The binding of adenosine 5’-monophosphate to liver glycogen phosphorylase \(a\) (EC 2.4.1.1) has been studied by size exclusion high performance liquid chromatography and isothermal titration microcalorimetry at pH 6.9 over a temperature range of 25 to 35 °C. The results are compared with those of the binding of the same nucleotide to the muscle isozyme and to liver phosphorylase \(b\). Calorimetric measurements in various buffer systems with different ionization levels suggest that protons are released during the binding of the nucleotide. The dimer of liver glycogen phosphorylase \(a\) has been shown to have two equal and independent sites for 5’-AMP, which would correspond to the activator sites identified in the muscle isozyme. The binding constants as well as the changes in Gibbs energy, enthalpy, and entropy per site for 5’-AMP binding were calculated at each temperature. The results show that the major contribution to the negative value of \(AG\) stems from the value of \(\Delta H\) in the range of 25 to 35 °C. The enthalpy change of binding is strongly temperature-dependent, arising from a large negative \(\Delta C_p\) of binding equal to \(-1.45 \pm 0.02 \text{ kJ K}^{-1} (\text{mol of 5’-AMP bound})^{-1}\), which suggests significant changes in the polar and apolar surfaces accessible to the solvent.

Glycogen is the carbohydrate reserve of most metabolically active cells in mammals. It is broken down to produce glucose 1-phosphate in response to the cells’ energy demands by the regulatory enzyme glycogen phosphorylase. In the liver glucose 1-phosphate is converted to glucose to provide fuel for extrahepatic tissues (1), while in muscle, glucose 1-phosphate is metabolized by glycolysis to provide ATP to meet the energy requirements for muscle contraction. The activation of phosphorylase is under hormonal, neuronal, and metabolic control. In response to either nervous or hormonal stimulation the enzyme is converted from the inactive \(b\) form to the active \(a\) form by covalent phosphorylation at serine 14 by the action of phosphorylase kinase. Phosphorylase seems to be activated in form by covalent phosphorylation at serine 14 by the action of the inactive muscle isozyme (3, 4) but is a weak activator of liver phosphorylase \(b\) and binds to it weakly and noncooperatively (5, 6). The phosphorylated forms do not depend upon 5’-AMP for their activity, although this can be enhanced by the addition of 5’-AMP in both the muscle (7) and liver (8, 9) isozymes.

The structure, function, and regulation of the muscle isozyme are better understood than are those of the liver isozyme (7, 10–13). In rabbit muscle phosphorylase crystallographic structures of both the \(a\) and \(b\) forms in their active and inactive states have been determined (14–18). A comparison of the active and inactive structures of the enzyme reveals that phosphorylation leads to the ordering of the N-terminal tail and disordering of the C-terminal tail (19, 20). The different binding sites identified in muscle phosphorylase are the catalytic site, the allosteric activator (N) site, the inhibitor (I) site, and the glycogen storage (G) site. 5’-AMP binds to the N and I sites in the phosphorylase \(b\) dimer of both rabbit muscle (18, 21, 22) and bovine liver (6). The muscle phosphorylated form shows high binding affinity for 5’-AMP to the N site (18, 23), this site alone being occupied at nucleotide concentrations of less than 2 \(\mu\text{M}\) (21, 24).

This work reports the results of studies of the binding of bovine-liver phosphorylase \(a\) to 5’-AMP using size-exclusion chromatography on HPLC (SE-HPLC)1 and high sensitivity titration microcalorimetry. To our knowledge, this is the first time that such a study, using calorimetry, has been carried out with the active form of liver phosphorylase. \(\Delta H\) was found to depend on buffer ionization heats, thus implying proton release upon binding. \(\Delta H\) on association has been measured at several temperatures to obtain the heat capacity change. The large negative \(\Delta C_p\) value obtained is discussed in terms of possible changes in the apolar and polar surfaces accessible to the solvent by the binding of 5’-AMP to phosphorylase \(a\). The results have also been compared with those of the binding of this nucleotide to liver \(b\) form and to the muscle isozyme.

**MATERIALS AND METHODS**

Glycogen phosphorylase \(b\) was prepared from bovine liver by the method described by Hwang et al. (25), with some modifications described by Cámara-Artigas et al. (26). The activity of liver glycogen phosphorylase \(b\) was determined by measuring the inorganic phosphate produced in the phosphorylase-catalyzed reaction of glycogen synthesis at 30 °C, according to the method described by Hedrick and Fischer (27), modified by Appleman et al. (28). This assay was performed in the presence of 0.7 \(\mu\text{M}\) Na\(_2\)SO\(_4\) and 1 \(\mu\text{M}\) 5’-AMP. Specific activities of the purified protein were 40–45 units/mg. In the absence of Na\(_2\)SO\(_4\), the specific activity was between 5 and 10 units/mg. Protein concentration was determined from absorbance measurements at 280 nm using the rabbit muscle enzyme absorbance coefficient \(E_{1\text{cm}}^{280}\) = 13.2 (29). The molecular mass of the dimer was taken as 194 kDa (13).

Phosphorylase \(b\) kinase (EC 2.7.1.38) from rabbit skeletal muscle

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1 The abbreviations used are: SE, size-exclusion; HPLC, high performance liquid chromatography; ITC, isothermal titration calorimetry; Pipes, 1,4-piperazinediethanesulfonic acid.
was obtained from Sigma. Bovine liver glycogen phosphorylase was obtained from phosphorylation of liver phosphorylase b kinase, according to the method described by Cohen (30). Liver phosphorylase a was isolated from the phosphorylation reaction mixture by chromatography on an affinity blue Sepharose column equilibrated in 5 mM Hepes, 1 mM EDTA, 1 mM 2-mercaptoethanol at pH 7 (buffer A). Approximately 250 ml of blue Sepharose in a 5 X 10-cm column were used for 100 mg of enzyme. All subsequent steps were carried out at 4°C and with a flow rate of 60 ml/h. The column was washed with two column volumes of buffer A. Phosphorylase a was then eluted with 10 ml 5'-AMP in buffer A. The 5'-AMP was used in the binding experiments (Pipes, Heps, or 2-glycerophosphate) at pH 6.9 and 4°C. The protein concentration was determined in the same way as that of phosphorylase b. The specific activity of the enzyme in the absence of both 5'-AMP and sulfate was always in the range of 60-65 units/mg at 50 mM Hepes, 1 mM EDTA. This value increased to about 75-80 units/mg in the presence of 5'-AMP.

The concentration of 5'-AMP was determined from absorbance measurements at 295 nm using a molar extinction coefficient of 15.4 10^3 M^-1 cm^-1 (31). Absorbance measurements were carried out in a Beckman DU-7400 spectrophotometer.

All chemicals were of the highest purity available and purchased from either Merck, Sigma, or Boehringer Mannheim. All solutions were degassed and clarified through a 0.45-μm Millipore filter before use. Standard liquid chromatographic materials used were blue Sepharose 6B, Superdex 200 HR 10/30, and Sephacryl G-25 HR 10/10 columns for fast protein liquid chromatography from Pharmacia Biotech Inc.

**Molecular Mass Determinations—Analytical HPLC was carried out on a Beckman apparatus.** Effluents were monitored with a diode array detector (DU168). The fast protein liquid chromatography column was Superdex 200 HR 10/30 from Pharmacia. The column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ribonuclease (13.7 kDa), and blue dextran (2000 kDa) from Sigma. The column was equilibrated in 50 mM Hepes, 50 mM KCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol (buffer B), and with or without 0.1 mM 5'-AMP, at pH 6.9 and 25°C. Two samples of phosphorylase a (11.6 mg/ml) were incubated with or without 0.4 mM of 5'-AMP in buffer B for 1 h at 25°C. After the incubation samples were then applied to the column at a volume of 100 μl.

**Measurement of Ligand/Enzyme Association by SE-HPLC—**The binding of the ligand to the enzyme was observed by SE-HPLC at 25°C, according to the method described by Hummel and Dreyer (32). HPLC was carried out on the apparatus described above. The fast protein liquid chromatography column was a Sephacryl G-25 HR 10/10 from Pharmacia. The dimeric enzyme concentrations were 0.2-0.3 mM in buffer B. The column was equilibrated and eluted with the concentration of 5'-AMP tested in buffer B, which varied from 3 μM to 120 μM. Phosphorylase a, equilibrated with a higher 5'-AMP concentration, was injected onto the column and the absorbance monitored. Phosphorylase a equilibrated at a lower 5'-AMP concentration, although still higher than that used to equilibrate the column, was then reloaded. A typical elution diagram is shown in Fig. 1. The bound ligand elutes with the protein as a positive peak. This is followed by another smaller positive peak, the area of which corresponds to the excess 5'-AMP remaining unbound to the eluted protein at the 5'-AMP concentration of the equilibrated column. With the amount of protein applied to the column, the two peaks in the elution diagram were well separated from each other. The concentration of bound 5'-AMP was determined from the difference between the excess of 5'-AMP added to the protein solution compared to the equilibrated solution and the 5'-AMP concentration corresponding to the second peak. The concentration of free 5'-AMP was that corresponding to the ligand concentration used to equilibrate the column.

**Calorimetric Experiments—**The titrations were performed on an isothermal titration calorimeter (ITC) designed, built and optimized in our laboratory. The characteristics of this instrument are similar to other calorimeters, such as those described by McKinnon et al. (33) and Freire et al. (34), but it is considerably less expensive to build. A full description of this calorimeter, together with a theoretical analysis of heat compensation technique in ITC, will also be published elsewhere. Electrical and chemical calibrations of the calorimeter's responses were made within the same range as those obtained in the calorimetric experiments. Electrical calibration was done by protonation of glycine with HCl in the lower pH range (35). The two calibration methods agreed with each other.

The titration experiments were carried out at 25, 30, and 35°C, as described elsewhere (36). The enzyme solutions and buffers were thoroughly degassed before being loaded into the sample and reference calorimeter cells (volume = 2.9 ml), respectively. Identical volumes (30 μl) of 5'-AMP solution and buffer were injected into the sample and reference cells, respectively. Dual injection compensates for most of the heat effects from nonchemical reaction sources. The heat effect arising from dilution of the 5'-AMP and noncompensated heat effects was measured in a separate experiment in which the sample and reference cells were filled with buffer solution. This concomitant heat effect was subtracted from the observed total heats. The thermal efficiency of the protein dilution was negligible in all cases. Phosphorylase a activity was routinely checked just before and after the calorimetric experiment. Similarly, the pH values of the buffer, 5'-AMP, and protein solutions were controlled at each temperature both before and after the binding reaction.

**Analysis of Calorimetric Titrations—**It is assumed that a dimeric protein has two identical and independent sites for binding the ligand, L, with its characteristic microscopic association constant, K, and molar enthalpy change of binding ΔH₀, and with n₁L being the number of protons taken up by the protein-ligand complex during the binding reaction. In a series of injections of ligand solution into the cell containing the protein, the heat released or absorbed for each injection, Q_n, should be proportional to the increase in the saturation fraction, ΔY, associated with the change in bound ligand concentration, according to the equation

\[ Q_{inj} = 2[P]V[ΔH_0 + n_1 L_1 ΔH_0]ΔY \]  

(Eq. 1)

where [P] stands for the dimer protein concentration in the calorimeter cell, V for the reaction volume, and ΔH₀ for the heat of buffer ionization. Because the sample volume, V, increases in each injection the protein concentration, [P], decreases throughout the titration, the equation above should be rewritten as

\[ Q_{inj} = 2[P]V[ΔH_0 + n_1 (L_1 ΔH_0)](1 + \frac{K[L]}{[L]}) \]  

(Eq. 2)

An iterative, nonlinear least-squares fitting of the experimental data, Q, for a given free-ligand concentration, [L], was used according to the Equation 2. In an ITC experiment only the total ligand concentration, [L]_T, and the total protein concentration, [P], are known. The free ligand concentration after each injection must be calculated from these according to

\[ [L] = [L]_T - [P] + 2K[P][L]_T + 4K[L][P]_T + 2KP - 1/K \]  

(Eq. 3)

Once the convergence criteria are satisfied the ΔH₀, K and n₁L values can be obtained for each temperature. A computer program for specific data analysis has been written including a function optimization based on the Levenberg-Marquardt algorithm (37).

**RESULTS**

**Molecular Mass Determinations—**Molecular mass determination was performed by SE-HPLC at 25°C. The elution volume of the enzyme and the 5'-AMP-protein complex from a Superdex 200 HR 10/30 column indicated that the phosphorylase a and its 5'-AMP complex have a molecular mass of approximately 190 kDa at concentrations in the range of 1 mg/ml. Thus, liver phosphorylase a is a dimer and there is no detectable evidence for association-dissociation phenomena caused by binding of 5'-AMP. The molecular mass of the dimer (8) in contrast to the rabbit muscle isozyme, which is a tetramer at concentrations of a few milligrams per ml (38).

**Measurement of 5'-AMP-Phosphorylase a Association by SE-**
HPLC—The binding of 5′-AMP to the liver-phosphorylase a dimer was observed as a function of the nucleotide concentration by SE-HPLC at 25 °C and pH 6.9 in buffer B. Dimeric enzyme concentrations were from 0.2 to 0.3 mM. As an example, Fig. 1 depicts some elution profiles for the reaction of 5′-AMP with phosphorylase a. The results of this binding study are displayed in Fig. 2 as a plot of the degree of binding, ν, versus free 5′-AMP concentration. Extrapolation of the upper part of the plot leads to a value of 2 mol of 5′-AMP bound per mol of dimeric enzyme at saturation. A Scatchard plot of these values clearly extrapolates to ν = 2 (data not shown) and no systematic deviations from linearity are detected. These results may be taken as evidence for the existence of two identical and independent binding sites. A Hill plot of these experimental values leads to a Hill coefficient of 1.1 ± 0.1, which indicates that binding is noncooperative. The fitting of the SE-HPLC data to this model gives the optimum value \( K = (8.8 ± 0.9) \times 10^4 \) M at 25 °C. The theoretical curve in Fig. 2 results from this estimated binding constant. If the curve in Fig. 2 is compared to that of the binding of 5′-AMP to liver phosphorylase b at 25 °C (6) it is evident that the a form of the enzyme has a higher affinity for 5′-AMP than the b form, since the value of \( K \) is approximately two orders of magnitude higher. However, the active forms of both the liver and muscle isozymes have a similar affinity, the latter having an association constant of \( (1.8 ± 0.2) \times 10^5 \) M⁻¹ (24).

**Calorimetric Titrations**—Fig. 3A shows data from the calorimetric titration of 2.9 ml of 46.5 \( \mu \)M dimeric phosphorylase a in buffer B with a ligand solution containing 2.71 mM 5′-AMP in the same buffer. There are 14 equivalent 30-μl injections (spaced at 5-min intervals) of 5′-AMP solution into the enzyme solution shown in the sample data. The control experiment (upper thermogram) also comprised 14 30-μl injections of nucleotide solution into the same buffer but without enzyme. Control injections represent the dilution heat of 5′-AMP and the heat effects from nonchemical reaction sources, which have not been compensated with dual injection. Injections 9–14 are very similar to the control injections and show that saturation has been reached. The integrated heats after subtraction of the small heat of the control experiment at each temperature are shown as the enthalpy per mole of 5′-AMP injected versus injection number in Fig. 3B.

Identical experiment to those described above in Hapes at 25 °C were carried out at 30 and 35 °C. As the temperature rises, the binding enthalpy becomes more exothermic. To measure ionization changes on binding, the calorimetric titration was done in three buffers with different ionization enthalpies, viz., 50 mM Hapes, 50 mM Pipes, 50 mM 2-glycerophosphate.
containing 50 mM KCl, 0.1 mM EDTA, and 0.1 mM 2-mercaptoethanol at pH 6.9. Since the reaction is more exothermic in Hepes buffer than it is in Pipes or 2-glycerophosphate at the three temperatures used, we conclude that there is net proton release during the binding reaction. Therefore, any analysis of the calorimetric data requires that the ionization heat of the buffers at each temperature must be known. (These values have been published by Christensen et al. (35).) The free 5'-AMP ligand also has buffering capacity at pH 6.9 (pK = 6.427 at 25 °C) (35), although its effect can be neglected because the 5'-AMP concentration is always considerably smaller than that of the buffer.

The SE-HPLC data referred to above show that 2 mol of 5'-AMP bind noncooperatively to phosphorylase a in the range of the ligand concentrations studied. Thus the cumulative amount of heat released per mol of dimer is a function of the saturation fraction, Y, according to

$$\Delta H = 2(\Delta H_b + n_{2D} \Delta H_{2D} - \frac{K_{[AMP]}}{1 + K_{[AMP]}}) \quad \text{(Eq. 4)}$$

and the parameters determined from the ITC data at pH 6.9 using a Levenberg-Marquardt algorithm (37) based on two independent binding sites in phosphorylase a, as described under "Materials and Methods," appear in Table I. The number of protons released by liver phosphorylase a subunit during 5'-AMP binding were 0.54 ± 0.03, 0.54 ± 0.02, and 0.52 ± 0.01 for 25, 30, and 35 °C, respectively. At 25 °C the K value obtained was equal within experimental error to that obtained by SE-HPLC at that temperature. The number of protons exchanged varied little with temperature and were more than when 5'-AMP binds to muscle isozyme (24). ΔG° and ΔS° for nucleotide binding obtained from the microscopic binding constants and ΔH, at each temperature are displayed in Table I. The standard state is that of 1 mol liter⁻¹. The calculation of thermodynamic functions implies the usual approximation of setting standard enthalpies equal to the observed ones. The temperature dependence of ΔH_b reveals a large negative ΔC_p of binding, with estimates of -1.45 ± 0.02 kJ K⁻¹ mol⁻¹ of 5'-AMP bound)⁻¹ obtained from the slope of a linear-regression analysis of the ΔH_b versus T data. In the temperature range studied, ΔC_p was found to be independent of temperature.

The equilibrium constants at the three temperatures studied should be initially consistent in terms of the van't Hoff equation. Thus, the K values at 30 and 35 °C can be recalculated from the value at 25 °C using the ΔH and ΔC values shown in Table I. The integrated form of the van't Hoff equation is

$$\ln \left( \frac{K(T)}{K(25)} \right) = \frac{1}{R} \frac{\Delta H_b(T)}{T} - \frac{\Delta H_b(25)}{T} + \frac{\Delta C_p}{R} \ln \frac{T}{T_0} \quad \text{(Eq. 5)}$$

Minor variations arose in the constant values thus obtained from the Equation 5 at 30 and 35 °C. Once the influence of the extent of protonation during the binding had been taken into account and the appropriate corrections to the accumulated heat data were made (Equation 4), three single sets of calorimetric values, ΔH_c, were obtained, one for each temperature.

### Table I

| Temperature (°C) | K × 10⁻⁴ | ΔG° (kJ mol⁻¹) | ΔH_b (kJ mol⁻¹) | ΔS° (kJ mol⁻¹ K⁻¹) |
|-----------------|----------|----------------|-----------------|-------------------|
| 25              | 8.5 ± 2.6| -28.1 ± 0.7    | -14.2 ± 0.5     | 47 ± 3            |
| 30              | 6.1 ± 0.9| -27.8 ± 0.4    | -21.6 ± 0.5     | 20 ± 2            |
| 35              | 5.7 ± 0.3| -28.1 ± 0.2    | -28.7 ± 0.2     | -2 ± 1            |

The uncertainties are standard errors in fitting of the curves.

The solid lines in Fig. 4 are those calculated with Equation 6, where the free concentration of 5'-AMP is given by Equation 3 for the thermodynamic parameters shown in Table I.

### DISCUSSION

The allosteric transition of rabbit muscle glycogen phosphorylase promoted by protein phosphorylation is known to be accompanied by two dimers associating to form a tetramer (39). Furthermore, changes in tertiary structure of muscle isozyme associated with effector binding are accompanied by changes in the quaternary structure (39). Nevertheless, some phosphorylases, such as those from pig muscle (40) and rabbit liver (8), are less prone to form tetramers on activation. If the binding of an effector produces a change in the state of protein oligomerization, an enthalpic contribution arising from this change would show up in the calorimetric data. To analyze the calorimetric data correctly it is first essential to know whether there is any change in the association state of the enzyme during binding. Thus we have investigated the aggregation state of bovine liver phosphorylase a and of the 5'-AMP-phosphorylase a complex using molecular weight chromatography and found them both to be dimers. So it would seem that liver isozyme activation is not accompanied by changes in the state of protein oligomerization. Barford and Johnson (39) have shown that there are many polar and charged residues in regions of the muscle phosphorylase structure that participate in the tetramer interface. The interactions at that interface are predominantly hydrogen bonds and salt bridges. For liver phosphorylase, the sequences of human (13) and rat (41) enzymes show that a total of nine substitutions occur in the tetramer contact regions and at least four are nonconservative (39). It appears likely that substitutions in these residues play a role in conferring the differential responses of muscle and liver isozymes to association/dissociation events controlled by effectors and protein phosphorylation.

The SE-HPLC measurements at 25 °C indicate that within the nucleotide concentration range studied 5'-AMP binds to two sites in the liver phosphorylase a dimer, which correspond to the activator sites identified in the muscle isozyme. The binding of nucleotide is noncooperative, which agrees with studies concerning the activation of liver phosphorylase a in others species (5, 8). A model which assumes two equal and independent sites also agrees well with the calorimetric meas-
urements made with different buffers at 25, 30, and 35 °C. Hill plots using the calorimetric data and assuming two sites resulted in straight lines with the following Hill coefficients: \( n_{Hill}(25 \, ^\circ \text{C}) = 1.08 \pm 0.02; \) \( n_{Hill}(30 \, ^\circ \text{C}) = 1.09 \pm 0.02; \) \( n_{Hill}(35 \, ^\circ \text{C}) = 0.92 \pm 0.02. \) The number and the noncooperative character of the binding sites shown by the bovine-liver isozyme seem to be the same as for the muscle isozyme. The affinity of 5'-AMP for liver phosphorylase a seems to be practically the same at all temperatures checked and is similar to that of muscle isozyme. The temperature dependence of \( \Delta H_0 \), \( T\Delta S^0 \), and \( \Delta G^0 \) of the 5'-AMP-enzyme binding exhibits a compensating behavior in that the enthalpy and the entropy changes decrease rapidly with temperature whereas the \( \Delta G^0 \) remains relatively constant with temperature. \( \Delta H_0 \) are negative at the three temperatures studied and \( \Delta S^0 \) is positive at 25 and 30 °C, changing sign in the physiological temperature range. Thus, the binding of 5'-AMP to liver phosphorylase a is driven fundamentally by enthalpy contributions. This behavior is analogous to that shown by muscle phosphorylase a (24).

Another contribution to the observed heats is the heat of buffer ionization or protonation produced by an exchange of protons during binding. For temperatures between 25 and 35 °C the protein-ligand system releases approximately 0.5 proton per binding site at the pH studied. The ionization changes during 5'-AMP-phosphorylase a binding can be attributed to a decrease in the pK in one or more groups of the nucleotide and/or enzyme on the complex forms. The \( \Delta H_0 \) values (Table I), although corrected for the enthalpy of buffer protonation, include the enthalpy change when 5'-AMP binds to the enzyme and the heat induced by proton release from the ligand-protein complex. Approximately the same numbers of protons are released over a temperature range of 25 to 35 °C. Although the functional groups responsible for proton release have not been identified, our results would seem to indicate that the contribution of ionization to \( \Delta H_0 \) is relatively small. In that case, several factors may well contribute to the experimental thermodynamic parameters (Table I): a net formation of hydrogen bonds and electrostatic interactions; changes in van der Waals interactions between specific groups; and changes in the hydration of ligand and protein groups.

5'-AMP binding to liver phosphorylase a involves a large negative \( \Delta C_p \). Heat capacity changes are usually interpreted as arising from hydrophobic interactions (42, 43). The results reported by Livingstone et al. (44), Brivanlou and Makhatadze (45), Murphy and Gill (46), and Spolar et al. (47) indicate that the \( \Delta C_p \), due to the burial of polar groups from water are positive, while the burying of apolar groups produces negative ones. Murphy and Freire (48) and Spolar and Record (49) have suggested that the \( \Delta C_p \) may be described as a phenomenon in terms of contributions from changes in apolar and polar area exposed to water, pointing out that changes in vibrational modes apparently contribute little to \( \Delta C_p \).

The application of Murphy’s approach (48) to our values indicates that the surface area buried on complex formation is one of 80% nonpolar surface (approximately 1250 Å²) and 40% polar surface (approximately 850 Å²) This accords with the idea that the 5'-AMP is practically buried in the active form of the muscle enzyme (50).

Since the amino acid sequences of human liver (13) and rat liver (41) phosphorylases are more than 80% identical to the rabbit muscle sequence (51) and the 5'-AMP binding site is highly conserved (13), several interactions postulated for the 5'-AMP-muscle phosphorylase a complex might also occur on the binding of 5'-AMP to liver isozyme. Crystallographic studies show several hydrogen bonding interactions, which may be taken as structural sources for the negative \( \Delta H_0 \) value. Two conserved arginines (Arg-309 and Arg-310) are involved in an extensive hydrogen-bonding network around the phosphate (16, 52, 53). Asp-42 in the cap of the other subunit is hydrogen bonded to the 2'-hydroxy of the ribose (16, 50). The net formation of hydrogen bonds produces a negative \( \Delta H_0 \), and should be accompanied by a negative \( \Delta S^0 \). It should be considered, however, that the formation of hydrogen bonds between 5'-AMP and the enzyme releases water. Before binding, these molecules were hydrogen bonded to protein and/or ligand and so one might well expect an unfavorable enthalpic component and a favorable entropic one due to the dissociation of water from protein or ligand, or both (54). A favorable van der Waals interaction between the adenine and Tyr-75 (23, 50) might contribute to a negative enthalpy change. The \( \Delta H_0 \), of 5'-AMP binding to liver phosphorylase a is not so negative as when 5'-AMP binds to muscle isozyme (24), and the absence of some hydrogen bonds between the adenine N6 amino group of 5'-AMP and amino acids in the 315 to 325 loop might contribute to this. Although the activator site residues in the rabbit muscle isozyme are highly preserved in human liver (13) and rat liver (41) phosphorylases, the 315–325 loop shows five or seven substitutions with respect to the muscle sequence (51). The loss of interactions between the liver enzyme and 5'-AMP is consistent with the observed increase in \( \Delta G^0 \) of about 2 kJ/mol compared to that in the muscle isozyme. Fersht et al. (55) have shown that a hydrogen bond contributes some 2.1–6.3 kJ/mol of stabilization energy to a complex.

Our results show that the binding of 5'-AMP to phosphorylase a is fundamentally enthalpy driven between 25 and 35 °C and is accompanied by the release of protons. Since the binding of nucleotide is noncooperative, the interaction does not produce any profound conformational change affecting the other subunit in the phosphorylase a dimer. Although 5'-AMP does not promote a shift in equilibrium from the inactive state to the active state in liver phosphorylase b (6), our results would seem to suggest that the binding of 5'-AMP to the active form of liver phosphorylase is similar to its binding to the muscle a form. The enthalpy and entropy changes on binding are very temperature dependent, arising from a large negative heat capacity change. The affinity of the nucleotide for the enzyme is, however, practically the same over the temperature range studied. The large negative \( \Delta C_p \) suggests significant changes in polar and apolar surfaces accessible to the solvent upon binding.

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