ON THE ALLOSTERIC INTERACTION BETWEEN 5'AMP AND ORTHOPHOSPHATE ON PHOSPHORYLASE b . QUANTITATIVE KINETIC PREDICTIONS .

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INTRODUCTION. The recent considerable developments of experimental and theoretical studies of regulatory enzymes has led to renewed interest in the very first such system to be discovered and studied in detail, namely muscle phosphorylase. The classical studies of Cori and his school had revealed the requirement of Ph b for 5'AMP. However in most of these early studies, the reaction was studied in the (non physiological) direction G 1 P + glycogen --➤ glycogen + P_i and, from these kinetic results, it was difficult to judge whether the characteristic effect exhibited by "typical" allosteric systems namely, homotropic interactions of the effector and/or substrates, did occur with this enzyme (Monod et al. 1965). More recent kinetic studies however have established the following facts :

1. Homotropic interactions occur in the binding of AMP.

2. At least in the presence of high magnesium concentrations, the affinity of the enzyme for 5'AMP increases with the concentration of P_i, and conversely. The binding of glycogen and G 1 P are also increased in the presence of AMP (Helmreich and Cori, 1964; Morgan and Parmeggiani, 1964; Lowry, 1967).

3. ATP acts as an antagonist of AMP (Madsen, 1964).

4. The binding of AMP promotes a conformational transition revealed by an increased affinity of the protein for the dye brom-thymol blue, and by an alteration of the spectrum of its bound PLP (Ullmann et al. 1964; Bresler et al., 1966).

These observations suggest that Ph b should be considered as an allosteric enzyme of the K class. If so, according to the "concerted transition" theory of Monod et al. (loc.cit.), the following predictions may be made :

a. The binding of P_i to the enzyme should also exhibit homotropic effects.

b. At saturation of P_i, the "requirement" for AMP should disappear.

c. The heterotropic interactions should be describable quantitatively in terms of alterations of the homotropic interactions.

RESULTS AND DISCUSSION. Initial velocity measurements, performed at various AMP and P_i concentrations are shown in Figures 1 and 2.

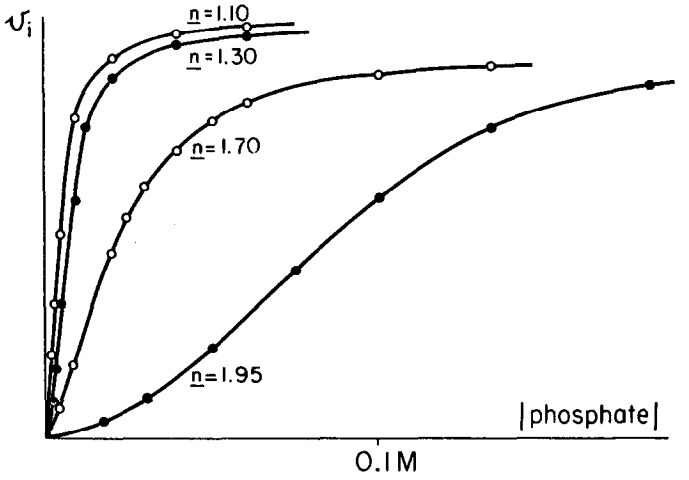

Figure 1. - Effect of P_i concentration on the activity of phosphorylase b at different levels of AMP in the presence of a constant concentration of glycogen (0.25%). Purification of reagents and measurements are done according to Helmreich and Cori (1964). However, in order to prevent agregation of the enzyme at high ionic strength, serum albumin is added at a final concentration of 0.2 mg/ml, glucose-6-phosphate dehydrogenase and phospho-glucomutase are also present in large excess (30 µg/ml and 15 µg/ml respectively). AMP concentrations are equal to 0, 10^{-5}, 3.10^{-5} and 10^{-4}M respectively.

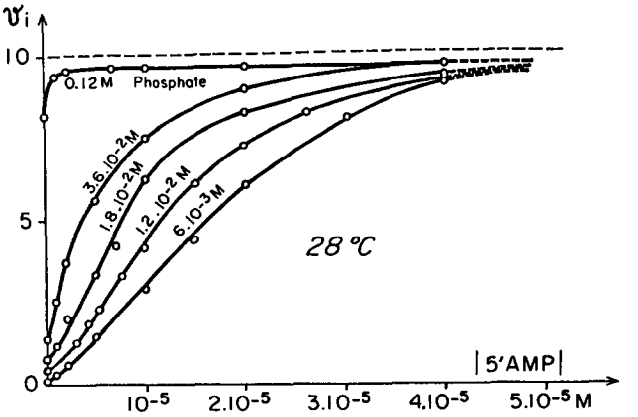

Figure 2. - Effect of 5'AMP on the activity of phosphorylase b at 28°C (same conditions as in figure 1).

We see that, at low AMP concentration, the rate concentration curve is strongly sigmoïdal with respect to phosphate, while, at high concentration of the effector, the curves tend towards an hyperbola. The cooperative effect of phosphate can be analyzed in terms of the Hill interaction coefficient. We find that, in the absence of AMP, the maximum value of this coefficient is 2.0 and decreases to 1 in the presence of 10^{-4}M AMP. The AMP curve is also sigmoïdal at low phosphate and is converted almost to a straight line at high phosphate concentration : in other words, at very high phosphate concentration, the classical requirement of phosphorylase b for 5'AMP disappears.

This behavior conforms therefore to the predictions of the theory for a "K" system where AMP is an allosteric activator and P_i an allosteric substrate.

It is well established that Ph b is a dimer, bearing two PLP prosthetic groups, and two phosphorylation sites. The maximum possible value of the interactions coefficient therefore should be 2. The fact that the Hill coefficient comes experimentally very close to this value is interpreted, according to the theory, to indicate that, of the two states accessible to the protein ("R" and "T") only one (we assume it to be R) has significant affinity for the two ligands, while the alternative state (T) is favored in the absence of the ligands. Such a system lends itself to a convenient method of analysis, using a function hereafter called the "quotient of states", i.e. the ratio of the amount of enzyme in the R state to that in the T state. We may write :

$$Q = \frac{|R|}{|T|} = \frac{R}{\bar{T}}$$

Considering Q as a function of AMP and phosphate, and assuming that the T state has no or negligible affinity for both, the following expression is easily derived from the theory :

$$Q = \frac{(1 + [P]/K_P)^2 \quad (1 + [A]/K_A)^2}{L_o} \qquad (1)$$

where : [P] = phosphate concentration

[A] = AMP concentration

K_P = dissociation constant of phosphate

K_A = dissociation constant of AMP

$L_o = \frac{[T]}{[R]}$ in absence of both phosphate and AMP, the concentration of any other ligand remaining constant.

Assuming initial velocities to be proportional to the binding func-

tion for phosphate, we write :

$$\bar{Y}_P = \frac{V}{V_M} = \bar{R}\,\frac{[P]}{K_P + [P]} \qquad (2)$$

where : $\bar{Y}_P$ = binding function for phosphate

V = initial velocity at any given concentration of phosphate and AMP

V_M = maximal initial velocity at saturation of both.

Now we define a new velocity constant V_a as the velocity observed at a given concentration of phosphate, and at saturation of AMP, for which $\bar{R} = 1$, and such that

$$\frac{V_a}{V_M} = \frac{[P]}{K_P + [P]}$$

We therefore have : $\bar{R} = \frac{V}{V_a}$

Using equation (1) and substituting the value of $\bar{R}$, we have :

$$\sqrt[2]{Q} = \sqrt[2]{\frac{V}{V_a - V}} = \sqrt[2]{\frac{1}{L_o}}\,\left(1 + \frac{P}{K_P}\right)\left(1 + \frac{A}{K_A}\right) \qquad (3)$$

At constant phosphate concentration, the square root of the quotient should be a linear function of AMP concentration, extrapolating on the abscissa at a point $[A] = -K_A$. Figure 3 shows that this is the case and that when different fixed concentrations of phosphate are used, the straight lines all extrapolate to the same point, as expected from the theory, one of the basic assumptions of which is the binding of phosphate and

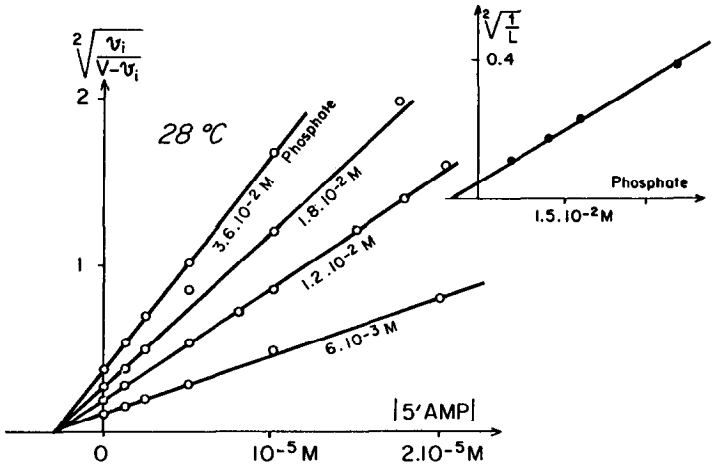

Figure 3. - Determination of the dissociation constant of 5'AMP with respect to the active state R of the protein. Effect of phosphate on the allosteric equilibrium.

AMP on the R state are independent. Moreover, the fit of the data to equation (1) may be tested using roots other than square ; taking roots 3 or 1, we see (Fig. 4) that the points are not interpolated by a straight line, and that the extrapolations do not converge at the same point on the abscissa. We conclude that the interactions involve two sites, as it should be, since the enzyme is known to be a dimer.

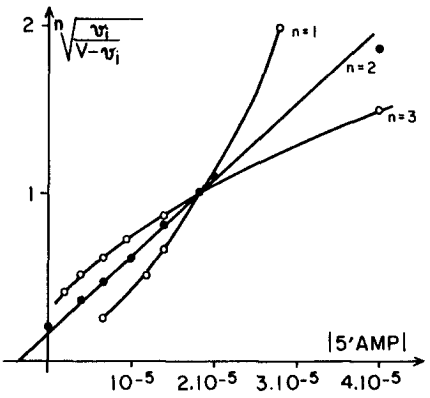

Figure 4. - Determination of the number of 5'AMP interacting sites.

Moreover, according to equation (3) the intercept on the ordinates are equal to

$$\sqrt[2]{\frac{1}{L_o}\left(1 + \frac{[P]}{K_P}\right)}$$

If these values are plotted with respect to phosphate concentrations, they fit a straight line, showing again that the binding of phosphate and AMP on the R state are independent. From these data, K_P, K_A and L_o can be estimated.

Therefore the data agree very well with equation (1), which was derived assuming that phosphate and AMP bind exclusively to the $\bar{R}$ state, and that the transition is fully "concerted". A significant contribution of the non exclusive binding or of hybrid states, would have resulted in deviations from straight lines in the plots given in Fig. 3.

In conclusion, we see that the kinetic behaviour of phosphorylase b is quantitatively described by a model based on the "concerted transition" theory with the following specifications :

a. Phosphorylase b is made up of two equivalent interacting protomers each bearing a single site for AMP and phosphate.

b. It may exist in two conformational states R and T the first of which has affinity for AMP (K_A = 3.10^{-6}M at 28°C) and for phosphate (K_P = 4.10^{-3}M) while the other state, predominant in absence of these ligands ($L_o \simeq 600$) has little or no affinity for them.

c. The transition is fully concerted, hybrid states do not occur or only in negligible amounts.

If the theory is correct these interpretations of kinetic results should be confirmed by direct determinations of the equilibria between the protein and its ligands and between the two states of the protein.

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