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AMP INTERACTION SITES IN GLYCOGEN PHOSPHORYLASE *b*

A THERMODYNAMIC ANALYSIS

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The binding of AMP to activator site N and to inhibitor site I in glycogen phosphorylase *b* has been characterized by calorimetry, potentiometry and ultracentrifugation in the pH range 6.5–7.5 at 25°C ($\mu = 0.1$). Calorimetric titration data of phosphorylase *b* with adenosine 5'-phosphoramidate are also reported at pH 6.9 ($T = 25$°C, $\mu = 0.1$). Calorimetric curves have been analyzed on the basis of potentiometric and sedimentation velocity results to determine thermodynamic quantities for AMP binding to the enzyme. The comparison of calorimetric titration data of AMP and adenosine 5'-phosphoramidate at pH 6.9 supports the hypothesis previously suggested that the dianionic phosphate form of the nucleotide preferentially binds to the allosteric activator site. The thermodynamic parameters for AMP binding to site N are as follows: $\Delta G^0 = -22$ kJ mol^{-1}, $\Delta H^0 = -34$ kJ mol^{-1} and $\Delta S^0 = -40$ J mol^{-1}K^{-1}. The binding of the nucleotide to site I was found to be strongly dependent on the pH. This behaviour may be explained in terms of coupled protonations of three groups having pK_a values of 6.0, 6.0 and 6.1 in the unbound form and 7.0, 7.5 and 7.2 in the enzyme-nucleotide complex. The thermodynamic parameters for nucleotide binding to site I for the enzymatic form in which all the modified groups are completely deprotonated or protonated have been calculated to be: $\Delta G^0 = -7.7$ kJ mol^{-1}, $\Delta H^0 = -28$ kJ mol^{-1} and $\Delta S^0 = -68$ J mol^{-1}K^{-1} and $\Delta G^0 = -28$ kJ mol^{-1}, $\Delta H^0_{II} = -10$ kJ mol^{-1} and $\Delta S^0_{II} = 61$ J mol^{-1}K^{-1}, respectively. These results suggest that attractive dispersion forces are of primary significance for AMP binding to activator site N, although electrostatic interactions act as a stabilizing factor in the nucleotide binding. The protonation states of those residues of which the pKa values are modified by AMP binding to site I highly influence the thermodynamic parameters for the nucleotide binding to this site.

1. Introduction

Glycogen phosphorylase *b* (EC 2.4.1.1), a dimer of molecular weight 194000 [1], is a key control enzyme of glycogen metabolism [2]. Phosphorylase *b* depends on AMP for catalytic activity [3]. AMP affects both the V_{max} and the K_m of the enzyme [4]. It has been shown that the binding of AMP to phosphorylase *b* is followed by conformational changes [5–8], as well as by an enhanced tendency of the enzyme to associate into tetramers [9,10].

AMP has two different binding sites on the enzyme monomer [7,11,12]. The first one is the allosteric activator site, called site N, which is close to the subunit/subunit interface and is on the opposite face of the protein with respect to the active site. The second one is the inhibitor site, called site I, which is located at the surface of the enzyme 10 Å away from the catalytic cleft where glucose and glucose 1-phosphate bind [11].

The interaction between AMP and phosphorylase *b* has been studied by several authors using calorimetric methods [13–16]. Unfortunately, the reported K_d and ΔH values, especially those for the nucleotide binding to the inhibitor site, are in

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Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid

disagreement. Recently, Cortijo and co-workers [16] have suggested that the biphasic shape of the calorimetric data may arise from the enzymatic conversion of AMP to IMP by traces of a contaminating AMP-aminohydrolase that may be present in purified enzyme preparations. These conflicting results suggest that the thermodynamics of AMP binding to phosphorylase *b* should be reinvestigated in more detail.

In the present work, the interaction of AMP with glycogen phosphorylase *b* has been studied in the pH range 6.5–7.5 ($T = 25°C$, $\mu = 0.1$). The influence of pH on the binding process and consequently on the thermodynamic parameters governing the interaction was analyzed by calorimetric, potentiometric titration and ultracentrifugation techniques. The absence of contaminating traces of AMP-aminohydrolase in the enzyme preparations was carefully checked in all enzymatic samples used during this work.

Calorimetric titration curves were analyzed on the basis of the information provided by potentiometric titration data and sedimentation velocity experiments. The thermodynamic parameters for the nucleotide binding, to either the activator (site N) or the inhibitor (site I) locus, were corrected for the contributions arising from the different processes that are associated with the nucleotide binding.

2. Materials and methods

Glycogen phosphorylase *b* was prepared from rabbit skeletal muscle by the methods of Krebs et al. [17], using β-mercaptoethanol instead of cysteine. The enzyme was recrystallized at least three times. Phosphorylase *b* crystals were freed from AMP by chromatography on a Sephadex G-25 column and by treatment with activated charcoal. The pH of all solutions was carefully checked before and after experiments.

The protein concentration was determined spectrophotometrically at 280 nm with an absorbance index $E^{1\%}_{1cm}$ of 13.2 [18]. The 260 nm/280 nm absorbance ratios of the enzyme solutions were always below 0.54.

Prior to use, the purity of all enzyme preparations used in this work was routinely checked in order to detect the possible presence of contaminating AMP-aminohydrolase [16]. Phosphorylase *b* solutions were incubated with 10 mM AMP (under the same conditions as for the calorimetric experiments) and their ultraviolet spectra were registered at different incubation times during a 2 h period, after diluting 1 : 100 with buffer. We could not detect any shift in the characteristic AMP absorption spectrum ($\lambda_{max} = 259$ nm) to shorter wavelengths, which would have indicated the conversion of AMP to IMP ($\lambda_{max} = 250$ nm) by the action of AMP-aminohydrolase [16].

Glycogen and AMP were purchased from Merck and phosphoglucomutase and glucose-6-phosphate dehydrogenase were obtained from Boehringer. All other chemicals were purchased from Sigma.

The buffer consisted of 50 mM glycylglycine or Pipes, 50 mM KCl, 0.1 mM EDTA and 0.6 mM β-mercaptoethanol, except for potentiometric titrations which were performed in 0.1 M KCl.

An enzyme concentration of 3 mg/ml was used in all experiments unless otherwise stated.

2.1. Calorimetry

Calorimetric measurements were performed at 25.00 ± 0.02°C with LKB flow and batch microcalorimeters, calibrated as previously described [19,20]. In the experiments performed with the flow microcalorimeter, a flow rate of 0.13 ml min^{-1} and a flow time of 15 min for reading the steady-state rate of heat evolution were employed. The dilution heat signal of the ligand was taken as the baseline for each experiment. In the batch microcalorimeter measurements the heat of dilution of the ligand was automatically subtracted in the reference cell. The heat of dilution of the protein solutions was determined in a separate run and subtracted from the total.

2.2. Potentiometry

Potentiometric titrations were performed with a Radiometer TTA3 pH-stat coupled with a Radiometer PHM28 pH-meter. The titrant, 0.01 M HCl, was added by means of a microburet in portions of 0.2–0.4 μl to 10 ml of phosphorylase *b*.

Proton uptake titrations were performed by mixing equal volumes of phosphorylase *b* and AMP solutions, both at identical pH and ionic strength, and titrating the resulting solutions back to the initial pH with 0.01 M HCl. All titrations were performed under a nitrogen atmosphere at 25°C and in 0.1 M KCl.

2.3. Ultracentrifugation

Sedimentation velocity experiments were performed using Schlieren optics in a Spinco model E analytical ultracentrifuge at 60000 rpm and at 25°C, in glycylglycine buffer. Sedimentation coefficients were corrected for the viscosity and density of the buffer relative to water at 20°C. The Schlieren patterns were resolved into their two components (dimer and tetramer) by using a 310 Dupont Curve Resolver. The constants of tetramerization were calculated from the area under each peak.

2.4. Activity assays

The initial reaction rates for the glycogen phosphorolysis reaction were determined by the procedure described by Helmreich and Cori [21]. The enzyme was discarded when a maximum specific activity lower than 33 U/mg was measured at 30°C. The reaction mixtures were composed of 1 mM AMP, 12 mM P_i, 16 mM glycogen in glucose residues, 9 mM magnesium acetate and 0.003 μM phosphorylase *b*, in glycylglycine buffer, pH 6.9.

3. Results

3.1. AMP binding to phosphorylase b

3.1.1. Calorimetric measurements

Figs. 1 and 2 show the experimental results for the calorimetric titration curves of phosphorylase *b* with AMP, at several pH values (6.5, 6.7, 6.9, 7.05 and 7.5) in glycylglycine buffer at 25°C. From pH 6.5 to 6.9 the calorimetric curves have a biphasic shape, possibly arising from the saturation of two different binding sites per monomer of enzyme [13,14], because AMP-aminohydrolase was not present in the enzyme samples. This behavior disappears at pH 7.05 and 7.5. In addition, the affinity and the enthalpy change associated with AMP binding to sites N and I are also a function of pH.

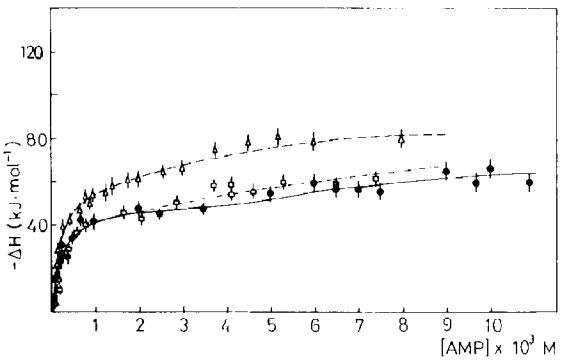

Fig. 1. Calorimetric titration of phosphorylase *b* with AMP in glycylglycine buffer at pH 6.5 (△), pH 6.7 (□) and pH 6.9 (●), ($T = 25$°C, $\mu = 0.1$). The plotted curves correspond to the theoretical fitting of the experimental data, using eq. 1, for the thermodynamic quantities reported in tables 2 and 3.

This dependence upon the pH might indicate a change in the ionization state of the enzyme induced by the nucleotide binding. Therefore, calorimetric measurements of AMP binding to the enzyme were performed at pH 6.9 in Pipes buffer (fig. 3) which has an enthalpy change of protonation ($\Delta H_p = -11.6$ kJ mol^{-1} [22]) very different from that of glycylglycine ($\Delta H_p = -44.3$ kJ mol^{-1}

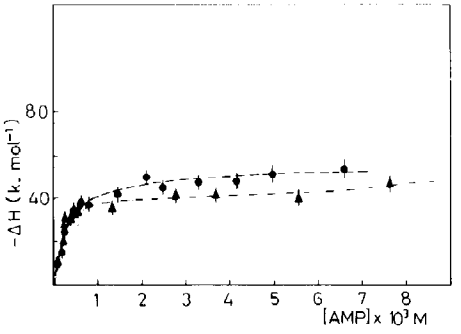

Fig. 2. Calorimetric titration of phosphorylase *b* with AMP in glycylglycine buffer at pH 7.05 (▲) and pH 7.5 (●), ($T = 25$°C, $\mu = 0.1$). The plotted curves correspond to the theoretical fitting of the experimental data, using eq. 1, for the thermodynamic parameters reported in tables 2 and 3.

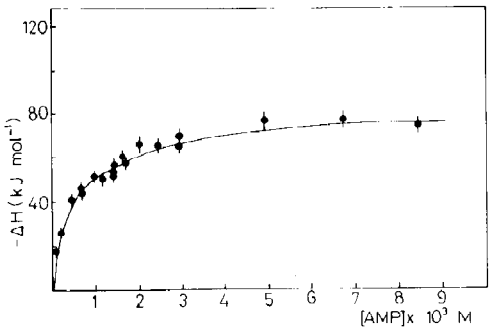

Fig. 3. Calorimetric titration of phosphorylase *b* with AMP in Pipes buffer at pH 6.9 ($T = 25°C$, $\mu = 0.1$). The continuous line was calculated as described in the text using eq. 1.

Table 1

Tetramerization constants of phosphorylase *b* in the presence of AMP

K_T^N and K_T^I are, respectively, the tetramerization constants at saturation of one or two AMP sites per monomer.

pH	K_T^N ($\times 10^{-3}$) (M^{-1})	K_T^I ($\times 10^{-3}$) (M^{-1})
6.5	8.0 ± 0.6	4.5 ± 0.3
6.7	7.6 ± 0.5	4.5 ± 0.3
6.9	7.2 ± 0.5	4.4 ± 0.3
7.06	6.2 ± 0.4	5.2 ± 0.4
7.2	5.2 ± 0.4	4.2 ± 0.3
7.4	4.5 ± 0.3	4.2 ± 0.3
7.5	4.5 ± 0.3	3.7 ± 0.3

[23]). These results clearly show that the apparent enthalpy change depended on the buffer.

3.1.2. Proton uptake

The proton uptake induced by formation of AMP-phosphorylase *b* complexes was investigated over the pH range studied at AMP concentrations of 1.4 and 8.4 mM. These concentrations of AMP almost saturate one or two binding sites per monomer of enzyme, respectively. The number of proton equivalents absorbed per mol enzyme monomer, ΔN_{H^+}, by formation of the nucleotide-enzyme complexes are shown in fig. 4A.

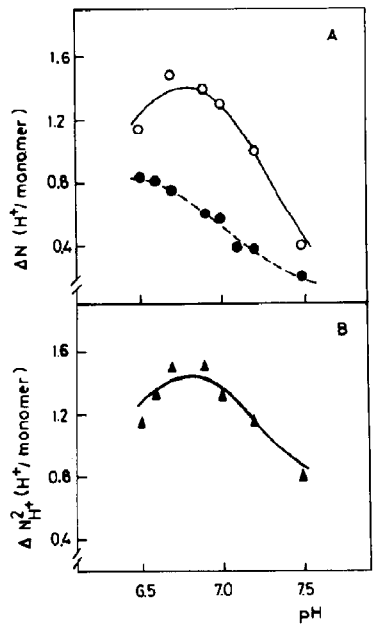

Fig. 4. Proton uptake by formation of AMP-phosphorylase *b* complexes ($T = 25°C$, $\mu = 0.1$). (A) ΔN, experimental data at 1.4 mM AMP (●) and 8.4 mM AMP (○). $\Delta N^2_{H^+}$, number of proton equivalents absorbed by the AMP-phosphorylase *b* complex at saturation of site I. The full curve in B was calculated using eq. 4 and assuming the dissociation constants reported in table 4.

3.1.3. Ultracentrifugation

The AMP-induced aggregation of the enzyme was studied in the pH range 6.5–7.5. Sedimentation velocity experiments show that in the presence of AMP, at 25°C, phosphorylase *b* (3 mg/ml) remains mostly as a dimer ($s_{20,w} = 8.4$ S), although a slight enhancement of the population of the tetrameric form ($s_{20,w} = 12.0$ S) is observed as AMP concentration rises to 1.2 mM; the tetramerization is also favored by acidic pH values. Further increases in the nucleotide concentration result in a partial reversal of enzyme association to a level which is practically independent of pH. The tetramerization constants of phosphorylase *b*, at saturation of one or two classes of binding sites per monomer of enzyme calculated as described in section 2, are reported in table 1.

3.2. Binding of adenosine 5'-phosphoramidate to phosphorylase b

Kinetic studies using several AMP analogs [7,24–27] have demonstrated the importance of the AMP diphosphate anion for nucleotide binding to

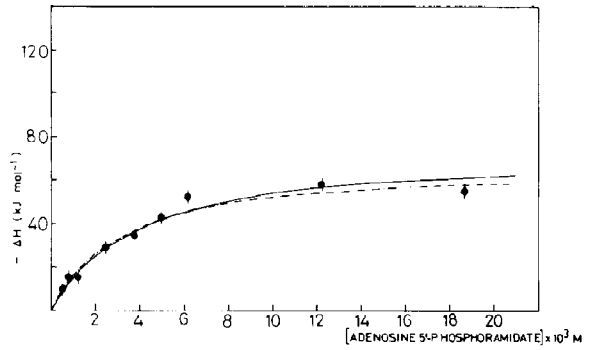

Fig. 5. Calorimetric titration of phosphorylase *b* with adenosine 5′-phosphoramidate in glycylglycine buffer at pH 6.9 ($T = 25°C$, $\mu = 0.1$). The full and broken lines were calculated as described in the text and considering one or two binding sites per enzyme monomer, respectively.

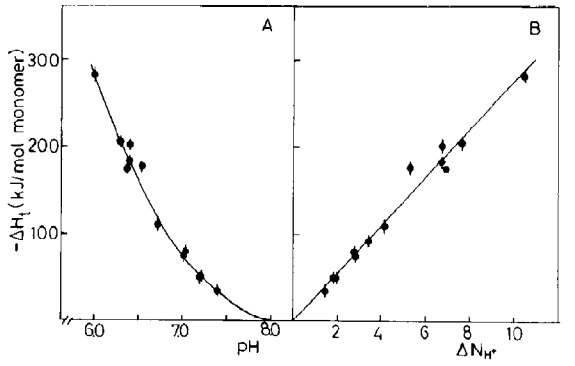

Fig. 6. Enthalpic titration curve of the protonable groups of the enzyme between pH 8.0 and 6.0 (A), $\mu = 0.1$ and $T = 25°C$. (B) Enthalpy change, ΔH_t, vs. number of proton equivalents absorbed by the protein from pH 8.0 to 6.0.

the activator site. Later results from X-ray diffraction studies show the existence of electrostatic interactions between the phosphate dianion of the nucleotide and three arginines (308, 309 and 242) at site N [2,11,36]. These data suggested that a preferential binding of the AMP diphosphate anion to site N is likely to occur. For this reason the binding of adenosine 5′-phosphoramidate to the enzyme has been studied by calorimetry in glycylglycine buffer (pH 6.9) at 25°C. Adenosine 5′-phosphoramidate, which binds to site N [27], has only a single negative charge on its phosphate moiety and thus provides an excellent AMP analog for testing the importance of the overall charge of the nucleotide phosphate in its binding to the enzyme. The experimental results obtained for the calorimetric titration curve of phosphorylase *b* with adenosine 5′-phosphoramidate, plotted in fig. 5, clearly show that also in the absence of substrates the loss of one negative charge on the phosphate group decreases the nucleotide affinity by a factor of ten.

3.3. Potentiometric and calorimetric titration of the ionizable groups of phosphorylase b

The ionization behavior of phosphorylase *b* has been characterized by calorimetric and potentiometric titration measurements between pH 6.0 and 8.0. At lower pH values the enzyme solutions became opalescent which indicated that some enzyme denaturation had occurred. The molar enthalpy change, ΔH_t, associated with the titration of the free enzyme from pH 8 to any other pH above 5.8 vs. pH and vs. the number of protons absorbed per mol protein determined by potentiometric titration has been plotted in fig. 6. The slope of the straight line (fig. 6B) yields a value of -27 ± 3 kJ mol^{-1}, for the apparent average protonation heat value of the enzymatic groups which are titrated in that pH region.

3.4. Analysis of results

The experimental results reported in this paper show that the heat evolved at each AMP concentration is a complex function of several contributions that should be taken into account in analyzing the calorimetric titration curves: the intrinsic binding to sites N and I, the enzyme tetramerization induced by the nucleotide, the change in the ionization state of such groups whose pK_a values are modified by AMP binding and ionization of the buffering species.

The calorimetric results of adenosine 5′-phosphoramidate binding to phosphorylase *b* show that the number of charges on the phosphate moiety highly influence the apparent affinity of the nucleotide towards site N, supporting the hypothesis that AMP binds to the activator site

mainly as the AMP diphosphate anion.

Calorimetric titration curves of AMP binding to phosphorylase *b* were analyzed by using the allosteric model of Monod et al. [28] to describe the nucleotide binding to the activator site (preferential binding of the AMP diphosphate anion was taken into account) and considering that the binding of the second molecule of nucleotide per monomer occurs when the activator site becomes saturated. It was also assumed that the protonations of those groups modified by the nucleotide are coupled to AMP binding. The enthalpy change associated with AMP binding to sites N ($i = 1$) or I ($i = 2$) may be then expressed as follows:

$$\Delta H = \sum_{i=1}^{i=2} \left\{ \left[\overline{E^iA}\right]\left(\Delta H^i_{app} + \Delta H_{buf}\Delta N^i_{H^+}\right) + \left[\overline{T^iA}\right]\Delta H^i_T \right\} + \frac{[H^+]}{[H^+] + K_{i,AMP}}\Delta H_{i,AMP} \quad (1)$$

where $[\overline{E^iA}]$ is the fraction of the enzyme having site i saturated; ΔH^i_{app} the enthalpy change associated with the interaction of AMP at the i-binding site of the enzyme, corrected for the buffering contributions; $\Delta N^i_{H^+}$ the number of proton equivalents absorbed at saturation of site i; ΔH_{buf} the enthalpy change per mol protons associated with the buffering action of the system; ΔH^i_T the tetramerization enthalpy at saturation of site N ($i = 1$) or both sites N and I ($i = 2$) and $[T^iA]$ the fraction of tetramer having one site (for $n = 1$) or two sites saturated (for $n = 2$). The last term in eq. 1 represents the contributions from the AMP-phosphate dissociation, arising from AMP binding to site N as the phosphate dianionic form; $K_{i,AMP}$ and $\Delta H_{i,AMP}$ are the dissociation constants and the enthalpy change for the dissociation of the second ionizable group of phosphate in the AMP molecule, respectively [29].

In Pipes (pH 6.9), the buffering capacity of the piperazine (pK_i 6.9 [22]) accounts for more than 90% of the total buffering capacity of the system and ΔH_{buf} is almost equal to the ionization enthalpy change of the piperazine. However, for the experiments performed in glycylglycine (pK_i 8.2 [23]), the contribution of the buffering capacity of AMP (pK_i 6.4 [29]) to the total buffering capacity of the system becomes significant below pH 7.5 at nucleotide concentrations above 0.5 mM. Thus, the ionization enthalpy changes of both AMP [29] and glycylglycine [23] contribute significantly to ΔH_{buf}, which should be calculated for each pH and AMP concentration as indicated in the appendix (section A3).

The values of K^i_{app} (the apparent dissociation constants of AMP), ΔH^i_{app} and L^1_0 (the transconformational constant for the enzymatic state stabilized by AMP binding to site N) were determined at each pH using eq. 1 in order to fit the calorimetric titration curves. The saturated fractions of the dimeric and tetrameric species were calculated as described in the appendix.

The tetramerization enthalpy at saturation of the two binding sites per monomer of enzyme ($\Delta H^2_T = -5 \pm 2$ kJ (mol monomer)$^{-1}$ [30]) makes the contribution of this term to eq. 1 insignificant. ΔH^1_T was taken as -39 ± 4 kJ (mol monomer)$^{-1}$ [30].

The values of $\Delta N^i_{H^+}$ were first assumed to be those experimentally determined from the potentiometric titration of the AMP-phosphorylase *b* complex formation at 1.4 mM AMP for site N ($i = 1$) and 8.4 mM AMP for sites N and I. Once the values of K^i_d and L^1_0 were determined, $\Delta N^i_{H^+}$ was corrected for the fractions of saturation of sites N and I at 1.4 and 8.4 mM AMP. This correction revealed that the protonations observed at 1.4 mM AMP were due to the saturated fraction of site I which is present at this concentration of nucleotide (fig. 4B). Apparently, it seems that the saturation of site N between pH 6.5 and 7.5 involves the ionization of the AMP-phosphate group arising from the preferential binding of the dianionic phosphate form of the nucleotide.

The set of values for K^i_{app}, ΔH^i_{app}, L^1_0 and $\Delta N^2_{H^+}$ which give the best fitting of the calorimetric data are reported in tables 2 and 3.

The thermodynamic quantities for AMP binding to site N are as follows: $\Delta G^{01} = -22.0 \pm 0.4$ kJ mol^{-1}, $\Delta H^{01} = -34 \pm 3$ kJ mol^{-1} and $\Delta S^{01} = -40 \pm 5$ J mol^{-1} K^{-1}.

The simplest scheme to account for AMP binding to site I in phosphorylase *b* is that the nucleo-

Table 2

Thermodynamic quantities for the binding of AMP to the activator site of phosphorylase *b* at 25°C

ΔH^0 is the enthalpy change corrected for the buffering effect and the P_i dissociation.

Buffer	pH	K^1_{app} ($\times 10^4$) (M)	$-\Delta H^0$ (kJ mol^{-1})	$\Delta N^1_{H^+}$ (P_i)	L_0
Glycylglycine	6.5	1.6 ± 0.2	33 ± 2	0.45	1.5 ± 0.2
	6.7	1.5 ± 0.2	33 ± 2	0.33	1.5 ± 0.2
	6.9	1.4 ± 0.2	34 ± 2	0.24	1.5 ± 0.2
	7.05	1.3 ± 0.2	33 ± 2	0.18	1.5 ± 0.2
	7.5	1.3 ± 0.2	36 ± 2	0.07	1.5 ± 0.2
Pipes	6.9	1.3 ± 0.2	37 ± 2	0.24	1.5 ± 0.2

tide binding and proton uptake are coupled and that the protonation of all residues modified by the nucleotide binding are independent of the free enzyme and of its complex with AMP. From these assumptions, the thermodynamic relationships for AMP binding to site I would be given by eqs. 2–4 [31].

$$\Delta G^2_{app} = \Delta G^0 - RT \sum_{j=1}^{n} \ln \frac{(1 + [H^+]/K'_j)}{(1 + [H^+]/K_j)} \quad (2)$$

$$\Delta H^2_{app} = \Delta H^0 \sum_{j=1}^{n} (f'_j \Delta H'_{p,j} - f_j \Delta H_{p,j}) \quad (3)$$

$$\Delta N^2 = \left\{ \sum_{j=1}^{n} (f'_j - f_j) \right\} - \frac{[H^+]}{[H^+] + K_{i,AMP}} \quad (4)$$

where ΔG^0 and ΔH^0 are the standard free energy and the enthalpy changes associated with the binding of AMP to site I, respectively, for an enzyme in which all the modified residues were unprotonated; f'_j and f_j are the fractional degrees of protonation of the j-th group and $\Delta H_{p,j}$ and $\Delta H'_{p,j}$ are, respectively, the enthalpies of protonation of the j-th group in the free and AMP-associated state of the enzyme.

The thermodynamic relations for the reaction of AMP with an enzyme in which all the modified residues were protonated are given by eqs. 5 and 6 [31].

$$\Delta G^{02}_H = \Delta G^{02} - RT \sum_{j=1}^{n} \ln(K_j/K'_j) \quad (5)$$

$$\Delta H^{02}_H = \Delta H^{02} + \sum_{j=1}^{n} (\Delta H'_{p,j} - \Delta H_{p,j}) \quad (6)$$

where K'_j and K_j are the ionization constants of the j-th residue of the AMP-associated and free states of the enzyme, respectively.

The analysis of the proton uptake by formation of the AMP-phosphorylase *b* complex (site I) in terms of eq. 4 showed that at least three groups were modified by the nucleotide binding. The K_j and K'_j values were allowed to vary over a wide range. The best fitting of the experimental data

Table 3

Thermodynamic quantities for the binding of AMP to the inhibitor site of phosphorylase *b* at 25°C

K^2_{app} and ΔH^2_{app} are the apparent thermodynamic estimates corrected for the buffering effect as described in the text.

Buffer	pH	K^2_{app} ($\times 10^3$) (M)	$-\Delta H^2_{app}$ (kJ mol^{-1})	$\Delta N^2_{H^+}$
Glycylglycine	6.5	0.5 ± 0.1	58 ± 3	1.55
	6.7	1.0 ± 0.2	59 ± 3	1.8
	6.9	2.0 ± 0.2	63 ± 3	1.7
	7.05	3.0 ± 0.3	57 ± 3	1.6
	7.5	7.0 ± 0.4	47 ± 3	0.85
Pipes	6.9	2.0 ± 0.2	64 ± 4	1.7

was obtained by assuming that the pK_j values of three groups were modified from 6.0 ± 0.1, 6.0 ± 0.1 and 6.1 ± 0.1 to 7.6 ± 0.1, 7.0 ± 0.1 and 7.1 ± 0.1, respectively.

The dependence of ΔG^2_{app} and ΔH^2_{app} upon pH was analyzed according to eqs. 2 and 3, respectively. They were assumed as K_j and K'_j values estimated from the fitting of the proton uptake using eq. 4. $\Delta H_{p,j}$ and $\Delta H'_{p,j}$ were approximated to their average values, $\overline{\Delta H}_{p,j}$ and $\overline{\Delta H}'_{p,j}$, in this pH range. The value of $\overline{\Delta H}_{p,j}$, was taken as -27 kJ mol^{-1} (the one derived from fig. 6B) and $\overline{\Delta H}'_{p,j}$ was allowed to vary within small limits. The best fitting of the experimental data (fig. 7) was obtained for $\Delta G^0 = -7.7 \pm 0.3$ kJ mol^{-1}, $\Delta H^0 = -28 \pm 2$ kJ mol^{-1} and $\overline{\Delta H'_{p,j}} = -21 \pm 2$ kJ mol^{-1}.

The thermodynamic quantities for AMP binding to phosphorylase *b* (site I) in the fully protonated state of the residues modified by the nucleotide were computed from ΔG^0, ΔH^0 and the average values of $\Delta H_{p,j}$ and $\Delta H'_{p,j}$, by using eqs. 5 and 6.

Table 4 summarizes the average values of the thermodynamic parameters for AMP binding to site I which provide the best fit obtained from the experimental data.

Calorimetric titration data for the interaction of adenosine 5′-phosphoramidate with phosphorylase *b* were analyzed considering one class of binding sites per enzyme monomer. The heat evolved at each ligand concentration may be expressed as follows:

$$\Delta H = \Delta H_{app}[\overline{EA}] \qquad (7)$$

where ΔH_{app} is the enthalpy of binding and $[\overline{EA}]$ is the fraction of the enzymic sites saturated by the ligand. $[\overline{EA}]$ was calculated in the same way as for the AMP binding to site I (see appendix). The best fitting of the experimental data was obtained for $\Delta H = -74 \pm 3$ kJ (mol monomer)$^{-1}$ and $K_{d,app} = 3.9 \pm 0.2$ mM. The value computed for the apparent enthalpy of binding is of the same order as those found at saturation by AMP (this paper)), adenine or adenosine [32], of both sites N and I in the enzyme. Thus, adenosine 5′-phosphoramidate might also bind to both sites N and I, as described

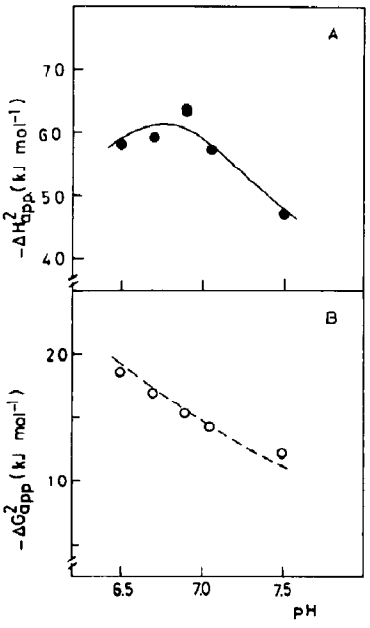

Fig. 7. Apparent thermodynamic parameters for AMP binding to site I vs. pH ($T = 25$°C, $\mu = 0.1$). The plotted curves in A and B were calculated by means of eq. 2 or 3 and using the thermodynamic parameters reported in table 4.

Table 4

Average values of the best fitting thermodynamic parameters describing the reaction of AMP with phosphorylase *b* at site I at 25°C, between pH 6.5 and 7.5

The term protonated or deprotonated state refers to the ionization state of the groups which are modified by the nucleotide binding.

pK_j	pK'_j	Phosphorylase *b*	
		Deprotonated state	Protonated state
6.0	7.6	$\Delta G^0 = -7.7 \pm 0.3$ kJ mol^{-1}	$\Delta G^0_H = -28.2 \pm 0$ kJ mol^{-1}
6.0	7.0	$\Delta H^0 = -28 \pm 2$ kJ mol^{-1}	$\Delta H^0_H = -10 \pm 2$ kJ mol^{-1}
6.1	7.1	$\Delta S^0 = -68 \pm 6$ J mol^{-1} K^{-1}	$\Delta S^0_H = 61 \pm 6$ J mol^{-1} K^{-1}

previously for several nucleotides, bases and nucleosides [7,11,12], with similar binding constants for the two binding sites, as occurs with adenine and adenosine at 25°C [32]. For this reason, the calorimetric data for the interaction of this AMP analog with the enzyme were also analyzed in terms of two binding sites per monomer of phosphorylase *b*. This adjustment was as good as that obtained for one class of binding sites (see fig. 5). The best fit of the calorimetric results was achieved for the following set of values: $\Delta H^1 = -30 \pm 2$ kJ mol^{-1}, $K^1_{app} = 1.5 \pm 0.2$ mM, $\Delta H^2 = -37 \pm 2$ kJ mol^{-1} and $K^2_{app} = 2.5 \pm 0.2$ mM. The enthalpy change evaluated for adenosine 5'-phosphoramidate binding to site N is the same as that for AMP binding to the activator site. On the other hand, the enthalpy change for this AMP analog binding to site I is in agreement with those found for adenine ($\Delta H^I_{app} = -33 \pm 2$ kJ mol^{-1}) and for adenosine ($\Delta H^I_{app} = -40 \pm 2$ kJ mol^{-1}) binding to the inhibitor site [32].

4. Discussion

Calorimetric and potentiometric data on AMP binding to phosphorylase *b* reported in the present work show a dependence of the apparent thermodynamic quantities upon the pH value that may be attributed mainly to the protonations of several groups induced by the nucleotide binding to site I.

As observed from the results of figs. 1–3 the existence of two binding sites per monomer might be overlooked on the basis of the calorimetric curves only, because the biphasic shape of the curves virtually disappears at pH > 7.0. This pH dependence of the calorimetric curve shape is higher for the experiments performed in glycylglycine buffer (fig. 2), since for this system, ΔH_{buf} depends strongly on the pH and on the AMP concentration.

The comparison of the calorimetric titration of phosphorylase *b* by AMP or by adenosine 5'-phosphoramidate at pH 6.9 supports the previous hypothesis suggesting the preferential binding of the dianionic form of AMP to the activator site. This would also explain that the activation parameters of adenosine 5'-phosphorothiodate (pK_a 5.3 [32]), which binds to the activator site as a dianion around neutrality, are similar to those of AMP [33]. Further, the fact that the binding of either AMP or adenosine 5'-phosphoramidate to site N (two binding sites are taken into account for the latter one) have the same enthalpy change (within experimental uncertainty) would indicate that the difference in their ΔG values for the activator site arises from the entropic contribution. Since attractive electrostatic interactions are usually followed by positive entropy changes and very low enthalpic changes, these results would also suggest that the number of negative charges on the phosphate moiety would be responsible for the difference in the affinity of both ligands. This is in agreement with crystallographic studies that have shown the existence of electrostatic interactions between the phosphate dianion and three arginines at the activator site [2,11].

The thermodynamic quantities found herein for AMP binding to the activator site N are similar to those reported from calorimetric experiments by several authors [13,15,16], since the enthalpy change of −33 kJ mol^{-1} computed for AMP binding has been corrected for the contributions arising from enzyme tetramerization and preferential binding of the AMP-phosphate dianionic form, as well as from the saturated fraction of site I which is present at AMP concentrations of about 1 mM. On the other hand, the values found for the average dissociation constant, K^1_{app}, and the Monod transconformational constant, L_0, are in agreement with the values reported by Steiner et al. [34] from equilibrium dialysis for the two stepwise equilibrium constants of the AMP binding to the activator site.

Thermodynamic parameters for AMP binding to site N show that this enzyme-nucleotide interaction is driven enthalpically, the molar entropy change being equal to −40 J mol^{-1} K^{-1}. The enthalpic character of this process may be attributed to the interaction between the base ring of the nucleotide and that of Tyr 75 [11,35,36], since attractive dispersion forces are usually characterised by negative enthalpic and entropic contributions [31].

The analysis of calorimetric results and proton uptake data, under saturation of site I by AMP,

indicate that three different groups of the protein may be modified in the AMP-bound state of phosphorylase *b*.

The thermodynamic parameters governing the interaction of AMP at the inhibitor site (table 4) show that for the phosphorylase *b* 'deprotonated state' (in terms of the ionization state of those groups which are modified by the nucleotide), the binding process is enthalpically driven. It thus appears that attractive dispersion forces may be of primary significance, which would agree with the reported stacking of the nucleotide base between Tyr 612, Phe 285 and Met 614 [36,38]. The protonation of the groups modified in the AMP-bound state of the enzyme makes the entropic contribution also favor the binding reaction.

Recent studies on AMP binding to phosphorylase *b* crystals [36] showed that the region of chain containing residues His 570 and Glu 571 may have a genuine conformational heterogeneity, which could be significant for catalysis. His 570 and Glu 571 are located near site I and C (the catalytic center) and may contribute to either or both sites [36]. Further, X-ray crystallographic studies on inhibitor binding to site I in phosphorylase *a* [38] have shown that the most significant structural change associated with this interaction is a shift of the His 570 side chain towards the interior of the active-site pocket and closer to the Asp 283 side chain. This increase in the hydrophobicity of the environment of His 570 may favor the formation of a His 570-Asp 283 hydrogen bond or ionic pair.

Although it would be rather speculative to assess which amino acid residues may be modified by nucleotide binding to this site (many abnormal ionizations have been reported in the neutral pH range), the crystallographic data above might suggest that His 570 might be one of the amino acid residues whose pK_a values are sensitive to the AMP binding to site I.

Finally, the thermodynamic results reported in the present paper point out the influence of binding protein-linked processes on the thermodynamic quantities and their relevance to account for the energetics of ligand-protein reactions. In this sense, AMP binding to site I constitutes a clear example of the role that proton uptake or release may play on binding processes, as found for 3'-CMP-ribonuclease A [39,40] and NAD^+-horse liver alcohol dehydrogenase interactions [41–43], or tetrameric hemoglobin oxygenation [44] and oligosaccharide reaction with lysozyme [45–48].

Appendix

A1. Calculation of the saturation fraction for AMP

The binding of AMP to the activator site (site N) of glycogen phosphorylase *b* can be described following the concerted allosteric model of Monod et al. [28]. The saturation fraction of site N for AMP, $[\overline{E^1A}]$, can be calculated using eq. A1, previously derived by Monod et al. [28].

$$\left[\overline{E^1A}\right] = \frac{\alpha + \alpha^2}{L_0 + (1+\alpha)^2} \qquad \text{(A1)}$$

$$\alpha = [A]/k^1_{app}$$

where L_0 is the transconformational constant in terms of the Monod nomenclature, and [A] the AMP free concentration and k^1_{app} its average dissociation constant.

The saturation fraction of site I (the inhibitor locus) has been calculated considering that the nucleotide binding to this site occurs when site N is saturated, as stated above. On the basis of this assumption, eq. A2 was derived from the expression for the average dissociation constant of AMP for this site, k^2_{app} (two binding sites per dimer of enzyme have been considered);

$$\left[\overline{E^2A}\right] = \frac{\gamma}{(1+\gamma)} \cdot \frac{\alpha + \alpha^2}{L_0 + (1+\alpha)^2} \qquad \text{(A2)}$$

$$\gamma = [A]/k^2_{app}$$

the second term on the right of eq. A2 represents the fraction of enzyme that may bind the second molecule of nucleotide per monomer of enzyme.

A2. Determination of the tetramer fraction induced by AMP

The concentration of tetramer formed in the presence of AMP, by saturation of site N, has

been calculated from the tetramerization constant, K_T^1, defined as:

$$K_T^1 = \frac{[T^1]}{[D^1]^2} \quad \text{(A3)}$$

where $[D^1]$ is the concentration of dimeric form which has only saturated its high-affinity binding site.

The total concentration of phosphorylase *b* molecules which have only saturated site N can be expressed in terms of monomer concentration as:

$$4[T^1] + 2[D^1] = [E^1A] - [E^2A] \quad \text{(A4)}$$

If $[D^1]$ is substituted in eq. A3 by the expression derived from eq. A4, the fraction of tetramer, defined as $[\overline{T^1}] = 4[T^1]/[E_t]$, can easily be derived.

$$[\overline{T^1}] = 4\frac{[T^1]}{[E_t]} = \frac{1}{2K_T^1[E_t]} \times \left\{1 + 2K_T^1([E^1A] - [E^2A]) \pm [1 + 4K_T^1([E^1A] - [E^2A])]^{1/2}\right\} \quad \text{(A5)}$$

A3. Determination of buffering capacities and the enthalpy change associated with the buffering action

The buffering effect of a component k in a system may be defined as

$$\beta_k = \left(\frac{\delta N_k}{\delta \text{ pH}}\right) C_k = 2.303 C_k \xi_k (1 - \xi_k) \quad \text{(A6)}$$

where C_k is the molar concentration of that component, N_k the number of protons absorbed by that component at a given pH and ξ_k the fraction of the acidic form [37]. The total buffering capacity of the system, β, is the sum of the buffering capacities for the individual component system. Similarly, the heat associated with this buffering action, ΔH_{buf}, may be expressed as

$$\Delta H_{buf} = \sum_k \Delta H_{p,k} b_k \quad \text{(A7)}$$

where $\Delta H_{p,k}$ is the enthalpy of protonation of the k component and $b_k = \beta_k/\beta$ is the relative buffering capacity of component k.

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