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IMMOBILIZATION OF PIG MUSCLE ENOLASE. STUDIES ON THE ACTIVITY OF SUBUNITS*

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The native dimeric form of enolase from pig muscle was immobilized on Sepharose 4B activated with cyanogen bromide. The amount of matrix-bound enolase, its specific activity and kinetic properties depend on the extent of gel activation with CNBr.

Only on the Sepharose activated with small quantities of CNBr the amount of protein which remained after treatment with Gdn·HCl was about 50% of the initially bound enolase, indicating that the enzyme was bound covalently to the matrix through a single subunit. The matrix-bound monomers obtained in this way were inactive and were unable to reassociate to dimers on addition of free subunits.

The matrix-bound monomers obtained after KBr treatment were inactive but retained the ability to reassociate into active dimers after addition of free subunits.

The results indicate that single matrix-bound subunits of pig muscle enolase are enzymatically inactive and dimeric structure is essential for catalytic activity.

Immobilization on several supports of some enzymes allows to their properties under conditions bearing more resemblance to conditions *in vivo* than experiments performed on isolated enzymes in dilute solutions. This results from the fact that, in the intact cell, many enzymes are found on the surface of the membranes or they form complexes with other enzymes. Moreover immobilization of enzymes may facilitate investigations on the relationship between quaternary structure of oligomeric enzymes and their catalytic action. This method developed by Chan [1] was successfully

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applied to study the activity of subunits of several enzymes [2]. An oligomer is attached to the insoluble matrix through a single covalent linkage. Dissociation of the protein followed by removal of noncovalently bound subunits produces immobilized monomers. Their properties can be studied without risk of spontaneous reassociation. Some enzymes e.g. aldolase [3, 4] and glyceraldehyde 3-phosphate dehydrogenase [5] which exist as oligomers in the native state, exhibit enzymatic activity in monomer form when bound to the Sepharose matrix.

The purpose of the present work was to elucidate the role of subunit interactions in the activity of pig muscle enolase. We attempted to answer the question whether single subunits of muscle enolase are enzymatically active. Enolase isolated from yeast mammalian muscle is a dimeric molecule with molecular weight in the range of 80000 - 100000 [6, 7]. The yeast enzyme dissociates easily into inactive subunits in the presence of monovalent ions [8]. On the other hand, it has been demonstrated, that highly diluted yeast enolase at 40°C dissociates into enzymatically active subunits [8]. However, this experiment has not so far been repeated with enolase from muscle.

In the present paper we describe the catalytic properties and stability of immobilized enolase from pig muscle. The catalytic properties of immobilized monomers were also studied.

MATERIALS AND METHODS

Reagents. Sepharose 4B and Sephadex were obtained from Pharmacia Fine Chem. (Uppsala, Sweden). CNBr was purchased from Merck (Darmstadt, F.R.G.) and guanidine hydrochloride (Gdn·HCl) from Fluka AG (Basle, Switzerland). 2-Phosphoglycerate (2PGA) was obtained from Boehringer (Mannheim, F.R.G.). ^{125}I was from IBJ (Świerk, Poland). Other chemicals were of analytical grade.

Enzyme preparation. Enolase from pig muscle was obtained by the procedure of Wolna *et al.* [9], except that the last crystallization step was replaced by ion-exchange chromatography as described by Rider & Taylor for rat muscle enolase [10]. The enzyme was homogeneous on polyacrylamide gel electrophoresis and its specific activity was 90 IU/mg at 25°C.

Enzyme assay. The catalytic activity of soluble enolase was determined as previously described [11] in a medium consisting of 0.05 M imidazole/HCl buffer, pH 6.8, 0.4 M KCl, 3 mM $MgCl_2$ and 1 mM 2PGA. The formation of the product phosphoenolpyruvate was monitored by changes in absorbance at 240 nm. To determine the activity of immobilized enolase, 0.1 ml of gel suspension was added to 3.1 ml of the assay mixture described

above. The absorbance was measured within the first 3 - 5 min, i.e. before the appearance of sedimentation.

Protein content. The protein concentration of soluble enolase was determined from absorbance at 280 nm using the extinction coefficient $A_{280}^{0.1\%} = 0.90$ [5]. Protein content of immobilized enolase was estimated from absorption measurement by determining the difference between the amount of protein added and that washed out of the gel after the coupling procedure. In some experiments, in which ^{125}I-iodinated-enolase was used, immobilized protein was determined from the specific radioactivity of the iodinated enolase. The results obtained by these two methods were in good agreement ($\pm 5\%$).

Iodination of enolase. The enzyme from pig muscle was iodinated by Greenwood *et al.* [12] and separated from unbound iodine and other compounds on a Sephadex G-25 column with 0.1 M $NaHCO_3$. The iodinated enolase had a specific activity of 90 IU/mg and a specific radioactivity of about 4×10^6 c.p.m./mg. Samples were counted in a scintilation counter of type US B-2 (Polon, Warsaw, Poland).

Immobilization of enolase on Sepharose 4B. Sepharose 4B was activated with different amounts of CNBr as described by Axen *et al.* [13] and combined with an equal volume of enolase (0.5 - 2.0 mg/ml). The suspension was gently stirred overnight at 4°C and filtered. The gel was then washed with 0.1 M $NaHCO_3$, pH 9.0, containing 1 M NaCl followed by 0.1 M $NaHCO_3$, pH 9.0, suspended in 0.1 M ethanolamine, pH 9.0, and left for 1 h at 4°C. Then the gel was washed with 0.1 M $NaHCO_3$, pH 8.3, containing 5 mM $MgSO_4$ and stored in this solution until used.

Immobilization of iodinated enolase was performed in the same way.

Dissociation of matrix-bound[1] *enolase with guanidine hydrochloride.* The MB-enzyme was diluted with 5 vol, of 6 M Gdn·HCl, pH 2 or 7, containing 1 mM EDTA and 10 mM dithiothreitol. The gel was filtered and the procedure was repeated twice more. The MB-protein was then incubated for 1 h at room temperature, washed five times in 4 vol. of 6 M Gdn·HCl, 0.1 M Tris/HCl, pH 7, 1 mM EDTA and 10 mM dithiothreitol, and then was renatured by exhaustive washing with 0.5 M Tris/HCl, pH 8.0, with 5 mM $MgSO_4$ followed by 0.1 M Tris/HCl, pH 8.0, containing 5 mM $MgSO_4$.

The MB-subunits of enolase were allowed to remain in this medium overnight at 4°C to assure full renaturation of the unfolded polypeptide chain.

[1] Abbreviations used: MB, matrix bound; Gdn·HCl, guanidine hydrochloride; 2PGA, 2-phosphoglycerate.

Denaturation and renaturation of soluble enolase with Gdn·HCl. Enolase in 0.05 M Tris/HCl buffer, pH 7.4, containing 1 mM EDTA and 1 mM dithiothreitol, was mixed with 4 vol. of 8 M Gdn·HCl, pH 2 or 7. Final concentration of enolase was 2 - 4 mg/ml. The mixture was incubated at room temperature and aliquots were withdrawn for activity determination. After 2 h the totally inactive protein was slowly added under gentle stirring to 100 vol. of the renaturation solution composed of 0.05 M imidazole, pH 7.5, 5 mM $MgSO_4$, 1 mM EDTA and 1 mM dithiothreitol. Aliquots of this mixture were assayed for activity.

Dissociation of enolase with KBr. The MB-enolase was suspended in 15 mM Tris/HCl buffer, pH 7.9, containing 5 mM EDTA, 10 mM 2-mercaptoethanol and 1 M KBr. The suspension was stirred overnight at room temperature and then filtered on a sintered glass funnel and washed five times with the same buffer containing no KBr.

Reassociation of MB-subunits with soluble subunits. A portion of 5 ml of packed gel with MB-subunits was suspended in 25 ml of renaturation buffer and continously stirred. At 10 min intervals 50 μl of ^{125}I-iodinated-enolase (2 mg/ml) in the same buffer containing 1 M KBr was added. Aliquots were withdrawn, washed and the amount of newly bound protein was determined by radioactivity counting.

Heat inactivation of MB-enolase. An 1:1 slurry of the MB-dimer in 15 mM sodium phosphate buffer, pH 7.0, containing 5 mM $MgSO_4$, was incubated at 65°C under gentle stirring. At equal intervals samples were assayed for enzymatic activity at 25°C.

Heat inactivation of soluble enolase was performed in the same way, at enolase concentration 0.14 mg/ml.

RESULTS AND DISCUSSION

Immobilization of enolase. The amount of enolase covalently bound to the Sepharose depends on the amount of CNBr which was used for activation of the support (Fig. 1). Prolongation of coupling time or increase of protein concentration were without effect. A plot of enzymatic activity of MB-enolase *versus* the protein content demonstrates that the activity increased gradually to reach the maximum at about 0.6 mg of bound protein per 1 ml of packed gel, and decreased at higher protein concentration (Fig. 2).

The ability of enolase to bind to activated Sepharose is relatively low. The specific activity was the highest in the samples with the lowest amount of bound protein (Table 1). The MB-enolase, when stored for 2 months at 5°C in 0.1 M $NaHCO_3$ and 5 mM $MgSO_4$ retained the activity.

Table 1

Properties of MB-enolase from pig muscle depending on the degree of activation of Sepharose with CNBr

CNBr	Protein content	Activity		Protein content after Gdn·HCl treatment*	K_m 2PGA
mg/ml gel	mg/ml* gel	units/mg gel	units/mg protein	%	mM
5	0.090	7.6	85	51.4	0.41
10	0.135	9.4	69.5	53.6	0.60
20	0.300	16.2	54.0	64.4	1.8
40	0.60	20.0	33.3	73.5	3.0
60	0.93	18.6	20.0	78.4	3.6
80	1.12	14.1	12.6	78.0	3.9
100	1.40	12.6	9.0	78.6	4.1
Soluble enzyme	–	–	90.0	–	0.28

* Protein content was estimated from the radioactivity of iodinated enolase

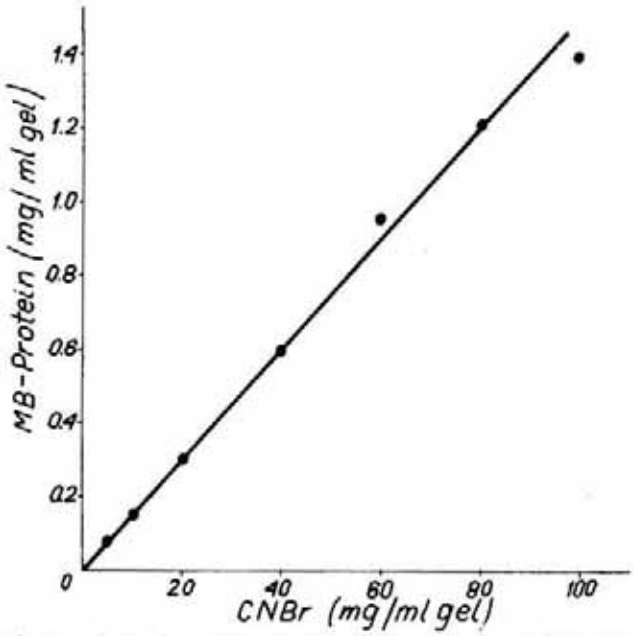

Fig. 1. The dependence of the immobilized protein content on the extent of activation of Sephadex 4B by cyanogen bromide. For details see Materials and Methods

K_m values for 2PGA increased with the amount of CNBr used for Sepharose activation (Table 1). Perhaps activation with a larger amount of CNBr facilitated binding of some molecules of enolase through both subunits, resulting in a hindrance in the catalytic process. High con-

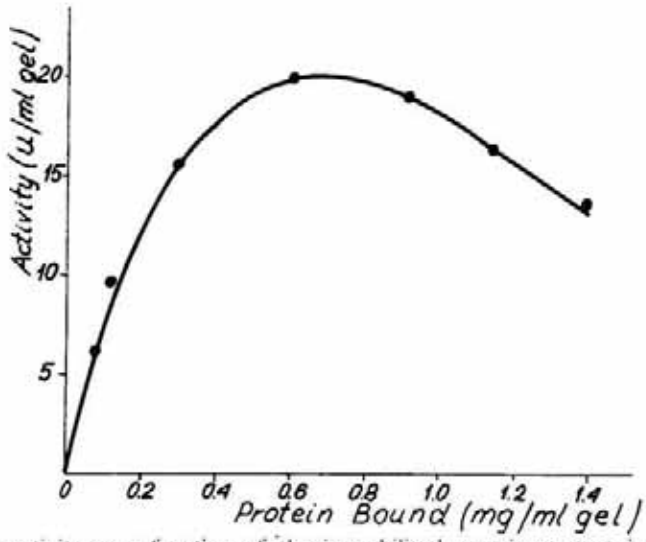

Fig. 2. Enolase activity as a function of the immobilized protein content in matrix-bound

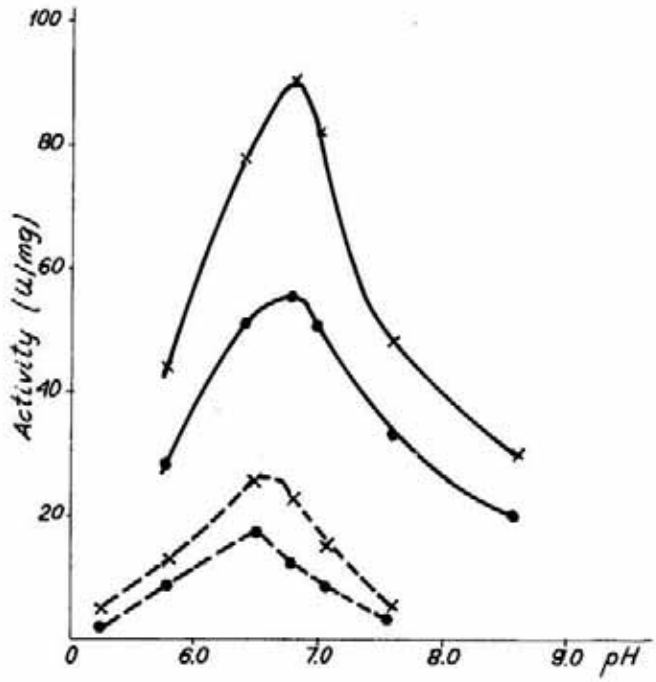

Fig. 3. The pH activity profile for native (×) and MB-dimer pig muscle enolase (○), 50 mM imidazole buffer (—), 75 mM phosphate buffer (- -). The reaction mixture and conditions were as described under Materials and Methods

centrations of Mg^{2+} and Mn^{2+} inhibited the MB-enolase in the same manner as was observed for the soluble enzyme [9]. The inhibition by 0.075 M phosphate and pH dependence in imidazole and phosphate buffers were identical for the soluble and MB-enolase (Fig. 3).

Heat inactivation of MB-enolase. The stability of MB-enzyme on heat treatment (65°C) was examined on the samples of enolase bound to low-activated Sepharose (10 mg CNBr/ml gel), and compared with that of soluble enolase. Soluble enolase was completely inactivated after 60 min but the MB-enzyme still exhibited 60% of initial activity (Fig. 4). Thus, it appears that enolase on the matrix makes its structure more resistant to heat treatment.

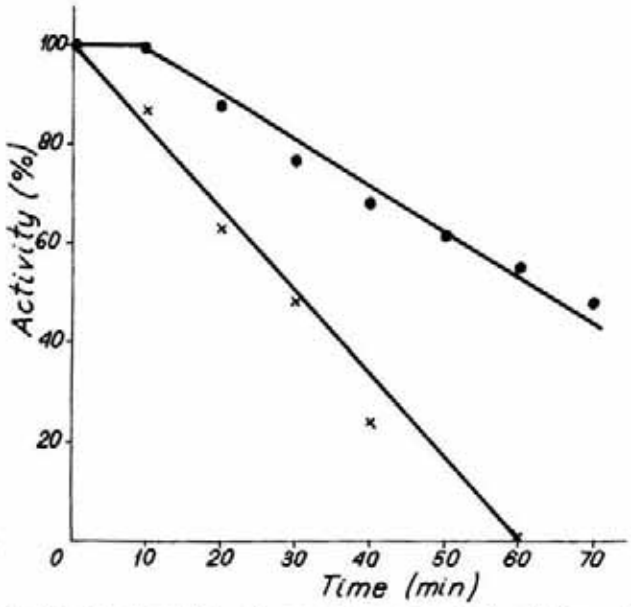

Fig. 4. Heat treatment of soluble pig muscle enolase (×) and immobilized dimer (●). The native and MB-enzyme were incubated at 65°C and assayed as described under Materials and Methods

Dissociation and reassociation of MB-enolase. The coupled enolase was treated with 6 M Gdn·HCl. The amount of bound protein which remined on the matrix after treatment with Gdn·HCl depended on the extent of activation with CNBr. Only the Sepharose activated with 5 or 10 mg CNBr per 1 ml of packed gel bound the enolase dimer to the solid support through one monomer, as upon treatment of this preparation with Gdn·HCl about half of the protein was removed from the matrix (Table 1).

The amount of immobilized and dissociated protein determined from the difference between the amount of protein introduced and found in the

filtrate after Gdn·HCl treatment, was verified in the experiments with iodinated ^{125}I-enolase. The results obtained by the two methods were very similar and reproducible. Though after iodination no changes in activity or other properties of the enzyme were observed, small changes in the structure of enzyme molecule due to incorporation of iodine can not be excluded. Therefore, the use of ^{125}I-enolase was restricted as far as possible.

Experiments on renaturation and reassociation were performed on the enolase bound to Sepharose through one subunit (low-activated Sepharose, 10 mg per 1 ml of packed gel). The sample was treated wih Gdn·HCl and then exhaustively washed with renaturation buffer. No activity was recovered which indicates that the MB-monomers obtained after Gdn·HCl treatment were inactive. Moreover, the reconstitution to dimer did not occur, as the MB-monomer titrated with free ^{125}I-enolase did not bind any radioactivity.

This is of variance with the results of Teipel & Koshland [14] who showed that rabbit muscle enolase, when denatured with 6 M Gdn·HCl, pH 2 or 7, recovered almost complete activity in renaturation buffer. Although the general properties of rabbit and pig muscle enzyme are identical [9], the experiments were repeated on enolase from pig muscle. The results were very similar — in the absence and presence of Mg^{2+} ions after 2 h of renaturation the specific activity was 15 and 70%, respectively of that of the native enzyme. The concentration of soluble enolase after renaturation was 100 - 180 μg/ml, slightly higher than in the experiments with MB-enzyme.

Treatment of MB-enolase with 3 M KCl known as an agent dissociating muscle enolase [5, 6, 7], caused neither liberation of protein nor any change in the activity. Incubation of MB-enolase in 1 M KBr in the absence of Mg^{2+} yielded the subunit-enolase-Sepharose derivative which contained a little more than 50% of initial MB-protein but exhibited only 5 - 8% of initial specific activity. Since the enolase seems to be attached by a single monomer (cf. Table 1), the remaining low activity can be ascribed to the interaction of some adjacently coupled monomers. The soluble muscle enolase, dissociated in KBr in the absence of Mg^{2+}, exhibited also some activity (14%). After dilution, which resulted in association to dimers, 82% of initial specific activity was restored. Similar results were obtained by Shimizu *et al.* [16] for the rat muscle enolase.

Only the KBr-treated MB-monomers retained the capacity for reassociation with free subunits of enolase, leading to recovery of about 70% of initial specific activity. As shown in Fig. 5, the binding of the labelled-subunits proceeded slowly but was complete. In control experiments no non-specific aggregation of soluble enolase to ethanolamine-blocked Sepharose or MB-enolase was observed.

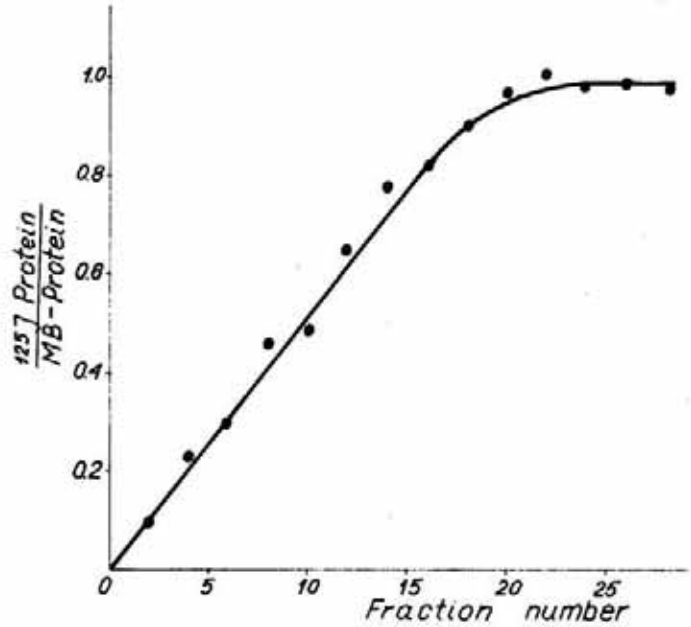

Fig. 5. Titration of MB-monomer enolase with soluble ^{125}I-labelled subunits. The ratio of ^{125}I-labelled protein to protein bound to the Sepharose is plotted as a function of the number of soluble subunits added. The titration was performed following the procedure described under Materials and Methods

Veronese *et al.* [15] found recently that enolase from *Baccillus stearothermophilus* immobilize on Sepharose 4B regained 40% of activity when dissociated with 6 M Gdn·HCl followed by extensive washing. Moreover, they reported that the terminal stability of MB-octamers and monomers did not differ from the stability of soluble enzyme. The enolase from thermophilic bacteria differes markedly from mammalian enolases in that it is octameric and its stability towards heat and protein denaturants is much higher [15]. These last properties of enolases thermophilic bacteria may have contributed to the observed partial reactivation of MB-subunits.

Although the activity of soluble subunits of yeast enolase was described [8], Brewer claimed that any dissociation of yeast enolase leads to inactivation [17].

In conclusion, the results of the present investigations have demonstrated that MB-subunits of pig muscle enolase are enzymatically inactive although they retain full ability to reassociate into active dimers. The dimeric structure of pig muscle enolase is essential for its catalytic activity.

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