PHOSPHORYLASE *b* TETRAMERIZATION INDUCED BY AMP AT 25°C

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1. Introduction

Several investigators [1] have shown that rabbit skeletal muscle phosphorylase *b* in the presence of 10^{-3} M AMP completely tetramerizes at 4°C. However, later work [2] showed only 5–10% of tetramerization under identical conditions, but, in the higher temperature region, 25–30°C. This difference in behaviour with increase in temperature has to some extent been clarified by Buc et al. [3] using 'temperature jump' studies. They found that one relaxation time disappeared in the temperature range, 4–25°C. Almost simultaneously, Ho and Wang [4], although they found no tetramerization at 25°C, did find by microcalorimetry that it begins to occur at 18°C, at which temperature a conformational change in the enzyme takes place [3,5]. Wang et al. [4,6], also by microcalorimetry, discovered the existence of two binding sites of differing affinity towards AMP. A little later the existence of these binding sites, at 4°C was also indicated by equilibrium dialysis studies [7].

In the present study, we report results obtained by microcalorimetric studies on the interaction, phosphorylase *b*–AMP. Our results differ from those of Ho and Wang [4] but, in a complementary study of the phosphate action on the previous interaction, we believe we can account for this difference.

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2. Materials and methods

Glycogen phosphorylase *b* (EC 2.4.1.1) was obtained by the method of Krebs et al. [8] as modified by Buc et al. [2]. The mol. wt. of the glycogen phosphorylase *b* monomer was taken as 92 500 [9]. The activity of phosphorylase *b* was determined by the procedure of Helmreich and Cori [10]. The purity of AMP (Merck) was checked by t.l.c. on PEI cellulose. The buffer used in all the experiments was 5×10^{-2} M glycil–glycine, 5×10^{-2} M ClK and 10^{-4} M EDTA, pH 6.9 [7].

Calorimetric measurements were performed using a LKB batch microcalorimeter at 25°C. In all the experiments the heat of dilution of the ligand was automatically cancelled using the reference cell. Separate experiments were made to determine the heat of the enzyme dilution which was found to be negligible. The final enzyme concentration, 2.66 mg/ml, was kept constant in all the experiments. The enzyme was used within one week after the third crystallization. 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. The enzyme concentration was the same as that used for the calorimetric experiments.

3. Results

The calorimetric titration of phosphorylase *b* with AMP, shows two distinct plateaus (fig.1), supporting the existence of two AMP binding sites of different

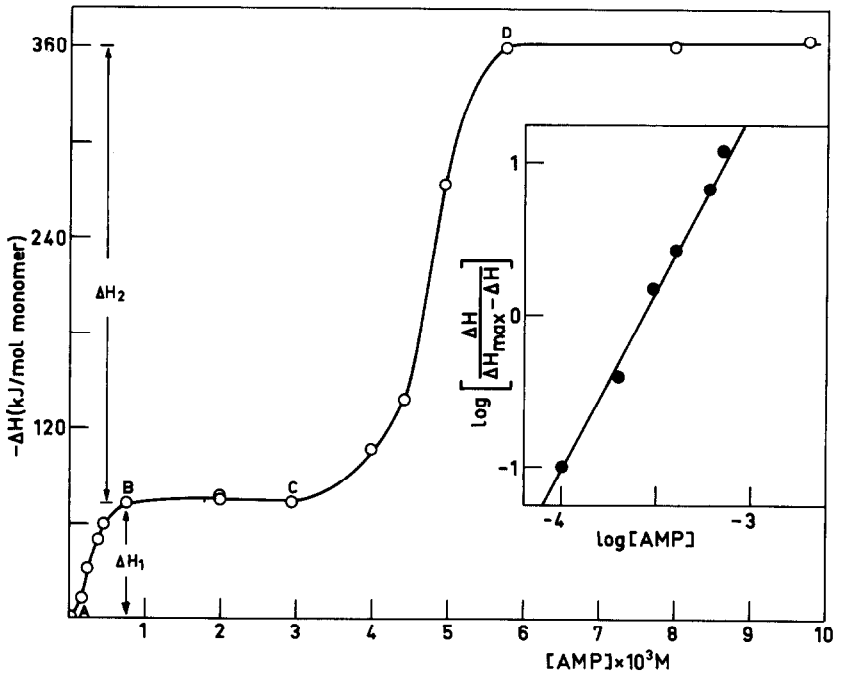

Fig.1. Enthalpy titration of phosphorylase *b* with AMP at 25°C. Phosphorylase *b* concentration kept constant at 2.66 mg/ml. Insert: A Hill plot of the data in the AB region.

affinity [4,6,7]. The first site has a half saturation constant of 2.5×10^{-4} M, in agreement with previous results [4]. The transition from the first to the second plateau occurs between 2.5×10^{-3} M and 6.0×10^{-3} M AMP. A Hill plot of our enthalpy values in the AB region is shown in the insert of fig.1. The slope of this leads to a value of $n = 2.5$, which supports the high cooperativity in the binding of AMP to the enzyme observed by other techniques [2,6,11] for the two strong affinity sites. Our experimental results give values of $-(77 \pm 4)$ kJ (mol monomer)$^{-1}$ and $-(284 \pm 12)$ kJ (mol monomer)$^{-1}$ for ΔH_1 and ΔH_2 respectively (fig.1). The enthalpy change of the first binding site is due to the AMP binding and to the associated conformational change of the enzyme. The extremely high enthalpy change for the second binding site cannot be explained in terms of a single binding process. It could be due to both a conformational change and an association process induced by AMP.

Ho and Wang [4] have reported for the interaction, phosphorylase *b*–AMP at 25°C, 5×10^{-2} M glycerophosphate buffer, ΔH values of -55 and -25 kJ (mol monomer)$^{-1}$ for the first and second binding sites respectively. Their ultracentrifuge experiments showed that the equilibrium dimer–tetramer is present at 10^{-3} M and 10^{-2} M AMP. The difference between our calorimetric results and those of Ho and Wang [4] probably arises because of the different buffers used. It has been postulated, on the basis of kinetic [11] and nanosecond fluorometry studies [12], that glycerophosphate interacts with the enzyme. In an attempt to clarify the difference between our work and that of Ho and Wang [4] we have studied the phosphate effect in the AMP–phosphorylase *b* interaction (see fig.2).

We find two differences between figs.1 and 2: (a) the AB region of fig.1, corresponding to the binding of AMP to the higher affinity site is maintained, although the enthalpy change of the interaction becomes endothermic. (b) In the presence of phosphate, the CD region of fig.1 is largely diminished.

Ultracentrifuge experiments have been done to determine whether the dimer–tetramer equilibrium

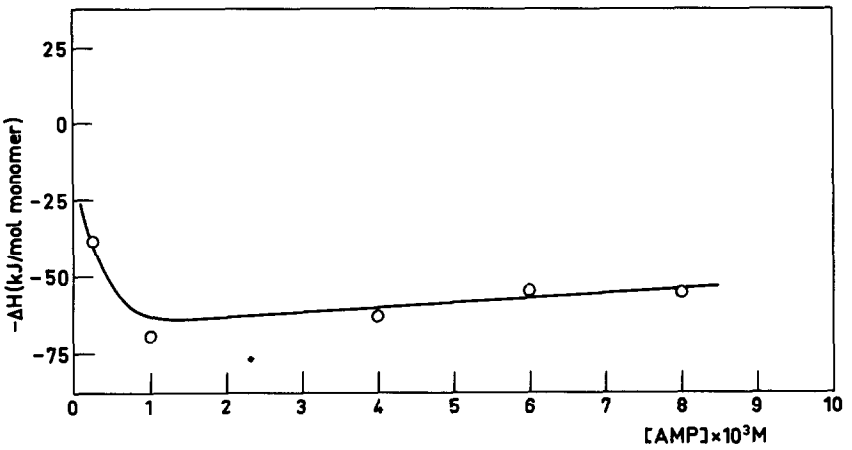

Fig.2. Calorimetric titration of the phosphate–phosphorylase *b* complex with AMP at 25°C. Conditions: [phosphorylase *b*] = 2.66 mg/ml and 2×10^{-2} M phosphate.

in phosphorylase *b* is altered in the presence of a saturation concentration of phosphate (5×10^{-2} M). We obtained the following results: (a) The enzyme in the presence of 2×10^{-2} M phosphate has a sedimentation coefficient of 8.6 S, which corresponds to a dimer. (b) In the presence of 2×10^{-2} M phosphate and 10^{-3} M AMP the sedimentation profile shows two peaks, one of 8.4 S and a broad one of 12.8 S, which correspond to the dimeric and tetrameric species (20% and 80%, respectively). (c) the phosphorylase *b* in the presence of 2×10^{-2} M phosphate and 10^{-2} M AMP also shows the existence of a dimer–tetramer equilibrium but, in this case, 60% tetramer 40% dimer. Fig.3 shows the pronounced effect of increasing phosphate concentrations upon the enthalpies of the AMP–phosphorylase *b* interactions.

The concentration of AMP was kept constant at the saturation values of the first (10^{-3} M) and second (10^{-2} M) AMP binding sites (fig.1). Both curves show that the enthalpy change decreases with increasing phosphate concentration. The difference in enthalpy between both curves reaches a constant value of (20–25) kJ (mol monomer)$^{-1}$ at 2×10^{-2} M phosphate. Ho and Wang [4] found the same enthalpy value for the difference between the first and second plateaus in the presence of glycerophosphate buffer. The difference between our present results and those of Ho and Wang is probably due to a strong interaction of the glycerophosphate with the enzyme via the phosphate.

4. Discussion

The above results can be interpreted in terms of the conformational equilibria proposed by Buc et al. [3] to explain the behaviour of phosphorylase *b* at 4°C. The relevant equilibria are:

$$E_2 \rightleftharpoons E_1 \rightleftharpoons E_3 \rightleftharpoons 1/2\ Q$$

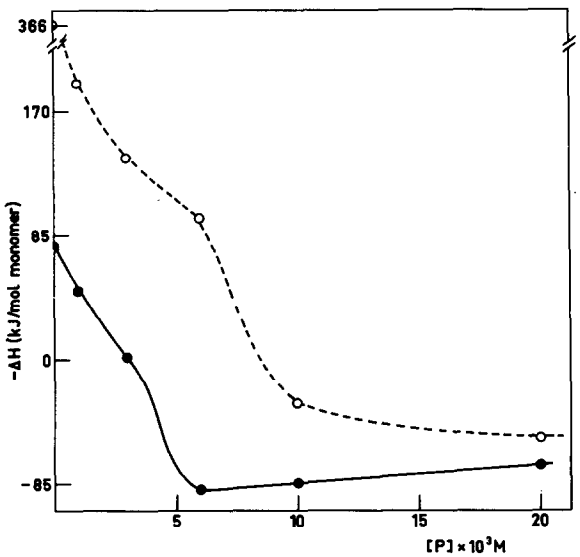

Fig.3. Interaction between AMP and complex phosphate–phosphorylase *b* at 25°C with increasing phosphate concentrations. The broken line corresponds to a final concentration of 10^{-2} M AMP and solid line to a final concentration of 10^{-3} M AMP.

where the symbols have the meanings given in reference [3]. When AMP binds to its stronger affinity sites in the enzyme the equilibrium moves allosterically to the E_1 state. But, when AMP binds to its weaker affinity sites, the equilibrium situation moves completely from the E_1 dimeric state to the tetrameric state (Q), a process which possesses great positive cooperativity. Buc et al. did not work at AMP concentrations in the saturation range (fig.1) of its weaker affinity sites and they did not therefore find the tetramer. On the other hand, Wang and co-workers [4,6] worked with glycerophosphate buffer. Our findings for the effect of phosphate on the AMP–phosphorylase *b* interaction agree with those obtained by Wang et al. for the AMP–phosphorylase *b* interaction in glycerophosphate. Thus, the enthalpy differences between both plateaus, 10^{-3} M AMP and 10^{-2} M AMP, in the presence of a saturation phosphate concentration or glycerophosphate buffer are (20–25) kJ (mol monomer)$^{-1}$. The ultracentrifuge patterns obtained in the presence of saturation phosphate concentration and at AMP concentration, 10^{-3} M and 10^{-2} M, agree well with those of Wang et al. [6]. In both cases a reversal of the tetramerization promoted at 10^{-3} M AMP occurs at 10^{-2} M AMP. These results can be explained if we consider that, in the above Buc equilibria sequence, phosphate displaces the enzyme conformation towards either E_1 or E_3. Phosphate thus facilitates the binding of AMP to its weaker affinity sites and the consequent tetramerization of the enzyme. In glycerophosphate the positive cooperativity almost completely dissapears. This indicates that in all probability phosphate leads to the E_3 dimeric conformation. If we suppose that the tetramer of phosphorylase *b* is stabilized by electrostatic interactions, like the tetramer of phosphorylase *a* [13], the reversal of the dimertetramer equilibrium of phosphorylase *b* caused by increasing the AMP concentration at a saturated phosphate concentration may be explained by the high buffer ionic strenth.

Acknowledgements

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