ANNA MALARSKA, ANNA MARCINKOWSKA, MARIAN WOLNY and KORNEL NOWAK

THE SITES OF CHYMOTRYPSIN ACTION ON THE PIG MUSCLE GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE DURING LIMITED PROTEOLYSIS*

Department of Biochemistry, Institute of Biochemistry and Biophysics, Medical School, Chalubinskiego 10; 50-368 Wroclaw, Poland

1. The sites of chymotrypsin action on glyceraldehyde 3-phosphate dehydrogenase [p-glyceraldehyde 3-phosphate : DNA+ oxidoreductase (phosphorylating), EC 1.2.1.12] was established; limited proteolysis by chymotrypsin results in lowering of the phosphorolytic activity of the enzyme without affecting its oxidative activity.

2. The low-molecular fraction of the chymotrypsin digest separated by Sephadex G-100 chromatography, was fractionated on Bio-gels. Determination of the amino acid composition of the nine peptides isolated, and of their amino acid sequence, permitted to relate cleavage of Leu-64, Trp-84, Leu-109, Leu-141, Phe-165, Lys-212, Val-239, Leu-242, Leu-271 (or Phe-315) bonds in the enzyme to the decrease of the phosphorolytic activity

Glyceraldehyde 3-phosphate dehydrogenase [p-glyceraldehyde 3-phosphate: NAD+ oxidoreductase (phosphorylating), EC 1.2.1.12] catalyses oxidation of glyceraldehyde 3-phosphate with concomitant phosphorylation to 1,3-glyceroyl diphosphate. Krimsy & Racker (1963) and Cantau et al. (1968, 1970) found that the dehydrogenase isolated from rabbit muscle retains after 30-min hydrolysis with chymotrypsin its full oxidative activity whereas the phosphorolytic activity decreases gradually in the course of hydrolysis.

This paper forms a part of studies on the structural changes of GPDH caused by limited proteolysis with chymotrypsin, associated with the changes in the enzymatic activities. Preliminary communications have

* This work was performed within the Project of the Ministry of Higher Education, Science and Technology, no. R 1.9.0.1.07 and 01.02.

† Abbreviations used: GPDH, glyceraldehyde 3-phosphate dehydrogenase; SDS, sodium dodecyl sulphate; PMSF, phenylmethylsulphonyl fluoride.

[383]

**MATERIALS AND METHODS**

Glyceraldehyde 3-phosphate dehydrogenase was isolated from pig muscle according to Elődi & Szőrenyi (1956); instead of several-fold crystallization, the enzyme was purified to homogeneity by CM-Sephadex chromatography (Nowak et al., 1976). Bound NAD was removed with Norit (Banaś et al., 1976), and the enzyme was used on the same day. The A_{280}/A_{260} coefficient was 1.90.

Glyceraldehyde 3-phosphate was prepared according to Szewczuk et al. (1961). Chymotrypsin was from Koch-Light (Colnbrook, Bucks, England); its activity with N-benzoyl-L-tyrosine ethyl ester (Serva, Heidelberg, F.R.G.) was 45 units/mg. D,L-Glyceraldehyde was from Fluka A.G. (Buchs S.G., Switzerland), p-nitrophenylacetate, monothioglycerol and phenylmethylsulphonyl fluoride from Sigma Co. (St. Louis, Mo., U.S.A.), and NAD from Reanal (Budapest, Hungary). Sephadex G-25 and G-100 were from Pharmacia (Uppsala, Sweden), Bio-Gel P2, P4, P6 from Bio-Rad Laboratories (Richmond, Calif., U.S.A.), cellulose MN 300 from Machery Nagel Co. (Düren, F.R.G.), polyamide plates from Schleicher & Schüll GmbH (Dassel, F.R.G.), Norit from Serva (Heidelberg, F.R.G.). The reagents for analysis of the amino acid sequence were purified according to Edman & Begg (1967). Other reagents were of analytical purity.

**Determination of glyceraldehyde 3-phosphate dehydrogenase activity.** The oxidative and phosphorolytic activities, as well as formation of 1-arseno-3-glyceroyl phosphate (arsenolytic activity) were calculated from changes in absorbance at 340 nm at 25°C, measured in various buffer systems (Banaś et al., 1976). The oxidative activity was measured in 40 mm-triethanolamine buffer, pH 8.6, containing 4 mM-EDTA, 25 mM-D,L-glyceraldehyde, 6 mM-monothioglycerol and 0.3 mM-NAD. The phosphorolytic activity was measured in the same buffer system supplemented with 4 mM-phosphate. The solution for determination of the arsenolytic activity contained: 50 mM-triethanolamine buffer, pH 8.6, 5 mM-EDTA, 10 mM-arsenate, 0.75 mM-NAD and 0.4 mM-D-glyceraldehyde 3-phosphate.

The esterase activity of the enzyme was determined in 10 mM-Tris/HCl, pH 8.3, containing 1 mM-EDTA, with 0.2 mM-p-nitrophenylacetate as substrate. The amount of p-nitrophenol formed was calculated from changes in A_{400} at 20°C. The amount of acyl enzyme formed during the first, rapid step of the reaction was calculated by extrapolation to zero time of the deacylation step, occurring according to steady-state kinetics (Park et al., 1961). The activity was measured using Unicam SP-800 spectrophotometer.
Digestion with chymotrypsin. GPDH was digested at 30°C in 50 mM-triethanolamine/1 mM-EDTA buffer, pH 7.9, or, when the reaction products were to be used for the amino acid sequence analysis, in 50 mM-NH$_4$HCO$_3$, pH 8.5. With either buffer, the effect of chymotrypsin on the activities of GPDH was the same. Concentration of GPDH was 10 - 15 mg/ml, and the amount of chymotrypsin added was 20 - 40 μg per 1 mg of the dehydrogenase. During incubation, appropriate portions of the digest were withdrawn, and oxidative, phosphorolytic and esterase activities were determined. For the assay of the arsenolytic activity, the withdrawn aliquots were diluted with 3 mM-NAD solution (1 : 100, v/v) to stop further action of chymotrypsin on GPDH.

After 30 min of digestion, when the arsenolytic and phosphorolytic activities dropped to about 30% of the initial values, hydrolysis was stopped by the addition of phenylmethylsulphonyl fluoride to a final concentration of 0.2 mM.

Polyacrylamide-gel electrophoresis. The electrophoresis was run in the presence of SDS, and the gels were stained with Coomassie blue R-250 according to Fairbanks et al. (1971).

Column chromatography. The product obtained by limited proteolysis of GPDH was separated on a Sephadex G-100 column (2 x 120 cm) in 0.4% acid ammonium carbonate. The elution was followed by measuring absorbance at 280 nm. The low-molecular fraction separated was then fractionated successively on four columns (0.9 x 120 cm) of Bio-Gel, used in the following order (column number being denoted by Roman numeral): (a), I, P6; II, P4; III and IV, P2; (b), I and II, P6; III and IV, P4. The peptides were eluted with 0.4% acid ammonium carbonate in 10% isopropanol solution. The elution was controlled by measuring absorbance at 215 nm.

Electrochromatography. The fractions obtained from Bio-Gel columns were further separated by electrophrophography on Whatman 3MM paper or on cellulose-coated glass plates. Paper electrophrophography was run in the first dimension by high-voltage electrophoresis in 0.12 M-pyridine/acetate buffer, pH 3.5, for 2 h at 50 - 75 V/cm in the Michl chamber (Michl, 1951), and in the second dimension by descending chromatography in the solvent system: n-butanol/acetic acid/water (12 : 3 : 5, by vol.) according to Katz et al. (1959). For separation of peptides on cellulose plates, the pyridine/acetic acid/acetone/water (1 : 2 : 8 : 40, by vol.) system, pH 4.4, was used for high-voltage electrophoresis, and in the second dimension the chromatograms were run in n-butanol/acetic acid/water/pyridine (15 : 3 : 12 : 10, by vol.), (Heiland et al., 1976). For preparative purposes, the electrochromatograms were developed with 0.1% ninhydrin solution in acetone. The peptides were extracted with 50% acetic acid and the solvent was removed by evaporation. For analytical purposes, the chromatograms were sprayed additionally with Pauly reagent (Easley et al., 1969).
Determination of N-terminal amino acids in peptides. The N-terminal amino acids were determined by the dansyl method of Gray (1967) and were identified by chromatography on polyamide plates (3.6 × 5 cm). The chromatograms were developed in the first dimension with 1.5% formic acid; in the second, with 10% acetic acid in benzene, and anew in the second dimension with a mixture of ethyl acetate, methanol and acetic acid (20 : 1 : 1, by vol.).

Analysis of the amino acid composition of peptides. This was determined after 18-h hydrolysis of the peptides at 105°C in 6 M-HCl. After hydrolysis, the samples were dansylated according to Gray (1967), and the dansyl derivatives of amino acids were identified on polyamide plates under the conditions used for determination of N-terminal amino acids.

Amino acid sequence of peptides. The isolated peptides were degraded by the method of Edman and dansylated (Gray, 1967); the dansyl derivatives of amino acids were identified by chromatography on polyamide plates.

RESULTS AND DISCUSSION

Glyceraldehyde 3-phosphate dehydrogenase is one of the few high-molecular-weight enzymes of well known primary and secondary structure. The full amino acid sequence of this dehydrogenase from lobster muscle was published by Davidson et al. (1967), and of the pig muscle enzyme by Harris & Perham (1968). The amino acid sequence of GPDH from yeast and bacteria is also known (Jones & Harris, 1972, and Biesecker et al., 1977, respectively). In 1976 Nowak et al. reported partial amino acid sequence of the enzyme from human muscle. The three-dimensional structure of GPDH from lobster (Buehner et al., 1974) and Bacillus stearothermophilus (Biesecker et al., 1977) was established on the basis of crystallographic studies. Moreover, the amino acids involved in the active centre, responsible for binding of subunits, as well as the amino acids located on the surface of the molecule, were identified (Olsen et al., 1975). The studies on the structure-function relationship were reviewed recently (Wolny, 1976; Nargadova, 1977). Observations of considerable interest were made by Krimsky & Racker (1963) and Cantau et al. (1968, 1970) on changes in GPDH activity during degradation of the enzyme with chymotrypsin. It was found that total oxidative activity of the dehydrogenase, and 30% of its phosphorolytic activity were retained even when the molecular weight was decreased to 60% of the initial value. These results prompted us to study in more detail changes in GPDH molecule after 30-min digestion with chymotrypsin.

Changes in the arsenolytic, oxidative, phosphorolytic and esterase activities of GPDH from pig muscle during digestion with chymotrypsin, are presented in Fig. 1. As can be seen, during the first 30 min of digestion, both the
arsenolytic and phosphorolytic activity declined sharply in an almost parallel manner, with pseudo-first-order kinetics, and $k^{\text{obs}} = 0.045 \text{ min}^{-1}$. At the same time, the oxidative activity was practically unchanged.

Similar changes were observed with the rabbit muscle enzyme (Krinsky & Racker, 1963; Cantau et al., 1968, 1970) the structure of which is almost identical with that of the pig muscle enzyme (Harris, 1970).

The changes in esterase activity showed a different pattern (Fig. 1); deacylation, which occurred at a slower rate and was linear with time, decreased slightly up to 60 min of incubation, whereas the amount of the acyl enzyme formed in the first step of the reaction, changed markedly: from 3.1 mol of acyl groups bound per mole of protein at 0-time to 2.50 mol after 30 min and 1.3 mol after 60 min of proteolysis. Thus, the deacylation rate was not limited by formation of the acyl enzyme. The results of kinetic studies of Lindquist & Cordes (1968) suggested that the same mechanism is involved both in the acylation and deacylation steps, with participation of the same amino acid residues in the active centre. Since the rate constant of the acylation reaction is higher by two orders of magnitude than the rate constant of deacylation, the decrease in the reaction rate at the first step due to proteolysis, does not affect the second step. A similar effect was observed with NAD, while adenyl nucleotides and photooxidation inhibit only the deacylation step (Francis et al., 1971).

The mixture digested for 30 min, in which the arsenolytic and phosphorolytic activities dropped to about 40% of the control value, was separated by Sephadex G-100 chromatography into three fractions showing absorbance at
280 nm (Fig. 2). The first pooled fraction which contained high-molecular-weight proteins, was not homogeneous despite the symmetrical shape of the elution profile, and the individual successive fractions, collected from the Sephadex column differed in the enzymatic activities and electrophoretic mobility in the presence of SDS. Since modifications of the conditions of chromatography did not improve the resolution, strong interaction between

![Graph showing chromatography](image)

Fig. 2. Sephadex G-100 column chromatography of the 30-min digest of glyceraldehyde 3-phosphate dehydrogenase with chymotrypsin. The column (2 x 120 cm) was eluted with 0.4 M NH₄HCO₃; fraction volume was 2 ml, elution rate 18 ml/h.

the peptides constituting this fraction could be anticipated. The second fraction contained only chymotrypsin; the fraction showed proteolytic activity when chromatographed without previous addition of PMSF. The third fraction contained low-molecular-weight compounds.

Krimsky & Racker (1963) subjected the digested protein to ultrafiltration in a sucrose gradient and obtained a very diffuse protein peak, also non-homogeneous with respect to the catalytic properties. They concluded, therefore, that the large, partly digested protein fragments are joined by non-covalent bonds with various low-molecular fragments.

It is difficult to explain the divergence between our results and those of Cantau et al. (1968) who on Sephadex G-100 column obtained a fraction of mol. wt. of about 60,000, almost completely devoid of phosphorolytic activity and with fully preserved oxidative activity.

To gain more knowledge on the changes of GPDH during limited digestion leading to changes in the enzymatic activities, we have focused our attention on the low-molecular peptides formed during digestion. Identification of these peptides allowed to establish the bonds split by chymotrypsin. The Sephadex G-100 fraction III (Fig. 2) which contained the low-molecular peptides, was resolved successively on the columns with Bio-Gel P2, P4 and P6 and by high-voltage electrophoresis on cellulose
Scheme 1. Separation of low-molecular peptides of glyceraldehyde 3-phosphate dehydrogenase after limited proteolysis with chymotrypsin. HVE, high-voltage electrophoresis.
Table 1

Partial sequence of low-molecular peptides isolated from glyceraldehyde 3-phosphate dehydrogenase of pig muscle after limited digestion with chymotrypsin

<table>
<thead>
<tr>
<th>Designation</th>
<th>N-Terminal sequence</th>
<th>Position in the GPDH sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Gly-Ile-Val-Glu-</td>
<td>166-169</td>
</tr>
<tr>
<td>C2</td>
<td>Lys-Gly-Gly-Ala-</td>
<td>110-113</td>
</tr>
<tr>
<td>C3</td>
<td>Gly-Asp-Ala-Gly-</td>
<td>85-88</td>
</tr>
<tr>
<td>C4</td>
<td>Thr-Cys-Arg-Leu-</td>
<td>243-246</td>
</tr>
<tr>
<td>C5</td>
<td>Lys-Ile-Val-Ser-</td>
<td>142-145</td>
</tr>
<tr>
<td>C6</td>
<td>Val-Ile-Asp-Gly-</td>
<td>65-68</td>
</tr>
<tr>
<td>C7</td>
<td>Ala-Val-Gly-Lys-</td>
<td>213-216</td>
</tr>
<tr>
<td>C8</td>
<td>Gly-Tyr-</td>
<td>272-273 or 316-317</td>
</tr>
<tr>
<td>C9</td>
<td>Val-Asp-Leu-</td>
<td>240-242</td>
</tr>
</tbody>
</table>

plates. Nine peptides were obtained (Scheme 1), and their purity was confirmed by finding a single N-terminal amino acid in each peptide (Gray, 1967). The amino acid composition of these peptides, and the sequence of four amino acids from the N-terminal were determined (Table 1). On this basis, the position of the isolated peptides in pig GPDH was established, and the probable sites of chymotrypsin action were identified (Fig. 3).

Fig. 3. The sites of chymotrypsin action on glyceraldehyde 3-phosphate dehydrogenase from pig muscle. For conditions of limited proteolysis see Materials and Methods. The bonds split by chymotrypsin are indicated by arrows, and the amino acids of the active centre (Olsen et al., 1975), by asterisks.
Digestion at Leu-64, Try-84, Leu-109, Lys-212 and Leu-271, that is cleavage of peptides C6, C3, C2, C7 and C8 (Scheme 1) probably does not cause significant changes in the catalytic centre as judged from their position in the tertiary structure (Olsen et al., 1975). On the other hand, splitting of peptide bonds at Phe-165 and Leu-141 and cleavage of the peptides C1 and C5, can change the conformation of the catalytic site, because Ser-148 and Cys-149, the amino acids directly involved in the reaction catalysed, are located close to the digestion site at Leu-141. Ser-148 binds inorganic phosphate in the active centre of GPDH from Bacillus stearothermophilus (Biesecker et al., 1977), and Cys-149 binds the substrate. Thus, digestion at Leu-141 can alter the position of Ser-148 and Cys-149 or, with concomitant splitting at the closely situated Phe-165, can even result in removal of these amino acids from the catalytic centre. Cleavage at Phe-165 may affect in a similar way the position of His-176, a cation residue in the catalytic centre (Buehner et al., 1974). These changes do not necessarily affect all subunits in a GPDH molecule.

Digestion of peptide bonds at Val-239 and Leu-242 lowers probably stability of the linkage between the subunits because Asp-241 of peptide C9 and Thr-243 and Arg-245 of peptide C4 are located in the plane at which subunits are joined (Buehner et al., 1974).

The dipeptide C8: Gly-Tyr could result from splitting of the bond either at Leu-271 or at Phe-315. If it is the 272-273 dipeptide, then, as mentioned previously, its cleavage should not cause significant changes in the catalytic centre. If, however, it is the 316-317 dipeptide, which contains Tyr-317, then its cleavage must lead to changes in the structure of the catalytic centre of GPDH (Olsen et al., 1975).

Assuming a close similarity of the three-dimensional structure between GPDH from lobster and pig, the action of chymotrypsin on the native GPDH from pig muscle would be restricted to those peptide bonds which are located on the surface of the tetramer, with the exception of the bonds at Val-239 and Leu-242. This suggests that during the first 30 min of digestion, GPDH does not dissociate into subunits. Changes in the conformation of active centre caused by the destruction of some peptide bonds located on the surface of tetramer did not hinder the acylation step, thus the reactivity of Cys-149 was preserved; however, the enzyme molecule was unable to carry out the deacylation step, i.e. the transfer of the acyl group from Cys-149 to the suitable acceptor (arsenate or phosphate). Further studies on the peptides composing the first fraction of the analysed chymotrypsin digest should be carried out to establish which part of the GPDH molecule is responsible for the deacylation step.
REFERENCES


MIEJSCA DZIŁANIA CHYMOTRYPSYNY NA DEHYDROGENAZĘ ALDEHYDU 3-FOSFOGLICERYNOWEGO Z MIĘŚNI WIERZOWYCH PODCZAS OGRANICZONEJ PROTEOLIZY

Streszczenie

1. Ustalono miejsca działania chymotrypsyny na dehydrogenazę aldehydu 3-fosfoglicerynowego [δ-aldehyd 3-fosfoglicerynowy: NAD oksydoreduktaza (fosforylująca), EC 1.2.1.12] z mięśni wieprzowych; ograniczona proteoliza chymotrypsyną powoduje obniżenie aktywności fosforolitycznej enzymu bez obniżenia aktywności utleniającej.


Received 22 August, 1979;
Revised 31 March, 1980.