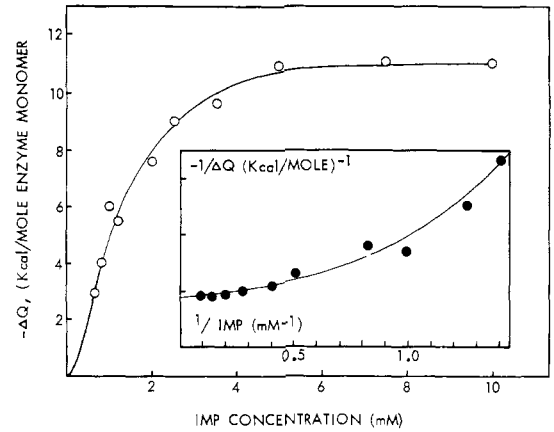

FIGURE 4: Calorimetric titration curve of phosphorylase *b* with IMP at 25°. The enzyme concentration used in the measurement was 4.5 mg/ml. Inset: double reciprocal plot for the data.

however, gives an upwardly curved line (Figure 4, inset) suggesting that the binding of IMP to phosphorylase *b* is cooperative. Although a determination of the intrinsic dissociation constants for IMP from the calorimetric titration curve is difficult, IMP concentration giving 50% of the heat at the plateau region may be taken as an estimation of the average apparent dissociation constant for IMP. The apparent dissociation constant, K_d, obtained from the data of Figure 4 is 1.2 mM. In a separate experiment, IMP activation of phosphorylase *b* was determined at 25° in the presence of 1% glycogen and 60 mM glucose 1-phosphate as a function of the nucleotide concentration. The double reciprocal plot of the activation with respect to IMP concentration showed a slight upward curvature and the nucleotide concentration giving 50% of the maximal activation was 1.30 mM. This value agrees well with the K_d of IMP determined calorimetrically suggesting that the interaction between phosphorylase *b* and IMP is not significantly affected by the substrates of the enzyme.

In addition to the determination of apparent K_d, the enthalpy of IMP binding may be calculated from the titration curve by assuming that there is one IMP binding site per phosphorylase *b* monomer. Figure 4 shows that the enthalpy of IMP binding is −11.1 kcal/mol.

Unlike AMP, IMP does not cause phosphorylase *b* to associate into a tetrameric species (Black and Wang, 1968). The molar heat of phosphorylase *b*–IMP interaction at near saturating concentration of the nucleotide is also independent of the protein concentration either at 18 or at 25°. When phosphorylase concentration was varied from 2 to 11 mg/ml and IMP concentration from 7.5 to 12.5 mM, the enthalpy of binding was found in the range of −11.8 to −10.7 kcal/mol at either 18 or 25°. An average value from ten measurements was −11.12 kcal/mol.

Since the apparent activation constant of IMP, K_{ass}, agrees with the apparent dissociation constant, K_d, determined by calorimetry, a van't Hoff plot for this kinetic parameter may also be used to obtain the enthalpy of IMP binding to phosphorylase *b*. The van't Hoff plot was linear and the enthalpy of the interaction was calculated to be −10.5 kcal/mol, in good agreement with the value determined from calorimetric data. For the determination of the kinetic parameter k_{ass}, initial velocities were measured with, in addition to IMP, 1% glycogen and 60 mM glucose 1-phosphate at pH 6.9.

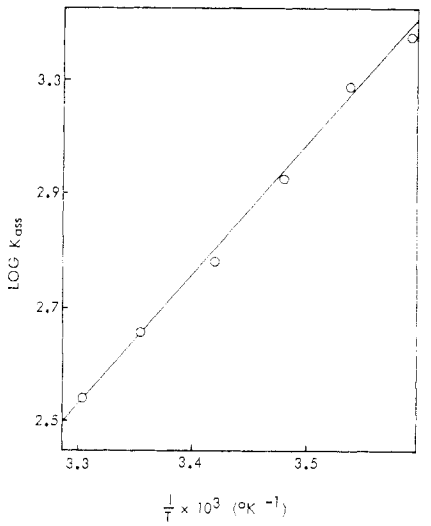

FIGURE 5: The van't Hoff plot for the interaction between phosphorylase *b* and IMP. The association constants, K_{ass}, were determined kinetically as the reciprocals of IMP concentrations providing 50% of the maximal IMP activation. Conditions for the kinetic study are described in the text.

Discussion

Glycogen phosphorylase *b* may undergo reversible association from a dimeric to a tetrameric species in solutions containing AMP (Appleman, 1962; Sealock and Graves, 1967). In the present study, both the enthalpy and the equilibrium constant for this enzyme association are evaluated from microcalorimetric results obtained at 18°. With these calorimetrically determined values, other thermodynamic parameters are calculated and shown in Table I. In view of the uncertainty in the equilibrium constant of the enzyme association, the thermodynamic quantities given in Table I are tentative (see Results). The standard entropy of the enzyme association may lie in the range of −280 to −260 kcal/mol. It can be seen that $\Delta G°$, $\Delta H°$, and $\Delta S°$ are all negative indicating that the driving force for this protein association is enthalpic. Thus, it suggests that hydrophobic interactions probably do not play a dominant role in the tetramer formation. Buc and Buc (1967) have studied the temperature dependence of this AMP induced phosphorylase *b* association by light scattering and ultracentrifugation and arrived at the same conclusion. Since they did not give the values for the various thermodynamic parameters in their report, a comparison between their results and those of the present study is not possible. However, the present results may be compared with the thermodynamic quantities of phosphorylase *a* association at 20° obtained by Huang and Graves (1970) (Table II). Similar to that of phosphorylase *b*, the association of phosphorylase *a* is also driven by enthalpic force. While phosphorylase *a* associates more readily than the AMP bound phosphorylase *b*, its enthalpy of association is less negative than that of the phosphorylase *b* association. Thus, the difference between the association properties of phosphorylase *a* and AMP bound phosphorylase *b* arises mainly from the entropy difference.

TABLE I: Thermodynamic Parameters for Phosphorylase Association.

Enzyme Species	Temp (°C)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)
Phosphorylase *a*[a]	20	−10	−60	−170
Phosphorylase *b*[b]	18	−7.1	−85	−268

[a] From Huang and Graves (1970). [b] In the presence of 0.5 mM AMP.

TABLE II: Thermodynamic Parameters for the Binding of Nucleotides to Phosphorylase *b*.

Nucleotide	Temp (°C)	$\Delta G°$ (kcal/mol binding)	$\Delta H°$ (kcal/mol binding)	$\Delta S°$ (eu/mol binding)
AMP	25	−4.9	−13.2	−27.9
AMP	18	−5.2	+2.6	+26.8
IMP	25	−3.9	−11.1	−24.2
IMP	18	−4.1	−11.1	−24.1

The complex relationships among the AMP binding, the conformational changes, and the reversible protein association of glycogen phosphorylase *b* have been thoroughly analyzed by Kastenschmidt *et al.* (1968a,b) employing kinetic, equilibrium binding, light scattering, as well as ultracentrifugal techniques. They have suggested that phosphorylase *b* shifts its conformation from a T state to a R state in the presence of AMP. At low temperatures, phosphorylase *b* may have an additional conformational state, R′, which has an AMP affinity even higher than that of R state. In addition, they have suggested that the tetramerization of phosphorylase *b* occurs predominantly with the enzyme in the R′ state. Although the calorimetric data in the present study do not lend themselves to a thorough quantities analysis according to the model of Kastenschmidt *et al.* (1968b), they agree with this model in that AMP shows different modes of interactions with phosphorylase *b* at different temperatures: 18 and 25°. Using the calorimetric data for the AMP binding to phosphorylase *b*, the thermodynamic quantities for this reaction have been calculated and shown in Table II. It is clear that the driving forces for AMP binding to the enzyme at 18 and 25° are qualitatively different, entropic and enthalpic, respectively, thus supporting the suggestion that different conformation states are stabilized by AMP at the two different temperatures.

From the preceding discussion, it seems logical to conclude that the interaction of phosphorylase *b* and AMP involves three separate reactions at low temperatures: the binding of the nucleotide, the conformational changes of the enzyme, and the reversible enzyme association. Kastenschmidt *et al.* (1968b) have calculated the enthalpies of AMP binding to phosphorylase *b* using the equilibrium binding data. In their analysis, they have found that ΔH's for AMP binding to the R and R′ forms of phosphorylase *b* are −16.9 and −3.8 kcal/mol, respectively (Kastenschmidt *et al.*, 1968b). In the present study, the calorimetrically determined ΔH's for the AMP binding to phosphorylase *b* at 25 and 18° are respectively −13.2 and 2.6 kcal/mol. Since AMP induces significant enzyme association at 18 but not at 25°, the predominant forms of phosphorylase *b* in the presence of AMP at the high and the low temperatures probably correspond respectively to the R and the R′ forms proposed by Kastenschmidt *et al.* (1968b).

It should be pointed out that studies of temperature effect on the phosphorylase *b*–AMP interaction have resulted in some

conflicting observations. Kastenschmidt *et al.* (1968a,b) and Buc and Buc (1967) have shown that association of phosphorylase *b* in the presence of AMP occurs to significant extent only at temperatures below 13°. On the other hand, ultracentrifugal patterns from several other laboratories (Appleman, 1962; Sealock and Graves, 1967; Wang *et al.*, 1968, 1970) as well as results in the present studies have demonstrated enzyme association at higher temperatures. The reason for this discrepancy is not clear. It has been documented, however, that the association of phosphorylase *b* may be influenced by many factors including the presence of substrates, buffer ions, salts, as well as polyvalent ions.

In addition to the interaction between AMP and phosphorylase *b*, the phosphorylase *b*–IMP interaction has also been examined by calorimetric measurements. Previous kinetic studies on the mechanism of IMP activation have led to the suggestion that IMP induces an enzyme conformation in phosphorylase *b* distinct from that induced by AMP (Black and Wang, 1968). Such suggestion is supported by the study using proton relaxation enhancement techniques (Birkett *et al.*, 1971). In Table II, thermodynamic parameters for the binding of IMP to phosphorylase *b* are also listed. As expected, the ΔG for the AMP binding is more negative than that for IMP binding. This difference in binding strength of the two nucleotides at 25° results mainly from their difference in the enthalpy of binding; *e.g.*, $-\Delta H$ for AMP binding being greater than that of IMP binding by 2 kcal/mol. Since AMP differs from IMP in containing an amino group purported to participate in hydrogen bonding in the enzyme–nucleotide (Okazaki *et al.*, 1968), this difference in the enthalpy of binding may be readily explained on the basis of this structural difference between the two nucleotides. Thus, if IMP and AMP do induce different conformational states in phosphorylase *b* at 25°, these conformational differences are not reflected in the thermodynamic properties of the enzyme–nucleotide interactions. In contrast, the thermodynamic properties of the nucleotide binding of phosphorylase *b* at 18° are significantly different for IMP and AMP (Table II). At this temperature the suggestion that IMP and AMP induce different phosphorylase *b* conformations is strongly supported.

From kinetic studies, it has been suggested previously that, unlike phosphorylase *b*–AMP interaction, phosphorylase *b*–IMP interaction is independent of glucose 1-phosphate concentration (Black and Wang, 1968). This suggestion is supported by the present study. The apparent association constant for the interaction between phosphorylase *b* and IMP is approximately the same when determined by the calorimetric measurement or by the kinetic method. In calorimetric measurements, the substrates are not present in the reaction mixture whereas the kinetic study employs near-saturating amount of the substrates. In addition, the enthalpies of IMP binding to phosphorylase *b* determined by calorimetric and kinetic methods are in good agreement, implying that other thermodynamic properties for the enzyme–IMP interaction are also independent of the substrates.

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