IMMOBILIZED DIMERS OF D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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1. Introduction

Immobilization of enzymes has proved useful for elucidating the problem of intersubunit interactions in the oligomeric proteins. A valuable approach to the study of matrix-bound protein subunits was developed by Chan and applied to investigation of a number of enzymes [1–3]. Recently we have bound tetrameric glyceraldehyde-3-phosphate dehydrogenase to Sepharose, the catalytic activity of the enzyme being retained to a considerable degree [4]. In the present communication we describe the method for dissociating the matrix-bound tetramer into enzymatically active dimers and characterize some of the properties of the immobilized dimers.

2. Materials and methods

Glyceraldehyde-3-phosphate dehydrogenase was isolated from rat skeletal muscle [5] and freed from bound nucleotide by gelfiltration on Sephadex G-50 and chromatography on a CM-cellulose column. Enzyme immobilization was carried out as follows. Sepharose 4B was activated according to Axen et al. [6] but using 20 mg CNBr per ml of packed gel. After activation, the Sepharose was washed with cold water, cold 0.1 M sodium bicarbonate (pH 9.0) and then with cold 0.1 M sodium phosphate buffer pH 8.0, containing 5 mM EDTA and 4 mM 2-mercaptoethanol.

20 ml of the gel suspension were added to 20 ml of the apoenzyme in the above phosphate buffer (14 mg of protein, specific activity 60–70 U/mg). After 18 hr (at 4°C with stirring) the excess protein, not covalently bound, was removed by washing with the above phosphate buffer. Washing was continued until no more protein eluted from the matrix, which was manifested by the disappearance of the enzymatic activity in the effluent.

The protein content of the immobilized enzyme was determined by the slightly modified method of Lowry [7] and occasionally by amino acid analysis of the matrix-bound derivatives. Enzymatic activity was determined spectrophotometrically at 340 nm. A small aliquot (0.05–0.1 ml) of the diluted suspension of the matrix-bound derivative was added to 2.9 ml of the assay mixture, containing 0.1 M glycine buffer pH 9.9, 5 mM sodium arsenate, 5 mM EDTA, 0.26 mM NAD at 25°C. The reaction was started by addition of glyceraldehyde-3-phosphate (final concentration 0.35 mM) and followed for 30 sec. Under conditions employed the assay was linear with the amount of the matrix-bound enzyme added.

3. Results and discussion

The main purpose of this work was to prove the suggestion that the inactivation of the rat skeletal muscle glyceraldehyde-3-phosphate dehydrogenase in the course of its reversible dissociation into dimers [8] is due to the conformational changes of the dimeric species, caused by dissociation conditions, and the reversal of these changes may give rise to the enzymatically active dimers.

In order to minimize alternations of the protein structure during dissociation sufficiently mild condition were found; as seen in fig. 1, treatment of the apoenzyme with adenine nucleotides at room temperature results in a rapid loss of activity.

Maximal inactivation is reached within 20–30 min, and no further loss of activity occurs. Evidently, an

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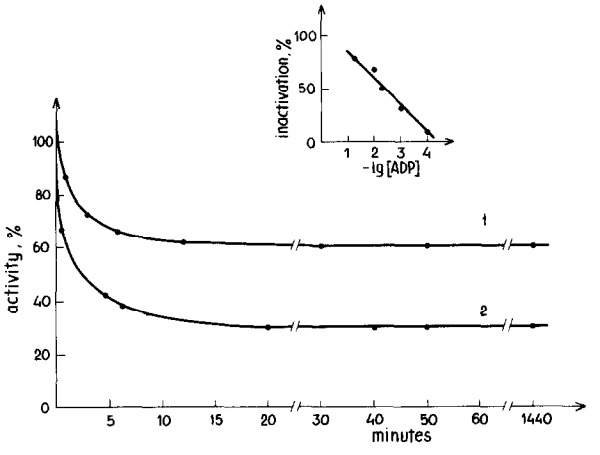

Fig. 1. Time course of the adenine nucleotide-induced inactivation of apo-glyceraldehyde-3-phosphate dehydrogenase. Enzyme samples (0.45 mg per ml) were incubated for indicated times at 20°C and pH 7.5 in 50 mM sodium phosphate buffer, 5 mM EDTA, 4 mM 2-mercaptoethanol, in the presence of 10 mM AMP (1) or 10 mM ADP (2). The activities were then assayed as described in Methods. Inset: Dependence of enzyme inactivation on concentration of ADP. Percent of inactivation at equilibrium is plotted against –lg of ADP concentration (M). Protein concentration, 0.5 mg per ml.

equilibrium is established between active and inactive forms of the enzyme. The dependence of the extent of inactivation at equilibrium on the concentration of ADP is shown in fig. 1, inset. It seems likely that the effect is due to the nucleotide binding in the active site of the enzyme, as suggested earlier [9,10], the extent of inactivation being dependent on the saturation of the nucleotide binding sites.

As shown in fig. 2, the inactivation is clearly dependent on protein concentration, which suggests that the dissociation is involved in the inactivation process. Assuming that in the presence of nucleotide an equilibrium is established, $T \rightleftarrows 2D$, where T stands for the active tetramer, and D for the inactive dimer, we calculated the value of equilibrium constant (K_D) with equation $K_D = \frac{D^2}{T}$. This value was found to correspond to 1.8×10^{-5} M and to 3.2×10^{-6} M in presence of 10 mM ADP and AMP, respectively.

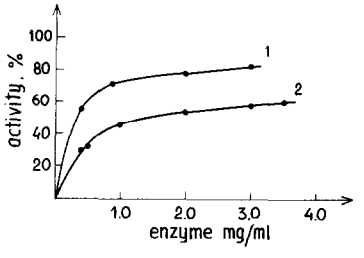

Fig. 2. Protein concentration dependence of the AMP (1) or ADP (2) induced inactivation of apo-glyceraldehyde-3-phosphate dehydrogenase. Inactivation was carried out for 60 min under conditions of fig. 1.

The good fit of the experimental data and the values, calculated on the basis of the proposed mechanism, suggests that AMP and ADP-induced inactivation is due to the enzyme dissociation into dimers, inactive in the presence of nucleotide. The appearance of the dimeric form was confirmed by sedimentation analysis.

The inactivation of glyceraldehyde-3-phosphate dehydrogenase by AMP or ADP can be completely reversed by removing the nucleotides with gel filtration. 0.3 ml of the reaction mixture, which had been incubated for 60 min under conditions of fig. 1 in the presence of 10 mM ADP (containing 0.15 mg enzyme with 30 percent of the initial activity) was passed through the column (6.0 × 1.2 cm) of Sephadex G-50 equilibra with 50 mM sodium phosphate buffer pH 7.5, containi 5 mM EDTA and 4 mM 2-mercaptoethanol at 20°C. Full recovery of activity was observed in the eluate free of nucleotide.

As to the mechanism of reactivation two possibilitie could be considered: 1) removal of the nucleotide is followed by the conformational changes in the dimers, with both the enzymatic activity and the capability of reassociating being regained. 2) The dimers free from nucleotide are inactive per se, but readily reassociate t give the active tetramer.

To distinguish between these two possibilities the dissociation was carried out with the matrix-bound enzyme. To this end, a column of 2 cm × 0.7 cm was filled with a suspension of the immobilized tetrameric glyceraldehyde-3-phosphate dehydrogenase (0.12 mg per ml of the packed gel) in 50 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA and 4 mM 2-mercaptoethanol at 20°C. A solution of ADP (50 mM) in the same buffer was slowly passed through the colu this procedure lasted 60 min; 10 ml of the nucleotide solution was used. The column was then extensively washed with the above buffer to fully remove the nucleotide and the dissociated protein.

Table 1
Protein content and activity of immobilized derivatives of glyceraldehyde-3-phosphate dehydrogenase

	Protein Content		Activity		Specific Activity	
	μg/ml	%	U/ml	%	U/mg	%
Bound tetramer	120	100	3.7	100	30.8	100
Bound dimer	55	45.8	1.55	43.1	28.3	91.8
Bound reassociated tetramer	120	100	3.24	87.6	27.0	87.6

As seen in table 1, nucleotide treatment released 54.2% of the protein from the matrix, which suggests dissociation of the tetrameric molecule, covalently bound to Sepharose, into dimers to have taken place. We do not know at present the number of sites of attachment of the enzyme to the matrix. However, the fact that a half of the bound protein can be readily dissociated under sufficiently mild conditions indicates that not more than two subunits are involved in the binding process; it is not excluded that the binding occurs through a single polypeptide chain. The effect of adenine nucleotides seems to specifically affect the inter-dimeric contacts in the molecule. It should be noted, that prolonged washing of the suspension of matrix-bound tetrameric enzyme with buffer devoid of nucleotide has no effect on the bound protein content.

Immobilization causes a decrease in the specific activity of the tetrameric enzyme; as seen in table 1, approx. 50% of the activity of the soluble enzyme (65U/mg) is retained. The specific activity of the bound tetrameric form was referred to as 100% and compared with the corresponding value for the immobilized dimeric species. As seen in table 1, nearly full retention of specific activity was observed with dimers bound to the matrix and freed of ADP. This suggests that in the absence of nucleotide the native conformation of the enzymatically active dimers has been regained.

The restoration of the native structure should be accompanied by the reappearance of the capability of reassociating with the native protein subunits. It seemed of interest therefore to see if the immobilized dimers would 'pick up' the native dimeric species existing in solution of the apoenzyme in equilibrium with tetramer

The experiments on reassociation were carried out as follows. Suspension of the immobilized dimers (0.8 ml) was added to 1.2 ml of the apoenzyme solution (0.6 mg/ml) in the 50 mM sodium phosphate buffe pH 8.0, containing 5 mM EDTA and 4 mM 2-mercaptoethanol, and incubated at 4°C with occasional stirring for 48 hr. The product was than extensively washed with the above buffer to remove any non-specifically bound protein. The data of table 1 show the 2-fold increase of the matrix-bound protein content with a concomitant increase in activity. This result confirmed

Table 2
Characteristics of matrix-bound dimers and tetramers of glyceraldehyde-3-phosphate dehydrogenase

	pH optimum of activity	Apparent K_m values (mM) for	
		NAD	PGA
Matrix-bound tetramers	9.9–10.1	0.043	0.17
Matrix-bound dimers	9.9–10.1	0.050	0.20
Soluble enzyme	8.9–9.2	0.015	0.12

the ability of the immobilized dimers to reassociate with the native subunits in solution. In a control experiment with matrix-bound tetrameric enzyme no increase in the amount of the immobilized protein was observed after 48 hr incubation in the apoenzyme solution.

The specific activity of the reformed tetramers approaches that of the immobilized native enzyme, suggesting the restoration of the native oligomeric structure of the dehydrogenase.

Assays were made in 0.1 M glycine buffer, containing 5 mM EDTA at 25°C. K_m values were determined at pH 9.9 (matrix-bound enzyme) and 9.2 (soluble enzyme). PGA, glyceraldehyde-3-phosphate. Some of the characteristics of the matrix-bound dimers, listed in the table 2, confirm the similarity of the catalytic properties of the tetrameric and dimeric forms of the enzyme.

In conclusion, the results of the present work provide evidence for the existence of enzymatically active dimeric form of glyceraldehyde-3-phosphate dehydrogenase, in agreement with the statement of Ovádi et al. [11]. We suggest that the loss of activity that accompanies enzyme dissociation into dimers under a variety of conditions, should be attributed to conformational changes in the dissociated species.

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