Identification of an arginine residue important for catalytic activity in the primary structure of D-glyceraldehyde 3-phosphate dehydrogenase

Studies with the rat skeletal-muscle enzyme

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(Received 14 July 1981/Accepted 21 July 1981)

The reaction of holo-(D-glyceraldehyde 3-phosphate dehydrogenase) (EC 1.2.1.12) from rat skeletal muscle with [^{14}C]butanedione in 0.05 M-NH $_4$ HCO $_3$, pH 8.0, resulted in modification (*) of two arginine residues per subunit with a concomitant loss of catalytic activity. From a tryptic digest of the modified protein two radiolabelled peptides were isolated, with the following sequences:

(1) Val-Ile-Ile-Asn-Ala-Pro-Thr-Ala-Asp-Ala(Glx, Met, Leu, Phe, Met)Gly-Val-Asx-Arg-Glx(His, Tyr)Ser-Lys

and

(2) Asp-Ala-Gly-Ala-Thr-Ile-Ala-Leu(Asx, Glx, Arg*, Phe, Val)Lys.

By comparison of the data with the known sequences of homologous enzymes, the localization of the modified residues was established. The first peptide was identified as corresponding to residues 116–139, the second to residues 293–306. Experimental evidence from this and previous studies suggests that arginine-134 is important for the catalytic activity of the rat muscle enzyme, being involved in structural rearrangements accompanying the organization of the active centre on the binding of coenzyme and substrate.

D-Glyceraldehyde 3-phosphate dehydrogenase, a tetrameric enzyme composed of identical subunits (Harris & Perham, 1965; Harrington & Karr, 1965), was previously shown to be inactivated by an arginine-specific reagent, butane-2,3-dione (Nagradova & Asryants, 1975; Nagradova *et al.*, 1976). Two arginine residues per subunit were found to be modified concomitant with the loss of the dehydrogenase activity in both yeast and rat muscle enzymes. Kinetic analysis of the data revealed that modification of a single arginine residue per subunit was responsible for the effect (Nagradova *et al.*, 1978a).

The microenvironment of the essential arginine residue was found to change on transition from apo- to holo-enzyme conformation; this was evidenced by a marked increase in the rate constant of butanedione-induced inactivation in the presence of NAD $^+$.

The effect was suggested to be caused by some movement of the essential arginine with respect to its position in the apoenzyme, introducing a positively charged guanidinium group in the active centre (Nagradova *et al.*, 1978a). This suggestion was in line with the hypothesis of Garavito *et al.* (1977) that a similarity exists between conformational changes induced by the binding of NAD $^+$ to lactate dehydrogenase, liver alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase. On the basis of crystallographic data, arginine-231 was suggested to be involved in structural rearrangements characteristic of the transition from the apo to holo conformation of the latter enzyme (Garavito *et al.*, 1977; Nagradova *et al.*, 1978a).

The present work was initiated to verify this suggestion. Our purpose was to reveal the localization of the butanedione-modified arginine residue in the primary structure of rat muscle

glyceraldehyde 3-phosphate dehydrogenase. The complete amino acid sequence of this protein is not known, but the structure of the *N*-terminal region of the molecule (Nagradova *et al.*, 1978b) and of the peptide containing the essential cysteine-149 (Baibakov *et al.*, 1977) closely resemble the corresponding parts of the polypeptide chain of the pig muscle enzyme (Harris & Perham, 1968). These facts, together with a high degree of homology which is known to exist between glyceraldehyde 3-phosphate dehydrogenases of different origin (Olsen *et al.*, 1975), justified our attempts to locate arginine residues modified with [^{14}C]butanedione within the covalent structure of the protein.

Materials and methods

Enzyme

D-Glyceraldehyde 3-phosphate dehydrogenase was isolated from rat skeletal muscle (Nagradova & Guseva, 1971). The enzyme was shown to be pure by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and by analysis of the *N*-terminal amino acid. Specific activity of the enzyme preparations assayed as previously described (Nagradova *et al.*, 1975) corresponded to 100 μmol of NAD^+ reduced/min per mg of protein. Protein concentrations were determined spectrophotometrically by using $A_{280}^{0.1\%}$ 0.83 for the apoenzyme.

Reagents

NAD^+ and EDTA were purchased from Reanal (Budapest, Hungary), and 2-mercaptoethanol, butane-2,3-dione, trypsin (1-chloro-4-phenyl-3-*L*-tosylamidobutan-2-one-treated) and chymotrypsin from Sigma (St. Louis, MO, U.S.A.). Formic acid was from Ciech (Warsaw, Poland), Sephadex G-50 from Pharmacia (Uppsala, Sweden), ninhydrin from Chemapol (Prague, Czechoslovakia), Cellex from Bio-Rad (Richmond, CA, U.S.A.). All other reagents were of analytical grade. Pyridine was distilled over KOH, and acetic acid over Cr_2O_3 . HCl was thrice distilled after boiling with SnCl_2 . [2,3- ^{14}C]Butanedione was synthesized (Snell & McIlvain, 1931) at a specific radioactivity of 0.6 $\mu\text{Ci}/\text{mmol}$ from [^{14}C]acetic acid (specific radioactivity 120 mCi/mmol ; Izotop, Leningrad, U.S.S.R.).

Enzyme modification by [^{14}C]butanedione

Modification of the enzyme (100 mg; 10 mg/ml) was carried out in a medium containing 0.05 M- NH_4HCO_3 , 5 mM-EDTA, 4 mM-2-mercaptoethanol, 150 μM - NAD^+ , pH 8.1–8.2, at 22°C. [^{14}C]Butanedione was added to a final concentration of 30 mM. The stock solution of [^{14}C]butanedione (250 $\mu\text{Ci}/\text{ml}$) was diluted before use with unlabelled reagent to give a ratio of 100 $\mu\text{Ci}/100\text{ mg}$ of protein. The time course of the reaction was followed by the

decrease in enzymic activity. The reaction was stopped at different time intervals (5 or 20 min in various experiments) by gel filtration of the mixture on a column (2 cm $\times$ 40 cm) of Sephadex G-50 (coarse grade) equilibrated and eluted with 0.5% formic acid or acetic acid. The concentration of the acid in the resultant solution was then raised to 30%, after which the mixture was frozen and freeze-dried.

Enzyme oxidation

Performic acid oxidation of the modified enzyme was carried out as described by Hirs (1956). Performic acid (1.5–2.0 ml) was added to 60 mg of freeze-dried protein. The mixture was kept for 60 min in the cold, then diluted 50-fold with water, frozen and freeze-dried.

Tryptic hydrolysis

A sample of freeze-dried butanedione-modified oxidized protein (80 mg) was suspended in 0.2 M-borate buffer, pH 8.2, to a final protein concentration of 6–8 mg/ml. Digestion was performed with a 1:100 (w/w) ratio of trypsin to glyceraldehyde 3-phosphate dehydrogenase, for 3.5 h at 37°C, with continuous stirring. A new portion of trypsin was then added in the ratio of 1:100 (trypsin/substrate) and the incubation continued for another 3.5 h. The completely soluble hydrolysate thus obtained was freeze-dried.

Chymotryptic hydrolysis

Freeze-dried peptide T-1 (see below) (about 50 nmol) was suspended in 100 ml of 0.05 M- NH_4HCO_3 , pH 8.0. Chymotrypsin was added in a ratio of 1:100 (enzyme/substrate) and the mixture was incubated at 37°C for 6 h with continuous stirring. The resultant solution was freeze-dried.

Amino acid analyses

Protein and peptides were hydrolysed at 110°C for 24 h in 5.7 M-HCl in evacuated sealed glass tubes. The analyses were performed with a Hitachi amino acid analyser model KLA-5 (protein samples), or with a LKB amino acid analyser model 3201 (peptide samples).

Peptide mapping

Two-dimensional peptide patterns of tryptic digests and purified peptides from tryptic digests were obtained as previously described (Harris & Perham, 1965; Vospelnikova *et al.*, 1977).

Localization of peptides on paper was determined with the use of ninhydrin. Arginine-containing peptides were detected by the Sakaguchi reaction. Radiolabelled peptides were identified by autoradiographs by using an Uran-1 apparatus in the Department of Analysis of Radiolabelled Materials,

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The chymotrypsin digest of peptide T-1 was analysed on 20 cm × 20 cm microcrystalline cellulose plates (Avicel 250 μm; Analtech, Wilmington, DE, U.S.A.). Electrophoresis was performed in pyridine/acetic acid/water (1:10:89, by vol.) at pH 3.5 for 20 min at 200 V and then for 100 min at 900 V, with subsequent chromatography in butan-1-ol/pyridine/water (1:1:1, by vol.) adjusted to pH 5.4 with acetic acid. The peptides were detected as fluorescent spots after the plate was sprayed with 3% pyridine in acetone and then with 0.001% fluorescamine (Fluram; Roche Diagnostics, Basel, Switzerland) in acetone. They were recovered from the plate by extraction with concentrated formic acid, vacuum-dried and subjected to peptide analysis.

Isolation of peptides containing modified arginine residues

Peptides of a tryptic digest (60–70 mg) were separated on a column (2 cm × 150 cm) of Sephadex G-50 (fine grade) equilibrated and eluted with 30% acetic acid. The effluent was monitored for absorbance at 280 nm and for radioactivity. Radioactivity was measured with a Nuclear Chicago mark 2 counter by using a dioxan-based scintillator [containing, per litre, 0.2 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 4 g of 2,5-diphenyloxazole, 60 g of naphthalene and 200 ml of ethanol]. Each vial contained 10 ml of scintillator and 0.1 ml of the sample to be analysed.

Column electrophoresis

Electrophoresis of the radiolabelled fractions was carried out on a 0.9 cm × 90 cm column packed with Cellex (Fryklund & Eaker, 1975) at 1000 V and 4 mA for 18 h in formic acid/acetic acid/water (1:4:45, by vol.), pH 1.9. The sample applied to the top of the column was allowed to penetrate the carrier until the distance from the surface to the upper front of the sample reached about 3 cm. Electrophoresis started at this moment. At the end of electrophoresis, elution with 30% acetic acid was performed, the rate being 0.5 ml/min; 1 ml fractions were collected. Samples (100 μl) were analysed for radioactivity and for the ninhydrin-positive material measured as absorbance at 570 nm (Hirs, 1967).

Amino acid sequence analysis

Automated sequential degradation was performed with a Beckman 890C sequencer with program no. 050972 and a solid-phase Rank Hilger sequenator, model APS 24. Amino acid phenylthiohydantoin and their trimethylsilyl derivatives were quantified by g.l.c. (Pisano *et al.*, 1972) on a Beckman GC-45 gas chromatograph. Occasionally,

the identification of amino acid phenylthiohydantoin was confirmed by t.l.c. analysis (Jeppsson & Sjoquist, 1972) of the material on 20 cm × 20 cm plates covered with silica gel (Merck, Darmstadt, West Germany). The amino acid sequence of chymotryptic peptides was determined by manual sequential analysis (Ambler, 1967). *N*-Terminal amino acids were identified by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) method (Gray, 1972).

Results

Identification of peptides containing modified arginine residues

Table 1 summarizes the results obtained by amino acid analysis of the butane-2,3-dione-modified enzyme at different steps of the treatment needed to make the analysis. Modification affected two of the ten arginine residues per subunit, which is in agreement with our previous findings (Nagradova *et al.*, 1976). The difference in the arginine content of control and modified enzyme samples persists throughout the procedure of tryptic-hydrolysate preparation. The product of modification appears to be sufficiently stable under the experimental conditions described.

Assuming that trypsin does not hydrolyse peptide bonds formed by the modified arginine residues (Yang & Schwert, 1972), we hoped to detect differences between the peptide maps of the modified and control enzyme preparations. Modifications of two arginine residues per subunit was expected to cause the disappearance of four peptides and the emergence of two new ones. Fig. 1 shows the results obtained by comparing the tryptic digests of control and modified enzyme preparations under strictly identical conditions. The differences are evident in the fractions of 'acidic' and 'neutral' peptides. A

Table 1. *Arginine content of [¹⁴C]butanedione-modified enzyme and products of its hydrolysis*

I, Holoenzyme modified with [¹⁴C]butanedione (time of incubation 20 min), and treated as described in the text. II, The same after performic acid oxidation. III, Tryptic digest of [¹⁴C]butanedione-modified oxidized enzyme. The amount of modified arginine residues was calculated by comparing the data obtained with modified and control enzyme preparations under similar conditions.

Sample analysed	Arginine content (mol/mol of tetramer)		Modified arginine residues (mol/mol of protein subunit)
	Control	Modified	
I	40.8	32.0	2.2
II	41.2	34.5	1.7
III	40.5	33.7	1.7

Fig. 1. Peptide maps of tryptic digests of a control enzyme sample (—) and of a [^{14}C]butanedione-modified enzyme (---)

(a) 'Basic' peptides; (b) 'acidic' peptides; (c) 'neutral' peptides. Shaded spots, new peptides which appear in the modified protein; cross-hatched spots, peptides which disappear in the modified protein. The arrows indicate radioactive peptides.

modified enzyme sample is characterized by the absence of four peptides (two in the 'acidic' fraction and two in the 'neutral' one); in addition, two new spots are seen on the 'neutral'-peptide map. Auto-radiography revealed radiolabelled material in these spots.

Isolation of peptides containing [^{14}C]butanedione-modified arginine residues

Fig. 2 shows the elution profile obtained by chromatography of the tryptic digest of the modified enzyme on Sephadex G-50. The fractions containing the main radioactivity (70% of the total) were pooled to give fraction T, which was subjected to further analysis by paper electrophoresis at pH 1.9. As shown in Fig. 3, two radioactive peaks were detected in this fraction. The presence of several radioactivity-containing peaks in Fig. 2 can probably be explained by a partial splitting of the product of arginine modification (which is unstable), the radioactive moiety being adsorbed on peptide material. The non-specific character of this binding is demonstrated by the fact that only two radioactivity-containing peptides were detected on peptide maps and after the analysis of fraction T by paper electrophoresis at pH 1.9.

An attempt was made to compare the rates of accumulation of the two fractions in Fig. 3 in the course of enzyme inactivation. The ratio of total radioactivity in peak F_1 to that in peak F_2 was found to be 3:4 in the preparations treated with [^{14}C]butanedione for 5 min; after 20 min incubation, the radioactivity became equally distributed between the two fractions. This result indicates that the most reactive arginine residue is localized in the peptide fraction which moves faster to the cathode under the

Fig. 2. Chromatography on a column of Sephadex G-50 (fine grade) of a tryptic digest of the enzyme modified with [^{14}C]butanedione

A portion (68 mg) of a tryptic digest of enzyme modified with [^{14}C]butanedione (incubation time 5 min) was dissolved in 3 ml of 30% acetic acid and applied on a column (2 cm $\times$ 150 cm) equilibrated and eluted with 30% acetic acid (flow rate 10 ml/h); 3 ml fractions were collected. The radioactive fractions were pooled as shown to give fraction T. $\bigcirc$, Radioactivity; $\bullet$, A_{280} .

conditions of Fig. 3. The data also suggest that the loss of enzymic activity, which was observed to reach 60% during 5 min of incubation, should be associated with modification of an arginine residue in this peptide fraction.

Fig. 4 shows the results of the chromatographic analysis of peptide fractions containing radioactivity. In addition to two radioactive peptides, fraction T contains two non-labelled peptides. None of the four spots was stained with the Sakaguchi reagent, which suggests that lysine residues are terminal residues in these peptides.

Fig. 3. Electrophoresis of fraction T (Fig. 2) on paper at pH 1.9

Freeze-dried fraction T was dissolved in 5 ml of formic acid/acetic acid/water (1:4:45, by vol.), and 0.05 ml of the resulting solution was applied on a sheet of paper (Whatman 3MM, 57 cm × 10 cm). Electrophoresis was carried out in the above formic acid/acetic acid/water mixture at 5000 V for 90 min. The sheet of paper was then cut into pieces (1.5 cm × 1 cm), which were analysed for radioactivity in scintillation fluid [a 1:1 (v/v) mixture of Triton X-100 (Sigma) and a toluene solution containing 5.0 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene per litre].

Fig. 4. Two-dimensional analysis of fraction T
The sample was treated as described in Fig. 3. After electrophoresis on a paper sheet (57 cm × 45 cm), chromatography was performed in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.). The arrows indicate radioactive peptides.

Preparative separation of the peptides was performed by electrophoresis on a column packed with Cellex in formate buffer, pH 1.9 (Fig. 5). Four peaks were revealed (T-1, T-2, T-3 and T-4), the radioactivity being localized in fractions T-1 and T-2.

Fig. 5. Column electrophoresis of fraction T
A sample (3 ml) of the solution of freeze-dried fraction T (see the legend to Fig. 3) was applied on a Cellex column and analysed as described in the Materials and methods section. ●, A_{570} ; ○, radioactivity.

Table 2. Amino acid compositions of peptides T-1 and T-2

The values for valine and isoleucine are underestimated, since the samples were hydrolysed only for 24 h. The values for glycine and serine are overestimated, probably owing to the presence of some amounts of these amino acids in reagents (Gray, 1972). The values given in parentheses are calculated on the basis of amino acid sequence determinations (see below).

Amino acid	Composition (mol of residue/mol of peptide)	
	T-1	T-2
Aspartic acid	2.8 (3)	2.3 (2)
Threonine	1.2 (1)	1.0 (1)
Serine	1.9 (1)	1.05 (0)
Glutamic acid	1.6 (2)	0.85 (1)
Proline	1.2 (1)	—
Glycine	2.5 (1)	1.85 (1)
Alanine	2.8 (3)	3.2 (3)
Valine	1.2 (2)	1.3 (1)
Isoleucine	1.0 (2)	0.7 (1)
Leucine	1.4 (1)	0.9 (1)
Tyrosine	0.4 (1)	—
Phenylalanine	1.0 (1)	0.6 (1)
Histidine	0.8 (1)	—
Lysine	1.0 (1)	0.85 (1)
Arginine	Traces (1)	Traces (1)
Methionine sulphone	1.6 (2)	—
Total	32.4 (24)	14.6 (14)

These fractions were demonstrated to be individual peptides by *N*-terminal amino acid determination. At the *N*-terminus of peptide T-1 valine was identified, and aspartic acid at the *N*-terminus of peptide T-2. The amino acid compositions of the two peptides containing modified arginine residues are given in Table 2.

Determination of amino acid sequences of peptides T-1 and T-2

The sequence of ten out of 24 residues of peptide T-1 was determined by automatic Edman degradation, and the presence of arginine in position 19 was established by a colour reaction with phenanthrenequinone. The following structure was obtained:

The analysis of the sequence between residues 11 and 19 was hindered, owing to a marked decrease in the yield of phenylthiohydantoin derivatives. The attempts to detect the location of the modified arginine residue by radioactivity were unsuccessful in these experiments. We suppose that the product of modification is unstable under the conditions of the Edman degradation procedure, and its splitting results in partial regeneration of arginine.

Further analysis was performed with a chymo-

tryptic digest of peptide T-1. Seven peptide fragments were detected in the digest, and three of them were obtained in a pure form. *N*-Terminal residues of these peptide fragments (c_1 , c_2 and c_3) were determined as valine, glycine and serine respectively. The analysis of the amino acid compositions of peptide fragments c_1 , c_2 and c_3 (Table 3) prompted us to suggest that fragment c_1 might derive from the *N*-terminal region of peptide T-1, fragment c_2 contains the radiolabelled arginine residue, and fragment c_3 is the *C*-terminal dipeptide Ser-Lys. This was confirmed by sequence determination. The structure of the *N*-terminal parts of peptide fragments c_1 and c_2 was found to be:

The localization of arginine was established by the phenanthrenequinone method. Taken together, the results enable us to propose the amino acid sequence for peptide T-1 as shown in Fig. 6.

The structure of peptide T-2, which contains the second arginine residue modified with [^{14}C]-butanedione, was established by means of automatic Edman degradation and on the basis of its amino acid composition; it proves to be the following:

Table 3. *Amino acid compositions of peptides isolated from the chymotryptic digest of peptide T-1*

Amino acid	Peptide fragments ...	Composition (mol of residue/mol of peptide)		
		c_1	c_2	c_3
Aspartic acid		2	1	—
Threonine		1	—	—
Serine		—	1	1
Glutamic acid		1	1	—
Proline		1	—	—
Glycine		—	1	—
Alanine		3	—	—
Valine		1	1	—
Isoleucine		2	—	—
Leucine		1	—	—
Tyrosine		—	1	—
Phenylalanine		1	—	—
Histidine		—	1	—
Lysine		—	1	1
Arginine		—	1	—
Methionine sulphone		1	—	—
Total		14	9	2

Discussion

Comparison of amino acid sequences of glyceraldehyde 3-phosphate dehydrogenases isolated from five species (Harris & Walker, 1977) demonstrates that although the total number of arginine residues varies from 9 in the lobster enzyme to 16 in the *Thermus aquaticus* dehydrogenase, seven of them occupy fixed positions. The presence of arginine residues in positions 10, 13, 17, 194, 197, 231 and 320 appears to be a characteristic feature of this enzyme. It is interesting, in this connection, that both reactive arginine residues modified in our study are located in the variable regions of the polypeptide chain. Further, rat muscle dehydrogenase is the only enzyme as yet sequenced that has an arginine residue in position 134, and this means that butanedione-modified arginine residues of the yeast and probably rabbit muscle enzymes must be located elsewhere in the amino acid sequence. Since each of the three enzymes was found to have an essential arginine residue sensitive to NAD^+ -induced conformational changes, we suppose that different portions of the polypeptide chain of the rat, yeast

	116	120	122a	125	130	135	138a	139
(a)	Val-Ile-Ile-Ser-Ala-Pro-Ala-Lys-Val-Glu-Asn-Ile-Thr-Val-Val-Met-Gly-Val-Asn-Gln-Asp-Lys-Tyr-Asp-Pro-Lys							
(b)	Ile	Thr	Ala-Lys-Gly-Glu-Asp-Ile-Thr-Leu	Ala-Lys-Gly-Glu-Asp-Ile-Thr-Leu	Met	His-Glu-Ala	Asp-Pro-Ser	
(c)	Val	Thr	Ser - Ser-Thr -Ala-Pro-Met-Phe	Ser - Ser-Thr -Ala-Pro-Met-Phe	Met	Glu-Val -Lys	Thr - Ser	
(d)	Val	Ser	Ser - Ala-Asp -Ala-Pro-Met-Phe	Ser - Ala-Asp -Ala-Pro-Met-Phe	Cys	Leu-Glu -Lys	Ser - Lys	
(e)	Ile	Ser	Ser - Ala-Asp -Ala-Pro-Met-Phe	Ser - Ala-Asp -Ala-Pro-Met-Phe	Met	His-Glu -Lys	Asp - Asn	
(f)	Val-Ile-Ile-Asn-Ala-Pro-Thr	- Ala-Asp -Ala (Glx, Met, Leu, Phe, Met) Gly-Val-Asx-Arg-Glx (His, Tyr) Ser	-	-	-	-	-	Lys

Fig. 7. Comparison of the amino acid sequences of residues 116-139 of glyceraldehyde 3-phosphate dehydrogenases from *B. stearohermophilus* (a), *T. aquaticus* (b), *baker's yeast* (c), *lobster* (d) and pig (e) with the structure of peptide T-1 derived from a tryptic digest of the rat muscle enzyme (f)

The data for (a)-(e) are taken from Harris & Walker (1977) and from Holland & Holland (1979). Sequence differences in enzymes from mesophilic organisms are shown in the boxed regions.