THE EFFECTS OF GLYCOGEN ON PHOSPHORYLASE b INTERACTIONS WITH 5'-AMP AND PHOSPHATE

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1. Introduction

Glycogen phosphorylase b (EC 2.4.1.1.) catalyses in vivo the α-1,4 glycosidic bond phosphorolysis of the glycogen molecule although this is a reversible process in vitro [1]. 5'-AMP is needed for this enzyme to show catalytic activity [2], a slight residual activity remaining in its absence [3]. The 5'-AMP activation process has allosteric characteristics [4]. Kinetic studies [5] have demonstrated that the reaction catalyzed by phosphorylase b follows a bi–bi random kinetic mechanism in both directions.

The research with usual spectrophotometric techniques is difficult due to light scattering by the glycogen solutions. Although isotopic exchange studies have been done [6,7] the catalytic process is not known in detail. The nature and situation of the different groups involved in the catalytic mechanism have not been established.

This work reports the calorimetric results obtained for the glycogen effects on the 5'-AMP and phosphate binding sites, all of them related to the catalytic process.

2. Materials and methods

Glycogen phosphorylase b from rabbit skeletal muscle was obtained by the methods of Krebs et al. [8] and Buc et al. [9]. The molecular weight of the glycogen phosphorylase b monomer was taken as 92 500 [10]. The activity of phosphorylase b was determined following the Helmreich and Cori procedure [11]. Glycogen was purchased from Merck. The purity of 5'-AMP (Merck) was checked by TLC chromatography on PEI-cellulose. The buffer used in all the experiments was glycyl–glycine, pH 6.9 [12].

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 only during the first week after the third crystallization. Sedimentation velocities were measured using an Analytical Ultracentrifugue Beckman Spinco Model E at 60 000 rev./min and 25°C. The enzyme concentration was the same as that used for the calorimetric experiments.

3. Results and discussions

Bresler and Firsov [13] have shown that the equilibrium dissociation constant of the phosphate–phosphorylase b complex reaches a steady value at 2.5×10^{-3} M glycogen (in glucose units). Previous kinetic studies [5,9] have been done at this polysaccharide concentration. To obtain comparative results our calorimetric studies were conducted at the same glycogen concentration. The enthalpy of

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phosphorylase–glycogen (2.5×10^{-3} M glucose units) interaction is highly positive (+112.9 ± 0.8 kJ/mol monomer, mean of the three experiments). According to reported results [5], this heat may be due to the displacement of the enzyme by glycogen towards a different conformational state [4]. The reported [5] equilibrium dissociation constant at 30°C of the phosphorylase b–glycogen complex, 4.6×10^{-3} M glycogen (glucose units), was obtained in glycerophosphate buffer, which interacts strongly with the enzyme [14]. So, we needed to know this Michaelis constant in glycyl–glycine buffer, which depends on the orthophosphate concentration. The calculated value from initial rate measurements is 5.0×10^{-3} M at 25°C. Using this and the equation: $-RT \ln K = \Delta H^\circ - T\Delta S^\circ$, an entropy change of −101 ± 2 e.u. (mol monomer) was estimated for the dissociation process.

Figure 1 shows the enthalpy titration of the phosphorylase b–glycogen (2.5×10^{-3} M glucose units) complex with phosphate. These experiments have been conducted in the absence of Mg^{2+} ions. In all of them the time to obtain zero heat effect was of the order of 10 min, similar to that reported for neutralization reactions [15]. The curve obtained is sigmoidal, which implies the existence of allosteric interactions due to glycogen because in its absence there is no cooperative effect. Hill plot data is given in the insert. The value, n=1.9, agrees with a high homotropic interaction between the two phosphate binding sites per dimer [16]. At this glycogen concentration the enzyme must be almost completely in the complexed form. The value measured for K_d, $6.3 \pm 0.7 \times 10^{-3}$ M, is of the same order as the one reported by Engers et al. [5] and by Buc [16] from kinetic studies. The high value observed for the heat in this curve may be due to nonspecific interaction between the phosphate and the positively charged groups at the enzyme surface.

Experimental enthalpies for the interaction between 5′-AMP and the phosphorylase b–glycogen (2.5×10^{-3} M glucose units) complex are plotted versus the 5′-AMP concentration in fig.2. The experimental data for the 5′-AMP–phosphorylase b interaction versus 5′-AMP concentration are also inserted [14]. Glycogen affects the 5′-AMP–phosphorylase b interaction in the following ways:

(a) As fig.2 shows the enthalpy of 5′-AMP inter-

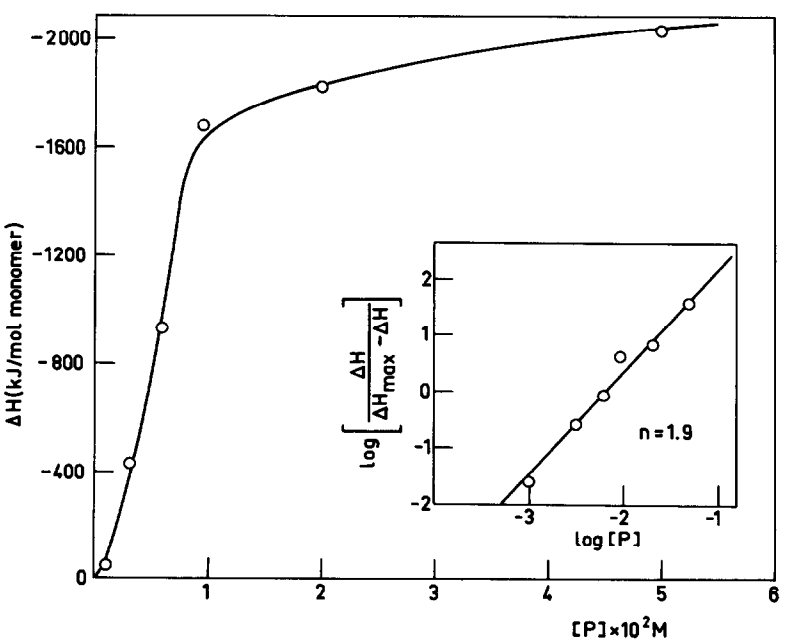

Fig.1. Enthalpy titration of phosphorylase b–glycogen complex with phosphate at 25°C. Insert: Hill plot of the data.

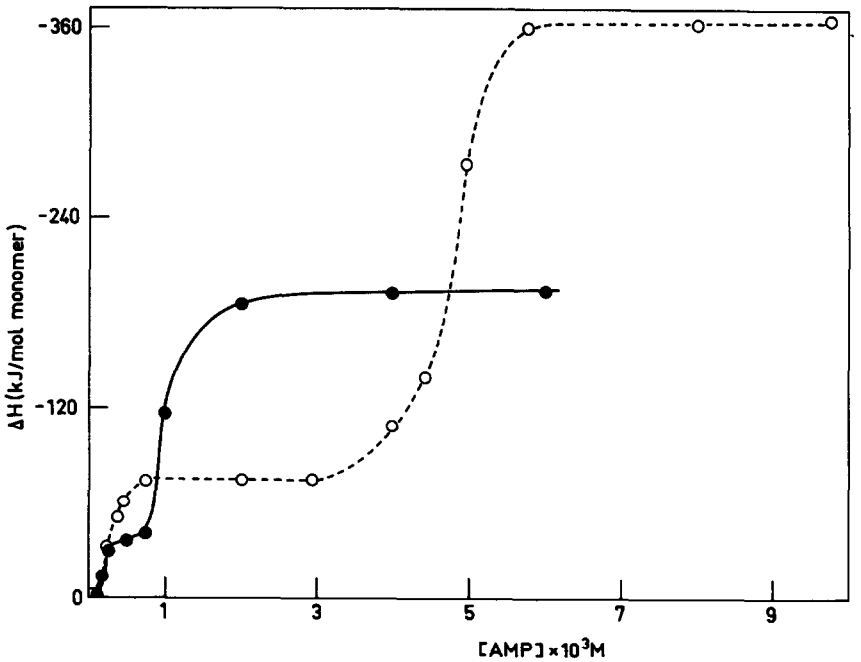

Fig.2. Calorimetric titration of phosphorylase b with 5′-AMP (○) and phosphorylase b–glycogen complex with 5′-AMP (●) at 25°C.

action in both plateaus is reduced by about 50%. The relevant enthalpies are respectively −37 ± 2 kJ/mol monomer and −77 ± 4 kJ/mol mono- with and without glycogen for the first two plateaus. This may be interpreted as a reduction in the 5′-AMP-induced conformational change, since the enthalpy of interaction between groups and atoms of 5′-AMP and its first binding site must be the same in both cases. The enthalpies for the second two plateaus, are respectively −150 ± 7 kJ/mol monomer and −284 ± 10 kJ/mol monomer. Here any possible explanation has to take into account the tetramerization of phosphorylase b [14]. Our ultracentrifugue experiments show that in the second plateau the tetrameric form is present. In the absence of glycogen [14] only the tetramer exists in the second plateau, but in the presence of polysaccharide two peaks appear. The first one, which contains nearly 60% of the enzyme, has a sedimentation coefficient equal to 14.06 S (tetramer) and the second one a sedimentation constant of 63.96 S. The latter peak is very broad and shows that there must be aggregate form, between the enzyme and the polysaccharide. If we assume that the phosphorylase molecules in solution are arranged along the glycogen chain, this could explain, the high entropy change obtained for the glycogen phosphorylase b interaction, the experimental observed aggregation, and also the great heat of phosphate–[phosphorylase b–glycogen] interaction.

(b) 5′-AMP affinity towards the weaker 5′-AMP binding site is greatly increased by the presence of glycogen. Other studies [14,17] indicate that this site is directly related to the association of the enzyme. The results of fig.2 support this. In the absence of glycogen this site is saturated between 3.0×10^{-3} M and 6.0×10^{-3} M 5′-AMP, while in its presence the saturation occurs in the range $0.75–2.00 \times 10^{-3}$ M 5′-AMP. This result should be correlated with the in vivo 5′-AMP concentration which reaches at maximum 3×10^{-4} M [2].

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