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PURIFICATION AND SOME PROPERTIES OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM CARP MUSCLE*

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Glyceraldehyde-3-phosphate dehydrogenase was purified from carp white muscle. On CM-Sephadex chromatography two well separated active peaks were obtained. Both of them show a single protein band on gel electrophoresis and have the same molecular and kinetic properties; they differ only by the amount of bound NAD, the enzyme in the second peak being coenzyme-free.

Significant differences were observed between the properties of carp and pig muscle enzymes. Glyceraldehyde-3-phosphate dehydrogenase from carp is more resistant to heat and proteolytic inactivation. Moreover NAD does not protect it against inactivation. Only one sulphhydryl group per subunit is able to react with 5,5'-dithiobis(2-nitrobenzoate), irrespective of the kind of the buffer.

The structure of glyceraldehyde-3-phosphate dehydrogenase from white muscle of carp seems to be more compact and therefore more inaccessible to some agents than that of the enzyme from pig muscle.

Within the last few years intensive studies were carried out on the adaptive mechanisms which enable optimal functioning of various organisms under variable conditions of the environment. The most interesting results have been obtained in comparative studies on homologous enzymes from endo- and ectothermic animals.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) has been purified from many sources. All animal enzymes so far investigated are of tetrameric structure, with subunit molecular weight of about 36 000 and very conservative amino acid sequence around the reactive cysteine 149 [1, 2]. Also their kinetic properties are very similar. However, glyceraldehyde-3-phosphate

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dehydrogenase from fish [3, 4] showed enhanced stability against heat and proteolysis.

Carp lives commonly in lakes and is grown in natural and artificial ponds on a commercial scale. The temperature of water in such ponds varies from nearly 0°C in winter to 25°C or more in summer. Purification of the carp muscle enzyme using acetone precipitation was described some years ago by Ludovicy-Bungert [5], but only electrophoretic mobility and sedimentation behaviour of the enzyme were studied.

In this paper a novel method of purification of glyceraldehyde-3-phosphate dehydrogenase from white muscle of carp is described. Moreover some properties of the purified enzyme were investigated.

MATERIALS AND METHODS

Materials. D-Glyceraldehyde 3-phosphate was prepared from fructose-1,6 bisphosphate according to Szeiczuk et al. [6]. For comparison, monobarium salt of D-glyceraldehyde 3-phosphate (Boehringer-Mannheim Biochemicals, F.R.G.) was also used. No differences were seen with either of these substrates. Sephadex and CM-Sephadex were from Pharmacia (Uppsala, Sweden). NAD, chymotrypsin, trypsin and 5,5′dithiobis(2-nitrobenzoate) were Sigma preparations. Bovine serum albumin, cytochrome c and Coomassie R 250 Serva Blue were purchased from Serva (F.R.G.). Enolase from pig muscle was a gift of Dr. E. Wolna and phosphoglycerate kinase from bovine heart was a gift of Dr. H. Siemieniewski from our department. Glyceraldehyde-3-phosphate dehydrogenase from pig muscle was obtained as described earlier [7].

Purification of carp muscle glyceraldehyde-3-phosphate dehydrogenase

Extraction. Fresh white muscle from carp was ground in a meat grinder and extracted for 20 h at 4°C with 2 vol. of extraction buffer (1 mM 4-morpholineethanesulphonic acid - NaOH, 2 mM 2-mercaptoethanol, 5 mM EDTA, pH 8.6). The slurry was centrifuged at 3 000 g for 60 min.

Heat treatment. The supernatant was heated with gentle stirring in a boiling water bath until the temperature of the solution reached 55°C. This temperature was maintained for 5 min, then the solution was cooled in an ice bath and centrifuged. The precipitate was discarded.

(NH₄)₂SO₄ precipitation. The precipitate formed at 0.60 - 0.83 saturation was collected by centrifugation and transferred into 1 mM 4-morpholineethanesulphonic acid - NaOH, 2 mM 2-mercaptoethanol, 5 mM EDTA, pH 6.5.
Chromatography on CM-Sephadex. The protein was applied to a CM-Sephadex column (3.2 x 15 cm) equilibrated with the same buffer. The column was washed with the buffer until the absorbance of the eluate at 280 nm was less than 0.02. The eluted protein was enzymatically inactive. The elution of the enzyme was carried out with a linear gradient of 0-0.35 M NaCl in standard buffer, pH 7.5, followed by a gradient of pH 7.5-8.5 in 0.35 M NaCl. As shown in Fig. 1, two peaks with enzymatic activity were resolved. The total activity of these peaks varied from preparation to preparation but the specific activity and other properties were identical. The protein of the peaks was pooled separately, precipitated with (NH₄)₂SO₄, stored at 4°C and dialysed against the appropriate buffer prior to use.

Protein determination and enzyme assay

Concentration of the enzyme was determined spectrophotometrically at 280 nm using a factor of 1.02 or 0.83 for the solution containing 1 mg/ml of holoenzyme or apoenzyme, respectively [8].

The assay was carried out at 25°C by following the formation of NADH at 340 nm in 50 mM triethanolamine buffer, pH 8.6, containing 5 mM EDTA, 10 mM arsenate, 0.3 mM NAD and 0.3 mM D-glyceraldehyde-3-phosphate. The reaction was started by addition of appropriate amount of the enzyme which gave the increase of absorbance at 340 nm not greater than 0.2/min.

In the “backward” reaction the assay mixture contained 50 mM triethanolamine buffer, pH 7.6, 1.1 mM ATP, 6.1 mM phosphoglycerate, 0.2 mM NADH, 0.9 mM EDTA, 2 mM MgSO₄ and 13 units/ml of 3-phosphoglycerate kinase. The change in absorbance at 340 nm was measured at 25°C.

Determination of NAD bound to the enzyme

The NAD content of the enzyme was determined as described by Seydoux et al. [9]. The firmly bound coenzyme was removed by charcoal treatment. The apoenzyme so prepared was indistinguishable from the apoenzyme prepared directly by CM-Sephadex chromatography (peak II).

Electrophoretic methods

Electrophoresis under non-denaturing conditions was performed in 7% polyacrylamide gel in 40 mM Tris/6 mM H₃BO₃/2 mM EDTA, pH 8.8. The gels were stained for protein with Coomassie R 250 Serva Blue. Glyceraldehyde-3-phosphate dehydrogenase activity was detected by immersing the
unstained gel into the assay solution containing 0.05 mg/ml phenazine methosulphate and 0.45 mg/ml nitroblue tetrazolium.

Disc electrophoresis in the presence of 0.1% sodium dodecyl sulphate was performed using gel reagents and buffer system as described by Weber & Osborne [10].

**Molecular weight determination**

The determination of molecular weight of glyceraldehyde-3-phosphate dehydrogenase was performed according to Andrews [11]. Molecular weight of the subunits was determined on polyacrylamide gel in the presence of sodium dodecyl sulphate.

**Determination of sulphhydryl groups**

The total number of sulphhydryl groups was measured by the method of Ellman [12] with 5,5'-dithiobis(2-nitrobenzoate) in 6 M urea. The reactivity of sulphhydryl groups in Tris or borate buffer was measured as described previously [13].

**Thermal inactivation of glyceraldehyde-3-phosphate dehydrogenase**

The enzyme samples (0.6 - 1.0 mg/ml) previously desalted against the extraction buffer, pH 8.6, were heated in a water bath at 55°C or 65°C. At time intervals aliquots were withdrawn and immediately assayed for activity at 25°C.

**Proteolytic inactivation of the enzyme**

The enzyme (0.8 mg/ml) in 1 mM 4-morpholineethanesulphonic acid - NaOH, 2 mM 2-mercaptoethanol, 5 mM EDTA and 30 mM CaCl₂, pH 8.2, was incubated with chymotrypsin or trypsin (0.18 mg/ml) at 35°C. Inactivation was monitored by assaying the residual enzyme activity at appropriate time intervals.

**RESULTS AND DISCUSSION**

**Purification of glyceraldehyde-3-phosphate dehydrogenase**

The procedure for isolation of glyceraldehyde-3-phosphate dehydrogenase from carp muscle outlined in Table 1 was developed on the basis of the
method described by Scheek & Slater [14] for the rabbit muscle enzyme, with some modifications. First, the extraction was prolonged to 20 h, because after 30 to 60 min, i.e. the time commonly used for mammalian muscle extraction, only about 60% of the total activity appeared in the solution.

The second modification concerned the heat denaturation step. As seen in Table 1, more than 50% of total protein was removed but the loss of glyceraldehyde-3-phosphate dehydrogenase activity was only about 13%.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Activity (units)</th>
<th>Activity (units/mg)</th>
<th>( \frac{A_{180}}{A_{260}} )</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>100</td>
<td>2,070</td>
<td>11,600</td>
<td>5.6</td>
<td></td>
<td>86.2</td>
</tr>
<tr>
<td>After heat treatment</td>
<td>90</td>
<td>920</td>
<td>10,000</td>
<td>10.9</td>
<td></td>
<td>67.7</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4 \ 0.60 - 0.83 \text{ sat.})</td>
<td>10</td>
<td>145</td>
<td>7,850</td>
<td>54.1</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>CM-Sepahdex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peak 1</td>
<td>12</td>
<td>8.7</td>
<td>1,650</td>
<td>190</td>
<td>1.35</td>
<td>58.2</td>
</tr>
<tr>
<td>peak 2</td>
<td>12</td>
<td>26.6</td>
<td>5,100</td>
<td>192</td>
<td>2.15</td>
<td></td>
</tr>
</tbody>
</table>

It should be emphasized that neither of the introduced modifications changed the essential properties of the purified enzyme. Their application was possible due to strong resistance of the carp enzyme to proteolysis and heat denaturation.

**Homogeneity and molecular properties**

The enzyme was eluted from CM-Sepahdex in two peaks (Fig. 1). Both peaks were well separated from glycolytic enzymes and haemoglobin and were homogeneous on rechromatography on CM-Sepahdex and Sephadex G-200. Electrophoresis in polyacrylamide gels in the presence or absence of sodium dodecyl sulphate yielded a single protein band. When the gels were stained for the enzyme activity, in each case a single band was observed at the position corresponding to that of the protein. The electrophoretic mobility of both active peaks was identical and did not differ from the mobility of the pig muscle enzyme.

Other properties of the two active peaks, their reactivity, the number of sulphhydryl groups and kinetic parameters were very similar and the only difference found was the amount of the bound coenzyme: the second peak
Fig. 1. Chromatography of glyceraldehyde-3-phosphate dehydrogenase from white muscle of carp on CM-Sephadex. The muscle extract prepared as described in the text was applied to CM-Sephadex column (3.2 x 15 cm) equilibrated with standard buffer, pH 6.5. The enzyme was eluted with a linear gradient of 0 - 0.35 M NaCl in standard buffer, pH 7.5 and a linear gradient of pH 7.5 - 8.5 in 0.35 M NaCl.

was practically coenzyme-free as determined enzymatically (less than 0.1 mol NAD/mol subunit) and from the $A_{280}/A_{260}$ ratio of 2.15. Therefore the protein from both peaks was pooled and after addition of NAD stored as holoenzyme in ammonium sulphate solution. However, the preparation in the form of apoenzyme was also very stable and at a concentration of about 10 mg/ml lost no activity during storage for two weeks at 4°C.

The extract of white as well as red muscle after 30 min and 20 h of extraction also gave each a single band when stained for the enzyme activity, thus only one unique form of glyceraldehyde-3-phosphate dehydrogenase exists in carp muscle, similarly as in muscle from other animals [1, 15].
It was found that, like all glyceraldehyde-3-phosphate dehydrogenases isolated from higher organisms, the carp muscle enzyme has the molecular weight of 144 000, is a tetramer and $M_r$ of the subunit is 36 000.

The total as well as specific activity of pure enzyme varied from preparation to preparation and correlated with the amount of activity units, which could be extracted from muscle. Similar variability was observed during purification of enolase from carp muscle and it was suggested to originate from the variable conditions of environment [16].

**Kinetic studies**

The pH optimum of glyceraldehyde-3-phosphate dehydrogenase from carp muscle was 8.6 in triethanolamine buffer used in the standard assay mixture. In the “backward” reaction, the optimum was at pH 7.4 - 7.6. Very similar pH-activity profiles have been obtained for the enzyme from other sources [1].

The Michaelis constants of the carp muscle enzyme were determined at 25°C in 50 mM triethanolamine buffer, pH 8.6 or 7.5, for the substrates of the “forward” or “backward” reaction, respectively. The results are summarized in Table 2.

<table>
<thead>
<tr>
<th>$K_m$ for</th>
<th>pH</th>
<th>Carp</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>8.6</td>
<td>70 μM</td>
<td>53 μM</td>
</tr>
<tr>
<td>d-Glyceraldehyde-3-phosphate</td>
<td>8.6</td>
<td>168 μM</td>
<td>210 μM</td>
</tr>
<tr>
<td>Arsenate</td>
<td>8.6</td>
<td>250 μM</td>
<td>120 μM</td>
</tr>
<tr>
<td>Phosphate</td>
<td>8.6</td>
<td>526 μM</td>
<td>345 μM</td>
</tr>
<tr>
<td>NADH</td>
<td>7.6</td>
<td>36 μM</td>
<td>32 μM</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>7.6</td>
<td>2.2 mM</td>
<td>1.62 mM</td>
</tr>
</tbody>
</table>

**Number and reactivity of sulphydryl groups**

The results, shown in Fig. 2, indicate that 5,5'-dithiobis-(2-nitrobenzoate) reacted rapidly with four equivalents of cysteine per 1 mole of native holo- or apoenzyme, with concomitant loss of the enzyme activity. A further increase of absorbance was extremely slow in Tris as well as in borate buffer. In 8 M urea three other cysteines per subunit, i.e. $15.6 \pm 0.3$ cysteines per tetramer, were found. This means that, with the exception of the
Fig. 2. Reaction of glyceraldehyde-3-phosphate dehydrogenase from carp and pig with 5,5'-dithiobis(2-nitrobenzoate). Concentration of the enzyme was 5 μM and that of 5,5'-dithiobis(2-nitrobenzoate) was 0.25 mM. The enzyme from carp in 50 mM Tris (△) and 40 mM borate (○) buffer, pH 8.3; the enzyme from pig in Tris (▲) and in borate (●) buffer, pH 8.3.

rapidly reacting catalytically active cysteines, the other cysteine residues are totally “buried”. A similar result has been reported for the sturgeon muscle enzyme [6]. In pig muscle glyceraldehyde-3-phosphate dehydrogenase, as shown for comparison, all four cysteines present in each subunit reacted in Tris buffer at different rates. In borate buffer two sulphydryl groups per subunit reacted rapidly, as reported previously [13].

**Heat inactivation**

The heat inactivation of glyceraldehyde-3-phosphate dehydrogenase from carp and pig muscle was studied over the temperature range of 55 - 65°C and at pH optimal for catalytic activity. At 55°C the enzyme from carp retained more than 90% of enzymatic activity after incubation for 100 min irrespective whether the coenzyme was presented or not (Fig. 3). The pig muscle enzyme kept at this temperature lost 60% (apoenzyme) or 40% (holoenzyme) of its activity after 80 min. At 65°C total inactivation of the pig muscle enzyme was observed as early as after 10 min. The rate
Fig. 3. Thermal stability at 55°C of glyceraldehyde-3-phosphate dehydrogenase from carp and pig. The protein (0.6 mg/ml) was dissolved in 1 mM 4-morpholineethanesulphonic acid - NaOH, 2 mM 2-mercaptoethanol, 5 mM EDTA buffer, pH 8.6. During incubation at 55°C aliquots were withdrawn and immediately assayed for enzymatic activity at 25°C. Solid line, and open symbols, the enzyme from carp muscle; dashed line, and closed symbols the enzyme from pig muscle. (○) and (●), holoenzyme (20 equivalents of NAD were added to the apoenzyme); (△) and (▲), apoenzyme.

The rate of inactivation by trypsin of carp muscle glyceraldehyde-3-phosphate dehydrogenase was very low as compared with that of the pig
muscle enzyme (Fig. 4). Similarly as in experiments on heat inactivation, there was no difference between the action of trypsin on the apo- and holoenzyme on the carp enzyme. On the other hand, the apoenzyme from pig muscle lost its activity completely within a few minutes. The presence of NAD markedly protected the pig muscle enzyme from trypsin inactivation.

The effect of chymotrypsin on both enzymes was very similar.

Although the basic molecular and catalytic properties of carp muscle glyceraldehyde-3-phosphate dehydrogenase resemble closely those of the

![Graph](image)

**Fig. 4.** Inactivation of glyceraldehyde-3-phosphate dehydrogenase from carp and pig muscle by trypsin. The reaction was performed in 1 mM 4-morpholineethanesulphonic acid - NaOH, 2 mM 2-mercaptoethanol, 5 mM EDTA buffer, pH 8.6. Preparations of enzymes (0.8 mg/ml) were incubated with trypsin (0.18 mg/ml) at 30°C. Aliquots removed from the incubation mixtures were tested for enzymatic activity. Solid line, enzyme from carp; dashed line, enzyme from pig muscle. (○) and (●), holoenzyme (20 equivalents of NAD were added to the apoenzyme). (△) and (▲), apoenzyme.
mammalian muscle enzymes, this protein exhibits some distinct features, i.e. high resistance toward heat and protease action and the lack of any protection by coenzyme against denaturation. The carp muscle enzyme differs markedly in these properties from fish enzymes studied by Leibman et al. [3, 4]. Studies on some bacterial enzymes led to the conclusion that a general correlation exists between the thermostability of proteins and their resistance to proteolysis and it has been suggested that thermostable proteins have a somewhat tighter or more folded structure [17]. The “buried” state of all sulphhydryl groups except the one which is essential for the enzymatic activity in carp muscle enzyme, seems to confirm this suggestion. Argos et al. [18] who studied thermal resistance of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase from mesophilic and thermophilic organisms found that substitution of some amino acids is responsible for the change of thermostability. By no means carp can be classified as a thermophilic organism, and although the glyceraldehyde-3-phosphate dehydrogenase from carp muscle differs in amino acid composition from the enzymes obtained from mammalian muscle (unpublished), there could be also other reasons for the observed resistance. The most important among them could be the necessity of adaptation of carp protein to function efficiently within a large range of temperature and other variables of the environment.

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REFERENCES

5. Ludovicy-Bungert, L. (1961) Isolement et properties de la 3-phosphoglyceraldehyde-


