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# THERMODYNAMICS OF NUCLEOTIDES BINDING TO PHOSPHORYLASE *b*

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The effects of several chemical modifications in the AMP molecule on its interaction with phosphorylase *b* are examined by microcalorimetry, equilibrium dialysis, light scattering and ultracentrifuge experiments. In this work we report the results obtained for eight AMP analogues corresponding to different substituents in the puric base or in the ribose, or to different positions of the phosphate. The thermodynamic properties of the interaction between the phosphorylase *b* and the above mentioned nucleotides are also reported. The following conclusions were obtained: a) Except for IMP and 2'dIMP all the nucleotides studied clearly show two types of binding sites in the enzyme. b) The interaction of the nucleotide with its weaker affinity binding site is highly dependent upon chemical alterations in the puric base. c) Both the amino group in C(6) and the N(1) of the adenine in the AMP seem to play an important role in the conformational transitions induced by the nucleotide on the enzyme. d) The tetramerization of phosphorylase *b* in the presence of $10^{-2}$ M AMP and in the conditions of the ultracentrifuge experiments is drastically affected by modifications in the ribose-phosphate part of the AMP molecule.

## 1. Introduction

Phosphorylase *b* from rabbit skeletal muscle (E.C. 2.4.1.1) is activated by AMP [1], this activation having allosteric characteristics [2]. Kinetic studies using AMP analogues [3,4] have shown that the three parts of the AMP molecule (adenine, ribose and phosphate) are needed to activate the enzyme. On the other hand, Mott and Bieber [5] showed that the importance of the different groups of the AMP molecule is not the same in the binding and in the activation processes.

It is known from microcalorimetric and ultracentrifuge data [7,8] that IMP, the other physiological nucleotide activating of the phosphorylase *b* [2], differs from AMP in its activation [6] and in the structural alterations produced by its binding to the phosphorylase *b*. In addition, IMP apparently recognizes only one type of binding site in the enzyme at 25°C [7] while AMP clearly shows two types of binding sites with different affinity towards this nucleotide [7,8]. The reasons for these differences still remain unknown. However, a phenomenological description of the phosphorylase *b* activation that considers the existence of different enzymatic conformational states induced by both nucleotides has been proposed to account for the above mentioned findings [9]. Furthermore, the microcalorimetric data of the AMP-phosphorylase *b* interaction show that an extension of the classical allosteric models is needed to describe this process [10].

In this paper we report microcalorimetric, equilibrium dialysis, light scattering and ultracentrifuge data on the interaction of eight AMP analogues with phosphorylase *b* in glycylglycine buffer, at 25°C. The calorimetric data have been analyzed following the hypothesis of the binding sites generation model [10]. The results show that there are clear differences in the enthalpy values of the interaction between the different nucleotides tested and the enzyme. These

differences lead us to several conclusions about the importance of the modified chemical groups of the AMP molecule in the allosteric effect associated to its interaction with phosphorylase *b*, and also about the relative importance of these groups for the interaction of AMP with its different types of binding sites.

## 2. Materials and methods

Glycogen phosphorylase *b* was prepared from fresh rabbit skeletal muscle by the method of Krebs et al. [11]. The enzyme was recrystallized three times and AMP was removed by passage through a column of Sephadex G-25 gel and then by treatment with activated charcoal. The $A_{260}/A_{280}$ ratios at pH 6.9 were always lower than 0.54. The enzyme concentration was measured spectrophotometrically using an extinction coefficient ($E^{1\%}_{1\,cm}$) at 280 nm of 13.2 [12]. The activity of phosphorylase *b* was determined using the procedure of Helmreich and Cori [13]. The molecular weight of phosphorylase *b* monomer was taken to be 97,500 [14]. AMP (Merck); $^6$N-methylAMP, 1-N-methylAMP and 6-chloropurine riboside 5'phosphate (6ClPRP) (Terra-Marine Bioresearch); IMP, 2'AMP, 3'AMP, 2'dIMP and 2'dAMP (SIGMA) were of the highest purity; it was checked chromatographically on PEI cellulose. The buffer used in all the experiments was $5 \times 10^{-2}$ M glycylglycine, $5 \times 10^{-2}$ M KCl and $2 \times 10^{-4}$ M EDTA, the final pH being adjusted to 6.9 with KOH. All other chemicals used were of high purity.

Equilibrium dialysis measurements were performed at 25°C according to Myer and Schellman [15], as previously done by Morange et al. [9] with this enzyme. At this temperature, equilibrium is obtained in less than six hours. Enzyme activity was checked after this treatment in several control experiments and no appreciable activity loss was found during the first week after the third crystallization.

Binding heats of AMP analogues to phosphorylase *b* were measured at 25.00 ± 0.05°C using an LKB 10700 Batch Microcalorimeter equipped with gold cells. A Keithley 150B Microvoltmeter coupled with an LKB recorder with electronic integrator were used to amplify and record output from the calorimeter. The calorimeter was calibrated as described elsewhere [16]. In all experiments the dilution heat of the ligands was automatically cancelled by the reference cell. In separate experiments the dilution heat of the enzyme solution was determined and subtracted from the mixing heat when significant.

Sedimentation velocity experiments were carried out with an analytical Ultracentrifuge (Beckman Spinco Model E) at 60,000 rev/min and 25°C using Schlieren optics.

Light scattering measurements were carried out at 25.00 ± 0.05°C with a model 42,000 SOFICA Photogoniodiffusometer equipped with a xylene thermostated bath. Calibration was made with benzene after Kratohvil et al. [17] with correction for the different refraction indexes of benzene and xylene [18]. At 25°C the 90° Rayleigh ratio of the 546 nm beam used for benzene was $16.3 \times 10^{-6}$ cm$^{-1}$ [19].

The d*n*/d*c* values of the phosphorylase *b* were measured under several experimental conditions with a differential interferometer LI 3, Carl Zeiss JENA.

The average molecular weight of the protein was calculated with the equation derived by Townend and Timasheff [20] for systems undergoing association-dissociation:

$$\frac{Kc}{R_{90°}} = \frac{1}{\bar{M}_w} + \frac{2Bc}{M_D} = \frac{1}{M_{app}} , \qquad (1)$$

where $K = 2\pi^2 n_0^2 (dn/dc)^2 / N\lambda^4$, $n_0$ being the buffer index; $N$, Avogadro's number; $\lambda$, the wavelength of the incident beam; $c$, the protein concentration in g/ml; $\bar{M}_w$, the average molecular weight; $M_D$, the molecular weight of the dimer; $B$, the second virial coefficient; and $R_{90°}$, the Rayleigh ratio at 90° of the incident beam.

To remove dust, the solutions were carefully filtered three times through Millipore HA (0.45 μm average pore diameter), this being followed by centrifugation for 2 hours at 16,000 g and 4°C.

All the solutions used in light-scattering and interferometric measurements were dialyzed overnight at 4°C against the adequate solvent. The cleaning method of the pipettes and cells used in light scattering experiments was that of Timasheff and Townend [21]. SOFICA high optical quality cylindric cells were used.

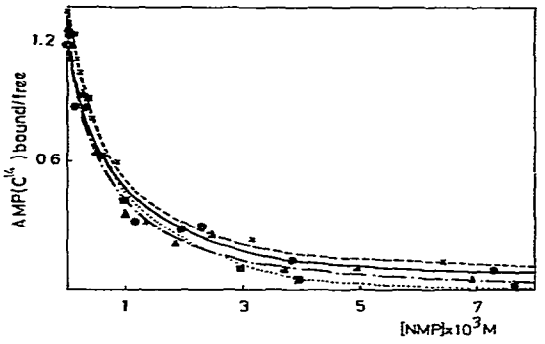


Fig. 1. Release of trace amounts of AMP $^{14}C$ by: • 1-N-methylAMP; ▲ $^{6}$N-methylAMP; X 6ClPRP; ■ IMP.

## 3. Results

### *3.1. Equilibrium dialysis*

Fig. 1 shows that all the AMP analogues in the base studied (i.e.: 6ClPRP, $^{6}$N-methylAMP, 1-N-methylAMP and IMP) completely displace the AMP-$^{14}C$ from its binding sites at 25°C. The displacement curves of AMP-$^{14}C$ by all the nucleotides mentioned above only overlap if the nucleotide concentration is reduced by a constant factor $f_A$ for each nucleotide; $f_A = K_{1/2upt}(NMP)/K_{1/2upt}(AMP)$, where $K_{1/2upt}(NMP)$ and $K_{1/2upt}(AMP)$ are the concentrations of NMP and AMP, respectively, needed to produce half the total displacement of AMP-$^{14}C$ bound to phosphorylase *b*. As shown earlier [9] this experimental finding indicates that the displacement of AMP-$^{14}C$ by the different nucleotides studied follows the same mechanism.

Fig. 2 shows the displacement of AMP-$^{14}C$ at 25°C by the analogues with chemical alterations in the ribose-phosphate part of the AMP, i.e. 2'AMP, 3'AMP, 2'dAMP and 2'dIMP. The deoxyderivatives displace only fifty per cent of the total amount of AMP-$^{14}C$ bound to the enzyme, while 2'AMP and 3'AMP completely displace the AMP-$^{14}C$ bound to phosphorylase *b*. Furthermore, fig. 2 also shows that low concentrations of 2'dAMP increase the amount of AMP-$^{14}C$ bound to the enzyme and that the displacement of AMP-$^{14}C$ by 2'dIMP starts at nucleotide concentrations higher than 4 mM.

Table 2 summarizes the values of $K_{1/2upt}(NMP)$ for all the nucleotides above mentioned.

### *3.2. Microcalorimetric data*

The results obtained for the enthalpic titration of the enzyme (2.7 mg/ml) with $^{6}$N-methylAMP, 1-N-methylAMP, 6ClPRP, IMP, 2'dAMP and 2'dIMP are given in figs. 3–8. The comparison between these results and those reported for AMP, 2'AMP and 3'AMP [10] lead us to the following experimental findings:

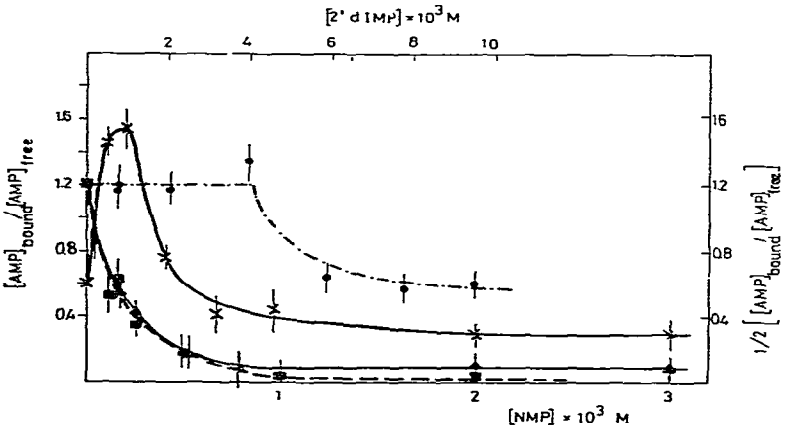


Fig. 2. Release of trace amounts of AMP $^{14}C$ by: ▲ 2'AMP, ■ 3'AMP, • 2'dIMP and X 2'dAMP. The scale in the right corresponds to 2'dAMP.

Table 1
Displacement of AMP-$^{14}$C by nucleotides at 25°C:
$K_{1/2\ upt}$: Nucleotide concentrations needed to produce half-displacement of AMP-$^{14}$C bound to phosphorylase *b*

| Nucleotide | $K_{1/2\ upt}$ | $K_a$ or $K_i$ [a] |
|---|---|---|
| AMP | $1.2 \times 10^{-4}$ | 1 |
| $^6$N-methylAMP | $1.2 \times 10^{-4}$ | 2 |
| 1-N-methylAMP | $4.2 \times 10^{-4}$ | 20 |
| 6ClPRP | $3.3 \times 10^{-4}$ | 5.5 |
| 2'AMP | $1.25 \times 10^{-4}$ | 34 |
| 3'AMP | $1.55 \times 10^{-4}$ | 37 |
| 2'dIMP | $4 \times 10^{-4}$ | 18 |
| IMP | $2.0 \times 10^{-4}$ | 32 |
| 2'dIMP | $4.5 \times 10^{-3}$ | 36 |

[a] Activation or inhibition constants, $K_a$ or $K_i$, respectively, taken from ref. [37].

a) Except for IMP and 2'dIMP, the titration of the enzyme with the AMP analogues has two distinct regions of saturation showing the existence of two types of binding sites with different affinities towards these nucleotides in phosphorylase *b*, as shown earlier with AMP [8,10].

b) The conclusions obtained from the double reciprocal plots of the enthalpic titration data of the enzyme (not shown) are:

— The absence of cooperativity in the saturation of phosphorylase *b* by IMP and 2'dIMP and in the saturation of the weaker affinity binding sites by 6ClPRP and $^6$N-methylAMP.

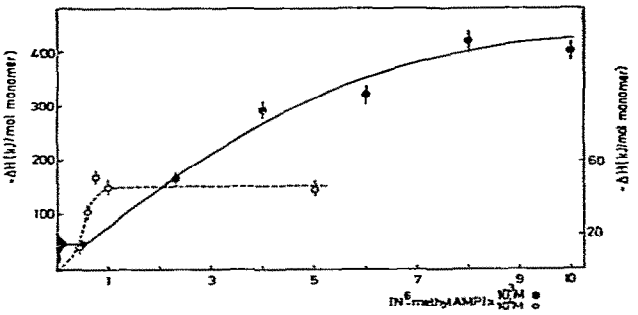


Fig. 3. Enthalpy titration of phosphorylase *b* with $^6$N-methyl-AMP. The solid line has been adjusted as indicated in the text. The dotted line is an amplification of the first plateau of the solid line (right side scale).

— The presence of positive cooperativity in the saturation of the higher and lower affinity binding sites for all the other cases.

The high interaction enthalpies of nucleotide with phosphorylase *b* (see figs. 3—8) have lead us to consider the possibility of formation of enzymatic aggregates induced by the binding of the nucleotide to the enzyme.

### *3.3. Ultracentrifuge and light scattering results*

At saturation of the first region of the microcalorimetric titration curves, all the studied nucleotides

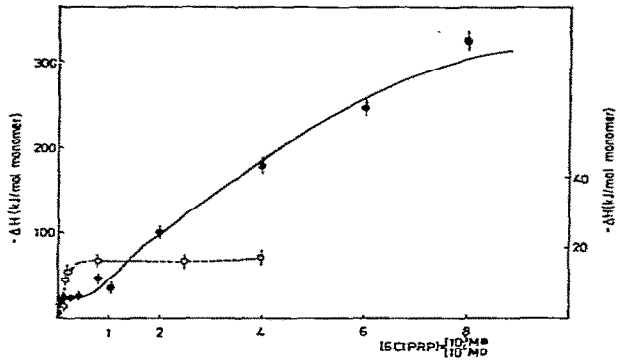


Fig. 4. Enthalpy titration of phosphorylase *b* with 6ClPRP. The solid line has been adjusted as indicated in the text. The dotted line is an amplification of the first plateau of the solid line (right side scale).

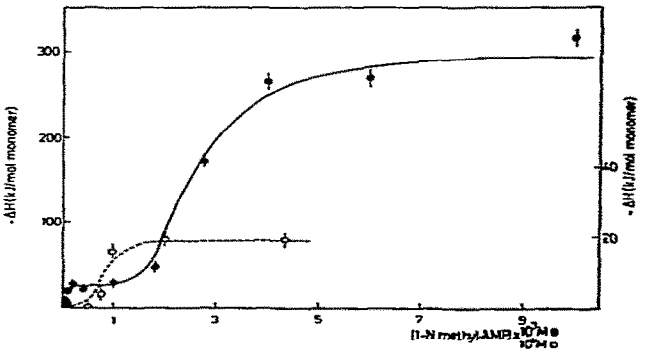


Fig. 5. Enthalpy titration of phosphorylase *b* with 1-N-methyl AMP. The solid line has been adjusted as indicated in the text. The dotted line is an amplification of the first plateau of the solid line (right side scale).

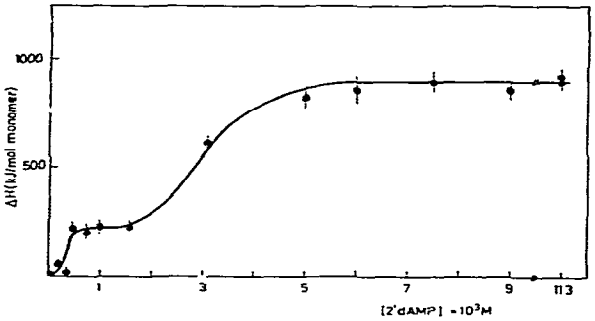


Fig. 6. Enthalpy titration of phosphorylase *b* with 2'dAMP. The solid line has been adjusted as indicated in the text.

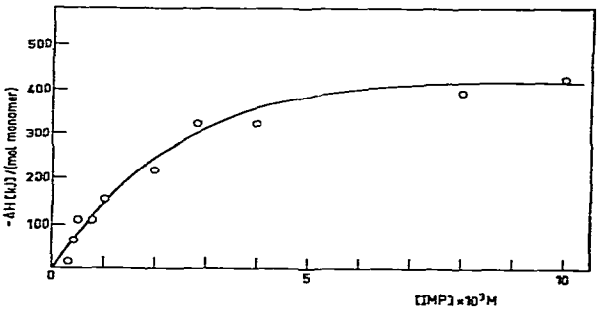


Fig. 7. Calorimetric titration of phosphorylase *b* with IMP.

stabilize a phosphorylase *b* dimer of (8.4–8.6)S although a small percentage of tetramer, less than 5% of the total amount of the enzyme, remains.

Phosphorylase *b* (2.7 mg/ml) in the presence of $10^{-2}$ M 1-N-methylAMP, 6ClPRP and $^{6}$N-methylAMP at 25°C shows a symmetric peak with a sedimentation coefficient of 12.8–13.2 S, corresponding to the tetramer of the enzyme [22]. In the presence of $10^{-2}$ M 2'AMP and 3'AMP at 25°C the enzyme has a symmetric pattern with a sedimentation coefficient of 9.1 S. This value was assumed to correspond to a modified dimer of the phosphorylase *b* [23]. At concentrations of $10^{-2}$ M of the deoxynucleotides 2'dAMP and 2'dIMP, and IMP the phosphorylase *b* is stabilized in a dimeric state which has a sedimentation coefficient of 8.6 S, the percentage of induced enzymatic tetramer in these conditions being less than 10%.

These experiments show that the 1-N-methyl AMP, 6ClPRP and $^{6}$N-methylAMP behave in the same way as AMP [24]. However, light scattering results [25] show the absence of an appreciable amount of phosphorylase *b* tetramer in the presence of $10^{-2}$ M AMP.

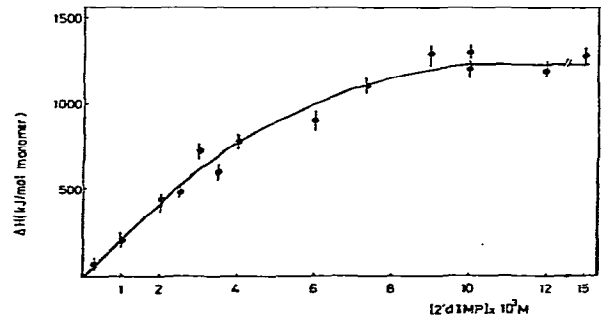


Fig. 8. Calorimetric titration of phosphorylase *b* with 2'dIMP.

To know the behaviour of these three nucleotides the following experiments were carried out. The $dn/dc$ value obtained for the enzyme in glycylglycine buffer was 0.183 ± 0.003, in agreement with literature data [26]. 1-N-methylAMP, 6ClPRP and $^{6}$N-methylAMP do not significantly alter the $dn/dc$ values of the enzyme solution. From these results and from the $I_{90°}$ values in the 4–0.5 mg/ml enzyme concentration range in a $10^{-2}$ M concentration of the above nucleotides an apparent molecular weight of the enzyme of (195,000–205,000) was obtained. The dissymetry ratios, $Z = I_{45°}/I_{135°}$, of the enzyme in the above conditions were in the range 0.99–1.02, indicating that this enzyme nearly behaves as a globular one, in agreement with small-angle X-ray scattering results [27].

The disagreement between ultracentrifugation and light scattering data obtained for phosphorylase *b* in the presence of 1-N-methylAMP, 6ClPRP or $^{6}$N-methylAMP can be explained if the centrifugal field could displace the equilibrium dimer ⇌ tetramer to the tetrameric form of the enzyme if the tetrameric form had a smaller specific volume.

### *3.4. Theoretical analysis of the calorimetric titration curves*

The calorimetric titration data which clearly exhibit two different saturation regions have been theoretically studied in terms of the binding sites generation model [10].

The experimental data of the first region of the titration curves of 1-N-methylAMP, 6ClPRP, $^{6}$N-methyl-

Table 2
Equilibrium constants calculated from the calorimetric titration curves

| Nucleotide | Site I | | | Site II | | |
|---|---|---|---|---|---|---|
| | $(1)L_{0,0}$ | $(1)K_R$ | $K_d$ | $(2)L_{0,0}$ | $(2)K_R$ | $(2)K_d$ |
| AMP [a] | 2000–5000 | $(2.5–4.5) \times 10^{-5}$ | – | 13000–15000 | $(8.0 \pm 0.5) \times 10^{-5}$ | – |
| $^6$N-methylAMP | 5–10 | $(2.5–3.1) \times 10^{-5}$ | – | – | – | $3.6 \times 10^{-3}$ |
| 1-N-methylAMP | 40<br>900–1000 [b] | $3 \times 10^{-5}$<br>$(2.0–2.1) \times 10^{-5}$ [b] | – | 1000±300 | $(1.0 \pm 0.1) \times 10^{-4}$ | – |
| 6-ClPRP | 5–10 | $(1.2–1.3) \times 10^{-5}$ | – | – | – | $(6.5 \pm 0.5) \times 10^{-3}$ |
| 2'AMP [a] | 100 | $(5.0 \pm 0.5) \times 10^{-5}$ | – | 1000–3000 | $(1.4–1.8) \times 10^{-4}$ | – |
| 3' AMP | 100 | $(5.0 \pm 0.5) \times 10^{-5}$ | – | 6000–10000 | $(1.5–1.7) \times 10^{-4}$ | – |
| 2'dAMP | 2000 | $3 \times 10^{-5}$ | – | 1000–3000 | $(1.0–1.2) \times 10^{-4}$ | – |
| IMP | – | – | $1.8 \times 10^{-3}$ | – | – | – |
| 2'dIMP | – | – | $3.7 \times 10^{-3}$ | – | – | – |

[a] From ref. [10]. [b] Calculated with Monod's Concerted Model [27] as indicated in the text.

Table 3
Thermodynamic properties of the phosphorylase *b*-nucleotide interactions at 25°C

| Nucleotide | Site I | | | Site II | | |
|---|---|---|---|---|---|---|
| | $\Delta G^0_I$ (a) | $\Delta H_I$ [b] | $\Delta S_I \times 10^3$ [c] | $\Delta G^0_{II}$ [a] | $\Delta H_{II}$ [b] | $\Delta S_{II} \times 10^3$ [c] |
| AMP [d] | –(16 ± 1) | –(120 ± 7) | –(347 ± 29) | –(12 ± 1) | –(240 ± 4) | –(765 ± 17) |
| $^6$N-methylAMP | –(23 ± 1) | –(45 ± 2) | –(75 ± 8) | –(14 ± 1) | –(400 ± 50) | –(1297 ± 171) |
| 1-N-methylAMP | –(21 ± 1) | –(21 ± 2) | ((0 ± 17) | –(15 ± 1) | –(275 ± 20) | –874 ±71) |
| 6ClPRP | –(23 ± 1) | –(17 ± 2) | (21 ± 17) | –(13 ± 1) | –(450 ± 50) | –(1464 ± 171) |
| 2'AMP [d] | –(19 ±1 ) | –(65 ± 2) | –(155 ± 8) | –(13 ± 1) | –(235 ± 12) | –(745 ± 42) |
| 3'AMP [d] | –(19 ± 1) | –(117 ± 8) | –(330 ± 33) | –(11 ± 1) | –(283 ± 20) | –(912 ± 75) |
| 2'dAMP | –(16 ± 1) | –230 ±10) | –(715 ± 38) | –(14 ± 1) | –(670 ± 25) | –(2201 ± 105) |
| IMP | –(16 ± 1) | –(425 ± 15) | –(1372 ± 54) | – | – | – |
| 2'dIMP | –(13 ± 1) | –1250 ±50) | –(4100 ± 167) | – | – | – |

[a] $\Delta G^0 = RT \ln {}^{(i)}K_T$, where ${}^{(i)}K_T = {}^{(i)}L_{0,0} \cdot {}^{(i)}K_R^i$ ($i = 1$ or 2) is the total dissociation constant of the complex; $\Delta G^0$ in kJ (mol monomer)$^{-1}$.
[b] $\Delta H$, in kJ (mol monomer)$^{-1}$. [c] $\Delta S$ in kJ (mol monomer)$^{-1}$ K$^{-1}$. [d] From ref. [10].

AMP and 2'dAMP were fitted to the eq. (2), derived to explain the experimental results obtained for the first region of the titration curves of AMP, 2'AMP and 3'AMP [10]

$$^{(1)}\Delta H_T/^{(1)}\Delta H_p = \alpha^2/(^{(1)}L_{0,A} + 1 + \alpha^2) \qquad (2)$$

and

$$^{(1)}L_{0,A} = {}^{(1)}L_{0,0}/(1 + 2\alpha).$$

Then, the saturation of these first regions of the titration curves mentioned above implies the existence of interdimer enzymatic interactions during the saturation by the nucleotide of its stronger affinity binding sites, these interdimeric interactions being destroyed when these binding sites are saturated.

Table 2 summarizes the calculated values for the allosteric constants $^{(1)}L_{0,0}$ and $^{(1)}K_R$ associated to the first regions of the mentioned titration curves. It should be pointed out that the concerted model [28] only fits, within experimental error, the data of the first region of the titration curve of 1-N-methylAMP. The calculated values of $^{(1)}K_R$ and $^{(1)}L_0$ for 1-N-methylAMP using the concerted model are also given in this table.

On the other hand, the saturation of weaker binding sites by 2'dAMP and 1-N-methylAMP can be described with the equation derived for the saturation by AMP, 2'AMP and 3'AMP of the weaker binding sites [10]:

$$^{(2)}\Delta H_T/^{(2)}\Delta H_p = (8[E_t]\,\alpha^4)^{-1} \times \{-(1 + {}^{(2)}L_{0,A}) + \sqrt{(1 + {}^{(2)}L_{0,A})^2 + [E_t]\alpha^4}\}^2 \qquad (3)$$

where

$$^{(2)}L_{0,A} = {}^{(2)}L_{0,0}/(1 + \alpha)^2.$$

The description of the weaker binding site saturation by 1-N-methylAMP using this equation implies the existence of strong interdimeric interactions during this process which disappear when these weaker binding sites are completely saturated.

For the enthalpic titration curves which do not show positive cooperativity the dissociation constant of the nucleotide-phosphorylase *b* complex has been calculated as previously done by Bjurulf et al. [16] for the enthalpic titration of lisozyme with N-acetylglucosamine.

The calculated constants for this nucleotide-phosphorylase *b* complexes are given in table 2.

Table 3 summarizes the thermodynamic properties of the interactions between the AMP analogues and phosphorylase *b* at 25°C.

## 4. Discussion

Except for IMP and 2'dIMP the microcalorimetric data of the interaction between phosphorylase *b* and the nucleotides can be described by the "binding sites generation" model [10].

The interaction of IMP with phosphorylase *b* shows only one region of enthalpic saturation, contrarily to the case of AMP, according to results obtained by Ho and Wang in glycerophosphate buffer [7].

Recently Kasvinsky et al. [29] have demonstrated the existence of two binding sites of IMP per phosphorylase monomer with a binding constant of the same order of that obtained by microcalorimetry.

There are important differences between the results of Ho and Wang [7] and ours, namely:

1. In the glycerophosphate buffer [7] the interaction of IMP with the enzyme shows positive cooperativity.

2. The enthalpy of interaction is quite different in the two buffers. The disagreements are due to the fact that glycerophosphate seems to stabilize a conformational state of the enzyme quite different from that stabiiized in glycylglycine [24,30].

The high entropic increments of table 3 seem to show the existence of important conformational alterations in the enzyme when interacting with the nucleotides. The values of $\Delta G^0$ for the saturation of both types of binding sites by the nucleotides show that the energetically most favourable saturation processes are those of the AMP analogues chemically modified in the base (6ClPRP, $^6$N-methylAMP and 1-N-methylAMP).

The low values of $\Delta S_1$ and $^{(1)}L_{0,0}$ for 1-N-methylAMP and 6ClPRP seem to indicate the existence of small conformational variations when they bind to their stronger binding sites in the enzyme.

The results given in the present paper indicate that the mechanism of saturation of the second class of binding sites in phosphorylase *b* exhibit a great specifity

towards the adenine of the AMP molecule, but it is practically insensitive to modifications in the ribose-phosphate part.

Besides, the N(1) and the chemical nature of the group in the C(6) of the puric base of the nucleotide should play an important role in enhancing conformational transitions in phosphorylase, as can be seen in figs. 3–8 and table 3; this supports the hypothesis of Morange et al. [9]. Recently Fletterick and Madsen [31] have demonstrated that the weaker affinity binding site of the nucleotide is the same as the stronger affinity binding site of adenine, in agreement with previous results [9]. The weaker affinity binding site of AMP is involved in enzymatic catalysis, as has been demonstrated by Kasvinsky et al. [29].

Except in the case of IMP, whose notorious differences with AMP in its action upon phosphorylase *b* we have already mentioned, the tetramerization of phosphorylase *b* by the nucleotides under the conditions of the ultracentrifugation experiments seems to be a consequence of the saturation by the nucleotide of its weaker affinity binding site. This tetramerization is rather insensitive to the alterations in the puric base, while it is enormously sensitive to modifications in the ribose-phosphate part of AMP.

A common factor to all AMP analogues in the ribose-phosphate part here studied is that the syn⇌anti equilibrium with respect to the AMP analogues in the puric base (AMP, 6ClPRP, 1-N-methylAMP and $^{6}$N-methylAMP) is displaced towards the syn conformation [32]. Since a high catalytic activity of phosphorylase *b* probably requires the binding of the nucleotide in an "anti" conformation [29,33], the capacity to induce tetramerization could be related to enzyme activation and to the conformation adopted by the nucleotide upon binding to the enzyme.

The different requirements to the chemical groups of the nucleotide molecule for the binding and for enyzmatic activation [5, and tables 1 and 2] show that the action of the nucleotide upon phosphorylase *b* has two clearly differentiated aspects: a structural one, i.e. the action upon certain regions of the enzyme, and a catalytic one, i.e. the action on the catalysis or on the right formation of the active center, each one of them governed by different parts of the AMP molecule.

The deoxyderivatives 2′dAMP and 2′dIMP give enthalpies of interaction with phosphorylase *b* much higher than all other nucleotides studied (figs. 7 and 8). The enthalpy contributions of the differential effects of protonation of the buffer to the total enthalpies of interaction could not be evaluated, due to the instability of phosphorylase *b* in distilled water [26]. Other two possible contributions to the high measured enthalpies of interaction could be: (a) the inespecif interaction of the phosphate of the nucleotide with the enzyme and (b) the formation of multimeric aggregates of phosphorylase *b* through the action of nucleotides. However, there is no appreciable amount of enzymatic aggregates at saturating concentrations of 2′dAMP and 2′dIMP, as the ultracentrifuge experiments reveal (see the results), and therefore hypothesis (b) can be disregarded.

The high values of $\Delta S$ obtained for these cases could be a consequence of the processes we have postulated in order to justify the high interaction enthalpies measured. Of course, these high values show the great complexity of the binding processes of the effectors to the enzyme.

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