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Preparation of the Peptide Containing the Essential Methionine Residue of Cytoplasmic Malate Dehydrogenase from Pig Heart

V. Leskovac

Abteilung Chemie, Ruhr-Universität, Bochum, West Germany and Department of Chemistry, University of Novi Sad, Yugoslavia

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The essential methionine residue in the active site of cytoplasmic malate dehydrogenase from pig heart has been labelled with (^{14}C)iodoacetate. The peptide, comprising this methionine residue, has been isolated and purified. The amino acid analysis showed that it is a hexapeptide of a composition: (Ser, Pro, Gly, Val, Met)-Arg.

In a previous paper[1] we were able to show that the inhibition of cytoplasmic malate dehydrogenase (L-malate : NAD oxidoreductase, E. C. 1.1.1.37) from pig heart with (^{14}C) iodoacetate results in the selective carboxymethylation of an essential methionine residue of the enzyme, and the formation of carboxymethyl-methionine sulfonium salt. The fingerprint analysis of the tryptic and chymotryptic digest of the labelled enzyme showed that the main portion of radioactivity was incorporated into only one peptide. The preparation and purification of this major radioactive peptide, containing the essential methionine residue, is outlined in the present paper.

Cytoplasmic malate dehydrogenase from pig heart was inhibited to 90% with (^{14}C) iodoacetic acid, and the labelled enzyme digested with trypsin and chymotrypsin. Incorporation of radioactivity was calculated to be 4.2 moles of (^{14}C) carboxymethyl-residues per mole of enzyme (M. W. 67,000), at 100% inhibition. The digest of the labelled enzyme was thereafter chromatographed on a Sephadex G-25 column (Fig. 1).

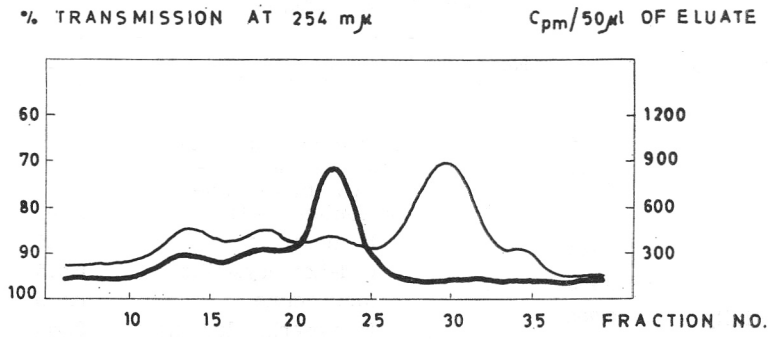

Fig. 1. Chromatography of the tryptic-chymotryptic peptides of (^{14}C) carboxymethylated malate dehydrogenase on Sephadex G-25 column, 40 × 2 cm. in water; fractions: 3 ml. ———— Transmission at 254 mμ ———— Radioactivity.

One fraction from the eluate, comprising 60% of total radioactivity, contained a single radioactive peptide, which was chromatographically and electrophoretically identical with the methionyl peptide previously identified as essential for the activity of cytoplasmic malate dehydrogenase[1]. This essential methionyl peptide was purified by a combination of preparative chromatrography and electrophoresis on thin-layers (Table I).

A portion of the pure peptide was hydrolysed with a mixture of proteolytic enzymes; the whole radioactivity showed to be associated with

TABLE I

Purification of the Essential Peptide from the Tryptic-chymotryptic Digest of (^{14}C) Carboxymethylated Malate Dehydrogenase

Purification step	μM of peptide	Yield %
1. (Proteolytic digest)	(3.0)	(100)
2. Chromatography on Sephadex G-25, (water, fractions 21—27).	1.8	60
3. Rechromatography on Sephadex G-25 (0.1 *M* ammonum-bicarbonate buffer).	1.5	50
4. Preparative electrophoresis on thin-layers in 4% formic acid.	0.6	20
5. Desalting on Sephadex G-10 column.	0.45	15
6. Preparative paper chromatography in pyridine-butanol-acetic acid-water (200 : 340 : 70 : 125).	0.4	13
7. Preparative paper electrophoresis in 4% formic acid.	0.25	8

The estimation of the quantity of the essential peptide was based on radioactivity; step 1. comprises the summ of all radioactive peptides in the digest. Chromatographic methods were previously described[1].

TABLE II

Amino Acid Composition of the Essential Methionyl Peptide of (^{14}C) Labelled Malate Dehydrogenase

Amino acid	μM	Nearest integer	Amino acid	μM	Nearest integer
Ser	0.172	1	Decomposition products of carboxymethyl-methionine sulfonium salt:	0.105	1
Pro	0.241	1	Homoserine	0.025	
Gly	0.290	1	CM-homocysteine	0.015	
Val	0.185	1	Methionine	0.025	
Arg	0.195	1	Homoserine lactone	0.040	
Radioactivity	0.200	1			

The model substances: homoserine, homoserine lactone and carboxymethyl-homocysteine were run independently on the amino-acid analyser for comparison.

(^{14}C) carboxymethyl-methionine sulfonium salt. The purified peptide, slightly basic on electrophoresis, was hydrolysed in 6 *N* hydrochloric acid, and the hydrolysate was analysed on an amino-acid analyser (Table II). It is evident that the carboxymethyl-methionine sulfonium salt was destroyed by acid hydrolysis[2]; only its decomposition products: homoserine, homoserine lactone, methionine and carboxymethyl-homocysteine, were identified on the chromatogram of the amino-acid analyser. Thus, as evidenced in Table II, the essential methionyl peptide of cytoplasmic malate dehydrogenase is a hexapeptide of the composition: (Ser, Pro, Gly, Val, Met)-Arg.

EXPERIMENTAL

Cytoplasmic malate dehydrogenase (50 mg.), purified from pig heart as previously described[3], with a specific activity of 600 U/mg. was inhibited to 90% (10% of activity remained) with (^{14}C)iodoacetate of a specific radioactivity of 0.05 *mC/mM*, at protein concentration of 33 mg./ml. and inhibitor concentration of 19 mg./ml. in 0.1 *M* sodium phosphate buffer *p*H 7.5. The excess of radioactivity was removed over a Sephadex G-25 column (40 × 2 cm.) in the same buffer. The labelled enzyme was denatured by the addition of solid urea until the final concentration of 8 *M*. After an incubation time of 3 hours at room temperature, the urea was removed by dialysis against water (3 l.), and than against 0.1 *M* ammonium-bicarbonate buffer (3 l.). The denatured enzyme was hydrolysed with a mixture of trypsin and chymotrypsin for 18 hours at 37°. Portions of proteolytic enzymes (1 mg.) were added after 0, 2, and 7 hours from the beginning of hydrolysis. The proteolytic digest was subsequently freeze-dried; the residue was dissolved in little 0.5% ammonia, applied to the Sephadex G-25 column, and washed with water (Fig. 1).

A total of 260.000 cpm were counted in the eluate (95% of the whole radioactivity applied to the column). The fractions 21—27 from the column had 60% of total radioactivity; they were pooled and freeze-dried. The residue contained only one radioactive peptide which was chromatographically (pyridine-butanol-acetic acid-water, 200 : 340 : 70 : 125) and electrophoretically (pyridine-water-acetic acid, 50 : 445 : 5, *p*H 6) identical with the methionyl peptide previously identified as essential in malate dehydrogenase[1]. This radioactive peptide was further purified (Table I) until it was chromatographically and electrophoretically pure. A small portion of the pure peptide was digested with a mixture of aminopeptidase M and pronase; the whole radioactivity derived from the digest was shown to be electrophoretically (pyridine acetate buffer *p*H 6, and 4% formic acid *p*H 2) identical with the model substance carboxymethyl-methionine sulfonium salt. Chromatographically pure peptide (0.2 μ moles) was hydrolysed in 6 *N* HCl in a sealed, evacuated glass tube for 20 hours at 100°. The hydrolysate was dried in a desiccator over KOH/H_2SO_4 and analysed on an amino-acid analyser (Technicon, Chertsy, England).

Other experimental conditions used in this work were the same as previously described[1].

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REFERENCES

1. V. Leskovac and G. Pfleiderer, *Z. physiol. Chemie* **350** (1969) 484.
2. H. G. Gundlach, H. W. Stein, and S. Moore, *J. Biol. Chem.* **234** (1959) 1754.
3. V. Leskovac, *Bull. Soc. chim. Belgrad* **34** (1969) 509.

IZVOD

Izolovanje esencijelnog metioninskog peptida iz aktivnog centra citoplazmatične malat dehidrogenaze iz svinjskog srca

V. Leskovac

Esencijelni metioninski ostatak u aktivnom centru citoplazmatične malat dehidrogenaze iz svinjskog srca markiran je sa (^{14}C)jodoacetatom. Peptid oko ovoga metioninskog ostatka je izolovan i prečišćen. Analiza aminokiselina pokazala je da je to heksapeptid sastava: (Ser, Pro, Gly, Val, Met)-Arg.

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