Thermodynamic Analysis of the Activation of Glycogen Phosphorylase b Over a Range of Temperatures*

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Equilibrium dialysis and isothermal microcalorimetry experiments have been carried out to characterize the thermodynamics of the binding of AMP to glycogen phosphorylase b (EC 2.4.1.1) at pH 6.9 over the temperature range of 25-35 °C. Thermal titrations were performed at each temperature in various buffer systems, which have afforded the calculation of the number of protons exchanged when the AMP binds to each site in the protein. Thermodynamic parameters were obtained for the binding of AMP to the two nucleotide and the two inhibitor sites of the dimeric enzyme. The former show positive cooperativity while the latter behave as independent binding sites. A positive ΔCp value was obtained for the AMP binding to the two N sites (1.3 and 1.4 kJ K⁻¹ mol⁻¹), while the ΔCp was negative for the binding to the I sites (1.9 kJ K⁻¹ mol⁻¹). The application of Sturtevant’s method to our data (Sturtevant, J. M. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2236–2240) and their comparison with a similar analysis undertaken with phosphorylase a (Mateo, P. L., González, J. F., Barón, C., Lopez-Mayorga, O., and Cortijo, M. (1986) J. Biol. Chem. 261, 17067–17072) has opened the way to some understanding of the thermodynamics of the allosteric transition in the protein.

Glycogen phosphorylase (EC 2.4.1.1) is one of the most complex macromolecules for which a high resolution atomic structure is available. The enzyme has two interconvertible forms, in which the difference between the inactive phosphorylase b and the active form, phosphorylase a, lies in the phosphorylation of the Ser-14 residue in the a form. Among the many effectors of the protein, AMP is the main allosteric activator for phosphorylase b and its interactions with the protein are of great importance. It is known from x-ray studies (1, 2) as well as from studies in solution (3–5) that the nucleotide binds to two distinct sites in the protomer, the allosteric activator site N and the inhibitor site I. Phosphorylase a also binds AMP in a non-cooperative manner (6–8) and increases its activity by 20–30%, reaching similar levels to those of the phosphorylase b-AMP complex (9). Several review articles on the biochemical and structural properties of phosphorylase have been published in recent years (10–13).

The binding of a ligand to a protein is usually accompanied by a rather large negative ΔCp, which implies characteristic temperature-dependent ΔH and ΔS values for the binding. In the case of allosteric, cooperative binding processes, the energetics of the binding is difficult to analyze due to the conformational transitions, which are responsible for the cooperative binding. The molecular interpretation of such an analysis is also complicated because one rarely has detailed structural information about the nature of those transitions. The most direct and effective technique for a thermodynamic investigation is that of isothermal microcalorimetry, although for these complex cooperative processes additional experimental methods are also required.

We have previously reported on the thermodynamic study of the binding of AMP to phosphorylase b at 25 °C (5), as well as that of AMP to phosphorylase a between 25 and 35 °C (8). We have now extended the temperature range of the former study to 30 and 35 °C by additional equilibrium dialysis measurements at 25 °C and by calorimetry at 30 and 35 °C using various buffers of different protonation heats at pH 6.9. The present work shows light on the thermodynamics of the binding of AMP to both the N and I sites, including the ΔCp values for each site, which have different signs. The data have been further analyzed by the method outlined by Sturtevant (14), and a comparison with the similar results for phosphorylase a (8) leads us to certain conclusions about the energetics of the conformational change in the protein and also to comment on the particular interactions and structural features responsible for the calculated thermodynamic functions of the binding.

MATERIALS AND METHODS

Enzymes and Chemicals—Glycogen phosphorylase b was prepared from rabbit skeletal muscle by the method of Fischer et al. (15, 16) with the modifications described by Krebs et al. (17). The catalytic activity of the enzyme was determined by Hedrick and Fischer’s assay (18). The preparations used had specific activities of 80–90 units/mg. Protein concentration was determined from absorbance measurements at 280 nm, using the absorbance coefficient ε132 = 13.2 (19). The molecular weight of the monomer was taken as 97,400 (20). The enzyme was crystallized at least three times. Phosphorylase b preparations were freed from AMP by chromatography on a Sephadex G-25 column equilibrated with 50 mM KCl, 0.1 mM 2-mercaptoethanol, 0.1 mM EDTA, 50 mM buffer solution (2-glycerophosphate, Pipes, or Tris), adjusted to pH 6.9. The A278/A362 ratio for the AMP-free phosphorylase b solutions was always below 0.53. Possible traces of AMP aminohydroxide (EC 3.5.4.6) (21) were eliminated by incubation with alumina C₃, as has been described elsewhere (22). [5⁻¹⁴C]AMP was obtained from the Radiochemical Center, Amersham, United Kingdom. AMP, alumina C₃, Pipes, and 2-mercaptoethanol were bought from Sigma, Tris and sodium 2-glycer-

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.
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The chemicals used were of the highest available purity. Distilled, deionized water was used throughout.

Techniques—The equilibrium dialysis experiments were carried out at 25 °C using a Dianorm equilibrium dialysis system, according to the method of Helmreich et al. (23). An LS 7500 Beckman scintillation counter was used in the dialysis experiments to calculate the saturation fraction of the enzyme with AMP. The fluorescence intensity of the protein, as a function of the enzyme concentration, both in the presence and the absence of AMP, was measured with an LS-5 Perkin-Elmer luminescence spectrometer with a thermostat. The protein concentration and enzymatic assays were measured with a Cary-210 spectrophotometer with the cells maintained at 25 °C.

Isotothermal calorimetry experiments were carried out at 30 and 35 °C using an LKB 2277 BioActivity monitor equipped with two flow units. Electrical and chemical calibrations were made in the same range as that which we obtained in the calorigraphic experiments themselves. The chemical calibrations were made by neutralizing Tris with HCl (24). Enzyme and AMP solutions were allowed to flow into the calorimeter at equal rates of 7 cm-h" in the majority of experiments, with the occasional change to check the completeness of the reaction.

All appropriate corrections for heats of dilution and mixing were applied. The heat effect of the enzyme dilution was negligible in all cases. The phosphorylase b activity was routinely checked prior to and after the calorimetric and dialysis experiments. The pH values of the several buffers, AMP, and enzyme solutions were controlled at each temperature before and after the binding reaction.

RESULTS

Fluorescence Determinations—Glycogen phosphorylase b shows two fluorescence transitions (25): one is caused by the protein moiety (excitation and emission maxima at 280 and 355 nm, respectively, and a quantum yield of 0.12), and the other is associated with its cofactor, pyridoxal 5'-phosphate (excitation maxima at 355 and 425 nm, emission maximum at 355 nm, and a quantum yield of 0.012). The fluorescence intensity in the emission maximum at 355 nm is quenched by the addition of Mg2+-AMP to the dimeric phosphorylase b (26), which promotes the tetramerization of the enzyme (27). We have used this fluorescence of the cofactor to determine any change taking place in the quaternary structure of the protein under conditions in which we have conducted the dialysis and calorigraphic experiments.

The enzyme was excited at 425 nm to keep the absorbance of the samples below 0.1. We then carried out intensity measurements of the pyridoxal phosphate fluorescence, both in the absence and in the presence of either 1 or 10 mM AMP, as a function of the enzyme concentration within the range 0.5–14 mg cm" at 25 and 35 °C. In all cases we obtained a good linear dependence between the fluorescence intensity and the protein concentration for the whole range investigated (linear regression coefficient higher than 0.998). Thus, there was no evidence for any intermolecular effects, i.e. detectable association-dissociation phenomena, dependent on the enzyme concentration for the above range.

Equilibrium Dialysis Measurements—The binding of AMP to phosphorylase b has been previously studied as a function of the activator concentration by equilibrium dialysis at 25 °C and pH 6.9 in Tris buffer, using 25 different experimental data (5). Since the present calorigraphic analysis relies critically on the precision and accuracy of the binding constants obtained from the dialysis study, we have carried out many more additional experiments to obtain more than 60 independent equilibrium dialysis data (compare, for example Fig. 1 here with Fig. 2 of Ref. 5) in order to calculate association equilibrium constant values with lower standard deviations.

In a system as complex as the present one, the analysis of the calorigraphic data requires the use of the binding constants obtained with other techniques (i.e. equilibrium dialysis). Thus, the information that it is possible to obtain from the calorigraphic study largely depends on the precision of the association constants used. All equilibrium dialysis data are given in a Scatchard plot in Fig. 1. As we have previously shown (5), the reaction of AMP with the phosphorylase b dimer can be considered as being the binding of AMP to two independent sets of sites. The high affinity sites show positive cooperativity according to their Hill coefficient (nH = 1.4 ± 0.04), while the low affinity sites can themselves be considered as independent of each other (nH = 0.96 ± 0.11). On this basis, the saturation fraction, Y, as a function of the free AMP concentration, is

\[
Y = \frac{1}{2} \frac{K_{\text{m}} \text{[AMP]} + K_{n} K_{\text{m}} \text{[AMP]}^{2}}{2 K_{n} \text{[AMP]} + 1} \quad (1)
\]

where K\text{m}\text{[AMP]} stands for the microscopic binding constants at the ith site, and K\text{n} = K_{n}.

The microscopic binding constant values given in Ref. 5 were used as the initial input values for the iterative Newton-Gauss method (28) to fit the total experimental data to the Equation 1. The optimum K\text{m}\text{[AMP]} values obtained are shown in Table I. These new constant values are approximately equal to those obtained previously, within their standard deviations. Nevertheless, they have minor standard deviations. The curve in Fig. 1 is the theoretical one using the Scatchard equation for the calculated binding constants (Table I).

Calorimetric Experiments—The enthalpy change during the binding of AMP to phosphorylase b was measured as a function of the activator concentration at two new different temperatures and with various buffers at pH 6.9. The buffer systems employed were 50 mM Tris and 50 mM 2-glycerophosphate at 30 °C and 50 mM Hepes, 50 mM Pipes, 50 mM Tris, and 50 mM 2-glycerophosphate at 35 °C. In all cases the buffer solutions were 50 mM KCl, 0.1 mM EDTA, and 0.1 mM 2-mercaptoethanol. The thermal experimental data determined at 30 and 35 °C are shown in Fig. 2, where the enthalpy change per mol of the phosphorylase b dimer is plotted as a function of the total concentration of AMP. The binding of AMP is exothermic at 30 and 35 °C, as it also is at 25 °C (5), giving rise to well defined monophasic curves (Fig. 2). The titration curve at 25 °C was the same regardless of the buffer system (5), and since the ionization heats of the buffers are different (29) no proton uptake or release seemed to occur throughout the binding at that temperature. At both 30 and...
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Table I
Apparent thermodynamic parameters for the binding of AMP to phosphorylase b at 25 °C and pH 6.9

<table>
<thead>
<tr>
<th>N sites</th>
<th>I sites</th>
<th>Total N sites</th>
<th>Total I sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_n \times 10^{3}$ (M⁻¹)</td>
<td>-4.0 ± 0.7</td>
<td>23.7 ± 5.5</td>
<td>0.37 ± 0.05</td>
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<tr>
<td>$\Delta G$ (kJ mol⁻¹)</td>
<td>-20.6 ± 0.5</td>
<td>-20.0 ± 0.6</td>
<td>-14.6 ± 0.3</td>
</tr>
<tr>
<td>$\Delta H$ (kJ mol⁻¹)</td>
<td>-19 ± 6</td>
<td>-74 ± 7</td>
<td>-39 ± 2</td>
</tr>
<tr>
<td>$\Delta S$ (J K⁻¹ mol⁻¹)</td>
<td>6 ± 20</td>
<td>-183 ± 25</td>
<td>-83 ± 6</td>
</tr>
<tr>
<td>$\Delta C_p$ (kJ K⁻¹ mol⁻¹)</td>
<td>1.3 ± 1.3</td>
<td>1.4 ± 1.7</td>
<td>-1.9 ± 0.5</td>
</tr>
</tbody>
</table>

35 °C, however, the heat evolved during the AMP binding depends upon the buffer system, due to a change in the protein's state of protonation, and we have found the extent of this change to be temperature-dependent. The analysis of the present experimental enthalpy data, $\Delta H_b$, thus requires that the heat of ionization of the various buffers used should be known at each temperature (8); these values have been reported in the literature (29).

As mentioned before, the AMP binding to phosphorylase b can be understood as the binding of a ligand, S, to a protein, P, with two sets of sites: the high affinity sites, N, and the low affinity sites, I. The binding at each temperature can then be represented by the following equilibria:

\[ P_N + S \rightleftharpoons P_NS \quad K_1 = 2K_m; \quad \Delta H_1 \]
\[ P_{NS} + S \rightleftharpoons P_{NS2} \quad K_2 = K_{m2}/2; \quad \Delta H_2 \]
\[ P_I + S \rightleftharpoons P_IS \quad K_3 = 2K_m; \quad \Delta H_3 \]
\[ P_{IS} + S \rightleftharpoons P_{IS2} \quad K_4 = K_{m4}/2; \quad \Delta H_4 \]

where $K_n$ and $K_m$ are the macroscopic and microscopic binding constants, respectively, and $\Delta H_i$ stands for the enthalpy change per mol of AMP bound to each site. $P_N$ and $P_I$ stand for the protein states when the high and low affinity sites are empty; $P_NS$ corresponds to the protein state with only one high affinity site empty, regardless of whether the low affinity sites are empty or not. The protein states $P_{NS}$ and $P_{IS}$ are defined similarly. In the binding of AMP to phosphorylase b, $K_m = K_{m2}$ and $\Delta H_2 = \Delta H_4$, since the two equal low affinity sites are considered as being independent, as was shown to be the case at 25 °C (5).

From equation 2, the enthalpy change per mol of enzyme (dimer), $\Delta H_b$, in a hypothetical buffer with zero ionization heat is given by

\[ \Delta H_b = \frac{\Delta H_b[P_NS] + (\Delta H_1 + \Delta H_3)[P_{NS}]_2}{[P_NS] + [P_{NS}]_2 + [P_{NS2}]} + \frac{\Delta H_b[P_IS] + 2\Delta H_3[P_{IS}]_2}{[P_IS] + [P_{IS}]_2 + [P_{IS2}]} \]

which can be expressed as the following:

\[ \Delta H_b = \frac{2\Delta H_bK_m[S] + (\Delta H_1 + \Delta H_3)K_mK_{m2}[S]^2}{1 + 2K_m[S] + K_mK_{m2}[S]^2} + \frac{2\Delta H_bK_{m2}[S]}{1 + K_m[S]} \]

As pointed out before, there is a net proton exchange, $n$, when the AMP binds to the enzyme (dimer). Let $n_1$, $n_2$, and $n_3$ stand for the number of protons exchanged by the system when AMP binds to the first and second high affinity sites and one of the low affinity sites, respectively. Then the $n$ value, which is itself a function of the saturation of the enzyme by AMP, can be expressed similarly to Equation 3 as

\[ n = \frac{n_1[P_NS] + (n_1 + n_2)[P_{NS2}]}{[P_NS] + [P_{NS}]_2 + [P_{NS2}]} + \frac{n_2[P_{NS2}] + 2n_2[P_{NS2}]}{[P_{NS2}]_2 + [P_{IS}]_2 + [P_{IS2}]} \]

or

\[ n = \frac{2n_1K_m[S] + (n_1 + n_2)K_mK_{m2}[S]^2}{1 + 2K_m[S] + K_mK_{m2}[S]^2} + \frac{2n_2K_{m2}[S]}{1 + K_m[S]} \]

At each temperature the number of protons exchanged by the protein-ligand system, $n$, will be released or taken up by the buffer present in the medium. In our case the situation is particularly complicated since, in addition to the buffer used in each case, the free ligand itself, AMP, has also buffering capacity at pH 6.9 (pK = 6.427 at 25 °C (29)). Moreover, the buffering effect of the free AMP will depend on its concentration, and it is clear that this effect cannot be neglected due to the AMP concentration range used compared to that of the buffer itself. Therefore the number of protons, $n$, will be equal to the sum of those exchanged by the buffer, $n_b$, and the free ligand, $n_0$:

\[ n = n_b + n_0 \]

The values of $n_b$ and $n_0$ are obviously not independent, and it can be shown that they follow an equation of the following form:

\[ n_0 = \frac{aq}{c} \]

where $a$, $b$, and $c$ are functions of the protein concentration, the pH, and the pK values and concentrations of both the buffer and the free AMP (see "Appendix").
Hence, the experimental calorimetric data, $\Delta H_n$, is given by

$$\Delta H_i = \Delta H_n + n_B \Delta H_B + n_A \Delta H_A$$  \hspace{1cm} (9)$$

where the new symbols, $\Delta H_B$ and $\Delta H_A$, stand for the ionization heats of the buffer and free ligand, respectively.

From Equations 7 and 8 the value of $n_B$ results as

$$n_B = \frac{1}{2c} \left[ cn - b - a + ((a + b - cn)^2 + 4abc)^{1/2} \right]$$  \hspace{1cm} (10)$$

with a similar equation for $n_A$ as a function of $n$. These two expressions for $n_B$ and $n_A$ can replace the corresponding values in Equation 9, leading to a $\Delta H_i$ which would depend on $n$ and $\Delta H_n$. Since these two latter values are given by Equations 6 and 4, we would finally obtain the experimental $\Delta H_i$ value at each temperature as a function of $K_m$, $\Delta H_n$, and $n_i$, for the three sites, $i = 1, 2, 3$.

At this point we would have three equations for $\Delta H_i$, corresponding to one for 25, 30, and 35 °C. Fitting all the experimental data to these three equations would involve dealing with a large number of unknowns. This analysis, however, can be simplified by taking into account some restrictions these values have to abide by and also by making some reasonable assumptions about the behavior of the system.

Thus, the three $K_m$ values at 25 °C are known from the dialysis experiments. In addition the enthalpy changes and the constant values for each site have to be consistent in terms of the Kirchhoff and the van't Hoff equations, respectively. The integrated forms of these equations for a constant $\Delta C_p$ value are

$$\Delta H(T_i) = \Delta H(T_1) + \Delta C_p(T_i - T_1)$$  \hspace{1cm} (11)$$

and

$$\ln \frac{K(T_i)}{K(T_1)} = -\frac{\Delta H(T_i) - \Delta H(T_1)}{T_1} + \frac{\Delta C_p}{R} \ln \frac{T_2}{T_1}$$  \hspace{1cm} (12)$$

The number of protons for the three sites, $n_1$, $n_2$, and $n_3$, have been shown to be zero at 25 °C within experimental error (5). These values, however, are not zero at 30 and 35 °C, as seen in Fig. 2, where an increasing proton-exchange effect from 30 to 35 °C can be clearly observed. Given the short temperature range investigated, we have assumed the $n_i$ values to have a linear dependence with temperature, i.e. $n_i(T_2) = n_i(T_1) + m_i(T_2 - T_1)$. This behavior is similar to that found for the binding of AMP to phosphorylase a (8). With this assumption and the above restrictions we can arrive at a general equation for the experimental $\Delta H_i$ values, including all the buffers and the three temperatures used, where the values to fit would be $\Delta H_i$ (at 25 °C), $\Delta C_p$, and $m_i$, for the three sites, $i = 1, 2, 3$. The experimental thermal data, which correspond to more than 150 independent calorimetric measurements, were fitted to the general equation by trial and error using the $\Delta H_i$ values at 25 °C previously reported (5). The $\Delta H_i$ (25 °C), $\Delta C_p$, and $m_i$ were optimized by the Newton-Gauss method (28). The curves in Fig. 2 are the theoretical ones using the calculated values. Thermodynamic parameters for the binding of AMP to phosphorylase b at 25 °C and pH 6.9, obtained from the $\Delta H_i$ and $K_m$, values, and including the $\Delta C_p$ values, are included in Table 1. The three enthalpy values at 25 °C compare well with those previously reported (5), which indicate the general data analysis carried out simultaneously for the three temperatures. Fig. 3 shows the variations of $\Delta H$ and $T \Delta S^*$ versus temperature and, as has been reported in the literature for many binding studies (14), the $\Delta G^*$ values remain practically constant for each site within the temperature range of 25–35 °C. The $m_i$ values obtained are $m_1 = -0.25 \pm 0.04$, $m_2 = 0.26 \pm 0.06$, and $m_3 = 0.05 \pm 0.02$, which lead to a negative $n_1$ value and positive $n_2$ and $n_3$ values for temperatures between 25 and 35 °C. Fig. 4 includes the three sets of data at 25, 30, and 35 °C after correction for the proton exchange thermal effects, together with the theoretical calorimetric binding curves also corrected for the proton effects, i.e. the $\Delta H_b$ values versus the free AMP concentration.

We should mention at this point that when dealing with ligand binding to multisite complex proteins the experimental data analysis is only possible if certain assumptions are accepted, i.e. the restrictions the system is supposed to obey. Only in this way can a set of individual binding parameters such as the one shown in Table I be arrived at. In our case the assumptions made were to consider the $\Delta C_p$ values as being constant (the norm for this type of study) and also the $m_i$ values, within the 25 to 35 °C range. Nevertheless, it should be pointed out, that when performing our data analysis, using the three $K_m$ values at 25 °C with either plus or minus one-half of their standard deviations (Table I), the optimal fitting practically leads to the same values for the rest of the thermodynamic parameters as those obtained here within their uncertainties (Table I).

**DISCUSSION**

The association of the phosphorylase b dimer caused by AMP in the presence of Mg$^{2+}$ has been reported.
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at 25 °C (26). Other authors (31, 32) have also detected aggregation of the enzyme when AMP binds at low temperatures, although not at 25 °C. If this were to be the case, an enthalpic contribution arising from the change in the association state (the extent of which would depend on temperature, AMP, and protein concentrations) would show up in the calorimetric data. We have already demonstrated (5), by carrying out thermal AMP titrations at different protein concentrations, that no phosphorylase association effects occur on the binding of AMP at 25 °C. Here we have also made use of the co-factor fluorescence intensity, in the absence of AMP and in the presence of 1 and 10 mM AMP, to confirm the nonexistence of intermolecular interactions at 25 and 35 °C, since this intensity is a very sensitive indicator of the association state of the protein (8, 26). In this way, we found a linear dependence between the fluorescence intensity and protein concentration within the range 0.5-14 mg cm⁻²² at both temperatures. Our thermal dilution experiments at 30 and 35 °C also agree with this conclusion. Therefore, it is apparent that no AMP-induced phosphorylase association/dissociation effects took place in our equilibrium dialysis and calorimetric binding experiments.

In order to analyze the calorimetric data correctly the first question that needs answering is whether there is any proton exchange during the binding. The usual way to do this is to carry out the thermal titration in various buffers of sufficiently different protonation heats. We have already shown, within experimental uncertainty, that there is no such exchange at 25 °C (5). This does not hold true, however, at 30 and 35 °C (Fig. 2), where two and four different buffers, respectively, were used; a similar behavior has been reported for the binding of AMP to phosphorylase a (8). It is worth noting that at both 30 and 35 °C at the AMP concentrations at which the different buffer titrature curves intersect (Fig. 2) the net proton exchange, n, is zero. This situation obviously implies a different proton behavior during the binding of AMP to the four sites, i.e. there should be simultaneous release and take up of protons depending upon each AMP bound.

In fact, for temperatures between 25 and 35 °C, the protein-ligand system releases protons when AMP binds to the first site and takes them up on binding to the three other sites. This means that one or several pK values, corresponding to some proton accepting groups of the enzyme-ligand system, decreases (i.e. become more acidic) upon binding of AMP to the first site, while these pK values increase when the effector binds to the other three sites. There are various reasons which may explain these changes in the pK values. Among these, a variation in the micropolarity of the chemical groups appears to us to be the most plausible explanation, given the high effect of the medium polarity (or water availability) on the pK values observed in analogous systems (30).

Once the protonation effect has been corrected, the thermodynamic parameters for the binding can be obtained, as explained under “Results.” Table I shows these values at 25 °C, including the ΔCp values, from which the calculation of any other parameter within the range 25-35 °C is straightforward. Fig. 3 illustrates the significant temperature dependence of the binding enthalpy and entropy, as might be expected from the ΔCp values. Despite the clear differences in the ΔH and ΔS° binding values/site, the affinity of AMP for each site, i.e. the corresponding ΔG° value, appears to be practically the same from 25 to 35 °C. This is another example of the so-called enthalpy-entropy compensation, common to many binding processes and, in fact, to practically any type of process dealing with biopolymers in an aqueous solution (14, 33).

The cooperative nature of the binding of AMP to the two N sites can also be seen in their ΔH and ΔS° values (see Fig. 3 and Table I). The most striking difference between the binding to the N and I sites resides in the fact that ΔCp is positive for both N sites and negative for the I sites. Negative ΔCp values are normal in binding studies (14), as is, for instance, the noncooperative binding of AMP to phosphorylase a (8). The positive ΔCp values for the N sites are, however, quite exceptional, and they are undoubtedly related to AMP’s highly cooperative binding to these sites and the AMP-induced structural change responsible for this cooperativity.

It is not possible to attempt any structural interpretation of the thermodynamic parameters for the binding of AMP to each N site, since the parameters for each site contain different contributions arising from the cooperative structural change (Table I). The total ΔH values for the AMP binding to the N sites at 30 and 55 °C are -79 ± 10 and -65 ± 14 kJ mol⁻¹ dimer⁻¹ respectively, while the corresponding ΔS° values are -112 ± 8 and -68 ± 15 J K⁻¹ mol⁻¹ (mol of dimer)⁻¹. The variation in these parameters with temperature is just the opposite to that found for the binding of AMP to the N sites of phosphorylase a (8) (obtained at the same temperatures, 25, 30, and 35 °C) with the corresponding ones in Table II, where ΔS° stands for the standard unitary entropy units (37, 38). It is worth comparing similar results for the AMP binding to the N sites of phosphorylase a (8) (obtained at the same temperatures, 25, 30, and 35 °C) with the corresponding ones in Table II, where it is noteworthy that the four parameters have opposite signs for the a and b forms of the enzyme. It is clear from the x-ray diffraction (39) and solution studies (40) that the remarkable differences between the AMP binding to the N sites in both enzymes cannot derive from the minor differences in the AMP interactions at both N sites and, therefore, must be put down to the conformational transition that takes place in phosphorylase b on the binding of AMP. Thus, a comparison of the data in our Table II and that of Table II in Ref. 8 should lead one to expect positive values for ΔCp(h), ΔCp(v), ΔS°(h), and ΔS°(v) and negative ones for ΔS°(h) for this transition, i.e. the activation process of the enzyme. From the sign in these parameters it seems that there should be a “loosening” of the structure with a concomitant increase in ΔCp(v) and ΔS°(v) on activation, from which the enzyme goes from the inactive to the fully active conformation. The positive ΔCp(h) and negative ΔS°(h) can reasonably be interpreted in terms of hydrophobic interactions; thus, the number of contacts between the apolar residues of the protein and the solvent should increase on enzyme activation. This conclusion agrees with the structural

### Table II

<table>
<thead>
<tr>
<th>T°C</th>
<th>ΔCp (h)</th>
<th>ΔCp (v)</th>
<th>ΔS° (h)</th>
<th>ΔS° (v)</th>
<th>J K⁻¹ mol⁻¹ (mol of dimer)⁻¹</th>
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</table>

Hydrophobic (h) and vibrational (v) contributions to the entropy and heat capacity for the binding of AMP to glycogen phosphorylase b at pH 6.9
information provided by Sprang and Fletterick (41), according to which there is a 2000-Å² island of solvent at the center of the subunit contact region; this central cavity, which can accommodate some 150-180 water molecules, which are inaccessible to the bulk of the solution in the inactive conformation, becomes deeper and narrower and opens up to the external solvent on activation, thus making for a more extended water-protein interaction, particularly for the first 75 N-terminal residues in both subunits (41).

The contribution of the allosteric conformational change would be the dominant one in the values of Table II for the AMP binding to the N sites. From these values it would be possible to conclude that for the dimeric enzyme some 60 internal vibrational modes would be activated as a result of the "intrinsically binding" (35) of 2 mol of AMP plus the concomitant conformational change (14), while there would be an increase of about 24 mol of "hydrophobic water" in the overall process (14). These numbers should be considered as a net balanced change in those water molecules since, on the other hand, there should be a loss of some hydrophobic water molecules due to the binding of AMP to the N sites, which Anderson and Graves (42) have described as being hydrophobic. An additional interaction expected from the $\Delta C_p$ and $\Delta S^*$ values would be the possible net formation or the strengthening of hydrogen bonds, which would also agree with the negative $\Delta H$ values. The overall binding of AMP to the N sites is thus favored by the enthalpy and vibrational entropy, which surmount the high hydrophobic entropy barrier, a barrier that results mainly from the above-mentioned conformational change.

The binding of AMP to the I sites has been shown to be noncooperative between 25 and 35°C, and therefore its thermodynamic parameters might be attributed to those of an intrinsic binding (35). The affinity of the nucleotide for these sites is between 1 and 2 orders of magnitude lower than that for the N sites, while we have again an example of the enthalpy-entropy compensation giving rise to a practically constant affinity throughout the above temperature range (Fig. 3). The $\Delta C_p$ for this binding has the usual negative value for this type of process (14) and compares well with that reported for the binding of FMN to the I site of phosphorylase $a$, $-1.7 \pm 0.5$ kJ K$^{-1}$ mol$^{-1}$ at 30°C (43), although in this case the $\Delta C_p$ was found to be a function of the temperature.

The negative $\Delta C_p$ can be interpreted, in terms of hydrophobic interactions, as being mainly due to a decrease in contacts between apolar protein groups and the solvent on the binding of AMP to the I sites. This interpretation, however, would also suggest a positive entropy change, but, in fact, $\Delta S^*$ is negative for this binding (Fig. 3). The application of Sturtevant's method (Table II) once again clarifies this point, given the positive value of $\Delta S^*(h)$. Here there seems to be a tightening of the structure with a loss of about 50 internal vibrational modes, with a liberation of approximately 15 mol of water that were previously in contact with apolar regions when 1 mol of AMP binds the I sites. From Table II it is now clear that the binding of the nucleotide to the I sites is both enthalpy- and hydrophobic entropy-driven between 25 and 35°C, overcoming the opposite vibrational entropy contribution. The negative $\Delta H$ should mainly come from the net formation of hydrogen bonds and of electrostatic pairs and, to a minor extent, from van der Waals' interactions.

Since the possible net formation of hydrogen bonds would make for a positive $\Delta C_p$ contribution, our estimation of the contribution of the hydrophobic effect to the binding might be a minimal evaluation of its relative importance.

Finally, it is interesting to note that in the overall binding of the four AMP molecules to the two N and two I sites of the dimer the net change in the water molecules in contact with the protein is very small, while there are also opposite effects to the rigidity or flexibility of the global protein conformation due to AMP's binding to both types of sites. This overall cancellation effect might be related to the enzyme activation on the binding of AMP to the N sites and to the inhibitory characteristics of the I sites.

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**APPENDIX**

In a solution with two systems with buffering capacity, B and S, the following equilibria exist:

\[
\begin{align*}
HB & \rightleftharpoons H^+ + B^- & K_b \equiv [H^+] [B^-] \\
HS & \rightleftharpoons H^+ + S^- & K_s \equiv [H^+] [S^-]
\end{align*}
\]  

where $K_b$ and $K_s$ are equilibrium constants, defined as

\[
K_b = \frac{[H^+] [B^-]}{[HB]} \quad \text{and} \quad K_s = \frac{[H^+] [S^-]}{[HS]}
\]  

The concentration of the components at equilibrium are given by

\[
\begin{align*}
[B^+] &= \frac{K_b C_b}{[H^+] + K_b}, \\
[HB] &= \frac{[H^+]C_b}{[H^+] + K_b} + [H^+]s, \\
[H^+]s &= \frac{[H^+]C_b}{[H^+] + K_b}
\end{align*}
\]  

where $C_b$ and $C_s$ are the total concentrations of B and S, respectively.

If a concentration of protons ([H$^+$]$_s$) is generated in the solution the concentration of all the components will change and a new equilibrium will be arrived at. The new concentration of all the components will then be given by

\[
\begin{align*}
[B^+]_s &= \frac{K_b C_b}{[H^+] + K_b} - [H^+]s, \\
[HB]_s &= \frac{[H^+]C_b}{[H^+] + K_b} + [H^+]s, \\
[H^+]s &= \frac{[H^+]C_b}{[H^+] + K_b} + [H^+]s
\end{align*}
\]  

where [H$^+$]$_s$ and [H$^+$]$_s$ stand for the proton concentrations taken up by the B and S systems, respectively, and [H$^+$] for the initial free proton concentration.

The proton concentration in the new equilibrium will be given by

\[
[H^+]_s = [H^+] + [H^+]_s - [H^+]_s
\]  

Substituting Equations A4 and A5 into Equations A2 applied to this new equilibrium, the ratio of $K_b$ to $K_s$ results in

\[
K_b = \frac{C_b[H^+] + [H^+]_s[H^+] + [H^+]_sK_b}{C_b[H^+] + [H^+]_s[H^+] + [H^+]_sK_b}
\]

Rearranging Equation A6, we have

\[
[H^+]_s = \frac{a[H^+]_s}{b + c[H^+]_s}
\]  

with $a$, $b$, and $c$ being the values given by

\[
\begin{align*}
a &= K_b C_b ([H^+] + K_b), \\
b &= K_b C_b ([H^+] + K_b)^2, \\
c &= (K_b - K_s)([H^+] + K_b)([H^+] + K_b)
\end{align*}
\]  

In our particular case, 1 mol of the protein-ligand dimer
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exchanges n protons with the two buffers present in the medium, and the proton concentration exchanged will be $nC_p$, where $C_p$ is the enzyme concentration.

Thus, $n_A$ and $n_B$ being now the number of protons released by B and S, respectively, we have

$$ [H^+]_{B} = -n_AC_p \quad \text{and} \quad [H^+]_{S} = -n_BC_p. \quad (A9) $$

Finally, by substituting Equations A9 into A7 and re-arranging, we obtain Equation 8 given in the text:

$$ n_S = \frac{an_A}{b + cn_B} $$

where $c = -c_nC_p$.

REFERENCES