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SUBSTRATE-INDUCED DISSOCIATION OF GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE DETECTED BY AFFINITY CHROMATOGRAPHY

STUDY OF SUBUNIT INTERACTIONS BY AFFINITY SORPTION

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Summary

All four NAD-binding domains of the tetrameric apoglyceraldehyde-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) displayed identical affinities ($K_d = 4.8 \cdot 10^{-5}$ M) towards the chromophore of Blue Dextran. The enzyme species which contained only the most firmly bound NAD coupled the dye through the remaining free sites with the same affinity as the apoenzyme.

The interaction of glyceraldehyde-phosphate dehydrogenase and Blue Dextran-Sepharose was characterized by the aid of an affinity sorbent batch technique. We found that the apparent dissociation constant varied with enzyme concentration showing preferential binding in dilute solutions. This suggests that the monomeric and/or dimeric form(s) of glyceraldehyde-phosphate dehydrogenase have higher affinity towards the sorbent than the tetramer. The apparent K_d of the enzyme-Blue Dextran-Sepharose complex at low enzyme concentration was similar to the K_i of the competitive inhibition of enzyme activity by free Blue Dextran, i.e. about 10^{-6} M.

Glyceraldehyde 3-phosphate increased the binding of the enzyme to the immobilized dye in chromatographic experiments and decreased the apparent dissociation constant in the batch system if enzyme solutions of around 10^{-6} M were examined. In more dilute solutions glyceraldehyde 3-phosphate had no effect on complex formation. These data suggest that glyceraldehyde 3-phosphate influences the interaction of the enzyme and the immobilized dye, first of all, by shifting the equilibrium of the different enzyme forms towards dissociation.

Introduction

Affinity chromatography is becoming an ever more appreciated tool for obtaining quantitative information on the interaction between enzymes and specific ligands. Thus equilibrium constants have been estimated by competitive zonal elution affinity chromatography [1,2]. The occurrence of subunit and protein-protein interactions has also been explored by affinity chromatography [3,4]. The theory underlying these determinations is in all cases rather complicated, since in affinity chromatography a heterogeneous system is operative [5,6].

Stellwagen and coworkers [7,8,9] have shown that Blue Dextran binds specifically to the NAD-binding domains of a variety of proteins. Immobilization of Blue Dextran through covalent linkage to Sepharose did not interfere with this specific complex formation. The enzyme bound to immobilized Blue Dextran could be displaced from the affinity column by low concentrations of the respective coenzyme.

Glyceraldehyde 3-phosphate dehydrogenase consists of four subunits [10] and can reversibly dissociate into dimers and monomers on dilution [11,12]. Data from the literature concerning the effect of its substrates NAD, glyceraldehyde 3-phosphate and P_i on the association-dissociation of the enzyme are rather contradictory [11—13]. Our kinetic investigations have suggested that the binding of glyceraldehyde 3-phosphate shifts the equilibrium towards the lower oligomeric forms during the course of catalysis [14].

In this paper a novel use of the sorbent Blue Dextran-Sepharose will be described for the study of the affinities of different oligomeric forms of glyceraldehyde-phosphate dehydrogenase to the immobilized dye. The results obtained by the affinity sorbent batch technique indicate that glyceraldehyde 3-phosphate promotes the dissociation of the tetrameric enzyme.

Materials and Methods

The experiments were carried out with four times recrystallized glyceraldehyde-phosphate dehydrogenase isolated from rabbit muscle [15].

NAD was a Reanal product. Glyceraldehyde 3-phosphate according to Szewczuk et al. [16]. Blue Dextran and Sepharose 4B were purchased from Pharmacia. All other chemicals were reagent grade commercial preparations.

The concentration of the blue chromophore (Cibacron blue F3GA) of Blue Dextran was determined spectrophotometrically at 615 nm using the absorption coefficient of 13 600 $M^{-1} \cdot cm^{-1}$ [17].

Sepharose 4B was activated with cyanogen bromide and derivatized with Blue Dextran according to the procedure of Ryan and Vestling [18].

The concentration of immobilized dye, used throughout the experiments calculated from the absorbance difference between Sepharose and Blue Dextran-Sepharose sedimented in 1 mm cuvettes (E_{615} = 13 600 $M^{-1} \cdot cm^{-1}$) was found to be $7 \cdot 10^{-4}$ M. The concentration of the enzyme was measured spectrophotometrically using $A_{280}^{0.1\%} = 1.0$ [19]. In all cases the enzyme solutions were gel filtered on a Sephadex G-50 column (25 × 1.2 cm) equilibrated with 50 mM Tris-HCl/1 mM EDTA/1 mM mercaptoethanol buffer, pH 7.5, to

remove $(NH_4)_2SO_4$. After this procedure the enzyme contains about 2.5 mol of firmly bound NAD per tetramer.

Enzyme activity was assayed spectrophotometrically at 25°C in the following reaction mixture, 2 mM NAD/2 mM glyceraldehyde 3-phosphate/10 mM P_i/ 50 mM Tris-HCl buffer containing 1 mM EDTA and 1 mM mercaptoethanol, pH 7.5. Under these conditions the molar activity of the enzyme was 230—250 kat/mol enzyme based on a tetrameric molecular weight of 145 000 [20].

All experiments were carried out in a standard buffer solutson 50 mM Tris-HCl containing 1 mM EDTA and 1 mM mercaptoethanol, pH 7.5.

In the chromatographic experiments Blue Dextran-Sepharose 4B columns (0.6 × 24 cm) were equilibrated at 4°C with the standard buffer solution also containing 2 mM glyceraldehyde 3-phosphate when indicated. The columns were saturated with enzyme solution of different concentrations. Fractions of 1.4 ml were collected at a flow rate of 4.2 ml/h. The unbound protein was eluted either by two bed-volumes of buffer or by buffer containing 2 mM glyceraldehyde 3-phosphate. Saturation of the columns was reached when the enzyme activity of the eluate became equal to that of the loading solution. The bound enzyme was displaced from the affinity column by a linear NAD gradient produced by 30 ml of standard buffer solution in the mixer and 30 ml of buffer solution containing 10 mM NAD in the container vessel. The recovery of enzyme activity after elution was higher than 90%. Control experiments showed that the enzyme did not bind to Sepharose 4B column.

Batch experiments were carried out in the standard buffer solution at 4°C. Aliquots (0.2—2 ml) of Blue Dextran-Sepharose 4B suspension (17 ml packed gel in 25 ml final volume) were poured into test tubes and 0.5—10 ml of enzyme solutions were added. The tubes were shaken until equilibrium was reached i.e. enzyme activity was constant in the supernatant. The equilibration time was 3 h. Then the suspensions were allowed to sediment and the enzymatic activity of the supernatant was determined. As a control, enzyme solutions of similar dilutions were kept in the absence of gel and assayed for activity after 3 h.

Opton DMR 21 and Cary 118 spectrophotometers were used. Difference spectra were recorded by using special 'tandem' cuvettes.

Results and Discussion

Kinetic and spectrophotometric studies of the binding of Blue Dextran to the NAD-binding domain

If Blue Dextran specifically binds to the dinucleotide fold of the enzyme, it should be a competitive inhibitor with respect to NAD [7]. To test this prediction the effect of Blue Dextran on the catalytic activity of glyceraldehyde-phosphate dehydrogenase was measured at low enzyme concentration (10^{-8} M). The Dixon plot of the experimental data (Fig. 1), as well as the Lineweaver-Burk plot, (not shown) indicates that the type of inhibition is competitive. The inhibition constant $K_i \approx 10^{-6}$ M indicates that the affinity of the chromophore for the dinucleotide fold is strong enough to permit study of the interaction between glyceraldehyde-phosphate dehydrogenase and immobilized Blue Dextran over a wide range of protein concentration. At higher Blue

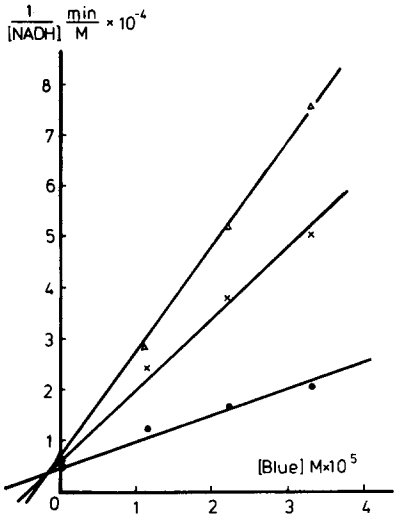

Fig. 1. Dixon plot of catalytic activity of glyceraldehyde-phosphate dehydrogenase in the presence of Blue Dextran. The apoenzyme (10^{-8} M) was preincubated with Blue Dextran and NAD until equilibrium. The reaction was started by addition of glyceraldehyde 3-phosphate. The concentrations of NAD were: ●——●, $1 \cdot 10^{-4}$ M; X——X, $9.5 \cdot 10^{-5}$ M and △——△, $7 \cdot 10^{-5}$ M. [Blue] is a concentration of Blue Dextran.

Dextran concentrations (data not shown) the Dixon plot becomes upward concave, which suggests the binding of additional dye molecules to binding site(s) of lower affinity.

This suggestion is supported by difference spectral measurements of the Blue

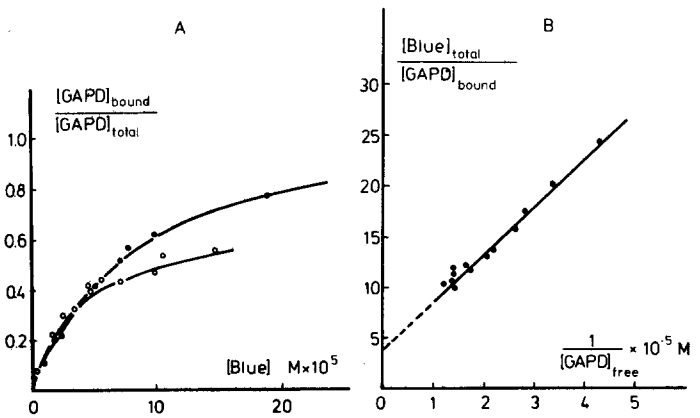

Fig. 2. Saturation curves of glyceraldehyde-phosphate dehydrogenase (GADP) with Blue Dextran. A, saturation curves of apoenzyme (●——●) and holoenzyme (○——○) in the presence of increasing concentrations of Blue Dextran as monitored by the difference absorbance at 660 nm. The concentrations of apoenzyme and holoenzyme (containing about 2.5 mol NAD per tetramer) were $1 \cdot 10^{-5}$ M. For the calculation $\Delta\epsilon_{660nm} = 4300\ M^{-1} \cdot cm^{-1}$ was used as determined from the limit value of the saturation curves of the apoenzyme. B, linearization of the saturation curve for the apoenzyme according to Wu and Hammes [21]. [Blue] is a concentration of Blue Dextran.

Dextran-holoenzyme complex at relatively high protein concentration (10^{-5} M). The complexing of Blue Dextran with both apo- and hologlyceraldehyde-phosphate dehydrogenases causes a red shift in the absorption spectrum of dye with a positive maximum in the difference spectrum at 660 nm. A similar phenomenon could be observed with M_4 lactate dehydrogenase and was attributed to the interaction of the dye with the apolar pocket in the NAD binding domain [8].

The absorbance difference at 660 nm characterizing apoglyceraldehyde-phosphate dehydrogenase-Blue Dextran or hologlyceraldehyde-phosphate dehydrogenase-Blue Dextran complex was measured in the presence of increasing concentrations of Blue Dextran (Fig. 2A). The limit value measured for the tetrameric apoenzyme gave a molar absorption coefficient of 4300 $M^{-1} \cdot cm^{-1}$. The experimental data for the apoenzyme plotted according to Wu and Hammes [21] (Fig. 2B) show that the binding of Blue Dextran to the apoenzyme can be characterized by a single apparent dissociation constant ($K_d = 4.8 \cdot 10^{-5}$ M). Although it has been reported [22] that Cibacron F3GA is inhomogeneous with respect to protein binding, the linearity of the Wu-Hammes plot argues for the homogeneity of the blue chromophore coupled to the Dextran molecules. Moreover, all binding sites of the enzyme seem to have identical affinities to the chromophore. The K_d value of about $5 \cdot 10^{-5}$ M is significantly higher than the K_i determined kinetically, which effect is probably due to the different protein concentrations. (see below). The apparent stoichiometry calculated from the extrapolated value is about 1 mol of dye per subunit.

If gel-filtered hologlyceraldehyde-phosphate dehydrogenase containing about 2.5 mol NAD per tetramer is tested, almost half of the binding sites can be saturated just as with the apoenzyme. (Fig. 2A). Saturation of the other binding sites with dye seems to be hindered by the firmly bound NAD.

Chromatographic experiments

Two sets of column chromatographic experiments were carried out. In one set the Blue Dextran-Sepharose column was equilibrated with standard buffer solution, in the other set the same buffer also contained 2 mM glyceraldehyde 3-phosphate. The columns were saturated with holoenzyme containing about 2.5 mM NAD per tetramer in the range of 10^{-7}—10^{-5} M enzyme concentration.

Elution profiles along a linear NAD gradient in the two types of experiment are shown in Fig. 3. The displacement of the enzyme from the column during the elution was tested by measuring the enzymatic activities in the fractions. From these it is evident that the amount of bound enzyme strongly depends on enzyme concentration and on the addition of glyceraldehyde 3-phosphate. The dependence on the loading enzyme concentration is obvious, since the bound enzyme is in equilibrium with the free one governed by the dissociation constant of the enzyme-immobilized Blue Dextran complex. If the experiment was carried out with 10^{-5} M apoenzyme instead of holoenzyme, the amount of the bound enzyme was practically the same. It follows that glyceraldehyde-phosphate dehydrogenase molecules couple to the matrix through binding site(s) not coupled by NAD.

In the presence of glyceraldehyde 3-phosphate much more enzyme was

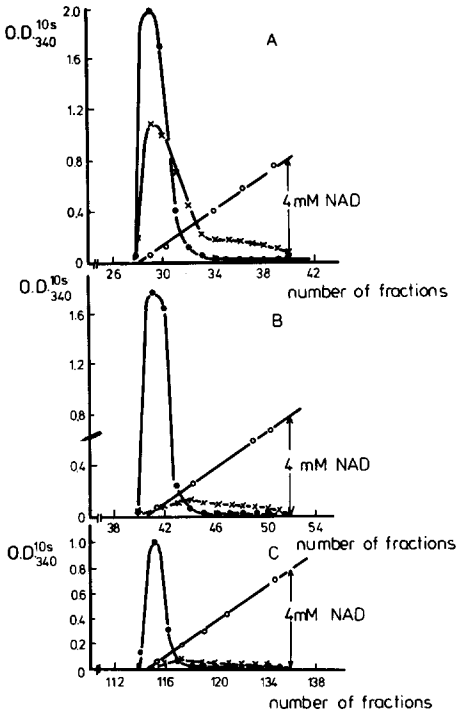

Fig. 3. Elution profiles of hologlyceraldehyde-3-phosphate dehydrogenase of different concentrations from a Blue Dextran-Sepharose affinity column by a linear NAD gradient (○———○). The loading of the column with enzyme was carried out in the absence (X———X) and presence (■———■) of 2 mM glyceraldehyde 3-phosphate. The initial enzyme concentrations were: A, 10^{-5} M; B, 10^{-6} M; C, 10^{-7} M. Activity assays were performed at 10^{-8} M enzyme concentration.

retained on the column. As demonstrated in Fig. 3, there was also a great difference in the elution profiles, depending on the presence of glyceraldehyde 3-phosphate. Whereas, the enzyme bound in the presence of glyceraldehyde 3-phosphate could be displaced from the affinity column by very low (about $4 \cdot 10^{-4}$ M) NAD concentrations, in the absence of glyceraldehyde 3-phosphate a considerable part of the bound protein was eluted only by high (about 1 mM) coenzyme concentrations (Fig. 3A).

This result indicates that the exposure of the coenzyme binding sites of glyceraldehyde 3-phosphate dehydrogenase is different in the absence and presence of the glyceraldehyde 3-phosphate substrate.

Affinity binding experiments in batch system

To obtain quantitative data for the interaction of enzyme and immobilized dye, we performed a series of affinity binding experiments in batch system.

Enzyme solutions of different concentrations were equilibrated with Blue Dextran-Sepharose in the absence and presence of 2 mM glyceraldehyde 3-phosphate. The concentration of unbound enzyme was determined by measuring the enzymatic activity of the supernatant. From the initial enzyme

concentration corrected for dilution the concentration of bound enzyme in the gel volume was calculated.

The effective concentration of Sepharose coupled dye was determined as follows. Blue Dextran-Sepharose was saturated with glyceraldehyde-phosphate dehydrogenase in a batch system. The difference in absorbance between sedimented Blue Dextran-Sepharose and sedimented Blue Dextran-Sepharose saturated with enzyme was measured at 660 nm. By the aid of the molar absorption coefficient of the enzyme-dye bond (see above) the concentration of effective binding sites in the gel volume was calculated and was found to be $4 \cdot 10^{-4}$ M.

The apparent dissociation constant of glyceraldehyde-phosphate dehydrogenase-Blue Dextran-Sepharose was determined at different enzyme concentrations on the basis of equation [1]:

$$K_d = \frac{([L]_{total} - [E]_{bound})[E]_{free}}{[E]_{bound}} \quad (1)$$

where $[E]_{free}$ is the concentration of unbound enzyme determined enzymatically in the supernatant, $[L]_{total}$ is the effective concentration of immobilized dye in the gel volume and $[E]_{bound}$ is the concentration of bound enzyme in the gel volume.

According to Scott [23] the gel volume containing the effective (dye) binding sites is two thirds of the actual sedimented gel volume.

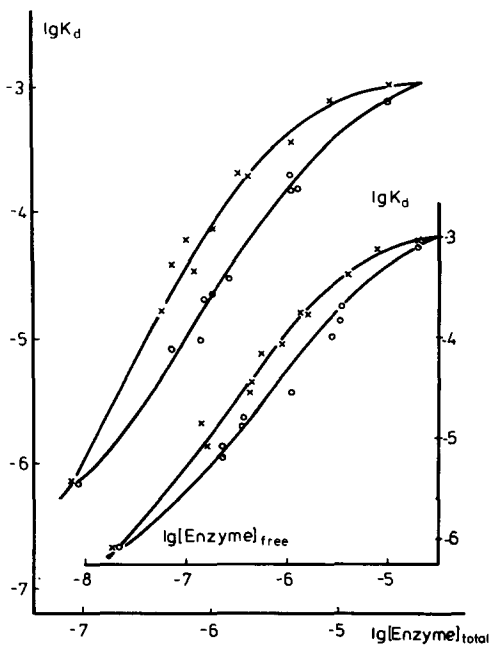

Fig. 4. Affinity binding of glyceraldehyde-phosphate dehydrogenase at different concentrations in batch system to immobilized Blue Dextran in the absence (X———X) and presence (●———●) of glyceraldehyde 3-phosphate (2 mM). The insert shows the dependence of K_d on the unbound (free) enzyme concentration.

It can be seen in Fig. 4 that the apparent dissociation constant of holo-glyceraldehyde-phosphate dehydrogenase-immobilized Blue Dextran varies markedly with enzyme concentration. If apoenzyme was used instead of holo-glyceraldehyde-phosphate dehydrogenase, qualitatively similar dependence of K_d on enzyme concentration was observed. This change in the apparent dissociation constant of the enzyme-ligand complex may be due to inhomogeneity of either the immobilized ligand or the enzyme itself. According to Fig. 2B, the chromophore bound to Dextran seems to be homogeneous. Since it is known that glyceraldehyde-phosphate dehydrogenase dissociates into subunits on dilution [11,12], the decrease in the enzyme-ligand dissociation constant towards low enzyme concentrations may be attributed to a shift in the equilibrium of the different oligomeric enzyme forms. This would mean that the dissociated forms of the enzyme bind preferentially to the immobilized Blue Dextran, which in turn may be due to some conformational change in the NAD binding domain brought about by the dissociation of tetrameric glyceraldehyde-phosphate dehydrogenase.

In the presence of 2 mM glyceraldehyde 3-phosphate the dissociation constant also decreases with enzyme concentration, moreover, it becomes more sensitive to enzyme dilution. It can also be seen from Fig. 3 and 4 that the effect of 2 mM glyceraldehyde 3-phosphate on the dissociation constant is the most pronounced around 10^{-6} M enzyme concentration. At low and high enzyme concentrations there is practically no difference in affinity of the enzyme to the matrix with and without glyceraldehyde 3-phosphate. This finding excludes the possibility that glyceraldehyde 3-phosphate affects the enzyme-dye interaction only through conformational changes without influencing enzyme dissociation. Though it is not shown in Fig. 4, at a fixed enzyme concentration (10^{-6} M) glyceraldehyde 3-phosphate concentrations higher than 2 mM elicited a further decrease in the dissociation constant of the enzyme-dye complex.

In another series of experiments 10^{-4} M NAD was added to the gel suspension equilibrated with 10^{-6} M enzyme. Under these conditions the enzyme was eluted from the gel similar to the chromatographic experiments (cf. Fig. 3). Then different concentrations of glyceraldehyde 3-phosphate up to 10 mM were added to the mixture. At 2 mM glyceraldehyde 3-phosphate no enzyme was rebound to the matrix. However, by increasing glyceraldehyde 3-phosphate concentration the readsorption of the enzyme could be achieved.

These data are consistent with our earlier kinetic results [14] which have suggested that the binding of glyceraldehyde 3-phosphate shifts the association-dissociation equilibrium of glyceraldehyde-phosphate dehydrogenase towards the formation of lower oligomeric forms. We can not decide whether this form(s) corresponds to the monomeric and/or the dimeric form. The dissociation constant of the enzyme-immobilized Blue Dextran complex determined in a batch system at low enzyme concentration, where it is independent of glyceraldehyde 3-phosphate, is $K_d = 7 \cdot 10^{-7}$ M (Fig. 4). This is in good agreement with the dissociation constant determined kinetically (cf. Fig. 1). We may conclude that a dissociation constant of about $K_d = 10^{-6}$ M characterizes the affinity of the dissociated enzyme form(s) to the dye. Furthermore, we suggest that the dissociation constant of about 10^{-3} M (Fig. 4), determined at high

protein concentration refers to the binding of the tetrameric form to the dye. Finally, we conclude that the affinity of the NAD-binding domains towards the blue chromophore as a coenzyme analogue is different in the various oligomeric forms of glyceraldehyde-phosphate dehydrogenase. Considering the results of chromatographic experiments (cf. Fig. 3), it seems conceivable that the ability of the enzyme to bind NAD is also dissociation-dependent [24].

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