HALF-OF-THE-SITES REACTIVITY IN IMMOBILIZED HYBRIDS OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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1. Introduction

Glyceraldehyde-3-phosphate dehydrogenases (GAPD) of various origins were shown to exhibit a half-of-the-sites reactivity upon modification of the essential cysteine residues [1–3]. The elucidation of the molecular mechanisms of this phenomenon and of its role in the enzyme function requires a detailed study of subunit interactions in the tetrametric enzyme. We have demonstrated that the isolated dimeric species, obtained by dissociation of matrix-bound rat muscle apo-GAPD, is capable of exhibiting a half-of-the-sites reactivity when modified with FDNB [3]. The tetrameric structure was therefore concluded not to be a prerequisite for the non-equivalence of the active centers of the dehydrogenase.

Here hybridization of GAPD on a solid support was used to study the contribution of various types of subunit contacts to the half-of-the-sites reactivity of the yeast enzyme.

2. Materials and methods

GAPD was isolated from baker's yeast by the method in [4] with slight modifications. The preparation of rat skeletal muscle GAPD was described [5]. Specific activities of the preparations varied between 150 and 200 μmol NADH .min^{-1} .mg protein^{-1}. The A_{280}/A_{260} ratio of the yeast enzyme was 1.9–2.0, which meant the absence of bound nucleotide. The apoenzyme of rat muscle GAPD was prepared by the use of gel-filtration and CM-cellulose chromatography [3]. Glyceraldehyde-3-phosphate was prepared by the method in [6]. NAD^+, ATP, EDTA and CM-cellulose were purchased from Reanal, dithiothreitol was obtained from Serva, Sepharose 4B and Sephadex G-50 from Sigma, polyethyleneglycol from Schuchardt. Immobilization of yeast GAPD was carried out as in [3] using CNBr-activated Sepharose (4 mg CNBr/ml packed gel). Determination of matrix-bound protein was performed spectrophotometrically in polyethyleneglycol [7]. Under conditions of our experiments, only one subunit of the tetramer was demonstrated to be covalently linked to the matrix (after 24 h treatment of the immobilized enzyme with 8 M urea followed by extensive washing the amount of the Sepharose-bound protein diminished to 25% of the initial value).

The immobilized enzyme activities were determined spectrophotometrically at 25°C in 50 mM Tris–HCl (pH 8.0) in the case of the yeast enzyme or in 100 mM glycine–NaOH (pH 9.9) in the case of rat muscle dehydrogenase. The assay mixture contained 1.5 mM glyceraldehyde-3-phosphate, 2.0 mM NAD^+, 5 mM sodium arsenate, 5 mM EDTA and 0.1 ml immobilized protein suspension (2–5 μg protein).

2.1. *Preparation of immobilized dimers of yeast dehydrogenase*

Immobilized apoenzyme (200 μg/ml of the packed gel) was incubated at 4°C in 50 mM ATP/0.15 M NaCl/5 mM EDTA/2 mM dithiothreitol (pH 7.3) over 1 h. The gel was then washed with 100 mM sodium phosphate/5 mM EDTA/2 mM dithiothreitol (pH 7.2). As a result of ATP-induced dissociation of the dehydrogenase, half of the matrix-bound protein became solubilized and was removed by repeated

washings. The specific activity of the dimeric and tetrameric enzyme covalently bound to Sepharose was found (after corrections for diffusion limitations [8]) to be similar to the activity of the enzyme in solution.

2.2. *Association of immobilized and soluble dimers*

Immobilized dimers (100 μg/ml of the packed gel) in 100 mM sodium phosphate/5 mM EDTA/2 mM dithiothreitol (pH 7.2) were incubated in the presence of an appropriate apoenzyme solution (yeast apo-GAPD, rat muscle apo-GAPD or carboxymethylated yeast apo–GAPD, each at 0.15–0.20 mg/ml). Incubation was performed at 22°C for 1 h. In the case of carboxymethylated yeast apoenzyme the mixture was occasionally cooled to –4°C and rewarmed to 22°C.

Reconstruction of the immobilized tetramers was followed by an increase of the matrix-bound enzyme content and activity.

2.3. *Carboxymethylation of the yeast enzyme*

The apoenzyme (6.95 μM in 0.1 M sodium phosphate/5 mM EDTA (pH 8.3) was incubated at 4°C with 1.8 mM iodoacetate in the presence of 0.5 mM NAD^+ or without it. After 2 h incubation, the reaction mixture was passed through a Sephadex G-50 column to remove the coenzyme and excess iodoacetate. Determination of the sulfhydryl groups present before and after the iodoacetate treatment [9] showed that 4 cysteine residues/tetramer were carboxymethylated in the presence of NAD^+, whereas only half of the active sites became modified in the samples containing the apoenzyme.

3. Results and discussion

The matrix-bound dimeric species were found to readily associate with dimers in solution. Complete reconstruction of the tetrameric structure could be achieved under mild conditions simply by incubating the immobilized dimers in diluted apoenzyme solutions. Addition of a dissociating agent, which is usually recommended to perform reassociation of matrix-bound and soluble enzyme subunits [10], appeared to be unnecessary. This may be explained assuming that interdimeric contacts in immobilized tetramer are stronger than in a tetramer in solution.

We suggest that the equilibrium between tetrameric and dimeric forms of the apoenzyme in solution is shifted in the presence of immobilized dimers in favour of the dissociated species, since a part of the soluble dimers become non-covalently bound to the matrix. Association between the soluble and immobilized dimers appears to be irreversible under our experimental conditions. This is evidenced by the fact that no dissociation of the matrix-bound tetramers was observed in the course of repeated extensive washings of the gel with a buffer. The process of recombination of matrix-bound and soluble dimers could be studied kinetically (in preparation) and was used to perform hybridization between various forms of GAPD.

Figure 1 and table 1 outline the variants of hybrid formation performed here. Incubation of the native matrix-bound dimers in a native apoenzyme solution resulted in reconstruction of a Sepharose-bound tetramer (a). Tetramer (b) was obtained by incubating the native immobilized dimers in a solution of the apoenzyme carboxymethylated at all the 4 active centers. In this case the reconstructed tetramer contains two subunits modified with the half-of-the-sites reagent. As shown in table 1, this tetramer retains 35% of activity.

Quite different is the situation with the soluble tetramer treated with 2 mol equiv. iodoacetate. In

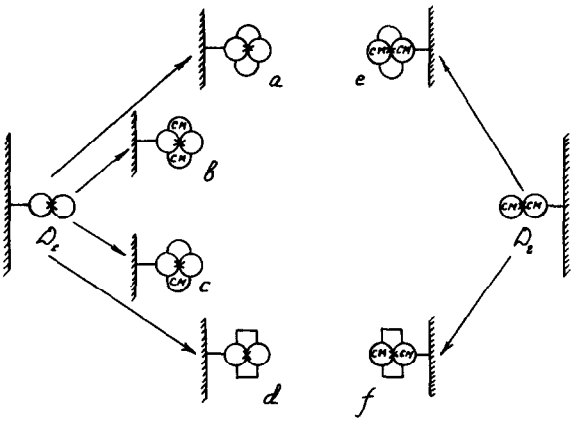

Fig.1. Association of immobilized dimers of yeast GAPD (D_1 and D_2) with soluble dimers. Subunits of the yeast and rat muscle enzymes are designated by circles and squares, respectively. CM, carboxymethyl group incorporated in the active center of the monomer. Subunit contacts in the 'functional dimers' are marked by '×'.

Table 1
Activity of various forms of GAPD

Soluble yeast apoenzyme	Activity (%)	Immobilized forms	Activity (%)
Native enzyme	100	tetrameric yeast apoenzyme	100
Enzyme with 4 carboxymethyl groups		D_1	50
		D_2	0
incorporated/tetramer	0	a	100
Enzyme with 2 carboxymethyl groups		b	35
		c	35
incorporated/tetramer	0	e	35
		Hybrid of the yeast and rat muscle enzymes	
Enzyme with 1 carboxymethyl group incorporated/ tetramer	20[a]	d	100[b]
		f	50[c]

[a] The data from [1]
[b] Activity calculated as the sum of activities of the 2 dimers. Assay conditions were optimal for each of them and different in the case of yeast and rat muscle species
[c] The data obtained at pH 9.9 and given in % of the immobilized rat muscle tetramer activity

Activity of immobilized forms of the yeast enzyme is given as % of the immobilized tetramer activity

agreement with the data in [1], it was found to be completely devoid of activity (table 1). To explain this discrepancy, we must assume the non-equivalence of the active centers of the tetramer in the reaction with iodoacetate. When the apoenzyme is incubated in solution in the presence of 2 molecules of the reagent/tetramer, one carboxymethyl group is probably incorporated per each of the 'functional dimers', leading to full inactivation. If however, both carboxymethyl groups are introduced in a single 'functional dimer', as is the case in variant (b) (fig.1), inactivation is not complete. The partial loss of activity observed thereby evidently reflects the effect transmitted from the modified to the native dimer.

We may therefore conclude that dimers which are formed upon dissociation of yeast apoenzyme, represent 'functional units' of the dehydrogenase molecule capable of exhibiting the half-of-the-sites effect. If the subunit contacts within 'functional dimers' had been cleaved upon dissociation, the tetramers of type (b) (fig.1) would have been completely inactive.

As seen in table 1, the tetramers which have been reconstructed by association of the native dimers with dimers containing one or two carboxymethylated subunits (fig.1, variants (b) and (c), respectively), have similar catalytic activities. This means that carboxymethylation of one of the subunits in a 'functional dimer' is sufficient to produce maximal inactivation of the neighbouring dimer. Modification of the second subunit gives no additional effect. The results were the same when a carboxymethylated dimer covalently linked to the matrix was reassociated with a native dimer in solution (table 1, variant (e)).

We come to conclusion that a dimer modified with the half-of-the-sites reagent is capable of transmitting the inactivating effect through inter-dimeric contacts. It remains to be elucidated if such effect bears on a single or both subunits of the neighbouring dimer. The results of this work are consistent with the conception in [1,2,11], according to which conformational changes induced by modification of the essential cysteine residues are involved in the realization of half-of-the-sites effect in GAPD.

However, our data clearly indicate that the subunit contacts which participate in the half-of-the-sites effect pre-exist in the apoenzyme, being the strongest among the 3 different types of inter-monomer interactions of GAPD molecule. The non-equivalence of active centers (induced or pre-existent) is conserved in the dimers formed upon dissociation of the tetramer.

Comparison of the half-of-the-sites effect in the yeast enzyme and in the hybrid tetramer composed of yeast and rat muscle dimers, has revealed a marked difference between them. As seen in table 1, variant (f) no change in the activity of the rat muscle dimer is observed upon its association with a carboxymethylated yeast dimer. Thus, the heterologous subunit association probably lacks some specific interactions involved in the realization of the cooperative effects in the GAPD molecule.

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