IMMOBILIZED D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE CAN EXIST AS A TRIMER

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1. Introduction

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPD) is a tetrameric enzyme composed of identical subunits which exhibit marked cooperativity [1,2]. To evaluate the contribution of various types of intersubunit contacts to site–site interactions in the oligomer, a comparative study of enzyme species differing in the number of subunits (tetramer, trimer, dimer and monomer) seems attractive. Using the technique of matrix immobilization, we have prepared catalytically active monomeric [3] and dimeric [4] forms of the dehydrogenase; the latter enzyme species manifested the phenomenon of half-of-the-sites reactivity [5,6] and the non-equivalence of NAD^+-binding sites [7].

The purpose of this investigation was to elaborate experimental conditions for preparation of an immobilized trimeric form of GAPD. We show that a trimer species of yeast GAPD is formed in the course of reassociation of a matrix-bound monomer and soluble dimers, which can be withdrawn from the solution and fixed in an immobilized state by means of interactions with a single subunit. Immobilized trimers served as a model for elucidation of the role of various types of subunit interactions in half-of-the-sites reactivity of yeast GAPD.

2. Materials and methods

The GAPD preparation from baker's yeast and immobilization of the enzyme on CNBr-activated Sepharose were done as in [6]. Packed gel volumes were determined after centrifugation at 1000 × *g* for 3 min in graduated centrifuge tubes. The stock suspension of the matrix-bound derivative was prepared by mixing equal volumes of gel and buffer A (0.1 M sodium phosphate, 5 mM EDTA, 2 mM dithiothreitol) (pH 8.3). The content of the matrix-bound protein was determined spectrophotometrically [8]. The activity of immobilized enzyme species was measured at 25°C in 50 mM Tris–buffer (pH 7.9). The standard assay mixture contained 1.5 mM glyceraldehyde-3-phosphate, 2 mM NAD^+, 5 mM sodium arsenate, 5 mM EDTA and 0.1–0.2 ml of a suitably diluted suspension of matrix-bound enzyme in a final volume of 3 ml.

Carboxymethylation of the soluble enzyme was performed in the presence of NAD^+ as in [6]. Glyceraldehyde-3-phosphate was prepared as in [9]. NAD^+, ATP, EDTA, fructose 1,6-diphosphate, Tris were purchased from Reanal. Sepharose 4B, Sephadex G-50, dithiothreitol were obtained from Sigma, polyethyleneglycol from Schuchardt. Urea (Sojuzreactiv, USSR, analytical grade) was twice recrystallized from ethanol.

2.1. *Preparation of matrix-bound monomers of yeast GAPD*

The procedure in [3] was somewhat altered: the modified method is given below. The suspension of immobilized tetramers was washed on a glass filter with a 10-fold vol. (with respect to the packed gel volume) of 8 M urea solution in buffer A at pH 7.55 and 23°C. The gel was then mixed with a 4-fold vol. of the above urea solution and incubated for 10 min at 30°C with gentle stirring. The gel was separated on a glass filter and incubated with urea for another 10 min. The procedure was repeated again, but this time the incubation lasted 1 h. The gel was then separated on a glass filter, mixed with a new portion

of the above urea solution and incubated for 10 min at 30°C; this procedure was repeated thrice. The gel collected on a glass filter was then washed at 4°C with a 5-fold vol. of 7 M urea solution in buffer A. This treatment was repeated with 6 M, 5 M, 4 M, 3 M, 2 M and 1 M urea solutions in buffer A (pH 7.55). Finally, the gel was washed with a 150-fold vol. of buffer A (pH 8.3).

2.2. *Association of immobilized and soluble monomers*

An apoenzyme solution (~3 mg/ml) in buffer A containing 8 M urea (pH 7.3) was incubated for 1 h at 23°C with continuous stirring. This ensured the dissociation of tetrameric enzyme into monomers. A suspension of immobilized monomers (1 vol. packed gel/5 vol. 0.03 M sodium phosphate, 5 mM EDTA, 2 mM dithiothreitol (pH 7.5)) was mixed with the above apoenzyme solution to obtain a 8-fold excess of soluble monomers over the immobilized ones. After a 30 min incubation at 23°C with gentle stirring, a new portion of the apoenzyme solution was added. This procedure was repeated twice. The gel suspension was then incubated for 14 h at 4°C and for 1 h at 26°C with gentle stirring. Finally, the gel was washed on a glass filter with buffer A at pH 8.3 and 23°C until no protein and enzyme activity were detected in the washings.

3. Results and discussion

We had shown that incubation of immobilized dimeric species in diluted apoenzyme solutions resulted in reassociation of matrix-bound and soluble dimers [7]. Apoenzyme exists is solution as a mixture of tetramers and dimers which are in equilibrium [10]. The complete reconstruction of the tetrameric enzyme form observed in [7] suggested that inter-dimeric interactions in an immobilized tetramer are somewhat stronger than in a soluble one, which leads to the 'withdrawal' of soluble dimers from the solution.

A similar approach was used here. The ability of an isolated monomeric species to reassociate with soluble subunits [3] justified our attempts to perform the reassociation between monomers and dimers. The procedure was as follows. The suspension of Sepharose-bound monomers was washed with a 8-fold vol. of 0.03 M sodium phosphate, 5 mM EDTA, 2 mM dithiothreitol (pH 7.5), at 23°C. A desalted apoenzyme solution in the above buffer (0.4 mg protein/ml) was then added in a volume equal to that of the packed gel. The mixture was incubated for 14 h at 4°C with gentle stirring and then for 1 h at 26°C. The gel was washed on a glass filter with buffer A at pH 8.3 and 23°C until no protein and enzyme activity were detected in the washings.

The data listed in table 1 [3] demonstrate the increase in the matrix-bound protein content and enzyme activity. The characteristics of the resulting immobilized species are close to the values expected if trimers were formed. To substantiate this conclusion, the alternative explanation of the results, namely, that immobilized enzyme species are a mixture of trimers, dimers and tetramers, had to be ruled out.

Table 1
Characteristics of matrix-bound species prepared under different conditions

Enzyme species	Protein (μg/ml packed gel)	Protein (%)	Activity (%)
1. Original tetramers	230 ± 5	100	100
2. Monomers	62 ± 2	27	26
3. Trimers[a]	166 ± 4	72.5	70
4. Product of ATP-induced dissociation of the original tetramer	115 ± 2	50	48
5. Product of ATP-induced dissociation of the trimer	67 ± 2	29	23.5
6. Reconstructed tetramers[b]	202 ± 4	88	84
7. Product of ATP-induced dissociation of the reconstructed tetramer	122 ± 2	53	48

[a] Reassociation was performed in an apoenzyme solution with no additions

[b] Reassociation was performed in an urea-containing apoenzyme solution

For other details see text

This would have been possible if apoenzyme solutions contained, besides tetramers and dimers, also monomeric species.

In order to test such a possibility, the matrix-bound enzyme preparations obtained in these experiments were treated under the conditions inducing the split of inter-dimeric contacts in GAPD molecule (incubation with ATP in the cold [11,12]). We had shown that dimers are the only product of dissociation under these conditions [7].

The following results were expected:

(i) If monomeric species capable of reassociating with immobilized monomers are present in the apoenzyme solution, matrix-bound dimers and tetramers will appear together with matrix-bound trimers. The treatment of this preparation with ATP in the cold will produce a mixture of immobilized monomers and dimers.

(ii) If trimers are the only matrix-bound species, the incubation with ATP in the cold was expected to solubilize non-covalently bound dimers and produce monomers attached to the matrix.

Experiments were performed with immobilized species which were regarded as trimers; immobilized tetrameric enzyme was treated similarly in a different sample. These samples were washed with a 20-fold vol. (with respect to that of the packed gel) of 0.15 M NaCl, 5 mM EDTA, 2 mM dithiothreitol (pH 7.4). The gel was then mixed with an equal volume of 0.15 M ATP in the above solution (pH 7.4) and incubated at 4°C with gentle stirring. The time-course of dissociation was followed by measuring the enzyme activity in aliquots taken from the incubation mixture. After the reaction was complete (a constant level of residual activity was established), the gel was washed with a 4-fold vol. of the above NaCl solution and then with buffer A (pH 8.3), at 4°C until no protein and enzyme activity were detected in the washings.

The data listed in table 1 (4) demonstrate that ~½ the matrix-bound protein goes into solution as a result of the incubation of the tetrameric enzyme species with ATP, suggesting its dissociation into dimers. As far as the other sample is concerned, the protein content and the activity of the matrix-bound product of ATP-induced dissociation are close to the values expected for a monomer (table 1, (5)). It should be concluded that the non-covalently bound dimer was split off in this case too.

Taken together, these results indicate that matrix-bound trimers of GAPD were obtained under our experimental conditions. The data also suggest that a diluted solution of yeast apoenzyme (~0.2 mg/ml) contains no detectable amounts of monomeric species capable of reassociating with matrix-bound monomers. To achieve the reconstruction of a tetrameric molecule, an immobilized monomer has to be incubated with an apoenzyme solution under denaturating conditions (see section 2), yielding soluble monomeric species.

The results of such an experiment are listed in table 1 (6). To confirm the suggestion that the reassociated species is a tetramer, it was subjected to ATP-induced dissociation. As seen in table 1 (7), only ½ the matrix-bound protein was split off in this case.

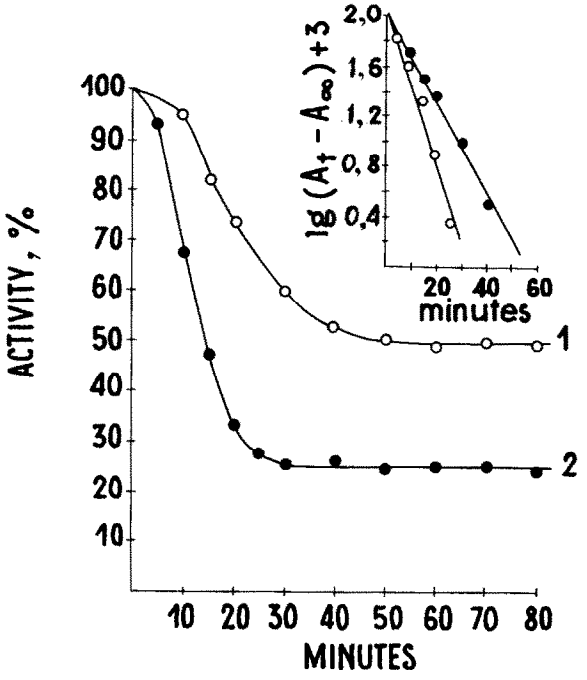

Fig.1. Time-course of cold inactivation of immobilized trimers (•) or tetramers (○) in the presence of ATP. (A) A suspension of matrix-bound enzyme species (3 ml packed gel mixed with an equal volume of 0.15 M NaCl, 0.15 M ATP, 5 mM EDTA, 2 mM dithiothreitol (pH 7.4)) was incubated at 4°C under continuous gentle stirring. The samples contained 750 μg immobilized tetramers (○) or 519 μg immobilized trimers (•). At fixed time intervals aliquots were taken for enzyme assay under standard conditions. Inset: Determination of the rate constant of inactivation. A_t represents the enzyme activity at time t and A_∞ is the constant level of activity remaining when the reaction was complete. The inactivation rate constants for tetramers (•) and trimers (○) were found to correspond to 0.073 min^{-1} and 0.138 min^{-1}, respectively.

It was expected that the inter-dimeric association in the immobilized tetramer would be stronger than the association between a matrix-bound monomer and a dimer in the trimeric species, since in the latter case some of the intersubunit contacts are lacking. Fig.1 shows that this does take place. It is seen that the matrix-bound activity is lost much faster in the case of trimer than in the case of tetramer. We had shown that incubation under these conditions affects the dimeric species which is split off [7].

To summarize the above results, we propose the following scheme illustrating the steps of the experimental procedure elaborated by us to prepare immobilized monomers, dimers and trimers (fig.2).

The immobilized trimeric species were used for a more detailed investigation of the phenomenon of half-of-the-sites reactivity exhibited by yeast GAPD. It became possible to examine the properties of the trimer composed of a native monomer and a dimer modified with a half-of-the-sites reagent, iodoacetate.

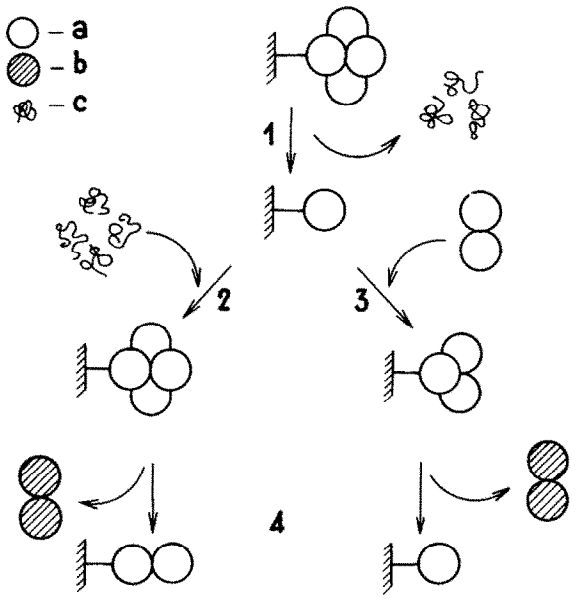

Fig.2. Schematic representation of the steps in the procedure employed to obtain different matrix-bound enzyme species. (1,2) Dissociation of an immobilized tetramer into monomers and reassociation of the unfolded subunits to reform the initial oligomeric molecule; (3) reassociation of a matrix-bound monomer with a soluble dimer; (4) dissociation of an immobilized tetramer or trimer under the mild conditions which ensure the rupture of inter-dimeric interactions in the tetramer. (a) Catalytically active conformation; (b) inactive conformation; (c) unfolded monomer.

We had shown that association of a matrix-bound native dimeric species with a soluble dimer, carboxymethylated at one active center causes a loss of ~½ the activity of the unmodified dimer; a similar effect was observed with a dimer carboxymethylated at both active centers [6].

This result indicated that the influence of the half-of-the-sites reagent is transmitted through inter-dimeric contacts in the tetramer, the modification of one of the active centers being sufficient to ensure the complete effect. It remained unclear however, if the effect was due to full inactivation of one of the subunits of the unmodified dimer or both subunits were partially affected.

We made an attempt to explore this problem by testing the ability of a carboxymethylated dimer to inactivate a single subunit originating from a neighboring dimer.

To this end, immobilized monomers were incubated in a solution of apoenzyme carboxymethylated at 4 cysteine residues/tetramer. The reassociation procedure was similar to that described above for an unmodified enzyme preparation. Table 2 summarizes the results of the experiment which included: dissociation of a matrix-bound tetramer into monomers; their reassociation with carboxymethylated soluble dimers and a second dissociation under specific conditions favourable for splitting off of the dimeric species.

It is seen that the binding of the modified dimer results in a nearly complete inactivation of a matrix-bound monomer. The residual activity (5–6%) is probably due to a minor amount of matrix-bound

Table 2
Demonstration of the reversibility of the effect of the half-of-the-sites reagent

Immobilized enzyme species	Protein (μg/ml packed gel)	Protein (%)	Activity (%)
1. Original tetramers	240 ± 4	100	100
2. Monomers	60 ± 2	25	24
3. Trimers	166 ± 3	69	5
4. Product of ATP-induced dissociation of trimers	72 ± 2	30	22

Inactivation of matrix-bound monomers by carboxymethylated dimers in the trimeric species and reactivation upon splitting off of the dimers. See text for details

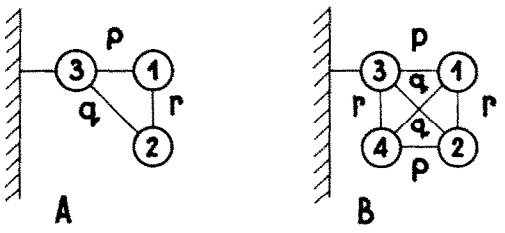

Fig.3. Schematic representation of an immobilized trimer (A) and tetramer (B). See text for details.

monomers which remained free due to incomplete reasssociation. The determination of protein content of the immobilized trimers (see table 2) confirms this interpretation.

The reversibility of the half-of-the-sites effect demonstrated in our studies is consistent with the conception of an induced character of this phenomenon [1]. Fig.3 schematically shows the structure of matrix-bound oligomeric enzyme forms organized as a pair of functional dimers or as an association of a functional dimer and a monomer. The whole body of evidence obtained in our studies [4–7] supports the conclusion that apoenzyme dissociation under mild conditions produces functional dimers.

According to [13] functional dimers comprise the *R*-axis related subunits; the contacts across this axis are designated in fig.3 as *r*. The communication between a matrix-bound monomer and carboxymethylated subunits 1 and 2 in a trimer (fig.3A) is realized therefore through two types of contacts, *p* and *q*. The results of this investigation suggest that these interactions are sufficient to completely inactivate subunit 3.

We may tentatively assume that the effect exerted by carboxymethylated dimer within the tetrameric molecule (fig.3B) also originates from the inactivation of a single subunit. The similarity of the influences of the dimers carboxymethylated at one or at both active centers [6] is consistent with this interpretation and suggests that the effect may be mainly transmitted through a single set of subunit contacts. A possibility is, however, not excluded that this phenomenon has a more complex character, i.e., modification of subunit 1 will inactivate subunit 3, but some additional influence may come from inactive subunit 2.

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