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A MODEL FOR THE BEHAVIOUR OF PHOSPHORYLASE *b*. THE GENERATION OF DIFFERENT BINDING SITES VIA INTERMEDIATE ENZYMATIC STATES

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The model given in this paper can be applied to enzymatic systems which have more than two conformational states in equilibrium and which clearly exhibit heterogeneity in the binding of one ligand. The model we propose makes possible quantitative interpretation of our experimental results and of those of many other workers as well. In some cases calorimetric, dialysis and kinetic magnitudes, when plotted against ligand concentration, give multiregional or "stepwise" curves. We suggest that such a behaviour arises because total occupation of one class of binding sites completely moves the enzyme towards a different conformational state in which the affinity for the ligand is greatly increased by the formation of a new class of binding sites. Our calorimetric results for the interaction between some nucleotides and phosphorylase *b* closely conform to our model

1. Introduction

Kinetic, calorimetric and dialysis data, in some cases lead to curves which show two or more regions of different saturation [1–7]. These cannot be theoretically interpreted on the basis of either the concerted model of Monod et al. [8] or of the sequential model of Koshland et al. [9]. Generally, these binding processes have different characteristics in the two regions, one of which exhibits positive cooperativity and the other negative cooperativity for the same ligand in oligomeric enzymes [10]. Generalization of the sequential model [11,12] only explains the "stepwise" curves obtained when the log of ligand concentration is represented in the X-axis, if it is assumed that there exist two binding sites of different affinity towards the ligand as a result of the formation of hybrids of the sub-units in different conformational states. Several authors [13–15] have shown that the macroscopic phenomena of "negative cooperativity" necessarily arise from the functional asymmetry of the oligomer. Further support for this hypothesis has been presented by Matthews and Bernhard [16] and Seydoux et al. [17]. Very recently, Viratelle and Seydoux [10] have proposed a different extension of the "concerted model" known as the "pseudo-conservative model", in which the principle of maximum symmetry is eliminated. Their extension explains the coexistence of both types of cooperativity, positive and negative, provided one of the two quaternary states in equilibrium has functional pairwise asymmetry. All the above models consider an equilibrium between two conformational states with different affinity towards the ligand.

In this communication we suggest a different extension of the generalized allosteric models which explains the multi-regional curves when either the ligand concentration or the log of the ligand concentration is represented in the X-axis. We should note that the above mentioned allosteric models only explain the case of multi-regional curves when any property of the system is plotted versus the log of the ligand concentration. In our approach we assume the presence of intermediate enzymatic conforma-

tional states which form a new set of binding sites of different affinity towards the ligand.

Glycogen phosphorylase *b* from rabbit muscle (E.C. 2.4.1.1.) is a known allosteric enzyme. AMP is indispensable to the catalytic activity of this enzyme [18]. The phosphorylase *b* activation process by AMP is clearly sigmoidal indicating strong positive cooperativity between two binding sites and heterotropic cooperativity between AMP and the substrates of the enzyme [19,20]. On the other hand, the different activations of AMP and IMP [21] indicate the existence of more than two conformational state equilibria, which can explain the observed differences with respect to the concerted model [22]. Buc et al. [23] proposed an alternative conformational situation based on their T-jump studies. Their model contains three conformational dimeric states and a tetrameric one, in equilibrium at 4°C. This scheme agrees very well with that of Radda et al. [24] deduced from NMR and ESR studies.

Recent microcalorimetric [1,2] and dialysis [25] studies have revealed the existence of two different affinity sites in phosphorylase *b* for AMP. Our model enables us to interpret the calorimetric results we have obtained for glycogen phosphorylase *b* from rabbit skeletal muscle. We call our model "the binding sites generation model".

2. The binding sites generation model

The hypothesis are:

a) For an oligomer of n-subunits there can be N-types of ligand binding sites per monomer. The total equivalent binding sites of each type are $a_i n$, a_i being a rational number. Therefore, the total ligand binding sites of all types is $n\Sigma_{i=1}^{N} a_i$. The coefficient a_i will not equal unity when cooperativity between more than n-centers exists (e.g. in aggregate or pseudoaggregate intermediate states).

b) The enzyme shows $(N+1)$ conformational states and N transconformational equilibria:

$$E_1 \rightleftharpoons E_2 \rightleftharpoons E_3 \rightleftharpoons E_4 \rightleftharpoons \ldots E_N \rightleftharpoons E_{N+1}$$

but in the absence of ligands the situation can usually be expressed in terms of an equilibrium between two states, because in most cases the enzymatic population of the other states can be considered as negligible.

c) ${}^{(i)}T$ and ${}^{(i)}R$ are the enzymatic states of the i-type binding sites with smaller and higher affinities towards the ligand respectively. If saturation by a ligand of its binding sites promotes long range intermolecular interactions, i.e. the formation of aggregate intermediate state with varying degrees of stability, then the allosteric transconformational constant which determines the relative population of the ${}^{(i)}T_0$ and ${}^{(i)}R_0$ unbounded forms, L_0, will be a function of the ligand concentration. The type of this function depends on the ligand-enzyme system. Therefore, we shall denote as $L_{0,0}$ and $L_{0,A}$ the classical allosteric constant L_0 in the absence and in the presence of a given concentration of the ligand A, respectively.

d) The "apparent generation" of a new class of binding sites $(i+1)$ requires the full saturation of class (i) of binding sites, which has higher affinity towards the ligand. So, the conformational state E_{i+1} is stabilized. This enzymatic state E_{i+1}, simultaneously acts as ${}^{(i)}R$ with respect to the $(i+1)$ class of binding sites if the saturation of these latter binding sites shows a sigmoidal shape.

The following parameters are needed to describe the enzymatic saturation behaviour by a ligand:

1) The N constants of the transconformational equilibria ${}^{(i)}L_{0,0}$ $(i = 1, 2, \ldots N)$ defined as the ratio between the concentrations of the ${}^{(i)}T_0$ and ${}^{(i)}R_0$ conformational states in the absence of the ligand, i.e.

$${}^{(i)}L_{0,0} = \lim_{[A]\to 0} {}^{(i)}L_{0,A} \qquad (1)$$

2) The $2N$ intrinsic constants of dissociation of the ligand to the different sites in the different conformational states:

$${}^{(1)}T \rightleftharpoons {}^{(1)}R$$

$${}^{(1)}k_T \qquad {}^{(1)}k_R \qquad {}^{(1)}R \equiv {}^{(2)}T$$

$$\ldots\ldots\ldots\ldots$$

$${}^{(i-1)}T \rightleftharpoons {}^{(i-1)}R$$

$${}^{(i-1)}k_T \qquad {}^{(i-1)}k_R \qquad {}^{(i-1)}R \equiv {}^{(i)}T$$

$${}^{(i)}T \rightleftharpoons {}^{(i)}R$$

$${}^{(i)}k_T \qquad {}^{(i)}k_R \qquad {}^{(i)}R \equiv {}^{(i+1)}T$$

$$\ldots\ldots\ldots\ldots$$

Using the above parameters, the saturation function,

$\overline{Y}_t$, for a ligand can be expressed:

$$\overline{Y}_t = (1/N)(\overline{Y}_1 + f_1\overline{Y}_2 + f_1f_2\overline{Y}_3 + \ldots\ldots + f_1f_2 \ldots\ldots f_{N-1}\overline{Y}_N) \quad (2)$$

where $f_1, f_2 \ldots\ldots f_{N-1}$ are the enzyme fractions which completely saturate the first, second ... $(N-1)$ types of sites respectively, and $\overline{Y}_t$ is the total saturation function. If there is high homotropic cooperativity between the different binding site types the values of f_i $[i = 1, 2, \ldots (N-1)]$ will be very similar to the values of $\overline{Y}_i$, the saturation function for every type of sites. This is so because the population for each partially saturated sites type of enzymatic states should be minimal. We can then write:

$$\overline{Y}_t = (1/N)(\overline{Y}_1 + \overline{Y}_1\overline{Y}_2 + \ldots + \overline{Y}_1\overline{Y}_2 \ldots \overline{Y}_N) = (1/N)\sum_{l=1}^{N}\prod_{i=1}^{l}\overline{Y}_i \quad (3)$$

The saturation function of the i-class of binding sites, $\overline{Y}_i$, can now be calculated. In general this function will be obtained as previously shown [8–12], except that the value of L_0 must be introduced in $\overline{Y}_i$ as a function of the ligand concentration if long range intermolecular interactions occurs during the saturation process.

We can greatly simplify the above if we consider that the binding of the ligand to the different binding sites is exclusive [8]. Then, only N intrinsic dissociation constants at the different states R, ${}^{(i)}k_R$, and N equilibrium constants, ${}^{(i)}L_{0,0}$ are needed.

3. $L_{0,A}$ dependence upon the ligand concentration

As stated above, the transconformational equilibrium constant, $L_{0,A}$, depends upon the ligand concentration if long range interactions between different oligomers are significant during the saturation of the protein by the ligand. These interactions will be denominated interoligomeric interactions, and will be described by ${}^{(i)}R_m \ldots {}^{(i)}R_p$, where m and p denote the number of bound ligand molecules per enzyme oligomer. In these cases the relative populations of the ${}^{(i)}R_0$ and ${}^{(i)}T_0$ states depend on the ligand concentration, since the ${}^{(i)}R_0$ or ${}^{(i)}T_0$ interacting molecules form intermediate enzymatic aggregates. We assume that these aggregates are not in equilibrium with their respective tautomeric forms.

If the ligand binds exclusively the ${}^{(i)}R$ state, ${}^{(i)}L_{0,A}$ can be written:

$${}^{(i)}L_{0,A} = \frac{[{}^{(i)}T_0]}{[{}^{(i)}R_0]_f + [{}^{(i)}R_0]_b} \quad (4)$$

where $[{}^{(i)}R_0]_b$ and $[{}^{(i)}R_0]_f$ refer to the ${}^{(i)}R_0$ concentrations bounded and unbounded by interoligomeric interactions to other oligomer, respectively. Taking into account the definition of ${}^{(i)}L_{0,0}$, eq. (4) adopts the form:

$${}^{(i)}L_{0,A} = {}^{(i)}L_{0,0}(1 + [{}^{(i)}R_0]_b/[{}^{(i)}R_0]_f)^{-1} \quad (5)$$

The $[{}^{(i)}R_0]_b/[{}^{(i)}R_0]_f$ ratio introduces the ${}^{(i)}L_{0,A}$ dependence upon the ligand concentration in eq. (5). Its algebraic form is a function of the saturation degree, P, of the enzymatic ${}^{(i)}R$ states necessary to produce relevant interoligomeric interactions and of the number of independent i-type binding sites, n. We shall consider for simplicity the case of the exclusive binding of the ligand, A, to the ${}^{(i)}R$ enzymatic state, following the hypothesis of the concerted model (8):

$${}^{(i)}T_0 \rightleftharpoons {}^{(i)}R \qquad {}^{(i)}L_{0,0} = \lim_{[A]\to 0} {}^{(i)}L_{0,A}$$

$${}^{(i)}R_0 + pA \rightleftharpoons {}^{(i)}R_p \qquad K_T = \frac{[{}^{(i)}R_0]_t[A]^p}{[{}^{(i)}R_p]}$$

$${}^{(i)}R_p + {}^{(i)}R_0 \rightleftharpoons {}^{(i)}R_p \ldots {}^{(i)}R_0$$

$$K_b = \frac{[{}^{(i)}R_p]_f[{}^{(i)}R_0]_f}{[{}^{(i)}R_p \ldots {}^{(i)}R_0]} \qquad (0 \leqslant p \leqslant n) \quad (6)$$

From the above expressions and considering the relation between ${}^{(i)}K_T$ and the intrinsic binding constants (8), it is easily obtained that

$$[{}^{(i)}R_p]_t = \binom{n}{p}[{}^{(i)}R_0]_t\alpha^p,$$

where α is the reduced ligand concentration ($\alpha = [A]/{}^{(i)}k_R$).

The stoichiometry of the third equilibrium shows that in this case

$$[{}^{(i)}R_0]_b = \sum_{p=0}^{p}[{}^{(i)}R_p \ldots {}^{(i)}R_0],$$

then the $[^{(i)}R_0]_b/[^{(i)}R_0]_f$ ratio can be easily deduced.

Systems with high $^{(i)}L_{0,0}$ have negligible concentrations of ensambled $^{(i)}R_0$ forms except for the cases with strong interoligomeric interactions, i.e. very low K_{bp}. In these latter cases $[^{(i)}R_0]_f$ will be negligible. Thus the expression of $^{(i)}L_{0,A}$ adopts the form:

$$^{(i)}L_{0,A} \approx [^{(i)}T_0]/\{[^{(i)}R_0 \dots {}^{(i)}R_0] + [^{(i)}R_1 \dots {}^{(i)}R_0] + \dots + [^{(i)}R_p \dots {}^{(i)}R_0]\}$$

using the assumed stoichiometric relation $[^{(i)}R_0]_b = [^{(i)}R_p \dots {}^{(i)}R_0]$.

Considering the definition of $^{(i)}L_{0,0}$ given above:

$$^{(i)}L_{0,A} = {}^{(i)}L_{0,0}\left\{1 + \frac{[^{(i)}R_1 \dots {}^{(i)}R_0]}{[^{(i)}R_0 \dots {}^{(i)}R_0]} + \frac{[^{(i)}R_2 \dots {}^{(i)}R_0]}{[^{(i)}R_0 \dots {}^{(i)}R_0]} + \dots + \frac{[^{(i)}R_p \dots {}^{(i)}R_0]}{[^{(i)}R_0 \dots {}^{(i)}R_0]}\right\}^{-1}.$$

Due to the high affinity between the enzymatic dimers we can assume that the $^{(i)}R_p \dots {}^{(i)}R_0$ concentration will be proportional to the molecular collisions between $^{(i)}R_p$ and $^{(i)}R_0$. Let us suppose that the energy difference between $^{(i)}R_i \dots {}^{(i)}R_0$ and $^{(i)}R_j \dots {}^{(i)}R_0$ is smaller than the thermal energy at 25°C. Then, we can write:

$$[^{(i)}R_p \dots {}^{(i)}R_0] = c[^{(i)}R_p]_t[^{(i)}R_0]_t,$$

where c is a constant in the usual experimental conditions (i.e., fixed temperature and total enzyme concentration) independent of the value of p.

For these latter cases we have considered two general situations:

I) The interoligomeric interactions vanish at saturation levels higher than a determined value, p. This behaviour is probably caused by the increase of the electrostatic repulsion forces between bound anionic or cationic ligands.

In these cases:

$$^{(i)}L_{0,A} = \frac{^{(i)}L_{0,0}}{1 + \binom{n}{1}\alpha + \binom{n}{2}\alpha^2 + \dots + \binom{n}{p}\alpha^p} \quad (p \leqslant n) \qquad (7)$$

II) The interoligomeric interactions tend to stabilize oligomeric aggregates. It should be pointed out an important difference between the case I when $p = n$ and the case II. Only in this latter case, for high cooperative saturation processes, the average molecular weight of the protein depend on the binding sites saturation by the ligand. In this situation the expression of $^{(i)}L_{0,A}$ is:

$$^{(i)}L_{0,A} = {}^{(i)}L_{0,0}/(1 + \alpha)^n. \qquad (8)$$

Fig. 1 shows the calculated values for the saturation function (Y_t) in an enzymatic system which apparently follows the concerted model with two subunits ($n = 2$) and with two different types of binding sites ($N = 2$). It has been assumed that the ligand binding is exclusive to the $^{(i)}R$ states. Several cases with different $^{(i)}L_{0,0}$ and $^{(i)}k_R$ have been analysed. The plots correspond to two allosteric equilibria between three conformational states, in agreement with our "binding sites generation model". Fig. 1 indicates that a system with two different types of binding sites sometimes shows a behaviour similar to the one expected on the basis of the Monod model [8]. To distinguish very clearly between both classes of binding sites in the saturation function it is necessary that $^{(i)}k_R \ll {}^{(i)}k_R$, that $^{(1)}L_{0,0}$ be high, that $^{(2)}L_{0,0} \gg {}^{(1)}L_{0,0}$, and also that interoligomer interactions are significant during the process, as will appear below for the system phosphorylase *b*-AMP.

4. Application of the above model to phosphorylase *b*-AMP calorimetric results

4.1. Experimental

Glycogen phosphorylase *b* from rabbit skeletal muscle was obtained by the method of Krebs et al. [26]. The molecular weight of the glycogen phosphorylase *b* monomer was taken to be 97,500 Daltons [27]. The activity of the enzyme was determined following Helmreich and Cori's procedure [28]. AMP was obtained from Merck and 2'AMP and 3'AMP were obtained from Sigma Chemical Co. The purity of the nucleotides was checked chromatographically on PEI cellulose. The buffer used in all the experiments was glycylglycine, pH 6.9 [25]. Calorimetric measurements were performed using an LKB batch microcalorimeter at 25°C as previously described [5].

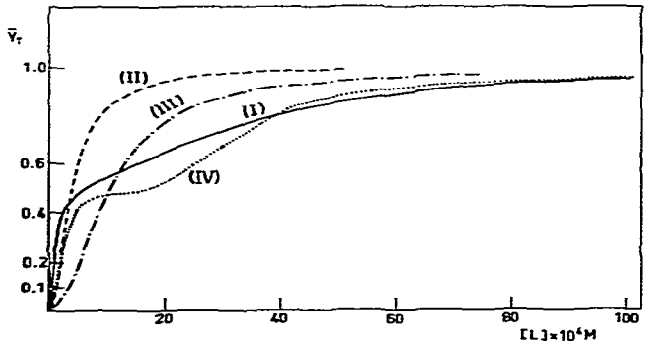

Fig. 1. Application of the model to a case with three conformational states in equilibrium with two types of binding sites ($N = 2$), and each one with two equivalent sites ($n = 2$). The curves I, II, and III correspond to different values of $^{(1)}k_R$, $^{(2)}k_R$, $^{(1)}L_{0,0}$ and $^{(2)}L_{0,0}$ for the case of $a_1 = a_2 = 1$, assuming that the saturation of each class of binding sites follows the concerted model. I. $^{(1)}k_R = 10^{-5}$, $^{(2)}k_R = 10^{-4}$, $^{(1)}L_{0,0} = 100$, $^{(2)}L_{0,0} = 1000$. II. $^{(1)}k_R = 10^{-5}$, $^{(2)}k_R = 10^{-4}$, $^{(1)}L_{0,0} = 1000$, $^{(2)}L_{0,0} = 10$. III. $^{(1)}k_R = 10^{-4}$, $^{(2)}k_R = 10^{-3}$, $^{(1)}L_{0,0} = 100$, $^{(2)}L_{0,0} = 1000$. IV. $^{(1)}k_R = 10^{-5}$, $^{(2)}k_R = 10^{-3}$, $^{(1)}L_{0,0} = 500$, $^{(2)}L_{0,0} = 5000$. $a_2 = 2$, $n = 2$ and $^{(2)}L_{0,A} = {}^{(2)}L_{0,0}(1+\alpha)^{-2}$. The saturation of the stronger binding sites is assumed to follow the concerted model.

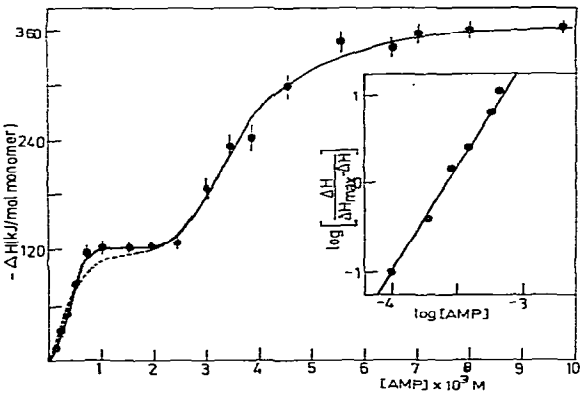

Fig. 2. Enthalpy titration of phosphorylase *b* with AMP at 25°C. The continuous line of the first region of the curve has been drawn using values of $(2.5-4.5) \times 10^{-5}$ and (5000–2000) for $^{(1)}k_R$ and $^{(1)}L_{0,0}$, respectively. The broken line of the first region of the curve corresponds to the calculated values of ΔH assuming that the saturation of this region follows the concerted model, the values of k_R and L_0 are given in the table 2. For the second region we have used a value of 5.0×10^{-4} for $^{(2)}k_R$ and a value of 5000 for $^{(2)}L_{0,0}$. For additional explanation see the text. Insert: Hill plot of the first region of the curve.

4.2. Results

Fig. 2 shows the experimental results obtained for the enthalpy of titration of phosphorylase *b* with AMP at 25°C. The plot has two quite different profiles which possess two distinct plateaus with high positive cooperativity in both regions.

Because of the clear separation between both plateaus each region can be studied separately. The equilibrium situation can be described by the conformational scheme:

$$D_1 \rightleftharpoons D_2 \rightleftharpoons D_3$$

where D_1, D_2, and D_3 are dimeric states. In our particular case $D_1 \equiv {}^{(1)}T$, $D_2 \equiv {}^{(1)}R \equiv {}^{(2)}T$ and $D_3 \equiv {}^{(2)}R$.

On the basis of the ultracentrifuge results, it has been concluded that the saturation of the weaker binding sites of the phosphorylase *b* by AMP at 25°C produces the total tetramerization of the enzyme, in glycylglycine buffer 5×10^{-2} M buffer [5] while the inhibitors, like 2'AMP and 3'AMP, do not (unpublished results). However, light scattering experiments recently carried out at 25°C in the same experimental conditions as those of the ultracentrifuge experiments reported elsewhere [5] show the absence of an appreciable amount of phosphorylase *b* tetramer at saturation of the weaker nucleotide binding sites by AMP. This discrepancy could be explained considering the probable alteration of the dimer ⇌ tetramer equilibrium by the ultracentrifugal field, since important changes in the specific partial volume have been shown [29] to take place in the enzyme solution between 1 and 3 mg/ml.

4.3. Equilibrium $^{(1)}T \rightleftharpoons {}^{(1)}R$

As a first and usual approximation [30,31] we can consider the enthalpy variation versus AMP concentration as the saturation function $\bar{Y}$. The Hill plot of ΔH for this region is practically linear ($n_H = 2.5$) [5]. This indicates that the population of the $^{(1)}R_1$ states can be neglected. Thus we can describe the first region by the equilibria:

$$^{(1)}T_0 \overset{^{(1)}L_{0,A}}{\rightleftharpoons} {}^{(1)}R_0$$
$$^{(1)}R_0 + 2A \overset{K}{\rightleftharpoons} {}^{(1)}R_2 \qquad (9)$$

where $^{(1)}T_0$ and $^{(1)}R_0$ are the enzymatic conformational states without any ligand molecule bound and $A \equiv$ AMP. $^{(1)}L_{0,A}$ is the transconformational equilibrium constant in the presence of ligand and K is the total dissociation constant of the first and second AMP molecules; ($K = K_1K_2 = {}^{(1)}k_R^2$ where $^{(1)}k_R$ is the intrinsic binding constant of AMP to the two equivalent binding sites of $^{(1)}R$, taking into account statistical factors [8]).

On the basis of the above conformational scheme we can express the total heat variation in the following form:

$$^{(1)}\Delta Q_T = 2^{(1)}\Delta H_b[^{(1)}R_2]_t + {}^{(1)}\Delta H_t\overline{(\Delta^{(1)}(R)[E_t]} \quad (10)$$

where: $^{(1)}\Delta H_b$ is the binding enthalpy of a molecule of AMP to the enzyme.
$^{(1)}\Delta H_t$ is the overall enthalpy associated to the transconformational change ($^{(1)}T \rightarrow {}^{(1)}R$) induced by AMP $^{(1)}\Delta Q_T$ is the total heat observed for a given AMP concentration. ($\Delta^{(1)}R$) is the difference between the molar fraction of the $^{(1)}R$ form of the enzyme in the presence of AMP and the fraction of the $^{(1)}R$ form in the absence of AMP. $[E_t]$ is the total enzyme concentration.

The value of $(\overline{^{(1)}R})$ can be estimated as:

$$(\overline{^{(1)}R}) = \frac{[^{(1)}R_0]_t + [^{(1)}R_2]_t - [^{(1)}R_0]_0}{[E_t]} \qquad (11)$$

where $[^{(1)}R_0]_t$ and $[^{(1)}R_0]_0$ are the enzyme concentrations in the $^{(1)}R_0$ form, in the presence or absence of AMP, respectively.

Previously reported L_0 transconformational values are high, ranging from 600 to 5000 under different conditions [20,22]. We can, therefore, to a first approximation assume that the $^{(1)}R_0$ enzyme concentration in either the presence or absence of AMP is negligible. Under these conditions eq. (10) may be reduced to the form:

$$^{(1)}\Delta Q_T/[E_t] = (^{(1)}\Delta H_t + 2^{(1)}\Delta H_b)(\overline{^{(1)}R_2}), \qquad (11a)$$

where $(\overline{^{(1)}R_2})$ is the molar fraction of the form $^{(1)}R_2$ for a given AMP concentration. The value of $(\overline{^{(1)}R_2})$ can be calculated as a function both of the AMP concentration and of the kinetic constant of the scheme set out above. We finally obtain eq. (12) as shown in the appendix A.

$$\frac{^{(1)}\Delta H_T}{^{(1)}\Delta H_p} = \frac{\alpha^2}{^{(1)}L_{0,A} + 1 + \alpha^2}. \qquad (12)$$

The experimental results obtained for this first saturation region of fig. 2 can be described alternatively by:

a) $^{(1)}L_{0,A} = {}^{(1)}L_{0,0}$,

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

Model (a) implies that the first region of saturation by AMP follows the concerted model and model (b) is one of those analysed above. A poor agreement of the experimental data with model (a) is observed. It should also be pointed out that model (a) cannot give a good agreement with experiment if the population of $^{(1)}R_1$ is considered, while model (b) does it without introduction of an appreciable change in the calculated allosteric constants. Moreover the value of $n_H > 2$ derived from the Hill plot of the data of this first saturation region cannot be explained by using the concerted model with only two independent binding sites [20,22]. Model (b) on the other hand, can justify this value of $n_H > 2$, as it implies the existence of interoligomeric interactions. The small percentage of $^{(1)}R_1$, the enzymatic species showing interoligomeric interaction (see the section 3), could explain the difficulty to distinguish between both situations.

Using our experimental results, we have obtained the parameters $^{(1)}k_R$ and $^{(1)}L_{0,0}$ in eq. (12) for both models (a) and (b) choosing the values of $^{(1)}k_R$ between 10^{-6} M and 10^{-3} M which give a best fit to a given value of $^{(1)}L_{0,0}$. The calculated values of $^{(1)}k_R$ and $^{(1)}L_{0,0}$ are given in table 2. These values agree with the allosteric parameters calculated from kinetic results [20,22,32,33].

In table 1 the relative populations of the different enzymatic states (see eq. (9)) are listed. These support the assumptions we have made in the interpretation of the data.

4.4. Equilibrium $^{(2)}T \rightleftharpoons {}^{(1)}R$

This equilibrium can be expressed as follows:

Table 1
Relative population of the different states involved in the kinetic scheme given for the first region of the AMP calorimetric curve (a)

AMP (M)	$^{(1)}R_0$ %	$^{(1)}T_0$ %	$^{(1)}R_2$ %	$^{(1)}R_1$ %
10^{-3}	0.1	3.5	91.8	4.6
7.5×10^{-4}	0.1	7.8	86.4	5.7
3.5×10^{-4}	0.2	43.4	49.4	7.0
1.75×10^{-4}	0.2	84.0	12.4	3.4

(a) Values have been calculated with $^{(1)}L_{0,0} = 5000$ and $^{(1)}k_R = 2.5 \times 10^{-5}$.

$$^{(2)}T_0 \rightleftharpoons^{(2)}L_{0,A}\ ^{(2)}R_0,$$

$$2\,^{(2)}R_0 + 4A \overset{K}{\rightleftharpoons} {}^{(2)}R_2 \ldots {}^{(2)}R_2,$$

where $^{(2)}T$ and $^{(2)}R$ are the dimeric states with, respectively, low and high affinity relative to the second class of binding sites for AMP. We have neglected participation of the $^{(2)}R_1$ state because of the abrupt change in the enthalpy plot, fig. 2, which clearly indicates the presence of high cooperativity in the process. The treatment of the above equilibrium is based upon the following assumptions:

i) The binding is exclusively directed to the $^{(2)}R$ state.

ii) The enzyme population of state $^{(2)}R_0$ is negligible.

Assumption (i) is justified because, although binding sites of the second type are saturated at high AMP concentration, we cannot exclude their participation in the catalytic process, as they are highly affected by the substrates, phosphate and glycogen [5,34] and, besides, because the phosphorylase *b* activation by AMP nearly follows the concerted model with exclusive binding to one state [20,35]. On the other hand, Ho and Wang [2] have already shown that tne two AMP molecules which produce tetramerization at 18°C bind to a dimeric state.

Assumption (ii) is justified if $^{(2)}L_{0,0}$ is high. This is effectively so, as is apparent from the abrupt change in fig. 2, and as will be seen later.

The observed heat of AMP binding to the second type of binding sites can be expressed in the following general form:

$$^{(2)}\Delta Q_T = {}^{(2)}\Delta H_t([{}^{(2)}R_0]_t + [{}^{(2)}R_2]_t) + 2\,{}^{(2)}\Delta H_b[{}^{(2)}R_2]_t. \quad (14)$$

This equation can be greatly simplified, on the basis of the above assumptions, to the form:

$$^{(2)}\Delta Q_T = 2(^{(2)}\Delta H_t + 2\,{}^{(2)}\Delta H_b)[{}^{(2)}R_2 \ldots {}^{(2)}R_2] \quad (15)$$

where $^{(2)}\Delta Q_T$ is the difference between the total heat observed for a given concentration of AMP and the maximum value of $^{(1)}\Delta Q_T$; $^{(2)}\Delta H_b$ and $^{(2)}\Delta H_t$ are respectively, the enthalpy of binding of AMP and of the induced transconformational change $^{(2)}T \rightarrow {}^{(2)}R$; $^{(2)}\Delta H_p$ is the enthalpy difference between the first and second plateaus of fig. 2, expressed in kJ (mol of dimer)$^{-1}$.

With the above assumptions eq. (16) can be derived (see Appendix B). It gives the nucleotide concentration dependence of the enthalpy of interaction between AMP and its weaker binding sites on the enzyme:

$$^{(2)}\Delta H_T/{}^{(2)}\Delta H_p = \{-(1 + {}^{(2)}L_{0,A}) + \sqrt{(1 + {}^{(2)}L_{0,A})^2 + 8[E_t]\alpha^4}\}^2/8[E_t]\alpha^4 \quad (16)$$

The experimental results conform to the above equation if $^{(2)}L_{0,A}$ is related with $^{(2)}L_{0,0}$ by the following equation:

$$^{(2)}L_{0,A} = {}^{(2)}L_{0,0}/(1 + \alpha)^2 \quad (16a)$$

where $\alpha = [A]/{}^{(2)}k_R$.

This mathematical requirement implies the existence of the interoligomeric interactions: $^{(2)}R_1 \ldots {}^{(2)}R_0$ and $^{(2)}R_2 \ldots {}^{(2)}R_0$ as shown above.

We have also tried to describe the curves by assuming $a_2 = 1$, i.e. interaction between only two binding sites in the enthalpic saturation process of the second region of the mentioned curves. We have also assumed $a_2 = 2$ but with $^{(2)}L_{0,0}$, i.e. existence of four weak binding sites per dimer without interoligomeric interactions during the saturation process. These assumptions do not give an acceptable fit.

We have varied the parameters $^{(2)}L_{0,0}$ and $^{(2)}k_R$ over the ranges (1–20 000) and (5×10^{-5} – 5×10^{-3}), respectively. The values which best fit the experimental results are given in table 2. The second region of the curve (fig. 2) has been drawn using these values of the $^{(2)}k_R$ and $^{(2)}L_{0,0}$.

Table 2
Phosphorylase b–nucleotide equilibrium constants at 25°C

Nucleotide	$^{(1)}L_{0,0}$	$^{(1)}k_R$	$^{(2)}L_{0,0}$	$^{(2)}k_R$
AMP	2000–5000 [a] 2000 [b]	(45.–2.5) × 10^{-5} 7.5 × 10^{-6}	13000–15000	(8.0–0.5) × 10^{-5}
2'AMP	100 [a] 700–1000 [b]	(5.0±0.5) × 10^{-5} (0.9–1.2) × 10^{-5}	1000– 3000	(1.4–1.8) × 10^{-4}
3'AMP	100 [a] 500–600 [b]	(5.0±0.5) × 10^{-5} (0.9–1.0) × 10^{-5}	6000–10000	(1.5–1.7) × 10^{-4}

[a] Values calculated with $^{(1)}L_{0,A} = {}^{(1)}L_{0,0}(1 + 2\alpha)^{-1}$. [b] Obtained with $^{(1)}L_{0,A} = {}^{(1)}L_{0,0}$.

Associated thermodynamics properties

Nucleotide	ΔG^0_I [a]	ΔH_I [b]	ΔS_I [c]	ΔG^0_{II} [a]	ΔH_{II} [b]	ΔS_{II} [c]
AMP	–(31.8 ± 0.8)	–(240 ± 15)	–(167 ± 13)	–(23.0 ± 0.5)	–(480 ± 8)	–(367 ± 7)
2'AMP	–(38 ± 1)	–(130 ± 4)	–(74 ± 4)	–(25 ± 2)	–(470 ± 24)	–(357 ± 21)
3'AMP	–(38 ± 1)	–(234 ± 16)	–(157 ± 14)	–(21 ± 2)	–(566 ± 40)	–(437 ± 34)

[a] $\Delta G^0 = RT \ln {}^{(i)}k_T$, where $^{(i)}k_T = {}^{(i)}L_{0,0}\,{}^{(i)}k_R^2$ (i = 1 or 2) is the total dissociation constant of the complex. The values of $^{(i)}L_{0,0}$ and $^{(i)}k_R$ used were those of the case a) of the upper table. ΔG^0 in kJ/mol dimer.
[b] ΔH, in kJ/mol dimer.
[c] ΔS, in cal (mol dimer)$^{-1}$ K^{-1}.

5. Enthalpy of binding of 2'AMP and 3'AMP to phosphorylase *b*

As has been previously reported (36) 2'AMP and 3'AMP are inhibitors of phosphorylase *b*, in opposition to AMP which is its strongest physiological activator. Both 2'AMP and 3'AMP completely displace AMP-^{14}C from its binding sites at 4°C [25] and at 25°C (unpublished results) and thus can be considered as formal analogues of AMP.

The enthalpy change accompanying the binding of 2'AMP and 3'AMP ot phosphorylase *b* was determined as a function of inhibitor concentration at 25°C. The results obtained are given in figs. 3 and 4. Due to the clear distinction between the two saturation regions, the curves can be analyzed as was previously done with AMP.

The continuous line in figs. 4 and 5 has been fitted to eq. (12) with $^{(1)}L_{0,A} = {}^{(1)}L_{0,0}(1 + 2\alpha)^{-1}$, for the first regions. Eq. (12) with $^{(1)}L_{0,A} = {}^{(1)}L_{0,0}$ poorly fits the experimental data for the first saturation regions of figs. 3 and 4; furthermore it gives $^{(1)}k_R$ values lower than those obtained for AMP

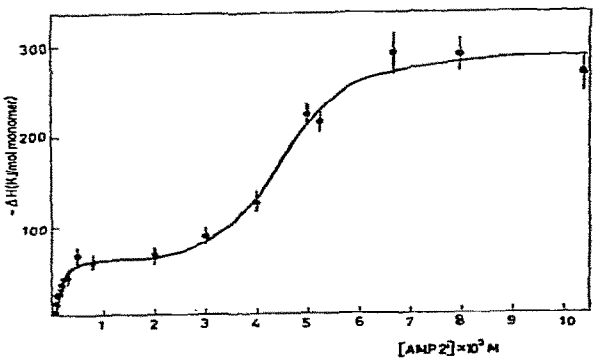

Fig. 3. Enthalpy titration of phosphorylase *b* with 2'AMP. The continuous line has been adjusted to the experimental data following eq. (12) with $^{(1)}L_{0,A} = {}^{(1)}L_{0,0}\,(1 + 2\alpha)^{-1}$, for the first region and eq. (17) for the second one. The values of the constant used are given in the table 2.

(see table 2). This situation is in disagreement with the kinetic and equilibrium dialysis data previously reported [36,37], and indicates that interoligomeric interactions probably occur.

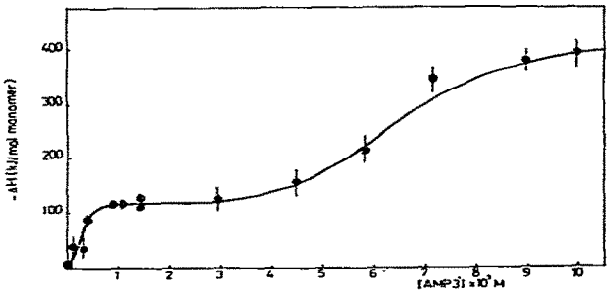

Fig. 4. Enthalpy titration of phosphorylase *b* with 3'AMP. The continuous line has been adjusted to the experimental data following eq. (12) with ${}^{(1)}L_{0,A} = {}^{(1)}L_{0,0}\,(1+2\alpha)^{-1}$, for the first region and eq. (17) for the second one.

The experimental data of the second regions have been fitted to eq. (11), obtained in Appendix B as indicated above for AMP. The constants ${}^{(1)}k_R$, ${}^{(2)}k_R$, ${}^{(1)}L_{0,0}$ and ${}^{(2)}L_{0,0}$, in each case, have been estimated from the best fit of the experimental results to the above equations, and are given in table 2.

6. Discussion

The equilibria between each two successive conformational states obey one of the stated allosteric models [8–12] only if long range interoligomer interactions are not present in the saturation process by the ligands.

The analysis of the microcalorimetric results obtained with phosphorylase *b* indicates the existence of interoligomeric interactions during its saturation by different nucleotides. These interdimer interactions induced during the saturation by the nucleotides of its binding sites may be explained in, at least, two different ways:

1) They are produced by attractive forces between proteinic regions.

2) They are produced by attractive forces arising from a nucleotide "bridging action" between dimers, possibly due to the interaction of different parts of the nucleotide molecule with different dimers.

The data given in this paper do not allow us to select which of the two assumptions is right, because the ${}^{(i)}R_0$ concentrations are very small for the studied samples.

The high Huggin's constants, and the enormous molecular dimensions of the phosphorylase *b* (obtained in glycylglycine buffer at 25°C from the viscosity equations derived for non-interacting systems (unpublished results)), further attest to the existence of interactions between phosphorylase *b* dimers. At the present time we are working to obtain a good knowledge about the nature of such interdimer interactions.

On the other hand, the interoligomeric interactions disappear above a given degree of saturation of binding sites by the studied nucleotides. This disappearance of the interoligomeric interactions may arise from the increase of the electrostatic repulsion forces between bound nucleotides molecules, as they are dianions.

These nucleotides induce great alterations in the system phosphorylase *b*-glycylglycine buffer, as can be inferred from the enthalpic and entropic changes (see table 2), i.e. the enzyme can have several thermodynamic states in solution. The high entropic changes also support the postulated different enzymatic conformational states in solution. The values of ΔG_I^0 and ΔG_{II}^0 demonstrate that the $D_1 \rightarrow D_2$ transition is energetically more favoured than the $D_2 \rightarrow D_3$ transitions, showing the relative high stability of the D_2 state, and also that its thermodynamic characterization under the experimental conditions used is possible.

It should be pointed out that the curve given in fig. 2 slightly differs from the one already published [5]. The differences are due to an oxidation process of the enzyme during the first week after the fourth crystallization, which we have now controlled (unpublished results).

The above analysis shows that the ratio ${}^{(i)}\Delta H_T / {}^{(i)}\Delta H_p$ can be considered as a saturation function, Y_i, when: (a) ${}^{(i)}\Delta H_b \gg {}^{(i)}\Delta H_t$ or (b) ${}^{(i)}k_R \ll {}^{(i)}k_T$, i.e. in the case of exclusive binding of the ligand, and very high ${}^{(i)}L_{0,0}$. On the contrary, the ${}^{(i)}\Delta H_T / {}^{(i)}\Delta H_p$ ratio can be considered as a state function if the two conditions are fulfilled, that ${}^{(i)}\Delta H_b \ll {}^{(i)}\Delta H_t$, and that ${}^{(i)}L_{0,0} \approx 1$ (this last equation is equivalent to ${}^{(i)}k_R \approx {}^{(i)}k_T$).

7. Concluding remarks

Perhaps two important differences between the here given allosteric formulation and the usually accepted models should be noted:

a) The existence of n ($n > 2$) enzymatic conformations which are thermodynamically stable.

b) The different types of binding sites must not necessarily be preformed in the enzyme, i.e. the effector molecules participate, in some, not yet known, fashion in the generation of new binding sites.

The first difference is a logical one if we consider the system enzyme-ligand from a thermodynamic point of view, because a series of intermediate states, successively nearer to the final state, is needed to explain the transition between very separate equilibrium thermodynamic states. Weber [38] has raised two important objections to the classical allosteric models:

1) These models establish a great and useful simplification of the thermodynamic approach only when the existence of an equilibrium between two conformational states is considered.

2) The tautomeric significance of the transconformational equilibrium can lead to disregard the influence of the enzyme concentration in the allosteric processes, and therefore to neglect the problems of aggregation, which, in some cases, have an important role in the enzymatic activation.

Both objections do not apply to our proposed formulation. We note that, when the second objection is taken account of in the first assumption of our model, it becomes necessary the study of the effect of the enzymatic concentration upon the allosteric interactions. Effectively, our preliminary results, already reported [39], have shown the importance of enzyme concentration. It is also evident that the detected intermediate states must not be taken as the only possible intermediate states for the system when it moves from the dimer stabilized in the absence of ligand to the tetramer. Probably other conformational enzymatic states, unstable in the above mentioned conditions, are induced. In other words, this allosteric formulation gives to the protein flexibility in solution a higher influence than the classical allosteric theories, as in it also the solvent influences selects the conformation of the protein.

The (b) difference implies to consider the enzyme molecules as part of the liquid system in which they are solved, i.e. that the liquid system can participate in the transmission of the cooperative effects. This is a logic point of view if we note the important differences previously reported in the enzymatic behaviour as a function of the working buffer [5]. These differences mainly affect the affinity of the enzyme towards its ligands, without appreciable changes in the maximum catalytic activity induced by them [22].

The presence of allosteric effect in monomeric proteins is an important problem for the classical allosteric models [40–43]. These "anomalous" cases could be explained, at least, by two different reasons:

I) The existence of allosteric intra-monomer effect between very separate proteinic regions [40–43].

II) That $a_i > 1$ is the only requirement for the existence of allosteric interaction between dynamically ensembled enzyme molecules, (i.e., formation of intermediate pseudo-aggregate during the enzymatic saturation by a ligand). This is the first hypothesis of our formulation.

Explanation I can be understood only if the inner proteinic regions are affected by the allosteric interactions. This implies then a high flexibility of the "core" of the protein, which allows some rotation or deformation to great submonomeric entities within a protein, i.e. "protomer", following Monod's terminology [8]. We think that in general it is difficult to accept this suggestion for globular, ellipsoidal or rod-like proteins, considering its physical properties.

Suggestion II seems to us the most plausible, because it only implies the participation of the enzymatic surface or regions nearest to it. This is the known case of the spatial situation of most of the detected allosteric enzymatic sites.

Appendix A

Taking into account the considerations given in the text, the total enzyme concentration, $[E_t]$, is given by the expression:

$$[^{(1)}R_0]_t + [^{(1)}R_2]_t + [^{(1)}T_0] = [E_t]$$

or simply:

$$(\overline{^{(1)}R_0})_t + (\overline{^{(1)}R_2})_t + (\overline{^{(1)}T_0}) = 1,$$

where $(\overline{^{(1)}R_0})_t$, $(\overline{^{(1)}R_2})_t$ and $(\overline{^{(1)}T_0})$ are the mole fractions of each enzyme form. Considering that:

$$[^{(1)}R_2]_t = [^{(1)}R_0]_t [A]^2/K$$

it can be written:

$$\overline{(^{(1)}R_0)}_t = \frac{1}{1 + {}^{(1)}L_{0,A} + [A]^2/K} \quad (A.1)$$

where ${}^{(1)}L_{0,A}$ has been defined in the section 3 of this paper.

But ${}^{(1)}\Delta H_t + 2{}^{(1)}\Delta H_b = {}^{(1)}\Delta H_p = -(240 \pm 15)$ kJ (mol dimer)$^{-1}$ is the enthalpy of the first plateau of the curve of fig. 2. Then expression (11) can be written:

$${}^{(1)}\Delta H_t = \frac{{}^{(1)}\Delta Q_T}{[E_t]} = \frac{{}^{(1)}\Delta H_p [A]^2/K}{1 + {}^{(1)}L_{0,A} + [A]^2/K} \quad (A.2)$$

As $K = {}^{(1)}k_R^2$ [8] and introducing $\alpha = [A]/{}^{(1)}k_R$ in eq. (A.2) we obtain:

$${}^{(1)}\Delta H_T/{}^{(1)}\Delta H_p = \alpha^2/({}^{(1)}L_{0,A} + 1 + \alpha^2) \quad (12)$$

Appendix B

The equilibrium constants for this case are:

$$K = {}^{(2)}k_R^4 = \frac{[{}^{(2)}R_0]_t [A]^4}{[{}^{(2)}R_2 \ldots {}^{(2)}R_2]}, \quad (B.1)$$

where $[{}^{(2)}R_0]_t$ refer to the total ${}^{(2)}R_0$ concentration, the constant K is easily related to ${}^{(2)}k_R$ by writing the (B.1) equilibrium step by step and taking into account statistical factors [8].

As in the case of AMP, due to the apparent high cooperativity of the process we have estimated the participation of the ${}^{(2)}R_1$ form to be negligible. Also by this reason we have considered that the relevant enzymatic populations are: ${}^{(2)}T_0$, ${}^{(2)}R_0$ and ${}^{(2)}R_2 \ldots {}^{(2)}R_2$, then:

$${}^{(2)}\Delta H_T/{}^{(2)}\Delta H_p = 2(\overline{{}^{(2)}R_2 \ldots {}^{(2)}R_2}) \quad (B.2)$$

where $(\overline{{}^{(2)}R_2 \ldots {}^{(2)}R_2})$ is the molar fraction of ${}^{(2)}R_2 \ldots {}^{(2)}R_2$.

On the other hand:

$$[{}^{(2)}R_0]_t + [{}^{(2)}T_0] + 2[{}^{(2)}R_2 \ldots {}^{(2)}R_2] = [E_t]$$

and by equilibrium equations it is easily derived:

$$[{}^{(2)}R_2 \ldots {}^{(2)}R_2] = \alpha^4 [{}^{(2)}R_0]_t^2 \quad (B.3)$$

and

$$\overline{(^{(2)}R_0)}_t = \{-(1 + {}^{(2)}L_{0,A}) + \sqrt{(1 + {}^{(2)}L_{0,A})^2 + 8\alpha^4 [E_t]}\}/4\alpha^4 [E_t] \quad (B.4)$$

where $(\overline{{}^{(2)}R_0})_t$ is the molar fraction of the total population, (free + bound), of ${}^{(2)}R_0$.

Substituting in equation (B.2) we finally obtain:

$${}^{(2)}\Delta H_T/{}^{(2)}\Delta H_p = \{-(1 + {}^{(2)}L_{0,A}) + \sqrt{(1 + {}^{(2)}L_{0,A})^2 + 8\alpha^4 [E_t]}\}^2/8\alpha^4 [E_t] \quad (17)$$

From the chemical equilibria of this Appendix and with the considerations given above for the ${}^{(2)}L_{0,A}$ dependence upon the ligand concentration, it is clear that:

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

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