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EVIDENCE FOR A SLOW TRANSITION LEADING TO COOPERATIVE BINDING OF 5'-AMP ONTO RABBIT MUSCLE PHOSPHORYLASE b AT 5°

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1. Introduction

We are currently studying the allosteric transitions of glycogen phosphorylase *b* from rabbit skeletal muscle. Four relaxation times can be detected in this system by various methods. In order to ascertain the relaxation time which corresponds to the quaternary change triggered by the allosteric activator, 5'-AMP, we have adapted to this problem the method of dialysis developed by Colowick and Womack [1]. This technique allows the determination of the extent of binding of diffusible molecules on proteins by the measure of the rate of diffusion of the radioactive ligand from from a mixture of enzyme and ligand at equilibrium. We have measured the rate of isomerisation of an allosteric enzyme caused by a sudden increase in the concentration of a diffusible effector in particularly favorable conditions where the relaxation time of the protein is longer than the time needed to reach the steady rate of dialysis. In the glycogen phosphorylase *b* system at 5°, the slowest relaxation step corresponds to the establishment of positive homotropic interactions between the two AMP sites of the dimer.

2. Material and methods

Phosphorylase *b* was prepared from rabbit muscle according to Krebs et al. [2]; tritiated 5'-AMP (17 to 19.5 Ci/mmole) was purchased from Schwarz Biochemicals and further purified by chromatography on DEAE paper in order to eliminate any contaminant adenosine, arising from radiolysis. Static measurements were performed as described in [3]. The apparatus used for the measurement of the rate of dialysis was initially designed by Dr. Jovin at the Max Planck Institute in Göttingen and is similar to the one described by Colowick and Womack [1]. The time course for approach to the steady state is exponential; the time constant τ' is proportional to $\frac{V}{f}$, f being the volume of buffer flowing per second through V, volume of the lower chamber. In order to minimize τ', the diameter of the mixing chamber was reduced to 8 mm and its depth to 4 mm. The chamber containing the enzyme is three times larger; they are both surrounded by a water jacket in order to maintain a constant temperature. The requirement for homogeneity of the eluate at the exit of the lower chamber limits the maximum buffer flow which can be used. In our device we can adjust it up to 2.4 ml per min, and reach values of τ' equal to 20 sec. Another constraint comes from the slow depletion of the upper chamber in radioactive ligand. This process is taking place at a rate constant equal to the ratio of the total number of cpm which have been placed in the upper chamber to the number of cpm which are collected per unit of time and is of the order of 400 min. Therefore relaxation times in the range 40 sec to 100 min can be measured by this method, if they lead to a significant change in the affinity of the protein for its effector.

Each experiment was carried out in the following way: 200 µl of a phosphorylase *b* solution at a concentration of 5×10^{-5} moles of sites per liter were incubated for 10 min in the upper chamber. After this time, 40 µl of tritiated AMP (representing 20×10^6 cpm and a final concentration of 1.5×10^{-6} M) were added to the upper chamber. Samples of the effluent

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degassed buffer were then collected during a period of time sufficient to reach a constant dialysis rate. Then, the concentration of the ligand was pertubed: 10 μl of unlabeled AMP were added, bringing the final AMP concentration in the range between 10^{-5} and 10^{-4} M and samples of the effluent buffer were collected until a constant diffusion rate of AMP was reached. When studying the relaxation of the enzyme, samples were collected for 30 min in order to ensure the completion of the process. Finally 10 μl of 0.1 M unlabeled AMP were added to the upper chamber. The new rate of diffusion corresponds to that obtained with labeled AMP alone in the upper chamber; it allows us to calculate after correction for dilutions, the concentration of free and bound 5′-AMP in the upper chamber at any time (cf. [1]). We use the following buffer (M): glycyl-glycine 6×10^{-2}, KCl 6×10^{-2}, EDTA 10^{-4} M, pH 7.5 at 4°.

3. Results

The amount of cpm collected per unit time at the exit of the lower chamber changes with time in three different experiments (fig. 1). The first plateau corresponds to the rate of dialysis imposed to the system by the enzyme when it is incubated in the presence of a constant and small amount of labeled AMP. At the time indicated by the first arrows the concentration of 5′-AMP in the chamber containing the enzyme is suddenly changed. A relaxation takes place. Then, a second change in AMP concentration is done which chases all the labeled AMP from the enzyme. The final rate of dialysis permits calibration of the whole curve. Some experiments (for example curve F) performed at a higher buffer flow allow us to decrease τ' and to confirm that the extrapolations made in dotted line in fig. 1 are correct. "Immediate" bindings of 5′-AMP (points $\alpha_1, \beta_1, \gamma_1$) can therefore be distinguished from final ones (points α_2, β_2). By immediate, we mean the extent of binding which is taking place before or within 20 sec, the dead time of the device. The representation of the extent of immediate and final binding is given in fig. 2, together with the results of static measurements.

The final extent of binding corresponds to the value expected from the static measurements; this saturation function $\overline{Y}_{(F)}$ is cooperative and can be characterized by the two empirical dissociation constants:

$$K_1 = 2\,\frac{[E][F]}{[EF]} = 2.7 \times 10^{-5}\ \text{M}$$

$$K_2 = \frac{[EF][F]}{2[EF_2]} = 1.0 \times 10^{-5}\ \text{M}$$

The Scatchard representation of the immediate binding is characterized by a single dissociation constant $K = K_1$. It is therefore a non-cooperative process. At saturation of 5′-AMP, it can be extrapolated to one site per phosphorylase *b* subunit. During the relaxation process, the total concentration of 5′-AMP remains constant in the upper chamber, and the representative point in the Scatchard plot moves along an hyperbola from the straight line which represents the initial binding to the ellipse which characterizes the final extent of binding.

In a control experiment, two enzyme AMP-mixtures, M_1 and M_2, corresponding to the same total concentration of protein and effector (6.2×10^{-5} M) were prepared. The only difference was that, in mixture M_2, no labeled AMP was present. Both mixtures were incubated for 40 min at 5°; mixture M_1 was placed in the upper chamber of the device; samples were collected from the lower chamber and then mixture M_2 was added. As a consequence of dilution of labeled AMP in the upper chamber, the amount of radioactivity collected per min in the lower chamber was decreased but no relaxation time other than the time constant of the device was observed, showing that the *exchange* between labeled and unlabeled AMP on the protein was taking place quite rapidly. The rates of diffusion observed before and after the addition of mixture M_2 corresponded exactly to the one determined by classical equilibrium dialysis.

The *relaxation times* τ depend on the concentration of free 5′-AMP, but not on protein concentration. For example:

τ = 4.5 min for $(AMP)_{free} = 4.7 \times 10^{-6}$ M

τ = 5.5 min for $(AMP)_{free} = 2.10 \times 10^{-5}$ M

These values are in close agreement with the results of the slowest isomerization step obtained by other techniques.

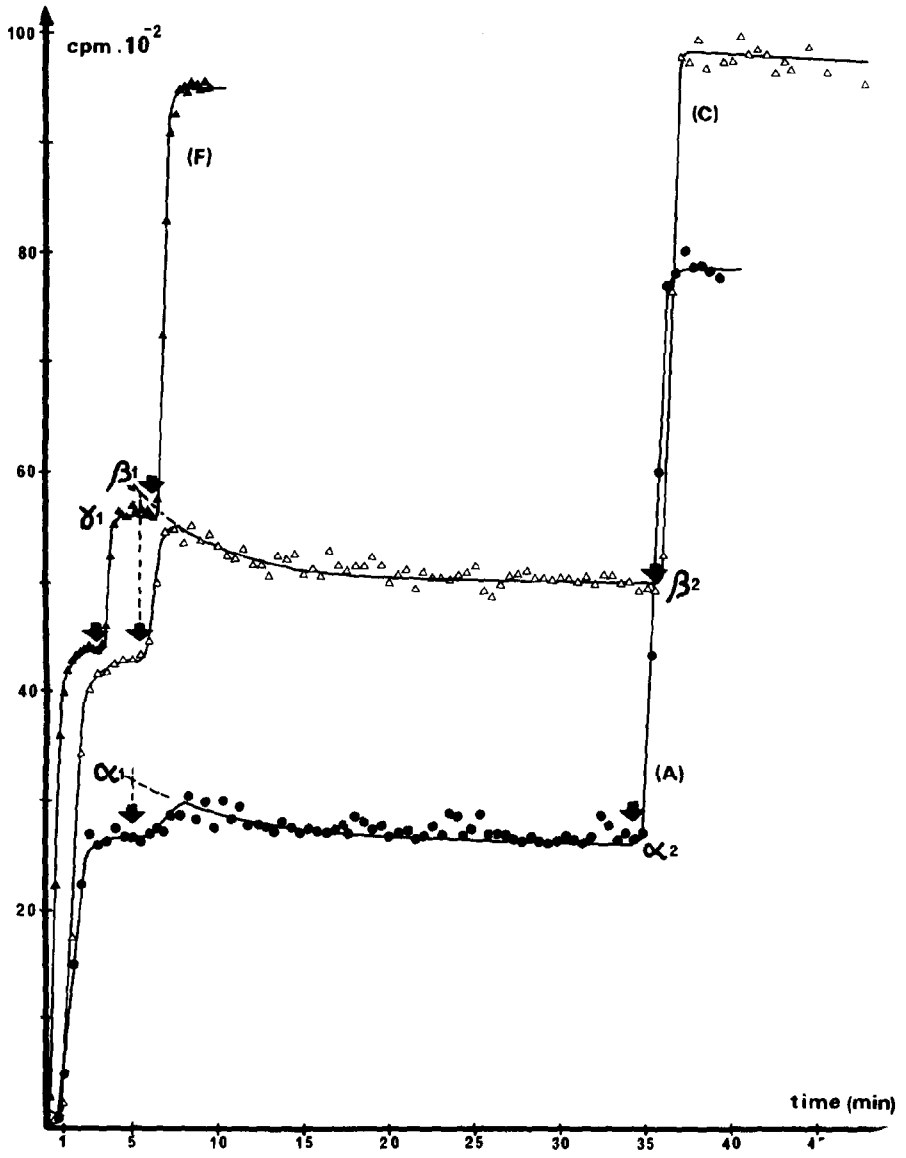

Fig. 1. Changes in the rate of dialysis with time for three different perturbations in 5′-AMP concentration. Labeled AMP (2 to 3×10^{-6} M) was added at zero time. On each curve, the first arrow represents the addition of unlabeled AMP (4×10^{-5} M for curves F and C, 10^{-5} M for curve A). The second arrow represents the addition of an excess of unlabeled AMP. In experiments A and C, the buffer flow was adjusted to 0.7 ml/min and the relaxation processes were followed (initial bindings corresponding to points α_1 and β_1, final bindings represented by values α_2 and β_2). In experiment F, flow of buffer was increased to 2.4 ml/min and the initial binding only was determined (plateau γ_1). Each sample was counted in Bray' solution, in an Intertechnique scintillation counter. The standard deviation of the measurements was less than 1%. For other details, see text.

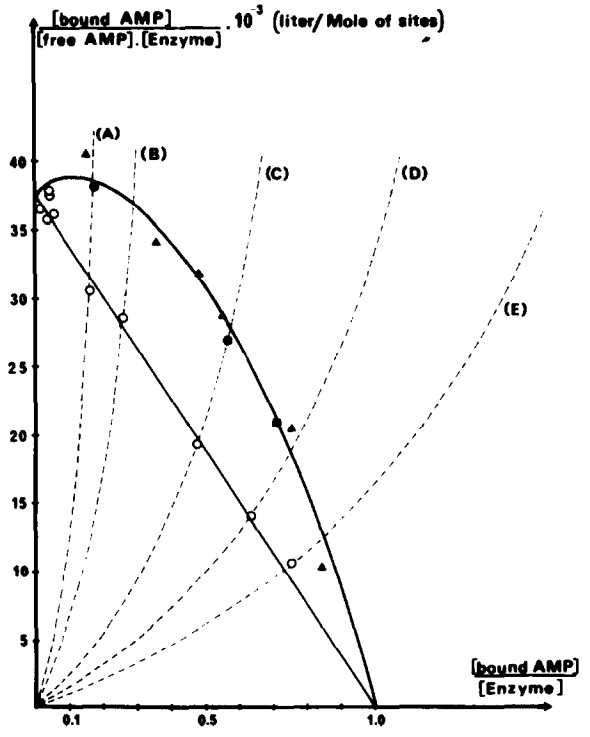

Fig. 2. Scatchard representation of the binding of 5′-AMP to phosphorylase *b* at 5°. The ellipse is the theoretical cooperative equilibrium saturation function determined for the values of the empirical dissociation constants given in the text:
▲: the extent of binding as determined by classical equilibrium dialysis;
●: the values obtained at the end of the relaxation process by the Colowick and Womack method;
○: the immediate binding of 5′-AMP;
■: a control experiment described in the text.
□: Hyperbolae A, B, C, D, E, are the loci of points where the mean AMP concentration remains constant. The relaxation process was studied along these curves.
Protein concentration, is determined as in [3].

4. Discussion

The slow relaxation which is observed can be assigned to an intramolecular isomerisation step, since it does not depend on protein concentration and since the exchange between bound and unbound labeled AMP appears in the control experiment to be quite fast.

The finding that the initial saturation function is non-cooperative has an important consequence for the interpretation of the mechanism of the allosteric transition of phosphorylase *b*. Let us assume that several enzyme conformations A_2, B_2, AB, C_2... having different affinities for 5′-AMP are initially present in equilibrium in this first incubation mixture which contains 10^{-6} M of free 5′-AMP. If a significant quantity of hybrid molecules of the AB type were initially present, an anti-cooperative contribution to the immediate binding is expected. This contribution appears to be undetectable and we shall neglect it. On the other hand, if a significant rearrangement between conformations A_2, B_2, C_2 was taking place before the transition corresponding to the slowest relaxation time, the relative amount of those conformations would change with AMP concentration according to mass action law and the initial binding function would be cooperative. Therefore (except if, by coincidence, the two effects which have just been mentioned were cancelling each other) we must assume that the relative weight of the predominant symmetrical conformations A_2, B_2, C_2 does not change until the slow relaxation time is taking place.

Since the initial saturation function $\overline{Y}_{(0)}$ extrapolates at high AMP concentration to one AMP site per subunit, binding of 5′-AMP must take place on all the conformations which are initially present in the mixing chamber. Within the resolution of our device, we can therefore distinguish two processes occurring after the increase of ligand concentration. First a binding process on conformations which are not allowed to reequilibrate, and then an isomerization from the forms which have a low affinity for 5′-AMP to the more affine ones. This last step is the only one which reestablishes the positive homotropic interactions between the two AMP sites of a given protein molecule. Since this second step takes place along a single relaxation, we must conclude that only two symmetrical conformations A_2 and B_2 were initially populated.

Although absorption techniques are more accurate for the measurement of relaxation times, we think that the present method has the advantage of following directly slow changes in affinity between effector molecules and enzymes. A combination of the two techniques will provide the simultaneous recording of conformational changes and modifications in ligand affinity.

Acknowledgements

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References

[1] S.P.Colowick and F.C.Womack, J. Biol. Chem. 244 (1969) 774.
[2] E.G.Krebs, D.S.Love, G.E.Bratvold, K.A.Trayser, W.L. Meyer and E.H.Fischer, Biochemistry 3 (1964) 1022.
[3] M.H.Buc and H.Buc, IVth FEBS Meeting Symposium on "The regulation of enzyme activity and allosteric interactions" Universitets Forlaget, Oslo (1968) 109.