GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM HUMAN MUSCLE: CLEAVAGE OF TRYPTOPHANYL PEPTIDES WITH O-IODOSOBENZOIC ACID *

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The new procedure of protein fragmentation with o-iodosobenzoic acid (Mahoney & Hermodson, 1979; Biochemistry, 18, 3810 - 3814) was used to split three tryptophanyl peptide bonds in glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from human skeletal muscle. The mixture obtained was separated into four homogeneous peptides by a one-step chromatographic procedure. High specificity of the cleavage reaction was proved by the amino acid analysis and determination of the N-terminal sequence of the peptide products. Both specificity and high yield obtained by this procedure confirmed its utility in the establishment of the protein primary structure.

Several methods have been proposed for cleavage of polypeptide chains at tryptophanyl peptide bonds (Needleman, 1970; Shechter et al., 1976; Ozons & Gerard, 1977). Most of these procedures involved either oxidative bromination or oxidative chlorination. However, both these methods are of limited use because of non-selective oxidation of many amino acid residues and low efficiency. NBS¹, used as an oxidative agent in these methods, is extremely reactive and splits not only the Trp but also Tyr and His bonds. BNPS-scatale (Omen et al., 1970) is a much more selective agent than NBS: bromination of tyrosine and histidine can be prevented by using

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¹ Abbreviations: GPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); NBS-scatale, N-bromosuccinimide; BNPS-scatale, 2-(2-nitrophenylsulphonyl)-3-methyl-3-bromo-indolene; Oi, o-iodosobenzoic acid; TLC, thin-layer chromatography; HVE, high-voltage electrophoresis; AA, amino acid.
a suboptimal amount of BNPS-scatole in the cleavage reaction, and after reduction and 5-alkylation of cysteine the side reactions are limited to formation of methionine sulfoxide. The yield varies from 15 to 60%.

The recently reported use of o-iodosobenzoic acid for cleavage of Trp bonds (Mahoney & Hermodson, 1979) offers much higher specificity of the cleavage reaction with the yield ranging from 70 to 100%.

The aim of the present work was to use this procedure in our comparative studies on the primary structure of glyceraldehyde-3-phosphate dehydrogenase [d-glyceraldehyde-3-phosphate:NAD\(^+\) oxidoreductase (phosphorylating), EC 1.2.1.12]. Specificity of cleavage was proved by the amino acid analysis of the separated peptide products.

MATERIALS AND METHODS

Reagents

Sephadex G-50 (superfine) was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). TPCK-Trypsin was obtained from Worthington Biochemical Corp. (Freehold, New Jersey, U.S.A.). o-Iodosobenzoic acid was purchased from Sigma Chem. Comp. (St.Louis, Mo., U.S.A.) and guanidine hydrochloride was from Pierce Chemical Co. (Rockford, Ill., U.S.A.). 2-Mercaptoethanol was a product of Organica (G.D.R.). Cellulose-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 & Schuell GmbH (Dassel, F.R.G.). 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

GPDH was prepared from human muscle as described by Baranowski & Wolny (1963). The contaminating myoglobin was removed, if necessary, on CM-cellulose as described by Nowak et al. (1976).

Carboxymethylation of GPDH and cleavage of tryptophanyl peptide bonds

The thiol groups in GPDH were carboxymethylated by the method of Crestfield et al. (1963), the protein was dialysed and lyophilized.

Carboxymethylated GPDH (30 mg) was dissolved in about 7 ml of 4 M-guanidine hydrochloride in 80% acetic acid. To this solution 30 mg of o-iodosobenzoic acid dissolved in the same solution was added and kept for 24 h at room temperature. The reaction was stopped by addition of 2-mercaptoethanol (10 μl). The reaction mixture was finally dialysed against 9% formic acid and lyophilized three times against water.
Isolation of peptides

*Column chromatography of tryptophanyl fragments.* The lyophilized mixture of peptides was redissolved in 9% formic acid and fractionated on a column of Sephadex G-50 SF (1.2 x 140 cm) equilibrated and developed with 9% formic acid, at a flow rate of 2 ml per hour; fractions of 4 ml were collected. The absorbance was monitored at 280 nm and the appropriate fractions were pooled and lyophilized.

*Digestion with trypsin.* Tryptic digestion of the peptides was performed in 0.5% ammonium bicarbonate buffer, pH 8.9, for 14 h, at 37°C (the substrate to trypsin weight ratio was 100:1). The digest obtained was freeze-dried.

*High-voltage electrophoresis and chromatography of tryptophanyl fragments*

*Analytical.* The mixture of peptides was dissolved in water and spotted on cellulose thin layer plate. In the first dimension, electrophoresis was run at pH 4.4 in pyridine/acetic acid/acetone/water system (1:2:8:40, by vol.) at 600 V and 28-32 mA for 2 h, and in the second dimension chromatography in n-butanol/acetic acid/water/pyridine (15:3:12:10, by vol.). The peptides were detected with ninhydrin, Pauly reagent (Heillman *et al.*, 1957) and with phenanthrenequinone (Easley *et al.*, 1969).

*Preparative.* The peptide sample dissolved in water was spotted onto the chromatography Whatman 3MM paper and high-voltage electrophoresis was run at pH 3.5 in pyridine/acetic acid/water (1:10:89, by vol.) at 55 V/cm, 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 four times with 10% solution of acetic acid and the solvents were evaporated under reduced pressure in a rotary evaporator at 40°C. The residues were dissolved in water and freeze-dried.

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

*N-Terminal analysis* of the purified peptides was performed by the dansyl procedure of Hartley (1970). The dansylated amino acids were then identified on micropolyamide sheets (3.6 x 5 cm) in the solvent systems of Chen (1976).

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 above.
RESULTS AND DISCUSSION

Our experiments involving N-terminal analysis (Nowak et al., 1975, 1976; Nowak & Banaś, 1980; Banaś et al., 1980; Banaś & Nowak, 1981) indicated that glyceraldehyde-3-phosphate dehydrogenase from human muscle is longer by two amino acids than the pig enzyme (Harris & Perham, 1968). For the purpose of comparison with GPDH from other sources, we kept the same numeration of amino acid residues as that used for GPDH from pig muscle. The two enzymes show high homology; three tryptophan residues are at the same position (84, 193, 310). The tryptophan-containing peptides in human and pig enzyme are of the same length except the first one (1-84) which is by two amino acid residues longer in the former.

Since it was not possible to establish the position of all amino acids in GPDH by tryptic digestion, chemical fragmentation was necessary. The o-iodosobenzoic acid treatment of protein results in a highly specific cleavage at the carboxyl group of tryptophan residues (Mahoney & Hermodson, 1979). Since these residues are rare in proteins, and in GPDH they are located at distant positions, cleavage of Trp bonds results in liberation of large peptides easy for sequencing. Thus, GPDH can be regarded as an excellent model for sequence analysis by the o-iodosobenzoic acid procedure of Mahoney & Hermodson (1980).

The general outline of the separation and isolation of the peptides obtained on cleavage of the enzyme with o-iodosobenzoic acid, is given in Scheme 1.

The reduced and S-carboxymethylated GPDH was treated with o-iodosobenzoic acid as described in Methods, and the mixture was fractionated on Sephadex G-50 SF column. Of the six peaks obtained (Fig. 1), the first three were close to each other. Since the amino acid composition

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Scheme 1. Isolation of tryptophan-containing peptides.
and the sequence established by the Edman degradation procedure were identical in all of them (Table 1), they could be considered as a single fraction and designated Oi-Ia, Oi-Ib and Oi-Ic. The reason for the separation of a single peptide into three subfractions remains obscure. As compared with the known sequence of the human muscle enzyme (Nowak et al., 1975, 1976), the peptide Oi-I should be located in positions between 194 and 310.

**Table 1**

**Amino acid composition and the N-terminal sequence by the Edman degradation of peptide Oi-I from GPDH of human muscle**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Composition</th>
<th>Amino acid</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>15</td>
<td>Met</td>
<td>1</td>
</tr>
<tr>
<td>Thr</td>
<td>6</td>
<td>Ile</td>
<td>3</td>
</tr>
<tr>
<td>Ser</td>
<td>6</td>
<td>Leu</td>
<td>10</td>
</tr>
<tr>
<td>Gly</td>
<td>11</td>
<td>Tyr</td>
<td>2</td>
</tr>
<tr>
<td>Pro</td>
<td>5</td>
<td>Phe</td>
<td>4</td>
</tr>
<tr>
<td>Glu</td>
<td>6</td>
<td>Lys</td>
<td>10</td>
</tr>
<tr>
<td>Ala</td>
<td>11</td>
<td>His</td>
<td>2</td>
</tr>
<tr>
<td>Val</td>
<td>11</td>
<td>Arg</td>
<td>3</td>
</tr>
</tbody>
</table>

N-Terminal sequence of peptides Oi-I by the Edman degradation procedure was: Arg - Asp - Gly - Arg ....
Fig. 2. Peptide maps of the tryptophan-containing peptides (A, Oi-II; B, Oi-III; C, Oi-IV; see Scheme 1) of GPDH from human muscle. The maps were obtained as described in Methods. The numbering is independent for each of the peptides.

The position of fragments Oi-II, Oi-III and Oi-IV were determined in more detail. The tryptic digests of these peptides were analysed by preparative fingerprinting (Fig. 2). In all the peptides isolated, N-terminal amino acids were determined and four degradations by the micro dansyl-Edman method were applied (Table 2). The sequence obtained (Tables 1 and 2) was compared with that previously obtained for GPDH from human muscle (Nowak et al., 1975, 1976) and pig muscle (Harris & Perham, 1968). The results proved that the Oi-II, Oi-III and Oi-IV peptides were chemically homogeneous and contained the amino acid residues -2 to 84, 85 to 193, and 311 to 332, respectively. Thus, together with peptide Oi-I, the complete molecule of the GPDH from human muscle was recovered.

**Table 2**

N-Terminal sequence of the peptides Oi-II, Oi-III and Oi-IV (see Scheme 1) from GPDH of human muscle after digestion with trypsin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>N-terminal amino acids</th>
<th>Peptide</th>
<th>N-terminal amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oi-II 1</td>
<td>Val-Ile-His...</td>
<td>Oi-III 1</td>
<td>Val-Asp-Ile...</td>
</tr>
<tr>
<td>2</td>
<td>Gly-Asp-Ala...</td>
<td>2</td>
<td>Ala-Ile-Thr...</td>
</tr>
<tr>
<td>3</td>
<td>Thr-Val-Asp...</td>
<td>3</td>
<td>Val-Gly-Val...</td>
</tr>
<tr>
<td>4</td>
<td>Ile-Ile-Ser...</td>
<td>4</td>
<td>Ala-Ala-Leu...</td>
</tr>
<tr>
<td>5</td>
<td>Ile-Val-Ile...</td>
<td>5</td>
<td>Phe-His-Gly...</td>
</tr>
<tr>
<td>6</td>
<td>Tyr-Asp-Asp...</td>
<td>6</td>
<td>Leu-Val-Ile...</td>
</tr>
<tr>
<td>7</td>
<td>Ala-Gly-Ala...</td>
<td>7</td>
<td>Asp-Pro-Ala...</td>
</tr>
<tr>
<td>8</td>
<td>Gly-Gly-Ala...</td>
<td>8</td>
<td>Ala-Glu-Asp...</td>
</tr>
<tr>
<td>9</td>
<td>Leu</td>
<td>9</td>
<td>Leu-Val-Thr...</td>
</tr>
<tr>
<td>Oi-IV 1</td>
<td>Tyr-Asp-Asp...</td>
<td>10</td>
<td>Ile-Gly</td>
</tr>
<tr>
<td>2</td>
<td>Val-Val-Asp...</td>
<td>11</td>
<td>Gly-Lys</td>
</tr>
<tr>
<td>3</td>
<td>Glu</td>
<td>12</td>
<td>Val-Lys</td>
</tr>
</tbody>
</table>
Major Fragments, cleavage at Trp

Gly - Oi-III (86 AA) - Trp 84
Gly - Oi-II (109 AA) - Trp 193
Arg - Oi-I (147 AA) - Trp 310
Tyr - IV (22 AA)

C-terminal peptide

Scheme 2. Schematic representation of the sequence of tryptophan-containing peptides in GPDH from human muscle.

The results obtained are summarized in Scheme 2, which shows the sequence of all the fragments analysed in glyceraldehyde-3-phosphate dehydrogenase from human muscle. The results indicate the advantage of the o-iodosobenzoic acid method for the sequence analysis of GPDH; this procedure can also be used e.g. for determination of the chemically modified amino acids.

REFERENCES


DEHYDROGENAZA ALDEHYDU 3-FOSFOGLICERYNOWEGO Z MIĘŚNI LUDZKICH: ROZBICIE TRYPTOFANOWYCH WIAZAN PEPTYDOWYCH KWASEM O-JODOZOBENZOESOWYM

Streszczenie

Nową metodę fragmentacji białek kwasem o-jodozobenzoesowym (Mahoney & Hermodson, 1979; Biochemistry, 18, 3810-3814) użyto do rozszczepienia trzech tryptofanowych wiązań peptydowych w dehydrogenazie aldehydu 3-fosfoglicerynowego (EC 1.2.1.12) z ludzkich mięśni szkieletowych. Otrzymaną mieszaninę rozdzieliło na cztery jednorodne peptydy w jednym chromatograficznym etapie. Wysoką specyficzność reakcji rozszczepienia udowodniono przez analizę składu aminokwasowego i oznaczenie N-końcowych sekwencji otrzymanych peptydów. Zarówno specyficzność, jak i wysoka wydajność metody potwierdzają jej przydatność w badaniach pierwszorzędowej struktury białek.

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