THE EFFECT OF 5'ADENYLIC ACID UPON THE ASSOCIATION BETWEEN BROMTHYMOL BLUE AND MUSCLE PHOSPHORYLASE b

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The classical studies of Cori and his school, confirmed and extended by the work of Fischer and Krebs have established the following facts concerning muscle phosphorylase b (Ph b) :

a. This enzyme (M.W. 242,000) is a dimer, made up of two, presumably identical, protomers(°°) (M.W. 125,000) (Keller and Cori, 1953; Madsen and Gurd, 1956).

b. Ph b is virtually inactive in the absence of 5'adenylic acid (5'AMP); the nucleotide however does not participate as an intermediate in the reaction (Cori and Green, 1943; Cohn and Cori, 1948).

c. At high protein concentration (above 4 mg/ml) 5'AMP provokes partial association of Ph b into a tetrameric form (Kent, Krebs and Fischer, 1958).

d. In presence of ATP and phosphorylase-kinase, Ph b is phosphorylated to phosphorylase a (Ph a) which is active in the absence of 5'AMP. Ph a, as normally isolated, is a tetramer (M.W. 495,000) (Krebs, Kent and Fischer, 1958).

e. There is one AMP binding site per protomer, both in Ph b and in Ph a. However, the affinity of the binding sites for AMP is

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(°°)- We use the terminology proposed by Changeux, Ullmann and Monod (1963).

much higher in Ph a than in Ph b (Madsen and Cori, 1957).

These observations suggest that the effect of 5'AMP upon Ph b is due to an allosteric (i.e. indirect) interaction between the nucleotide binding sites and the active sites of the protein.

Since it seems reasonable to assume that allosteric interactions in general are mediated by conformational alterations of the protein (Monod et al., 1963), we have attempted to find an experimental test for this assumption in the case of Ph b.

A first possibility was suggested by the observations of Kent, Krebs and Fischer : namely that the activation of Ph b in presence of AMP was directly correlated with the "associative" effect of the nucleotide. In a first series of experiments we therefore tested whether a significant variation of sedimentation velocity occured when 5'AMP was added to relatively low concentrations of Ph b (ca. 0.5 mg/ml). As shown in Fig. 1, no effect of 5'AMP upon the sedimentation

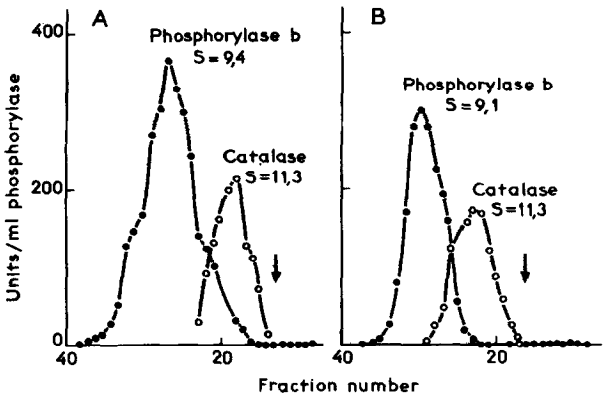

Figure 1. - Sedimentation of phosphorylase b in a sucrose gradient. 0.5 mg of phosphorylase b (free of 5'AMP) is centrifuged in a linear sucrose gradient (5-20 %) 7 h at 39,000 rpm. A : Centrifugation in the absence of AMP. B : Centrifugation in the presence of 10^{-3} M 5'AMP. The arrow indicates the theoretical position of the tetramer.

of Ph b is detectable under these conditions, although the allosteric effect (i.e. the activation of Ph b by 5'AMP) is fully demonstrable under the same conditions of protein concentration and temperature. The two sets of observations, concerning respectively the associative and the activating effects of the nucleotide are not entirely comparable, since glycogen and glucose-1-phosphate (G1P) are present in the activity tests while they are absent in the centrifugal runs. However the results suggest rather strongly that the activation of Ph b is not directly correlated with, and dependent upon, its association into a tetrameric form.

Another method of possibly revealing a conformational effect of 5'AMP upon Ph b was suggested to us by the recent very interesting observations of Antonini et al. (1963). These authors have found that the rate of association between the dye bromthymol blue (BTB) and hemoglobin is profoundly affected by O_2 pressure and they have presented convincing evidence that this effect is directly related to the (allosteric) heme-heme interactions in this protein. It therefore seemed of particular interest to test whether another, entirely different, allosteric system would exhibit a similar effect.

As shown in Table I when Ph b (1.0 mg/ml) is dialyzed to equilibrium against BTB (2×10^{-5}M) on a sephadex column, association of the protein with the dye is observed. Moreover the amount of protein-bound BTB is increased about three fold in the presence of 5'AMP at saturating concentration (10^{-3}M). The effect is specific for AMP, since it is not observed with 2'3'AMP which is known to exert no activating effect upon Ph b. Using BTB binding as a test, at various AMP concentrations, the apparent affinity of the nucleotide for Ph b may be roughly determined (Fig. 2). The concentration corresponding to half-saturation is about the same as the apparent K_D of AMP as estimated from enzyme-activity measurements. Ph a also associates with BTB.

TABLE I

Association of BTB with phosphorylase

	BTB fixed moles/mole protein(°)
Phosphorylase b - AMP	1.17
" + 5'AMP	3.22
" + 2'3'AMP	1.14
Phosphorylase a - AMP	3.6
" + 5'AMP	3.25

(°) - Of 242,000 M.W. unit.

1 mg/ml of protein is dialyzed against BTB 2×10^{-5}M, on a column of sephadex G-25. The protein-bound BTB is measured, after alkalinization, at 620 mμ.

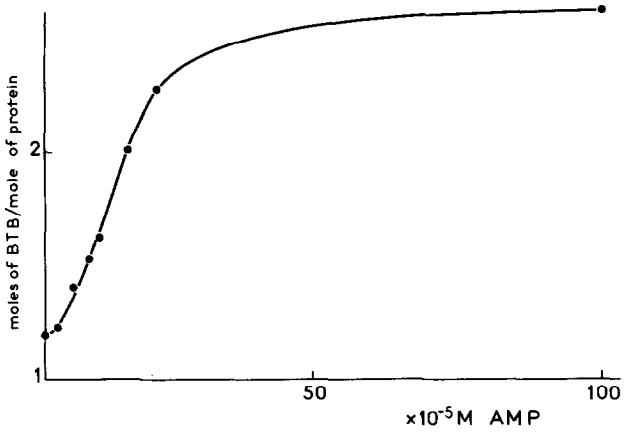

Figure 2. - BTB-binding as a function of 5'AMP concentration. 1 mg/ml protein is dialyzed on a sephadex G-25 column preequilibrated with 2×10^{-5}M BTB, containing 5'AMP at different concentrations.

In fact, as shown also in Table I, it takes up about three times more dye than does Ph b under the same conditions of protein and dye concentration. However, although 5'AMP is known to bind to Ph a even more strongly than Ph b (Madsen and Cori, 1957), it does not modify the affinity of Ph a for the dye.

These observations show that the effect of 5'AMP upon the association between Ph b and the dye is directly correlated with its allosteric effect. Further indications as to the nature of the binding between the protein and BTB may be derived from the fact that a pronounced shift in color is observed upon mixing the dye with the protein, in the absence of AMP, and a further shift occurs as soon as AMP is added. The relative amounts of the acid (yellow) and conjugate base (blue) forms of BTB may be calculated from spectrophotometric measurements at two wave-lengths (440 mμ and 620 mμ). Comparisons of such determinations with the results obtained by equilibrium dialysis indicate that, at pH 7.0, over 95 % of the protein-bound dye is in the acid form (assuming, naturally, that the pK of the free dye is unchanged). This means that the BTB-binding "sites" on the protein have a much higher affinity for the acid than for the conjugate base. This is further confirmed by the fact that the amount of protein-bound BTB increases when the pH is lowered, i.e. when the relative concentration of the acid form becomes larger. Now, when a phase separation is performed between amyl alcohol and a buffered solution of BTB, at pH 7.0, almost all the dye goes into the organic phase, and the spectrum of the latter corresponds to that of a water solution of BTB at pH 2. This predictable result (since the acid corresponds to the unionized form of the dye) strongly suggests that BTB associates mainly with apolar, water-repellent, areas or groups in the protein.

It may be concluded that in the presence of its allosteric effector 5'AMP, the enzyme-protein undergoes a reversible alteration which results in increasing its capacity to bind certain lipophilic ligands.

Since the AMP-induced alteration does not occur with Ph a, it cannot be ascribed to a direct interaction between the dye and the bound nucleotide itself; it clearly depends upon a transition of state which is undergone by Ph b, but not by Ph a, in the presence of 5'AMP.

This interpretation is further justified by the fact that, in the absence of AMP, Ph a is more "lipophilic" than Ph b in that it binds more BTB. It may therefore be considered that the "state" which is stabilized by AMP in Ph b already obtains in Ph a.

The significance of these observations is greatly strengthened by the fact that closely similar findings should have been made with an entirely different system (hemoglobin-oxygen) whose only analogy with the present one is that, in both cases, allosteric interactions between distinct specific binding-sites are known (hemoglobin) or presumed (Ph b) to occur. We might mention that we have tested the effect of specific ligands upon the binding of BTB by various enzyme-proteins which show no evidence of allosteric interactions (β-galactosides with β-galactosidase; ATP or glucose with hexokinase). The results were negative, as they were with Ph a. The BTB binding effect may therefore turn out to constitute a fairly widely applicable, as well as very simple, test of the capacity of a specific ligand to induce a transition of state in a protein. Where it occurs it may also be used, as we have seen, as an independent method of studying the binding of an allosteric effector to a protein.

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