LOCALIZATION OF ARGINYL RESIDUES MODIFIED
WITH BUTANEDIONE IN GLYCERALDEHYDE-3-PHOSPHATE
DEHYDROGENASE FROM PIG MUSCLE*

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Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from pig muscle was
inactivated by incubation with butanedione in triethanolamine buffer, pH 8.3.
The inactivation was reversible after short treatment with butanedione; it became
irreversible after 12-15 h, with a concomitant loss of two arginy1 residues per
subunit. The modified enzyme was digested with TPCK-trypsin and the peptides
were purified by chromatography and electrophorography.

Two new peptides were obtained as the result of modification. From their
partially determined sequence the modified arginy1 residues were identified as
Arg-13 and Arg-231 in the primary structure of pig muscle enzyme.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.12), (GPDH) is com-
posed of four identical subunits of molecular weight 36 500 (Harrington &
Karr, 1965). The amino acid sequence of the enzyme from pig muscle
(Harris & Perham, 1968), lobster (Davidson et al., 1967), human muscle
(Banaś et al., 1981), yeast (Jones & Harris, 1972), Bacillus stearothermophilus
(Biesecker et al., 1977) and from Thermus aquaticus (Hocking & Harris, 1980)
is known. The three-dimensional structure of lobster muscle (Buehner et al.,
1974) and Bacillus stearothermophilus enzymes (Biesecker et al., 1977) has
been determined. Two arginy1 residues, Arg-10 and Arg-231, the positions
of which in the known sequence are conserved, were shown to be involved
in coenzyme- or substrate binding, respectively, and to be important for
the catalytic mechanism (Buehner et al., 1974; Biesecker et al., 1977).
Nagradova & Asryants (1975) and Nagradova et al. (1976) showed that

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glyceraldehyde-3-phosphate dehydrogenase from yeast and from rat skeletal muscle were inactivated in the presence of butanedione; the inactivation was accompanied by modification of two arginy1 residues per subunit. However, it was deduced from the kinetics of inactivation that only a single arginy1 residue was essential for activity. Since it was shown that the modified residue was involved in formation of the substrate-binding site, the authors suggested that it is Arg-231 which is essential for the activity (Nagradova et al., 1978).

Similar kinetic effects of modification of pig muscle enzyme by butanedione were observed in our laboratory: the estimated reaction order with respect to inhibitor concentration suggests an approximately equimolar inhibitor-enzyme interaction (A. Marcinkowska and M. Wolny, unpublished).

According to Riordan (1973) the reaction between arginine and butanedione proceeds in several stages. The first step is reversible and the complex formed is unstable in alkaline solution. Further steps leading to a stable compound are slow in slightly alkaline solution but rapid in acid solutions. The resistance of the final complex to various chemical treatments including acid hydrolysis followed by amino acid analysis were first reported by Riordan (1973) and confirmed by other authors (Ehrlich & Colman, 1977). Therefore it could be assumed that the peptide bond formed by the modified arginy1 residue will be resistant to the action of trypsin.

MATERIALS AND METHODS

Reagents. D-Glyceraldehyde-3-phosphate was prepared according to the method of Szewczuk et al. (1961). Bio-Gel and polyacrylamide gels were purchased from Bio-Rad Laboratories (Richmond, California, U.S.A.). Cel lulose - pulver MN 300 for thin-layer chromatography was from Macherey Nagel (Düren, F.R.G.). TLC - Ready Plastic Sheets F 1700 Micro-Polyamide were from Schleicher & Schüll GmbH (Dassel, F.R.G.). 2,3-Butanedione was from Koch-Light Laboratories Ltd. (Colnbrook, England). TPCK-Trypsin (trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone) was from Worthington Chemical Corp. (Freehold, N.Y., U.S.A.). Reagents for the amino acid sequence analysis were purified as described by Edman & Begg (1967). All other chemicals used were of analytical grade.

Isolation of glyceraldehyde-3-phosphate dehydrogenase. The enzyme was prepared from pig muscle as described by Elődi & Szörenyi (1956) with the modification of Bloch et al. (1971). Enzymatic activity was assayed in the presence of 0.3 mm glyceraldehyde-3-phosphate, 0.3 mm NAD\(^+\), 3 mm-sodium arsenate and 10 mm-EDTA in 0.05 m-triethanolamine buffer, pH 8.6, at 25°C.

Concentration of protein was measured spectrophotometrically, \(\varepsilon_{280}^0 = 1.02\). Visible and ultraviolet spectra were recorded with a Unicam SP 800 spectrophotometer.
Modification of arginine residues with 2,3-butanedione. The crystals of glyceraldehyde-3-phosphate dehydrogenase from pig muscle were collected by centrifugation and dissolved in 1 mm-dithioerythritol containing 1 mm-EDTA and 5 mm-Tris/HCl buffer, pH 7.6. This mixture was left for 30 min till complete reduction of thiol groups. The solution obtained was passed through a Sephadex G-25 column (2 x 50 cm), equilibrated with the 0.05 m-triethanolamine buffer containing 1 mm-EDTA, pH 8.3. The eluted protein was added to freshly prepared butanedione solution in the same buffer. The final concentration of the enzyme was 20 μM, and of butanedione 35 mM. To stop the reaction and stabilize the reaction product, HCl was added to the final concentration of 0.6 M. The solution was allowed to stand for 1 h, then it was dialysed against water and next against 0.5% NH₄HCO₃ and lyophilized.

Digestion with TPCK-Trypsin. Tryptic digestion of GPDH, after its oxidation by performic acid, was performed in 0.5% ammonium bicarbonate buffer, pH 8.9, for 14 h, at 37°C. The enzyme to trypsin ratio was 100:1 by weight. The digest obtained was freeze-dried.

Isolation of peptides. The lyophilized mixture of peptides (50 mg) obtained after digestion with trypsin was redissolved in 0.5% NH₄HCO₃ containing 10% isopropyl alcohol, and was applied to four columns (1 x 150 cm) connected together with peristaltic pumps; two of them were filled with Bio-Gel P-60 (100 - 200 mesh), the remaining two with Bio-Gel P-10 (100 - 200 mesh). The columns were eluted with 0.5% NH₄HCO₃ containing 10% isopropyl alcohol, at a flow rate of 4.8 ml/h at room temperature. The absorbance of the fraction obtained was monitored at 215 nm (Unicam SP-500) and the appropriate fractions were pooled and lyophilized.

The pooled fractions containing arginine residues (IV, V, VI) were chromatographed on two connected columns (1 x 150 cm) filled with Bio-Gel P-6 (100 - 200 mesh) and with Bio-Gel P-2 (100 - 200 mesh), equilibrated with 0.5% NH₄HCO₃ containing 10% isopropyl alcohol, at a flow rate of 4.8 ml/h at room temperature. The absorbance of the fractions was monitored at 215 nm. The obtained fractions were lyophilized and analytical and preparative maps were made.

High voltage electrophoresis and chromatography of obtained peptides. Analytical. The mixture of peptides (200 μg) was dissolved in 10 μl of deionized water and spotted onto the cellulose MN 300 thin-layer plates. In the first dimension, electrophoresis was run at pH 4.4 in the pyridine/acetic acid/acetonewater system (1:2:8:40 by vol.) at 600 V and 28 - 32 mA for 2 h at 15°C. The plate was dried and thin-layer chromatography was run in the second dimension using the buffer system containing n-butanol/acetic acid/water/pyridine (15:3:12:10, by vol.). The peptides were detected with ninhydrin and arginine residues with phenanthrenequinone (Easley et al., 1969).

Preparative. For preparative purposes the mixture of peptides was separated by electrophoresis in pyridine/acetic acid/water (1:10:89, by vol.)
of pH 3.5 at 55 V/cm, on Whatman No. 3 MM papers, followed by descending chromatography in n-butanol/acetic acid/water (12:3:5, by vol.). The dried chromatogram was sprayed with 0.05% ninhydrin in n-butyl alcohol and heated at 60°C for 10 - 15 min to visualize the spots. The peptides were eluted with 10% solution of acetic acid, the respective extracts from four chromatograms were pooled and the solvent mixture was evaporated under reduced pressure in a rotary evaporator at 40°C. The residues were dissolved in deionized water and freeze-dried.

Amino acid and N-terminal analysis. Amino acid composition of peptides was determined, after acid hydrolysis (6 m-HCl, 24, 48 and 72 h, 105°C, in vacuum) on a AAA-881 amino acid analyser (Microtechna, Praha) in the two-column system.

N-Terminal analysis of the purified peptides was performed by the dansyl procedure of Hartley (1970). The dansylated amino acids were identified on micropolyamide sheets (3.6 × 5 cm) in the systems I, II and III of Chen (1976): system I — 1.5% formic acid in water; system II — 10% acetic acid in benzene; system III — ethyl acetate/methanol/acetic acid (20:1:1, by vol.).

Micro dansyl-Edman technique. The micro dansyl-Edman procedure of Peterson et al. (1972) was used with the modifications described by Nowak et al. (1977) and the dansylated amino acids were identified as described above.

RESULTS AND DISCUSSION

Modification of arginine residues of GPDH from pig muscle leads to inactivation which is highly reversible if the reaction mixture is passed through Sephadex G-25 column. Also strong dilution of the reaction mixture (more than 100-fold) leads after some time to almost complete reactivation. However, the reactivation takes place only when the contact between enzyme and butanedione was of relatively short duration. After 12 - 15 h of treatment the inactivation is irreversible (A. Marcinkowska and M. Wolny, unpublished).

To find out whether the irreversible inactivation was due to transformation of the unstable complex into a stable compound, or to modification of further Arg residues in the enzyme molecule, glyceraldehyde-3-phosphate dehydrogenase from pig muscle was modified by three parallel procedures:

Sample A, control: immediately after mixing of butanedione with the enzyme, the reaction was terminated by acidification.

Sample B: the reaction was interrupted after 10 min. After that time the enzyme was inactivated by 85% and the inactivation was reversible.

Sample C: the mixture of enzyme and butanedione was left for 20 h in darkness. Full, completely irreversible inactivation took place.

Further procedures were identical for all three samples: after dialysis, lyophilization, oxidation with performic acid, trypsin digestion and Bio-Gel
column chromatography, peptide maps were obtained (see Materials and Methods and Scheme 1). As it was expected, the number and mobility of peptides varied. The most important are the differences in the number of Arg-containing peptides: from the ten arginyl peptides in sample A, that is the number consistent with the Arg content in native enzyme, after irreversible modification their number was reduced to eight in sample C.

![Scheme 1. Isolation from glyceraldehyde-3-phosphate dehydrogenase from pig muscle of tryptic peptides containing an arginine-residue.](image)

![Fig. 1. Chromatography of the tryptic digest of GPDH from pig muscle after 10 min modification with 2,3-butanedione (procedure B). The digest was separated by gel filtration in the four-column system: two Bio-Gel P-60 and two Bio-Gel P-10. The columns were eluted with 0.5\% \( \text{NH}_4\text{HCO}_3 \) containing 10\% isopropanol. flow rate 4.8 ml/h at room temperature.](image)
Analysis of the amino acid composition also showed a decrease in the number of Arg from 10.1 in sample A to 7.8 in sample C. The content of other amino acids was unchanged.

The mixture of trypsin peptides was resolved chromatographically on Bio-Gel P-60 and P-10 columns (Fig. 1). Analysis of particular peaks by means of analytical peptide maps indicated that the Arg-containing peptides were present only in fractions IV, V and VI, and, therefore, only these fractions were subsequently purified by chromatography in Bio-Gel P-6 and P-2 columns, as shown in Fig. 2. After separation of the peptide mixture the number of detected arginine peptides in sample B was raised to 12. This means that two new peptides have been formed (see Table 1.

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**Fig. 2.** Separation of fraction B-V. a. The pooled fractions marked as peak V in Fig. 1 were separated on Bio-Gel P-6 column. b. The peak B-V-2 pooled as indicated in Fig. 2a was separated on Bio-Gel P-2 column.
and Fig. 3) as a result of modification of arginyl residues by butanedione: one peptide instead of R-8 and R-9 and another instead of R-2 and R-3. Determination of N-terminal sequence and amino acid composition of these peptides enabled us to localize them in the known amino acid sequence (Harris & Perham, 1968), as shown in Table 1. Peptide R-8 is terminated by Arg-231, the participation of which in the active centre was demonstrated by Buehner et al. (1974). Peptide R-2 contained Arg-13, whereas Buehner postulated participation of Arg-10 in the active centre for the lobster enzyme. Thus, an apparent discrepancy seems to exist between our results and the active center model provided by Buehner et al. (1974). A helix structure was demonstrated for the peptide chain section between amino acid residues No. 6 and No. 13 (...-Asn-Gly-Phe-Gly-Arg-Ile-Gly-Arg-...), both for the enzyme from lobster muscle (Buehner et al., 1974) and Bacillus stearothermophilus (Biesecker et al., 1977) Arg-10 and Arg-13 are thus separated from one another by one helix jump. The three-dimensional structure of the pig muscle enzyme is not known till now. Although the sequence of this section of peptide chain (No. 6 to No. 13) is identical in the lobster and pig muscle enzymes, it can not be ruled out that the secondary structures are different in the two enzymes, which may cause also a difference in the accessibility of the two arginines.
Table 1
Peptides containing arginine residue, isolated from the tryptic digest of oxidized glyceraldehyde-3-phosphate dehydrogenase from pig muscle

In all fragments the degradation of peptides was carried out using the direct method of Edman as modified by Peterson et al. (1972) and the dansyl-Edman technique, and the dansylated amino acids were identified as described in Methods. The sequences obtained are in good agreement with the data of Harris & Perham (1968).

<table>
<thead>
<tr>
<th>Fragments</th>
<th>N-Terminal amino acid</th>
<th>Number of amino acid residues</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>Val</td>
<td>8</td>
<td>3 - 10</td>
</tr>
<tr>
<td>R-2</td>
<td>Ile</td>
<td>3</td>
<td>11 - 13</td>
</tr>
<tr>
<td>R-3</td>
<td>Leu</td>
<td>4</td>
<td>14 - 17</td>
</tr>
<tr>
<td>R-4</td>
<td>Ala</td>
<td>8</td>
<td>70 - 77</td>
</tr>
<tr>
<td>R-5</td>
<td>Arg</td>
<td>1</td>
<td>115</td>
</tr>
<tr>
<td>R-6</td>
<td>Leu</td>
<td>3</td>
<td>192 - 194</td>
</tr>
<tr>
<td>R-7</td>
<td>Asp</td>
<td>3</td>
<td>195 - 197</td>
</tr>
<tr>
<td>R-8</td>
<td>Leu</td>
<td>7</td>
<td>225 - 231</td>
</tr>
<tr>
<td>R-9</td>
<td>Val</td>
<td>14</td>
<td>232 - 245</td>
</tr>
<tr>
<td>R-10</td>
<td>Leu</td>
<td>14</td>
<td>307 - 320</td>
</tr>
<tr>
<td>Newly-formed peptides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-2 + R-3</td>
<td>Ile</td>
<td>7</td>
<td>11 - 17</td>
</tr>
<tr>
<td>R-8 + R-9</td>
<td>Leu</td>
<td>21</td>
<td>225 - 245</td>
</tr>
</tbody>
</table>

The appearance of new peptides in sample B apart from the peptides existing in sample A provides evidence that even after a short time of the reaction with butanedione leading to reversible inactivation, both arginines: 13 and 231 are parallely, but not completely modified. Since sample B after modification was found to contain only 15% of the activity, thus, assuming a linear relationship between the activity and extent of Arg modification, the ratio of modified to native Arg should be about 6:1. Consequently, the ratio between peptides “formed” and “vanishing” should be the same, however, no such large difference was found. This indicates either equal or similar modification rates of both Arg residues. Thus, it could be suggested that in each dimer only one Arg-13 and one Arg-231 are simultaneously modified in the same or in the different subunits of a given dimer, which leads to reversible inactivation of whole enzyme molecule. For the irreversible inactivation, the modification of both Arg residues would be required in all four subunits.

After the manuscript had been prepared, the paper of Vospelnikova et al. (1982) appeared. They localized the two modified arginyl residues in rat muscle GPDH: one in position 134, and the second in the peptide containing residues 301 to 315. The comparison of their results with ours is difficult, since the whole amino acid sequence of rat muscle enzyme is not known. Arginyl residues in these positions are not present in the pig muscle nor in other known glyceraldehyde-3-phosphate dehydrogenases.
REFERENCES


POŁOŻENIE RESZT ARGINYLOWYCH
W DEHYDROGENAZIE ALDEHYDU 3-FOSFOGLICYERYNOWEGO
MODYFIKOWANYCH BUTANDIONEM

Streszczenie

Dehydrogenaza aldehydu 3-fosfolicyerynowego (EC 1.2.1.12) z mięśni wieprza ulega inaktywacji po inkubacji z butandionem w buforze trójetanoloaminowym, pH 8.3. Inaktywacja, odwracalna po krótkim działaniu butandionu, staje się nieodwracalna po 12-15 godzinach z równoległym ubytkiem dwóch reszt arginylowych na podjednostkę. Modyfikowany enzym trawiono TPKK-trypsyną, peptydy oczyszczono chromatograficznie i elektrochromatograficznie.

W wyniku modyfikacji uzyskano dwa nowe peptydy. Na podstawie częściowo oznaczonej sekwencji, zmodyfikowane reszty arginylowe zidentyfikowano jako Arg-13 i Arg-231 w strukturze pierwotnej enzymu z mięśni, wieprza.

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