USE OF IMMOBILIZED ENZYMATICALLY ACTIVE MONOMERS OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE TO INVESTIGATE SUBUNIT COOPERATIVITY IN THE OLIGOMERIC ENZYME

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1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase is a tetrameric enzyme composed of chemically identical subunits. A considerable body of evidence indicates that the active sites of the oligomer are functionally non-equivalent; site–site interactions are supposed to be involved in the catalysis [1–2]. To elucidate the molecular mechanism of subunit cooperativity we prepared a dissociated (dimeric) form of the dehydrogenase, stabilized by immobilization on the solid support. It was shown to fully retain the catalytic activity, half-of-the-sites reactivity [3,4] and the non-equivalence of the NAD^+-binding sites (in press). Thus, a 'functional dimer' of the dehydrogenase was demonstrated to exist in the absence of any contacts with a neighboring dimer.

We further succeeded in preparation of an isolated matrix-bound monomer of the yeast enzyme, which retained the full specific activity of the original tetramer (submitted). It became possible therefore to compare the properties of immobilized native monomers of the dehydrogenase and their associated forms (dimers or tetramers) differing in the number and character of intersubunit contacts. This approach was used here to clarify the contribution of subunit interactions to the coenzyme-induced changes, affecting the microenvironment of essential arginine residues.

2. Materials and methods

2.1. *Materials*

Glyceraldehyde-3-phosphate was prepared by the method in [5]. NAD^+ and EDTA were purchased from Reanal, ATP from Merck, dithiothreitol and 2,3-butanedione from Serva. Sepharose 4B and Sephadex G-50 were obtained from Sigma. Glyceraldehyde-3-phosphate dehydrogenase isolated from baker's yeast [6] had A_{280}: A_{260} 1.9. It was immobilized on CNBr-activated Sepharose 4B via a single subunit [4].

2.2. *Preparation of immobilized enzyme dimers*

Dissociation of the matrix-bound tetrameric form of the enzyme into dimers was carried out as in [7]. The procedure included washing of the matrix with 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol (pH 7.2), incubation at 4°C in the presence of 50 mM ATP and an extensive washing of the gel to remove split protein.

2.3. *Preparation of immobilized enzyme monomers*

The procedure, detailed elsewhere, consisted of washing the immobilized tetramer with 8 M urea, 0.1 M sodium phosphate, 5 mM EDTA, 2 mM dithiothreitol (pH 7.6), incubation in the above solution, washing the gel with urea solutions, concentration of which decreased stepwise, and a final extensive washing with a buffer to remove the dissociating agent and split protein.

2.4. *Characterization of the immobilized preparations*

The amount of matrix-bound protein was determined spectrophotometrically in poly(ethylene)glycol [8]. The activity of immobilized enzyme was assayed at 25°C in 50 mM sodium pyrophosphate, 1.5 mM glyceraldehyde-3-phosphate, 2 mM NAD^+, 5 mM sodium arsenate, 5 mM EDTA (pH 8.4). The sample (final vol. 3 ml) contained 0.1 ml suitably diluted suspension of the immobilized enzyme. Specific

activity of immobilized tetrameric, dimeric and monomeric forms of the enzyme corresponded to 60, 55 and 60 U/mg protein, respectively. The stock suspensions of the immobilized enzymes were prepared by mixing equal volumes of gels packed by centrifugation (1000 × *g*, 3 min) and buffer (0.1 M sodium phosphate, 5 mM EDTA, 2 mM dithiothreitol (pH 8.3). Accurate samples were taken from the stirred suspensions for activity and protein determinations.

2.5. *Modification of arginine residues of the soluble and immobilized enzyme preparations*

Incubation was carried out at 20°C in 0.1 M medinal buffer (pH 8.2) containing 5 mM EDTA, 2 mM dithiothreitol and 18 mM 2,3-butanedione, at constant stirring. At fixed time intervals aliquots were removed for activity determination in the standard assay system. In the case of immobilized enzyme preparations, the gel was collected on a glass filter and washed with 0.1 M medinal buffer, 5 mM EDTA, 2 mM dithiothreitol (pH 8.2). The gel was then mixed with an equal volume of the same buffer. 0.02–0.04 ml of 2,3-butanedione solution was added to this suspension to achieve 18 mM final conc. reagent.

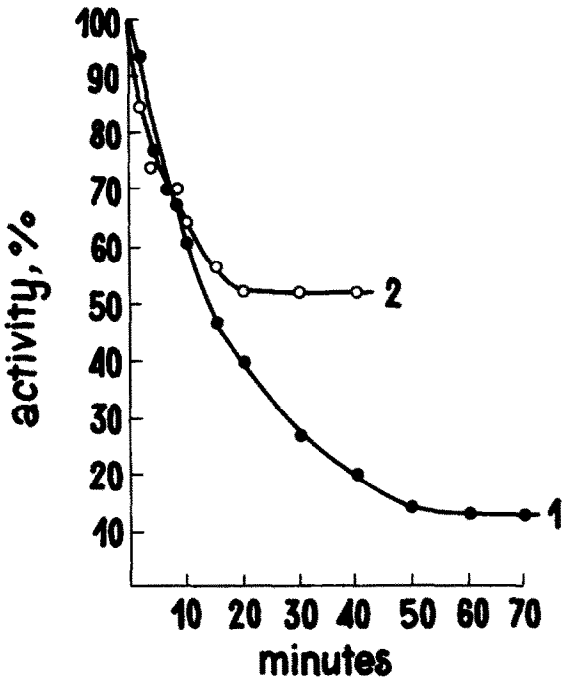

Fig.1. Inactivation of the soluble yeast enzyme in presence of 2,3-butanedione. *Apo*-enzyme (0.8 mg/ml) was incubated with no additions (1) and in presence of 0.28 mM NAD^+ (2). Similar results were obtained when NAD^+ content was increased up to 1.0 mM. Activities are expressed as % of the unmodified control.

3. Results and discussion

We have demonstrated that modification with 2,3-butanedione of 2 arginine residues/subunit yeast glyceraldehyde-3-phosphate dehydrogenase inactivates the enzyme, but does not abolish its capability of binding NAD^+. Experimental evidence led us to conclude that functional arginine residues are located in the catalytic region of the active center, near the binding site of the nicotinamide portion of the coenzyme [9].

As shown in fig.1, NAD^+ alters the time-course of enzyme inactivation in the presence of the arginine-specific reagent. Inactivation of the holoenzyme is initially accelerated, then it slows down and a constant level of residual activity is established, which is markedly higher than the level observed in the case of apoenzyme (fig.1 (1)). It appears that the binding of NAD^+ partially protects the yeast dehydrogenase against 2,3-butanedione-induced inactivation, in sharp contrast with the results obtained with the rat muscle enzyme [10], as well as with the rabbit muscle one (unpublished).

The inactivation of the both muscle apoenzymes in the presence of 2,3-butanedione was shown to be significantly accelerated by NAD^+, the effect of which appeared to be positively cooperative [10]. To explain the different effects of NAD^+ on the microenvironment of essential arginine residues of the yeast and skeletal muscle dehydrogenases, two possibilities may be considered:

1. The conformational changes induced by the binding of coenzyme differently affect the microenvironment of the essential arginine residues in the yeast or muscle monomer;
2. Subunit interactions in the oligomeric forms of the yeast and muscle enzymes differ in such a way, that the transition from *apo*- to *holo*-conformation induces the non-identical accessibility of arginine residues in neighboring subunits, which is only evident in the case of yeast dehydrogenase. As a consequence, NAD^+ partially 'protects' this enzyme from inactivation.

To discriminate between these possibilities, we compared the effect of NAD^+ on the inactivation of

the oligomeric and monomeric forms of the yeast enzyme. This became possible with the use of immobilized monomeric, dimeric, and tetrameric dehydrogenase species. Fig.2 demonstrates that the time-course of inactivation of immobilized tetramer in the presence of 2,3-butanedione, and the effect of NAD^+ on this process are very similar to the effects observed with the soluble enzyme.

Another situation is observed, however, with the immobilized monomeric form (fig.3). In this case, the 'protective' effect of NAD^+ practically disappears, and the transition from *apo-* to *holo-*conformation is characterized by a marked acceleration of 2,3-butanedione-induced inactivation. This result is noticeable, since it indicates that in the absence of specific subunit interactions the NAD^+-induced conformational changes affecting the microenvironment of essential arginine residues are similar in the yeast and muscle monomers. In fact, the effect of NAD^+ is analogous with its effect on the 2,3-butanedione-induced inactivation of the rat muscle dehydrogenase [10].

We conclude that the process of organization of the active center upon coenzyme binding is characterized by the increase in accessibility of functional arginine residues; the effect is similar in yeast and muscle glyceraldehyde-3-phosphate dehydrogenase. This rearrangement may be a consequence of a conformational change which introduces a positively charged quanidinium group into the catalytic region of the active center, as suggested in [10,11].

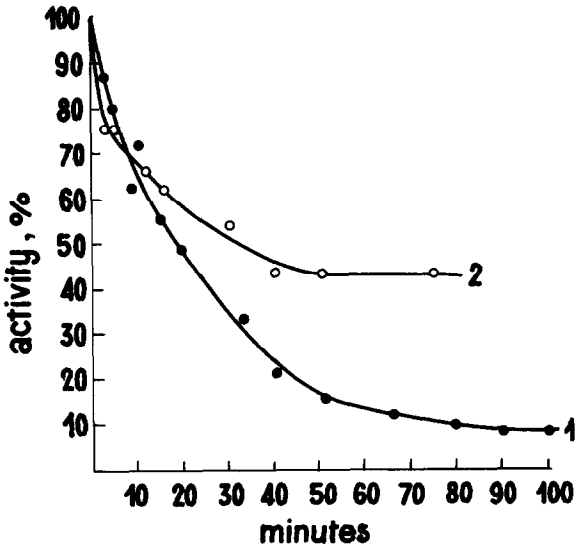

Fig.2. Inactivation of the immobilized tetrameric yeast enzyme in presence of 2,3-butanedione. *Apo*-enzyme suspension, containing 0.2 mg protein was incubated in 2 ml final vol. with no additions (1) and in presence of 0.25 mM NAD^+ (2).

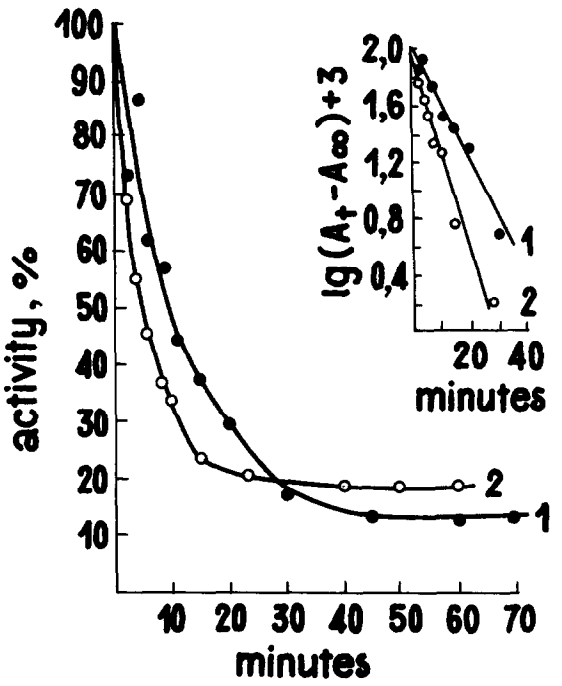

Fig.3. Inactivation of the immobilized monomeric yeast enzyme in presence of 2,3-butanedione. (A) Apoenzyme suspension, containing 0.075 mg protein, was incubated in 3 ml final vol. with no additions (1) and in presence of 0.25 mM NAD^+ (2). (B) Determination of the rate constant of inactivation. A_t 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 *apo-* and *holo*-enzyme were found to correspond to 0.083 min^{-1} and and 0.18 min^{-1}, respectively.

The association of individual subunits into oligomeric structure markedly affects the environment of essential arginine residues. Yeast and muscle enzymes behave differently in this case. In fact, the binding of NAD^+ was demonstrated to increase the accessibility of arginine residues in all active centers of the tetrameric rat muscle enzyme [10], whereas the yeast dehydrogenase in the *holo*-form is only partially inactivated by 2,3-butanedione. The data in fig.1, 2 suggest that subunit association in the yeast tetramer renders a part of active centers less accessible for modification by the arginine-specific reagent.

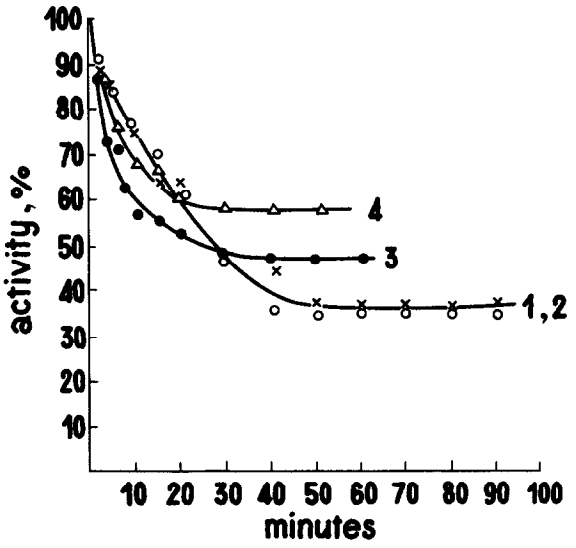

Fig.4. Inactivation of immobilized dimeric and tetrameric forms of the yeast enzyme in presence of 2,3-butanedione. Incubation mixture contained in 3 ml final vol. a suspension of immobilized apodimers (0.15 mg protein) or *apo*-tetramers (0.3 mg protein): (1,2) *apo*-enzymes; (3) *apo*-dimers in presence of 0.25 mM NAD^+; (4) *apo*-tetramers in presence of 0.25 mM NAD^+. The extent of inactivation in the presence of a fixed concentration of 2,3-butanedione was observed to vary depending on the enzyme preparation. In experiments illustrated in fig.1–3 and 4, respectively, two different enzyme preparations were used, therefore the constant level of residual activity varied.

To evaluate the contribution of different types of subunit interactions to the non-equivalence of active centers of the oligomer we compared the effect of NAD^+ on the 2,3-butanedione-induced inactivation of tetrameric and dimeric forms of the enzyme. The time-courses of inactivation of *apo*-dimers and *apo*-tetramers are similar (fig.4 (1,2)). This indicates that subunit association in the *apo*-form does not induce any non-equivalence of active centers revealed by the method employed.

The transition into *holo*-conformation is accompanied by the appearance of an active-sites asymmetry, which is characterized by the diminished accessibility of a part of essential arginine residues for modification. The effect is apparent in the dimeric form (fig.4 (3)); it is more pronounced however in the case of tetrameric subunit association (fig.4 (4)).

Fig.4 also shows that 2,3-butanedione-induced inactivation of the apo-forms of the enzyme is accelerated in presence of NAD^+, similarly to the situation observed with an isolated monomer (fig.3). The effect appears therefore to be complex. We believe that it includes the intra-subunit 'organization' of an active center, on the one hand, and the inter-subunit cooperativity, on the other. Arginine residues affected by modification are probably involved in the arrangement of substrate in the productive complex with *holo*-enzyme [10] or in the transformation of this complex. It seems likely therefore that the ligand-induced asymmetry of active centers revealed here may be of functional significance, reflecting catalytic cooperativity between subunits.

In conclusion, the results of the present investigation have demonstrated that isolated matrix-bound dissociated species may be useful in studies on molecular mechanisms of subunit cooperativity in an oligomeric enzyme.

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