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STUDY OF SUBUNIT INTERACTIONS IN IMMOBILIZED D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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Summary

Under conditions which cause dissociation of soluble tetrameric glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) into inactive dimers, immobilized apoenzymes from yeast and rat skeletal muscle coupled to CNBr-activated Sepharose via one subunit retain 50% of matrix-bound protein with unaltered specific activity. The solubilized dissociated species are inactive.

Two molecules of NAD^+ (NADH) firmly bound to the immobilized rat muscle tetramer can prevent the dissociation. Immobilized dimer was demonstrated to bind one molecule of coenzyme with high affinity. Using various combinations of immobilized and soluble rat muscle and yeast dimers, we succeeded in reconstituting tetramers, containing one molecule of NAD^+ bound either to a matrix-linked or to a non-covalently bound dimer. In the latter case, the dissociation of the tetramer was completely prevented. This suggests that the binding of a single coenzyme molecule is sufficient to stabilize the interdimeric contacts provided the neighbouring dimer is stabilized independently.

Such stabilization is produced by the covalent binding of one of the subunits comprising the dimer to the matrix. The structure of the dimer as a whole becomes resistant to the action of the dissociating agent. The effect appears to be cooperative and similar to that of NAD^+ or NADH. The dissociation of the immobilized tetramer is, most likely, the result of conformational changes, affecting the structure of the non-covalently bound dimer. Any factor, capable of preventing these changes, would stabilize the interdimeric contacts. The latter conclusion is substantiated by the effect of specific antibodies, which prevent the dissociation of the immobilized tetramer by forming a complex with the dimer, non-covalently bound to the matrix.

The evidence obtained in the present investigation supports the conclusion that the isolated dimer of glyceraldehyde-3-phosphate dehydrogenase represents a relatively independent structural and functional 'unit' of the enzyme. It can be stabilized in a catalytically active form by interactions other than those involved in inter-dimeric contacts in the tetramer.

The kinetics of the association of immobilized and soluble dimers have been studied. Association rate constants were determined for homologous (yeast-yeast, rat-rat) and heterologous (yeast-rat, yeast-rabbit) dimer combinations. The binding of one molecule of specific antibody to the immobilized dimer was shown to increase the rate constant of association.

Introduction

Different manifestations of the non-equivalence of the active centers of tetrameric glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) ('half-of-the-sites reactivity' [1,2], cooperativity towards the binding of NAD^+ [3—6] suggest that subunit interactions play an important role in enzyme functioning. This justifies the attempts to elucidate the contribution of different inter-monomer interactions to the enzyme activity, stability, and regulation. Using the approach of matrix-bound derivatives [7], we could demonstrate that the dimeric form of dehydrogenase produced by dissociation of the apotetramer is catalytically active. Similar results were obtained with the enzyme bound to the matrix covalently [8] and non-covalently, by adsorbing it on specific antibodies immobilized on a solid support [9]. Recently we presented evidence of the 'half-of-the-sites reactivity' in an isolated dimer [10].

These results are not consistent with the conclusions derived from the experiments with the soluble enzyme. In fact, dissociation of the tetramer in solution under sufficiently mild conditions produces dimers devoid of catalytic activity [11—14]. One of the purposes of the present investigation was to reveal the reason for disparity in the results obtained with soluble and immobilized enzymes. To this end, a detailed study of dissociation of matrix-bound yeast and muscle tetramers was carried out under the conditions previously used to dissociate these enzymes in solution.

Another aim was to examine the possibility of the non-equivalence of NAD^+-binding sites in an isolated dimer. The major part of the work is devoted to the study of interaction between immobilized and soluble dimers of various origin.

Experimental procedure

Materials

Glyceraldehyde-3-phosphate dehydrogenase was isolated from baker's yeast by the method of Krebs [15], from rat skeletal muscle as previously described [16] and from rabbit skeletal muscle by the method of Elödi et al. [17] with subsequent purification on a DEAE-cellulose column. The specific activities of the preparations were between 150 and 200 μmol/min per mg. The yeast enzyme preparation had an absorbance ratio (A_{280nm}/A_{260nm}) of 1.9 to 2.0.

The rat muscle apoenzyme was prepared as previously described [10]. To obtain the rabbit muscle apoenzyme, the crystals were dissolved in 100 mM sodium phosphate/5 mM EDTA (pH 7.2) to a protein concentration of 20 mg/ml. The solution was treated with 5 mg of activated charcoal/mg of enzyme for 25—30 min at 20°C with constant stirring. The charcoal was then removed by centrifugation, and the protein solution was passed through a column of Sephadex G-50. This procedure yielded an enzyme solution with the A_{280nm}/A_{260nm} ratio of 1.92. The concentration of protein in solution was estimated spectrophotometrically at 280 nm. The molecular weights of the enzymes were assumed to be 144 000.

Glyceraldehyde 3-phosphate was prepared by the method of Szewczuk et al. [18]. NAD^+, NADH, EDTA and DEAE-cellulose were purchased from Reanal, ATP and ADP from Merck, dithiothreitol from Serva, Sepharose 4B and Sephadex G-50 from Sigma, poly(ethyleneglycol) from Ferak and Schuchardt.

Preparation and characterization of the immobilized enzymes

The coupling of the rat muscle and yeast apoenzymes to CNBr-activated Sepharose was performed as previously described [10]. The content of the matrix-bound protein was determined spectrophotometrically in poly(ethyleneglycol) [19], a special calibration curve was constructed for each determination.

The low degree of activation of Sepharose 4B (4 mg CNBr/ml of the packed gel) was used to favour the covalent attachment of the tetrameric molecules to the matrix via a single subunit. To prove that it really occurs, the immobilized enzyme was treated with a denaturing agent to remove non-covalently bound subunits. 5 ml of 8 M urea containing 5 mM EDTA were added to 1 ml of the packed gel and incubated at 20°C for 20 h with constant stirring. The gel was then exhaustively washed on a sintered glass filter with the above urea solution (20 ml) and then with water (60 ml). The content of the immobilized protein was found to correspond to 25% of the starting value.

A stock suspension of the matrix-bound derivative was prepared by mixing equal volumes of gel and buffer A (100 mM sodium phosphate containing 5 mM EDTA and 2 mM dithiothreitol, pH 7.2). Accurate samples were taken from the stirred suspension.

The activity of immobilized enzyme preparations was measured spectrophotometrically at 25°C in 50 mM Tris-HCl (pH 7.8—8.0) in the case of yeast dehydrogenase, and in 100 mM glycine-NaOH (pH 8.7—8.9 or 9.7—9.9) in the case of rabbit and rat muscle enzymes, respectively. The standard assay mixture consisted of 1.5 mM glyceraldehyde 3-phosphate/2 mM NAD^+/5 mM sodium arsenate/5 mM EDTA and 0.1 ml of a suitably diluted suspension of matrix-bound enzyme in a final volume of 3 ml. The reaction was started by addition of a small volume of glyceraldehyde 3-phosphate and proceeded for 15—30 s.

The specific activities of the immobilized enzymes obtained under optimal conditions were, on the average, 60% of those of the corresponding soluble enzyme preparations. The difference, however, markedly diminished if the pH of the assay mixture was changed to decelerate the reaction. At pH 6.6, when the yeast glyceraldehyde-3-phosphate dehydrogenase activity is only 40% of the optimal value, immobilized and soluble preparations had nearly equal specific activities.

This means that the measured enzyme activity of matrix-bound species may be influenced by substrate diffusion between the solution and the catalytic sites; the slower the reaction, the less the difference between specific activities of free and bound enzymes. We performed a quantitative evaluation of diffusional effects on the immobilized glyceraldehyde-3-phosphate dehydrogenase reaction, using the method described [20].

The procedure involves determination of the effectiveness factor, η, which characterizes the ratio of the observed enzyme activity to the activity that could be measured in the absence of diffusion limitations.

$$\eta = \eta_e \cdot \eta_i$$

where η_e and η_i are external and internal effectiveness factors, respectively. These values were determined graphically [20]. The intrinsic Michaelis constants of the bound rat muscle enzyme for NAD^+ and glyceraldehyde 3-phosphate were assumed to be 0.043 and 0.17 mM, respectively [8]. The external transport coefficients, h, for glyceraldehyde-3-phosphate and NAD^+ were calculated to be $4.4 \cdot 10^{-3}$ cm $\cdot$ s^{-1} and $3.5 \cdot 10^{-3}$ cm $\cdot$ s^{-1}, respectively. The diffusion coefficients for glyceraldehyde-3-phosphate and NAD^+ were assumed to be $5 \cdot 10^{-6}$ cm^2 $\cdot$ s^{-1} and $3 \cdot 10^{-6}$ cm^2 $\cdot$ s^{-1}, respectively [21].

The value of η_e was found to be close to unity for both glyceraldehyde 3-phosphate and NAD^+, which suggests that the transport of the substrates from the solution to the surface of the gel is not affected by diffusion limitations. As to η_i, it was different for the two substrates.

For NAD^+ it approached 1, whereas for glyceraldehyde 3-phosphate η_i was found to be 0.6. It follows that

$$\eta = \eta_e \cdot \eta_i = 0.6$$

which means that the measured activity corresponds to nearly 60% of its value obtained in the absence of diffusion effects.

The difference in specific activities of matrix-bound and soluble enzyme preparations can thus be explained by diffusion limitations. The covalent immobilization of the dehydrogenase molecule via 1 subunit seems to have no effect on the catalytic activity of the enzyme.

Dissociation of immobilized glyceraldehyde-3-phosphate dehydrogenase into dimers

1. Yeast enzyme. Immobilized apoenzyme (90 μg/ml of the packed gel) was washed on a sintered-glass filter with 150 mM NaCl/5 mM EDTA/2 mM dithiothreitol (pH 7.2), using 10—15 ml of the solution per ml of gel.

The gel was packed by centrifugation and mixed with an equal volume of the above NaCl solution, which was supplemented with 100 mM ATP. The samples were cooled to 4°C and incubated at that temperature with constant stirring. At fixed time intervals aliquots were removed for activity determination in the standard assay system. After the constant level of the residual activity was attained, the matrix was separated from the mixture on a sintered-glass filter and washed with buffer A until no absorbance at 280 nm was detected in the washings. The content and activity of the immobilized protein were then determined.

2. Rat skeletal muscle enzyme. (a) Immobilized apoenzyme (60 μg/ml of the packed gel) was washed and treated as described for the yeast enzyme, but incubation at 4°C was performed without ATP.

(b) Immobilized apoenzyme (60 μg/ml of the packed gel) was mixed with an equal volume of buffer A, containing 100 mM ADP, and incubated at 22°C, as described above. After a constant level of the residual activity was attained, the gel was treated as indicated for the yeast enzyme.

Reassociation of immobilized dimers with dimers in solution

Tetrameric apoglyceraldehyde-3-phosphate dehydrogenase is known to dissociate reversibly into dimers under non-denaturing conditions, the proportion of the dimeric form in solution depending on the protein concentration [22—24]. Therefore, it became possible to perform reassociation between immobilized and soluble dimers under mild conditions by mere incubation of the matrix-bound derivative in diluted apoenzyme solutions. Various combinations of immobilized and soluble dimers were used to obtain homotetrameric and hybrid molecules. The procedure was as follows. 8—10 ml of packed Sepharose gel containing 45 μg of immobilized dimers/ml were mixed with an equal volume of the apoenzyme solution in buffer A. The suspension was rapidly stirred and incubation continued at 22°C with continuous stirring. At fixed time intervals aliquots (about 1 ml) were withdrawn and extensively washed with buffer A to remove soluble protein. The enzymatic activity of the samples was then measured and occasionally the protein content was also estimated. The incubation was stopped after a constant level of immobilized protein content and activity was attained. The association between immobilized yeast and soluble muscle dimers was followed by measuring the activity of the immobilized protein at pH 10.0. Under these conditions, the yeast dimer is inactive [25], and the activity of the immobilized enzyme is directly related to hybrid formation. When measured under optimal conditions, the specific activities of rat or rabbit muscle dimers in the tetrameric hybrid molecules were found to be similar to the activities of immobilized homotetramers.

Studies of association kinetics

The reaction between matrix-bound and soluble dimers may be written as

$$D_b + D_f \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} T_b$$

where D_b, D_f and T_b represent the concentrations of bound dimers, soluble dimers and bound tetramers, respectively; k_{+1} and k_{-1} are association and dissociation rate constants. Under our experimental conditions the dissociation reaction is very slow as compared to the association reaction. This conclusion is based on the observation that a matrix-bound tetrameric enzyme remains stable in the course of repeated washings with a buffer; no measurable splitting of the immobilized protein is observed under non-denaturing conditions. This is contrary to the behaviour of a soluble apo-tetrameric form which dissociates reversibly into dimers under mild conditions.

Diluted apoenzyme solutions were used as a source of soluble dimers in experiments on association. Using appropriate concentrations of the immo-

bilized dimeric species and an apoenzyme solution, it became possible to perform association under pseudo-first-order conditions. The existence of tetramer $\rightleftharpoons$ 2 dimers equilibrium ensured the maintenance of soluble dimer concentration (D_f) at a nearly constant level, in spite of the removal of a portion of soluble dimers from the solution in the course of association with the matrix-bound derivative.

It may be written then

$$dT_b/dt = k_{+1} \cdot D_f(D_b - T_b)$$

and

$$(D_b - T_b) = D_b \cdot e^{-k_{+1} \cdot D_f \cdot t}$$

or

$$\ln(D_b - T_b) = \ln D_b - k_{+1} \cdot D_f \cdot t$$

A plot of $\ln (D_b - T_b)$ vs. t should yield a straight line with the slope equal to $-k_{+1} \cdot D_f$.

Analogous treatment of the data was executed by Smith and Skubitz [26] in the study of kinetics of interactions between antibodies and haptens.

The values of D_b and T_b were determined experimentally from the analysis of protein content and activity of corresponding immobilized species. D_f was calculated for various apoenzyme concentrations from the expression

$$K_d = \frac{D_f^2}{T_f}$$

where T_f represents the soluble tetramer concentration, and K_d is a dissociation constant. K_d for rat skeletal muscle apoenzyme was assumed to be close to the value reported for rabbit muscle dehydrogenase and equal to 0.5 μM [22]. The dissociation constant for the tetramer-dimer interconversion of the yeast enzyme has not been reported in the literature. There are indications that it does not differ much from K_d for muscle apoenzyme [23] or may be markedly lower [27]. Consequently, we made calculations using $K_d = 0.5$ μM and repeated them with $K_d = 0.05$ μM.

The combination of immobilized glyceraldehyde-3-phosphate dehydrogenase with antibodies

Rabbit antibodies that are specific to rat skeletal muscle glyceraldehyde-3-phosphate dehydrogenase were obtained as previously described [28]. The procedure for producing rabbit antibodies to the yeast enzyme will be reported elsewhere. Both types of antibodies were specific for the corresponding antigens and gave no cross-reaction. The amount of antibodies in the serum was estimated with the use of an immunosorbent prepared by immobilizing the antigen on CNBr-activated Sepharose 4B (200 mg CNBr/ml of the packed gel). The sample of serum to be analyzed was passed through a column containing a fixed quantity of the antigen. After saturation has been achieved (as judged by the appearance of antibodies in the effluent), the immobilized enzyme-antibody complex was analyzed for protein content. The data obtained were used to calculate the amount of antibodies in a given volume of antiserum.

Complexes of immobilized antigen with a different number of antibody molecules were obtained as follows. Matrix-bound tetramers (90 μg/ml of the packed gel) or dimers (45 μg/ml of the packed gel) were washed with 150 mM NaCl/5 mM EDTA/2 mM dithiothreitol (pH 7.2). The gel was then packed by centrifugation and mixed with an equal volume of antiserum, which contained a 10—20-fold excess of antibodies with respect to the antigen protein. Incubation was carried out for 30 min at 20°C with constant stirring and then continued for 10—12 h at 4°C with occasional stirring. The immobilized tetrameric form was then washed with the above solution, whereas the matrix-bound dimeric form was washed with buffer A, until no protein could be detected in the washings. The number of antibody molecules bound to an antigen molecule was estimated on the basis of the immobilized protein content. It was established that a matrix-bound tetrameric enzyme forms a complex with three antibody molecules, and an immobilized dimer combines with a single antibody molecule. The binding of specific antibodies had no effect on the activity of the immobilized enzyme preparations.

The immobilized yeast dimer in complex with one antibody molecule was used for reassociation with soluble dimers that were free of antibodies. The matrix-bound derivative (145 μg/ml of the packed gel) was incubated for 2 h at 22°C with the yeast apoenzyme solution in buffer A (final protein concentration, 0.15 mg/ml). The gel was then washed with buffer A to remove the soluble protein. The protein content in the matrix-bound derivative was found to be 190 μg/ml of the packed gel. This indicates that the tetrameric form of the enzyme has been reconstructed, since the antibody-free dimeric form contained 45 μg of protein per ml of the packed gel.

Preparation of immobilized hybrid tetramers in complex with antibodies

A hybrid of yeast dimer covalently bound to the matrix and a rat muscle dimer were used to form three types of complexes with antibodies:

(1) Only the yeast dimer was in the complex with a rabbit anti-yeast antibody;

(2) Only the rat muscle dimer was in the complex with two molecules of rabbit anti-rat antibodies;

(3) Each of the dimers was bound to a corresponding specific antibody — one antibody molecule was in the complex with the yeast dimer and two molecules with the rat muscle dimer. The complex of the first type was obtained by incubation of immobilized yeast dimer bound to a specific antibody in the presence of rat muscle apoenzyme, as described above. To prepare the complex of the second type, an immobilized hybrid free of anti-yeast antibodies was incubated with antiserum produced against rat skeletal muscle dehydrogenase. The content of immobilized protein determined after the completion of the reaction indicated that the rat muscle dimer in the hybrid tetramer binds two molecules of specific antibodies. The complex of the third type was obtained by a combination of the procedures described above.

Results

Effect of various factors on dissociation of the immobilized glyceraldehyde-3-phosphate dehydrogenase into dimers

Previously we have shown that incubation of rat skeletal muscle apoenzyme at 4°C in 150 mM NaCl or at 20°C in the presence of adenine mononucleotides results in its dissociation into inactive dimers; inactivation is completely reversible when the dissociating agents are removed [13,14]. Fig. 1A illustrates the results obtained with an immobilized rat apoenzyme under similar conditions. Incubation under both sets of conditions leads to a 50% loss of the immobilized enzyme activity. No further inactivation was observed upon prolonged incubation. The determination of the protein content in the matrix-bound enzyme showed that the inactivation was accompanied by the splitting and solubilization of half of the immobilized protein.

Fig. 1B shows the time course of the inactivation of the immobilized yeast dehydrogenase under the conditions previously developed by Deal et al. for dissociation of the enzyme in solution [11,12]. It can be seen that, similar to rat muscle enzyme, yeast dehydrogenase loses 50% of its matrix-bound activity and protein content, with no further changes taking place when incubation is prolonged. We may conclude that dissociation of immobilized and soluble dehydrogenases occur under similar conditions. It should be pointed out that in both cases these conditions differ for rat muscle and yeast enzymes, being more 'rigid' for the latter.

The important feature of the dissociation of the immobilized tetramer is that only one of the resulting dimers loses its activity. The dimeric species which remains bound to the matrix is fully active under conditions causing inactivation of the dimer that goes into solution.

As shown in Fig. 2, NAD^+ and NADH markedly affect the dissociation of the immobilized enzyme. The ability of apoenzymes isolated from skeletal muscle

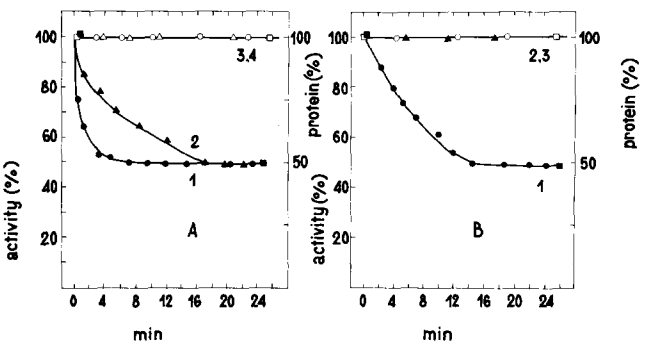

Fig. 1. Dissociation of immobilized apoglyceraldehyde-3-phosphate dehydrogenase from rat muscle (A) and yeast (B) into dimers. (A) Activity of the immobilized enzyme after incubation in 150 mM NaCl at 4°C (▲) or at 22°C (△) and in 100 mM sodium phosphate at 22°C with 50 mM ADP (●) or without (○). Percentage of protein bound to the matrix in samples 1, 2 (■) and 3, 4 (□). (B) Activity of the immobilized enzyme after incubation in 150 mM NaCl at 4°C with no additions (○) or in the presence of 50 mM ATP (●). ▲, incubation with ATP, but at 22°C. Percentage of protein bound to the matrix in samples 1 (■) and 2 and 3 (□). Other details are given in the text.

to bind 2 mol equivalents of NAD^+ or NADH with high affinity [3,29—31] was used to prepare an immobilized tetramer containing two firmly bound coenzyme molecules. To this end, a series of samples of immobilized rat muscle apoenzyme (30 μg of enzyme in 1 ml of suspension) were incubated for 2 h at 22°C in 150 mM NaCl/5 mM EDTA/2 mM dithiothreitol (pH 7.2) in the presence of 2 mol equivalents of NAD^+ or NADH per tetramer. The gel was then packed by centrifugation and washed with the above solution. In a parallel experiment incubation was carried out in the presence of an excess of NAD^+ or NADH and was followed by washing to remove unbound nucleotide.

As seen in Fig. 2, two coenzyme molecules (oxidized or reduced) per tetramer protect it completely from cold dissociation in the presence of 150 mM NaCl. Similar results were obtained with the samples incubated in the presence of 2 mol equivalents or an excess of coenzyme. It seemed likely that one 'tight' coenzyme binding site exists in each of the dimers. The saturation of this site with NAD^+ or NADH renders the whole dimer stable, thus protecting it from conformational changes which lead to dissociation.

To verify this suggestion, a suspension of the immobilized dimeric form (15 μg of enzyme/ml) in 150 mM NaCl/5 mM EDTA/2 mM dithiothreitol/20 mM sodium phosphate (pH 7.2) was incubated in the presence of 1 or 2 mol equivalents of NAD^+ per dimer. After 2 h incubation at 22°C with continuous stirring, the unbound nucleotide was washed from the gel, packed by centrifugation and mixed with an equal volume of a rat muscle apoenzyme solution (0.4 mg/ml). The suspension was stirred for 1.5 h at 22°C to achieve reassociation of the immobilized and soluble dimers. The gel was then packed by centrifugation and soluble protein washed from it. The reconstruction of the immobilized tetramer was confirmed by activity and matrix-bound protein determinations.

The amount of NAD^+ firmly bound to this tetramer must depend on the coenzyme-binding properties of the isolated dimer. If the immobilized dimeric

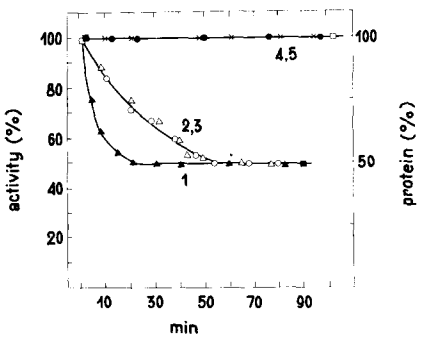

Fig. 2. The effect of NAD^+ and NADH on the dissociation of the immobilized tetramer of glyceraldehyde-3-phosphate dehydrogenase from rat muscle. Incubation in 150 mM NaCl at 4°C was performed with the following preparations of the immobilized tetramer: apoenzyme (▲), apoenzyme pretreated with 2 mol equivalents of NAD^+ (●) or NADH (X) prior to incubation. (△) and (○) refer to the samples containing the immobilized tetramers prepared by the association of the soluble apo-dimers with the immobilized dimers pretreated with one (△) or two (○) equivalents of NAD^+ per mol of dimer prior to reassociation. The data represent the fraction of activity remaining after fixed intervals of incubation. The immobilized protein content in samples 1—3 (■) and 4, 5 (□).

species were able to firmly bind NAD^+ to both active centers, the incubation in the presence of 2 mol equivalents of coenzyme followed by reassociation with soluble dimers would yield a tetramer which is completely stable under the conditions of cold inactivation (see Fig. 2, samples 4 and 5). If the isolated dimer contained only one 'tight' coenzyme binding site, the incubation in the presence of one or two mol equivalents of NAD^+ would give similar results.

Fig. 2 shows that the second alternative is valid. One molecule of the coenzyme per tetramer retards inactivation and dissociation, but does not prevent them. The full protection is achieved only by two firmly bound NAD^+ (NADH) molecules, which are, apparently, located in different dimers (the one covalently bound to the matrix and the dissociable one).

As shown in Fig. 3, the dissociation of the immobilized enzyme can also be prevented by other factors that are capable of stabilizing the native protein conformation. Rabbit antibodies that are specific for yeast dehydrogenase have no influence on the catalytic activity (unpublished data), but, by analogy with anti-rat muscle antibodies [28,32], were expected to protect the enzyme molecule from cold inactivation and dissociation. The results in Fig. 3 demonstrate the effect of antibodies bound either only to the covalently bound dimer (sample 2) or to both dimers (sample 1). It is seen that complete stabilization is only achieved in the second case.

Reassociation of immobilized and soluble dimers

Association kinetics. Fig. 4 shows the time course of association between the immobilized and soluble dimers that is registrated by increase in matrix-bound activity and protein content. Full reconstruction of the tetrameric enzyme is

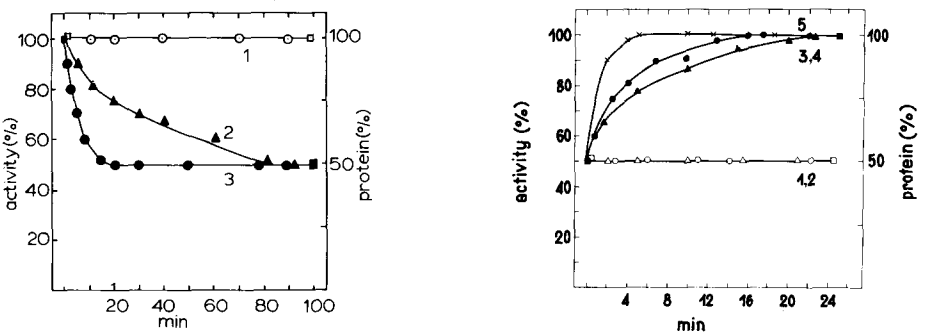

Fig. 3. Effect of specific antibodies on the dissociation of the immobilized tetramer of yeast apoglyceraldehyde-3-phosphate dehydrogenase. Immobilized apoenzyme (●) and its complex with one (▲) or three (○) molecules of specific antibodies per tetramer was incubated at 4°C in 150 mM NaCl containing 50 mM ATP. The data represent the percentage of activity at indicated time intervals. Amounts of immobilized enzyme protein in samples 1 (□), and 2, 3 (■). The details are given in the text.

Fig. 4. Time-course of association reaction between the immobilized dimers and the soluble dimers. The samples 1, 2: immobilized dimers of the rat muscle dehydrogenase (0.1 μM) were incubated with no additions (○) and in the presence of 0.2 mg/ml apoenzyme from rat muscle (●). In the samples 3—5 the immobilized dimers of the yeast dehydrogenase (0.15 μM) were incubated with no additions (△) and in the presence of 0.16 mg/ml yeast apoenzyme (▲). The sample (X) is similar to (▲), but the immobilized dimers are in complex with specific antibodies (one molecule of antibody per dimer). The data represent the activity of the immobilized protein as a percentage of the matrix-bound enzyme protein in the samples 1, 2 and 3—5, respectively.

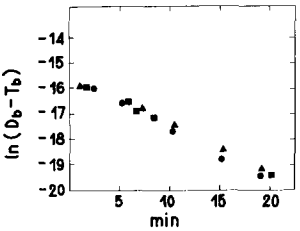

Fig. 5. Kinetics of the association reaction between immobilized and soluble dimers plotted according to pseudo-first-order derivation (see text). The combination occurred between the immobilized dimeric form of the yeast dehydrogenase and soluble dimers of the yeast (●), rabbit muscle (■) and rat muscle (▲) apoenzymes. D_b = 0.15 μM, D_f = 0.38 μM (●), 0.50 μM (■) and 0.49 μM (▲).

accomplished within 15—20 min. An antibody molecule bound to the immobilized dimer markedly accelerates the association reaction (curve 5).

Similar experiments were performed to determine the kinetics of association between immobilized yeast dimers and the soluble rat muscle and rabbit muscle dimers. Fig. 5 represents the results obtained with various dimer combinations using pseudo-first-order analysis of the data. The association rate constants are summarized in Table I.

Effect of various factors on dissociation of the immobilized hybrids of glyceraldehyde-3-phosphate dehydrogenase

The immobilized hybrids that had resulted from association of matrix-bound and soluble dimers were used for investigation of the influence of interdimeric interactions on the stability of the tetramer. As shown in Fig. 1, the experimental conditions which cause dissociation of rat muscle and yeast apoenzymes are different. It seemed to be of interest to compare the stabilities of tetramers composed by the combination of homo- or heterodimers. This was performed with hybrids of the immobilized yeast dimer and a non-covalently bound dimer of the muscle type. In agreement with the results described above, incubation

TABLE I

ASSOCIATION RATE CONSTANTS FOR VARIOUS COMBINATIONS OF IMMOBILIZED AND SOLUBLE DIMERS OF APOGLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

Rate constants (k_{+1}) were calculated as described in the text. The dissociation constant for the tetramer-dimer interconversion of the rat and rabbit muscle apoenzymes (K_d) was assumed to be 0.5 μM. In the case of yeast apoenzyme calculations have been made using K_d = 0.5 μM (a) or 0.05 μM (b).

Immobilized dimer	Soluble dimer	Rate constant × 10^{-4} ($M^{-1} \cdot s^{-1}$)
Yeast	yeast	0.8 [a] 1.1 [b]
Yeast dimer in the complex with one antibody molecule	yeast	2.3 [a] 3.2 [b]
Yeast	rabbit muscle	0.58
Yeast	rat muscle	0.50
Rat muscle	rat muscle	1.20

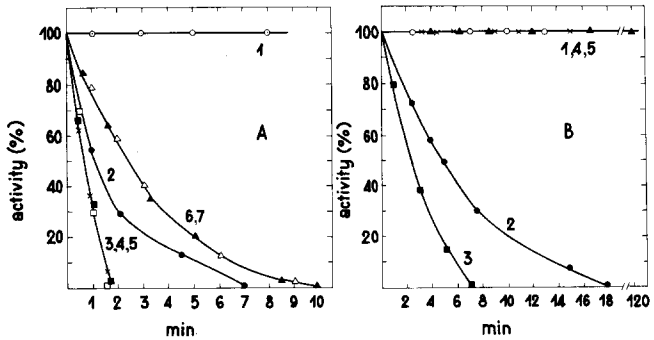

Fig. 6. Inactivation and dissociation of the immobilized hybrids of glyceraldehyde-3-phosphate dehydrogenase. (A) Activity of the immobilized enzyme after incubation at 22°C in 100 mM sodium phosphate containing 50 mM ADP (○), yeast apoenzyme, (●), rat muscle apoenzyme. Samples 3 and 4, hybrids of the immobilized dimeric form of the yeast dehydrogenase and the non-covalently bound dimers of the rat muscle (■) or rabbit muscle (□) enzymes. Sample 5 (X) is similar to 3 (■), but the dimer of the yeast enzyme was in complex with one molecule of specific antibody. (△) is the same as (■), but the dimer of the rat muscle dehydrogenase was in complex with two molecules of specific antibodies. (▲) is similar to (△), but the dimer of the yeast enzyme was in complex with one molecule of specific antibody. (B) Activity of the immobilized enzyme after incubation at 4°C in 150 mM NaCl. Yeast apoenzyme (○), rat muscle apoenzyme (●), the hybrid of the immobilized dimeric form of the yeast dehydrogenase and the non-covalently bound dimer of the rat muscle enzyme (■). In the sample 4 (X) the dimer of the rat muscle enzyme was in complex with two molecules of specific antibodies; in the sample 5 (△) the hybrid was in complex with one molecule of NAD^+. Details are given in the text. The results in A and B are expressed as percentage of the activity of the non-covalently bound dimer. The amount of the immobilized protein remained unchanged in the samples 1 (A) and 1, 4, 5 (B), but decreased in the samples 3—7 (A) and 2, 3 (B). After 20 min incubation it was found to correspond to 50% of the initial content.

under dissociating conditions did not affect the activity of the dimer bound to the matrix covalently.

The results shown in Fig. 6 represent inactivation of a non-covalently associated dimer. It occurs under conditions which cause dissociation of a rat muscle homotetramer, but are too mild to dissociate yeast enzyme. Under both sets of experimental conditions (Fig. 6A and B), the hybrid tetramer proves markedly less stable than the rat skeletal muscle enzyme. Similar results were obtained with the hybrids of yeast and rat or yeast and rabbit muscle dimers, respectively (Fig. 6A, samples 3 and 4).

To get more information about subunit interactions in the hybrid molecules, we studied the effect of ligands that are capable of interacting specifically with one of the dimers composing the tetramer. It became evident that the binding of one antibody molecule to the yeast dimer has no influence on the rate of hybrid inactivation (Fig. 6, samples 5 and 7), in contrast with the results obtained with the yeast homotetramer (Fig. 2).

Dissociation of the hybrid is retarded (Fig. 6A, sample 6) or prevented (Fig. 6B, sample 4) in case specific antibodies are bound to the rat muscle dimer. To substantiate the conclusion that dissociation of the immobilized tetrameric enzyme depends upon the factors affecting the stability of a dimer which is not bound to the matrix covalently, we performed the following experiment. A hybrid bound to the matrix via the yeast dimer was used. The immobilized enzyme (90 μg/ml of the packed gel) was incubated with an equal volume of

150 mM NaCl/5 mM EDTA/2 mM dithiothreitol (pH 7.2) in the presence of one equivalent of NAD^+ per tetramer. After 3 h incubation at 22°C with continuous stirring, the suspension was rapidly cooled to 4°C and the incubation continued at this temperature. The subsequent procedure is given in the legend to Fig. 6B. The control samples incubated in the absence of NAD^+ were observed to rapidly lose their catalytic activity and to dissociate; they were similar to sample 3 (Fig. 6B). The coenzyme completely protected the hybrid molecule from inactivation and dissociation under the conditions reported in Fig. 6B (sample 5). This effect can be attributed to the binding of NAD^+ to the rat muscle dimer, since the affinity of the yeast dehydrogenase for NAD^+ is several orders of magnitude lower than that of the muscle enzyme [6].

Discussion

It is suggested that the dissociation of immobilized glyceraldehyde-3-phosphate dehydrogenase proceeds according to the scheme given in Fig. 7. Subunit contacts preserved upon dissociation into dimers are shown by thick lines. The first step probably involves change in native conformation of the non-covalently bound dimer which precedes its splitting into solution. The dimer involved in the covalent bond with the matrix is stabilized and remains active under the dissociating conditions. The existence of such a stabilizing effect may explain the dissimilarity of results obtained in the studies of the matrix-bound and soluble enzyme dissociation. An isolated dimeric species is capable of exhibiting the dehydrogenase activity provided that its native conformation is maintained.

When dissociation is performed in solution, stabilization can be achieved by the binding of coenzyme. Previously, we have shown that one molecule of NAD^+ or NADH bound per dimer completely prevents the cold inactivation accompanied by dissociation [13]. The results of the present work demonstrate another type of stabilization. Covalent binding to the matrix does not involve the active site region of the apoenzyme, since full catalytic activity is preserved. Nevertheless, a striking similarity exists between the stabilizing effect of coenzyme, on the one hand, and that of immobilization on Sepharose, on the other. In both cases, only 1 subunit of a dimer interacts with the stabilizing agent, but the structure of the whole dimer is affected.

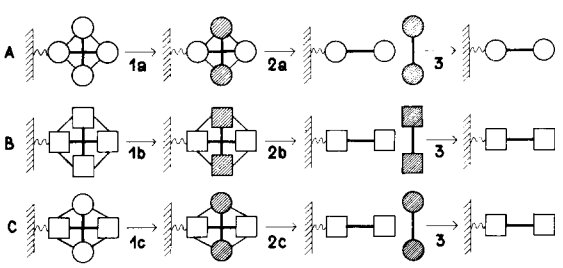

Fig. 7. Suggested mechanism of the disssociation of matrix-bound apoglyceraldehyde-3-phosphate dehydrogenase. A, rat muscle apoenzyme; B, yeast apoenzyme and C, hybrid of the yeast and muscle enzymes. 1a, 2a, 1c, 2c-incubation in 150 mM NaCl at 4°C or in 50 mM ADP at 22°C. 1b, 2b-incubation in 150 mM NaCl plus 50 mM ATP at 4°C. 3 represents the removal of the split protein. Open and shadowed symbols represent active and inactivated species, respectively.

The intradimeric contact regions designated in Fig. 7 by thick lines, therefore, appear to be involved in the realization of cooperativity between enzyme subunits. The non-equivalence of the NAD^+ binding sites of an isolated dimer revealed in the present investigation is in accord with this conclusion. The immobilized dimer of rat muscle dehydrogenase was previously shown to exhibit the 'half-of-the-sites reactivity' [10].

All these facts suggest that an isolated and stabilized dimeric species represents a 'functional unit' of the enzyme molecule. We have presented evidence that each of the 2 dimers can be stabilized independently, by factors other than specific protein-protein interactions realized in a native tetramer. Maintenance of an active conformation may be attained by covalent attachment to the matrix, by interactions with specific antibodies or by the binding of NAD^+ or NADH. The similarity of the stabilizing effects of NAD^+ and NADH previously observed in our study on dissociation of the enzyme in solution [13] and confirmed for immobilized dehydrogenase (Fig. 2) is in contrast with the results of Osborne and Hollaway [33,34], who reported the "loosening of the tetrameric structure of glyceraldehyde-3-phosphate dehydrogenase in the presence of NADH". Two molecules of firmly bound coenzyme were found to completely protect the tetramer from dissociation under the conditions employed in our studies. These results are not consistent with the conclusion of other authors [24,33], that the stabilization of the tetrameric structure of glyceraldehyde-3-phosphate dehydrogenase is only achieved upon binding the third NAD^+ molecule. They are however in accord with the findings of Fenselau [35] and support the model of the tetrameric enzyme organization proposed by this author.

The results of the present work may be discussed in connection with the X-ray crystallographic studies on the lobster hologlyceraldehyde-3-phosphate dehydrogenase [36—38]. The subunit interactions across each of the axes (P, Q and R) were found to considerably differ in the number of amino acid residues which compose the corresponding contact regions. The strongest subunit interactions exist across the P-axis; it was therefore suggested that dissociation would produce the P-axis related dimers [33,38]. In this case, the formation of a cooperative functional dimer would be excluded, since subunit cooperativity is mainly accomplished through the contacts of the R-type [36—38].

Our data indicate that the subunit contacts, which are the strongest in apoenzyme, are responsible for cooperative effects. It seems likely that it is the R-axis related dimer that forms upon dissociation of the apoenzyme. This conclusion does not contradict the results of the crystallographic studies obtained for holoenzyme. The binding of NAD^+ was demonstrated to considerably alter the strength of subunit interactions in the tetramer [13,14,22,27, 33], so it is quite possible that the number and character of contacts across P, R and Q axes would be different in apo- and holoenzymes.

The evidence obtained in the present study supports the conclusion that the hybridization of glyceraldehyde-3-phosphate dehydrogenase is achieved by combination of 'functional dimers'. In a hybrid tetramer each of the dimers may act independently, under conditions which are optimal for the corresponding parent enzyme. Under similar conditions, hybrid molecules dissociate

much more readily than the corresponding homotetramers.

This may result from a deficiency of complementary interactions between dimers in the hybrids. An incomplete 'fitting' of amino acid residues involved in the association of yeast and muscle dimers probably accounts also for the result shown in Fig. 6, sample 5. Contrary to the effect observed with the yeast tetramer, no transmittance of the influence of the antibody via interdimeric contacts was detected in the hybrid. It remains to be elucidated what alterations in the cooperativity between binding sites of dehydrogenase are brought about by substitution of heterologous interdimeric interactions for homologous ones. The work on clarification of this point is in progress.

Some comments are to be made concerning the experimental approach used in the present study to obtain matrix-bound dissociated forms of the enzyme. It differs from the method developed by Chan [7,39] and successfully employed by others [40—42] in two respects. Firstly, dissociation of the immobilized oligomer is performed under conditions which are sufficiently mild to preserve the native structure of the dimer covalently bound to the matrix. Consequently, it becomes unnecessary to renature the immobilized dissociated form (the procedure included in the original method [7]), and the only purpose of repeated washings of the matrix is to remove the soluble protein.

The second difference relates to the reassociation conditions. According to the customary procedure [7,40], small samples of a solution of dissociated enzyme (previously incubated with a denaturing buffer) are added to a suspension of immobilized subunits in a renaturing buffer. Then the reconstruction of an immobilized oligomer proceeds slowly, the first step being a refolding of soluble subunits followed by their reassociation with the immobilized species. In the case of glyceraldehyde-3-phosphate dehydrogenase the association of matrix-bound and soluble dimers was found to take place in the absence of any denaturing agent, by incubation of the immobilized species in an apoenzyme solution. The process is supposed to proceed according to the scheme given in Fig. 8. The complete reconstruction of the tetrameric structure of an immobilized enzyme observed in our experiments may be explained assuming that the interdimeric contacts in a matrix-bound tetramer are stronger than the contacts in a soluble tetramer.

The equilibrium would then be shifted in favour of formation of an immobilized enzyme. The inability of a matrix-bound tetramer to dissociate under non-denaturing conditions (upon repeated extensive washings with a buffer) is in accord with the above assumption. Evidently, this method of reassociation is only applicable under the conditions illustrated in Fig. 8. If association between immobilized and solid dimers is weaker than between the dimers in solution, no reconstitution of a matrix-bound tetramer will occur. A situation of this kind probably arises in the system; immobilized rat muscle dimer-soluble yeast apoenzyme. Our attempts to achieve reassociation under these conditions were unsuccessful. When, however, immobilized yeast dimers were incubated in the rat muscle apoenzyme solution, the hybrid tetramer formed readily. These results suggest that the dissociation constants of the tetramer-dimer interconversion are different for yeast and muscle apoenzymes, with the interdimeric contacts being stronger in the former. We may tentatively assume,

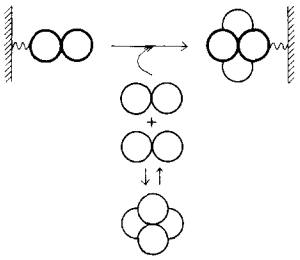

Fig. 8. Association of the immobilized and soluble dimers of glyceraldehyde-3-phosphate dehydrogenase. The contacts formed between a matrix-bound and soluble dimer are stronger than those established between two soluble dimers.

that of the two K_d values for the yeast apoenzyme, that were used to calculate the association rate constants (Table I), the value 0.05 μM is closer to the actual figure than the value 0.5 μM.

In conclusion, we would like to emphasize the convenience of using matrix-bound enzyme subunits for hybridization. The main advantage of this method is the possibility to perform separate dissociation of the partner oligomers, under conditions which are specific for each of the two enzymes. This may help to achieve hybridization between enzymes which differe markedly in the stability of the tetrameric structure. A partial denaturation of the more labile component, which accompanies hybridization performed in the mixtures of soluble enzymes [43,44], is thus avoided.

Furthermore, hybridization on a solid support is preferable since hybrid species are easier to isolate. They can be separated from other components of the reaction mixture by a simple washing of the matrix. This is not the case in the studies on hybrid formation in solution. Since the difference in the electrophoretic mobility between partner oligomers is a prerequisite for isolation and even detection of hybrid species, the method has a restricted applicability. Thus, some combinations of glyceraldehyde-3-phosphate dehydrogenases of various species could not be analyzed because the parent enzymes had very similar electrophoretic migration patterns [43].

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