PHOSPHORYLASE *b*: THE CRITICAL DEPENDENCE OF A TWO-STATE CONFORMATIONAL TRANSITION UPON THE ENZYME CONCENTRATION

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1. Introduction

The interaction between glycogen phosphorylase *b* (EC 2.4.1.1) and AMP has been studied by different experimental methods such as kinetic studies [1], equilibrium dialysis [2], spectrophotometric studies [3], NMR and ESR [4]. The dissociation constants obtained by these methods do not agree, possibly because the enzyme concentration range is large, 0.01 mg/ml (activation kinetics) to 8 mg/ml equilibrium dialysis) or higher, 20 mg/ml in differential spectroscopy [5].

In the present paper, we have studied the effect of the enzyme concentration on the enthalpy of the AMP-phosphorylase *b* interaction. The results obtained are very different from those reported by Ho et al. [6] and raise interesting questions.

2. Materials and methods

Glycogen phosphorylase *b* was prepared from rabbit skeletal muscle by the method of Krebs et al. [7] as modified by Buc et al. [8]. The enzyme was recrystallized three times and AMP was removed by passage through a column of Sephadex G-25 gel and then treated with activated charcoal. The A_{260}/A_{280} ratios at pH 6.9 were always lower than 0.54. The enzyme concentration was measured spectrophotometrically using an extinction coefficient ($E^{1\%}_{1\,cm}$) at 280 nm of 13.2 [9]. The activity of phosphorylase *b* was determined using the procedure of Helmreich and Cori [10]. The molecular weight of phosphorylase *b* monomer was taken as 92 500 [9]. The purity of AMP (Merck) was checked chromatographically on PEI cellulose. The buffer used in all the experiments was 5×10^{-2} M glycyl-glycine, 5×10^{-2} M KCl and 2×10^{-4} M EDTA; the final pH was adjusted to 6.9. All chemicals used were of high grade. Calorimetric measurements were made in an LKB batch microcalorimeter at 25.00 ± 0.05°C. The calorimeter was calibrated as described elsewhere [11]. In all experiments the heat of dilution of the nucleotide was automatically cancelled by the reference cell. In separate experiments the heat of dilution of the enzyme solution was determined and subtracted from the heat of mixing when significant.

The enzyme was used only one week after the third crystallization [7]. Sedimentation velocities were measured with a Spinco model E analytical ultracentrifuge (60 000 rpm and 25°C) using Schlieren optics.

3. Results

As has been previously reported [12], the calorimetric titration of phosphorylase *b* with AMP shows two distinct plateaus, supporting the existence of two AMP binding sites of different

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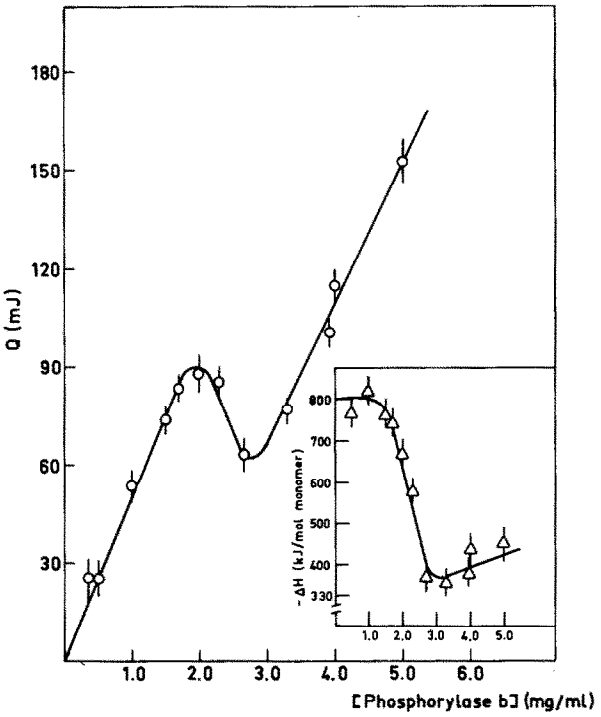

Fig.1. The dependence of heat evolved in the phosphorylase *b*-AMP interaction upon the enzyme concentration. The experiments were carried out at 25°C. The AMP concentration was kept constant at 10 mM. Insert: The enthalpy change of the above interaction versus enzyme concentration.

affinity [13–15]. These experiments were carried out at constant enzyme concentration (2.66 mg/ml) and the concentration of nucleotide was varied from 5×10^{-5} M to 10^{-2} M.

To find out whether the enzyme concentration affects the above mentioned results we studied calorimetrically the interaction between AMP and phosphorylase *b*. In our experiments the nucleotide concentration was maintained constant at the saturation of the second binding site (10^{-2} M AMP) and the enzyme concentration was varied from 0.25 mg/ml. Fig.1 shows how the heat evolved depends upon the enzyme concentration. The interesting point is that we obtain two straight lines, with a break in the concentration range: 1.5 to 2.66 mg/ml. The plot shows all the characteristics of a conformational transition between two enzymatic states. From the slopes of the two straight lines we derive an enthalpy change of –(794 ± 40) kJ/mol monomer for the enzyme concentration range: 0 to 1.5 mg/ml and an enthalpy change of –(397 ± 30) kJ/mol monomer for enzyme concentrations higher than 2.66 mg/ml. From the insert of fig.1 we obtain an enthalpy of transition of +(400 ± 80) kJ/mol monomer. At enzyme concentrations higher than 2.66 mg/ml in the presence of 10^{-2} M AMP phosphorylase *b* is in a tetrameric state [12]; we believe that the enthalpy change (–(397 ± 30) kJ/mol monomer) in the concentration range: 2.66 to 5 mg/ml is mainly due to tetramerization of the enzyme and is in close agreement with the value reported by Ho et al. [6] (–(360 ± 15) kJ/mol monomer) for the tetramerization of phosphorylase *b* at 18°C.

At enzyme concentrations lower than 1.5 mg/ml in the presence of 10^{-2} M AMP our ultracentrifuge experiments give sedimentation coefficient of 9.1 S, that can be assigned to a modified dimer. Phosphorylase *b*, at concentrations of 1 and 2.66 mg/ml in glycyl-glycine buffer alone has a sedimentation coefficient of 8.3 S, a value which corresponds to the dimeric form of the enzyme. The enthalpy value obtained for the AMP-phosphorylase *b* interaction at enzyme concentrations lower than 1.5 mg/ml is mainly due to the AMP binding and to the induced conformational change.

4. Discussion

Our results can be understood if we assume that at enzyme concentrations lower than 1.5 mg/ml, the phosphorylase *b* moves to a conformational state unable to tetramerize in the presence of 10^{-2} M AMP. This is supported by our results for the titration of phosphorylase *b* with DTNB, which also explain the high heat of transition. The enthalpy of formation of an S-S bond is ΔH_f^0 (S-S) = –(284 ± 4) kJ/mol [16].

The two-state transition reported in this work, was not detected by Ho et al. [6] who used the same experimental technique but worked with a different buffer (glycerophosphate) and at a different AMP concentration (10^{-3} M), corresponding to the first binding site. As we have previously reported [12] the glycerophosphate displaces the enzyme to a conformational state very similar to the one produced by

effect of phosphate. There are, two possible explanations for this: (a) the conformational state induced by the glycerophosphate conceals the enzymatic transition, or (b) the transition does not affect the binding sites of highest affinity towards AMP.

The present results explain the discrepancies which arise when experiments are performed at different enzyme concentrations.

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