THE EFFECT OF SUBSTRATES ON THE ASSOCIATION EQUILIBRIUM OF MAMMALIAN D-GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

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1. Introduction

Mammalian D-glyceraldehyde 3-phosphate dehydrogenase, mol. wt 145 000 [1,2], is composed of four identical subunits [3] and it crystallizes with 3.5–4 mol of NAD per mol of tetrameric enzyme [4]. The reversible dissociation of the enzyme into dimers at neutral pH in 0.1 M phosphate buffer at 5°C, below 500 μg per ml enzyme concentration has been reported [5]. In a previous paper [6] we presented a detailed analysis of the molecular weight distribution of the enzyme containing its original NAD complement in the 50–500 μg per ml enzyme concentration range at 5°C. A monomer–dimer–tetramer equilibrium was found and under 50 μg per ml concentration the monomeric form of the enzyme was predominant.

D-glyceraldehyde 3-phosphate dehydrogenase catalyzes the reversible conversion of glyceraldehyde 3-phosphate into 1,3-diphosphoglyceric acid in the presence of inorganic phosphate and NAD.

The purpose of our investigation was to study the effect of the various substrates on the association equilibrium of the monomer–dimer–tetramer system and to determine the degree of association of the catalitically active enzyme form.

2. Materials and methods

Four times recrystallized GAPD from porcine skeletal muscle [7] was dissolved in 0.1 M glycine buffer, pH 8.5. To remove ammonium sulfate the enzyme solution was gelfiltered on a Sephadex G-25 column equilibrated with the same buffer. The bound coenzyme (NAD) was removed from the enzyme by charcoal treatment, which resulted in an A_{280}/A_{260} absorbance ratio higher than 1.9; this corresponds to less than 5% residual NAD content [4]. Enzyme concentration was determined spectrophotometrically, by using the absorption coefficient $A_{280}^{0.1\%} = 0.89$ for the apoenzyme. All operations with the enzyme were carried out at 5°C.

NAD was a Reanal product of 95% purity.

GAP was prepared from fructose 1,6-bisphosphate [8] and the contaminating inorganic phosphate was removed by ion exchange chromatography.

The concentration dependence of the number-, weight- and Z-average molecular weights was measured by the meniscus depletion method [9]. The sedimentation equilibrium runs were carried out in a MOM 3170 ultracentrifuge equipped with Rayleigh interferometer optics. The initial protein concentration was about 200 μg per ml, the solution column height in the ultracentrifuge cell varied between 0.2 and 0.3 cm. The appropriate rotor speed and the time required to attain the equilibrium were calculated according to Van Holde and Baldwin [10], 10 to 24 h runs were made with a rotor speed of 16 000 to 30 000 rev/min. The protein concentration at different positions of the cell was calculated from the interference fringe displacements. The partial specific volume was 0.733 ml per g, as measured pycnometrically.

Analytical band centrifugation experiments on the

Abbreviations: GAPD, D-glyceraldehyde 3-phosphate dehydrogenase; GAP, glyceraldehyde 3-phosphate; DPGA, -1,3 diphosphoglyceric acid; P_i inorganic phosphate.

active enzyme–substrate complex were performed in a Spinco model E analytical ultracentrifuge equipped with an automatic digital absorption scanning system of high precision [11], as described by Cohen et al. [12].

The sedimentation of the active enzyme–substrate complex band can be observed through the reaction catalyzed by the enzyme, i.e. by measuring the absorption at 365 nm due to the conversion of NAD to NADH according to the reaction:

$$GAP + NAD^+ + P_i \rightleftharpoons DPGA + NADH + H^+$$

The substrate solution contained 10 mM Na_2HPO_4, 2 mM NAD and 2 mM GAP in 0.1 M glycine buffer pH 8.5. The enzyme solution (10 μl) containing $2 \times 10^{-8} - 10^{-7}$ g enzyme was layered onto approx. 70 μl of the substrate solution in the sector of the rotating ultracentrifuge cell. The transfer of the enzyme solution occurred at about 2000 rev/min. Centrifugation runs were carried out at 5 and 20°C in an AN-D rotor at 56 000 rev/min. Absorption scannings on the rotating cell were made every 5 min.

The calculation of the sedimentation coefficient of the active enzyme–substrate complex was made according to Cohen and Mire [13] by using the differences of successive absorption scannings. The slope of the semilogarithmic plot of the difference maxima versus time is proportional to the sedimentation coefficient of the active enzyme-substrate complex (fig.1).

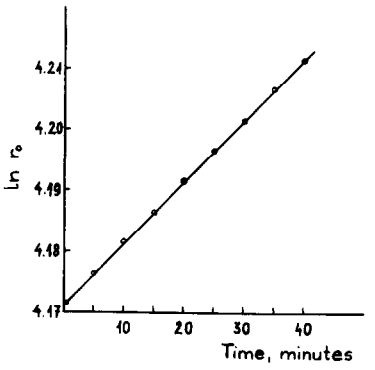

Fig.1. Active enzyme band centrifugation. Plot of the logarithm of the positions of the active band versus time. Rotor speed: 56 000 rev/min, temperature: 5°C, protein concentration 2 μg per ml, substrate concentrations: 2 mM GAP + 10 mM Na_2HPO_4 + 2 mM NAD in 0.1 M glycine buffer, pH 8.5. $s_{20,w} = 7.46$ S.

3. Results and discussion

High speed sedimentation equilibrium experiments were performed with NAD-free enzyme. A Rayleigh interference pattern is shown in fig.2. The apparent number-, weight- and Z-average molecular weights were calculated over the 50–500 μg per ml enzyme concentration range, as described earlier [6]. The concentration dependence of the different average molecular weight values and the calculated distribution of the weight fractions of the monomer, dimer and tetramer are presented in fig.3a and 3b,

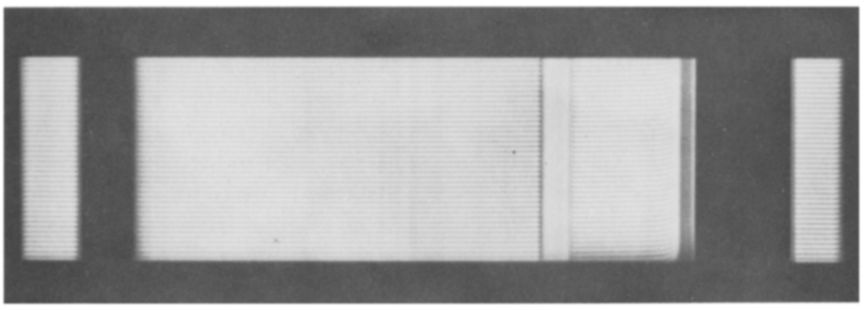

Fig.2. Rayleigh interference pattern of a meniscus depletion experiment with apoenzyme. Initial protein concentration 0.185 mg per ml, rotor speed: 24 000 rev/min, temperature: 5°C.

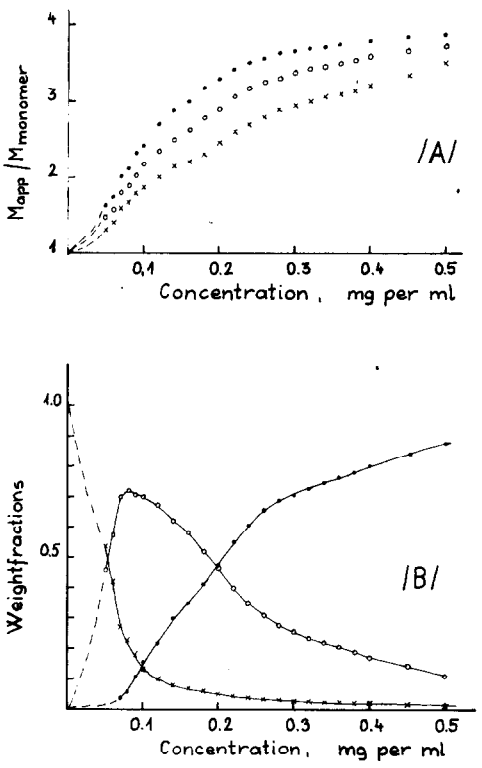

Fig.3. Concentration dependence of the apparent number- (X-X-X), weight- (o-o-o) and Z-average (•- •-) molecular weights (A) and the distribution of the monomeric (X-X-X), dimeric (o-o-o) and tetrameric (•- •-) weight fractions (B) as a function of total protein concentration in the case of apoenzyme in 0.1 M glycine buffer, pH 8.5, at 5°C.

respectively. All three average molecular weights decrease with decreasing concentration. At the lower end of the concentration range the values approach the molecular weight of the monomer (36 300) and at the upper end that of the tetramer (145 000).

In the assay mixture of GAPD the enzyme concentration generally used is a few μg per ml. In this enzyme concentration range the equilibrium of the monomer–dimer–tetramer system is shifted towards the monomeric form (fig.3). In order to determine the association state of GAPD in the presence of substrates we performed analytical band centrifugation experiments on the active enzyme-substrate complex as described in Materials and methods. The enzyme concentration range covered was 2–10 μg per ml. The sedimentation coefficient obtained for the active front was $s_{20,w} = 7.50 \pm 0.40$ (fig.1). If one compares this value with that obtained for the tetrameric form of GAPD ($s_{20,w} = 7.82 \pm 0.08$; cf. [14]) there is no doubt that the active front corresponds to the tetramer.

The specific activity of the enzyme in the active front was calculated from the amount of NADH produced during a given period of time. From the quantity of NADH produced in the band centrifugation experiment and that measured spectrophotometrically with the same amount of enzyme present in the ultracentrifuge cell, the specific activities were 23 000 ± 2500 and 26 000 ± 1000 mol NADH per mol GAPD per min, respectively. The initial rate calculated from the first 10 sec was the basis of comparison, since the enzyme front in the band centrifugation experiments always meets fresh substrate solution due to sedimentation. The values compared are identical within the limits of error. The slightly decreased specific activity of the active band may be explained by the adsorption of the enzyme on capillary surfaces and by the diffusional broadening of the enzyme front during the experiment, in which case optimal substrate concentration is no more available for the tail of the enzyme band. From the activity data we can conclude that the tetrameric front carries all the activity introduced into the ultracentrifuge cell.

Although the apoenzyme dissociates at the microgram level, in the presence of all substrates involved in the enzymic reaction, the enzyme exists and reacts in the tetrameric form. The effects of various substrates and substrate combinations were investigated in order to reveal their influence on the association process. Meniscus depletion equilibrium experiments were carried out like in the case of apoenzyme. The substrate concentrations were the same as in the band centrifugation experiments. The various apparent average molecular weight distributions and from these values the weight fractions of monomer, dimer and tetramer were calculated over the 50–500 μg per ml enzyme concentration range as described earlier [6].

Fig.4 shows that inorganic phosphate ions and GAP, whether applied alone or together, have only a slight effect on the association equilibrium of the subunits. At 2 mM NAD concentration, when all

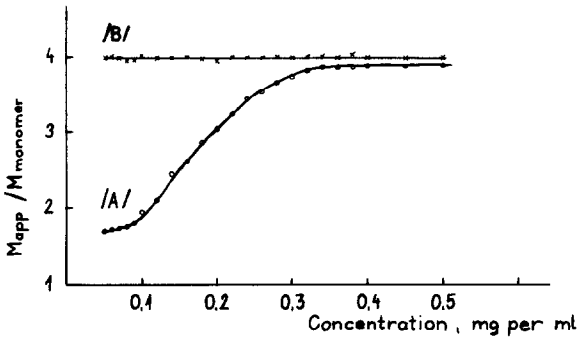

Fig.4. Apparent weight-average molecular weight as a function of total protein concentration in the case of apoenzyme in the presence of GAP or inorganic phosphate or both (A), and in the presence of excess NAD (B). For experimental details see text.

four coenzyme binding sites are occupied [15], a mol. wt of 146 000 was measured, even at 50 μg per ml enzyme concentration (fig.4). Considering the results summarized in fig.4 we may state that the coenzyme (NAD) is responsible for the association of the subunits in the enzyme assay mixture.

In our previous work [6], when the holoenzyme was investigated without the addition of excess NAD, we found that at 50 μg per ml enzyme concentration – when only half of the binding sites are occupied because of the partial release of bound coenzyme [15] – there was no essential change in the association equilibrium of the subunits compared to that of the apoenzyme. Therefore it can be concluded that the second two tightly bound coenzyme molecules play the decisive role in the association process, i.e. in the stabilization of the tetrameric structure of GAPD in the enzyme assay mixture.

Our results corroborate the suggestion recently made by Osborne and Hollaway [16], on the basis of hybridization studies with yeast and rabbit GAPD-s, that the decrease in hybrid formation in the presence of NAD is due to the displacement of the dimer–tetramer equilibrium towards the tetrameric form. Their results also show that the structural changes only take place on the binding of the third NAD molecule.

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