Amino acid sequence of the tryptic peptide containing the catalytic-site thiol group of bovine liver uridine diphosphate glucose dehydrogenase

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The catalytic-site thiol groups of UDP-glucose dehydrogenase from bovine liver were carboxymethylated with iodo[2-¹⁴C]acetate or with iodoacetamidofluorescein. After the residual thiol groups were carboxymethylated with iodoacetate, the proteins were digested with trypsin. The ¹⁴C-labelled peptide from the carboxymethylated enzyme was purified to homogeneity by successive thick-layer chromatography on silica gel, paper electrophoresis and chromatography, and column chromatography on Bio-Gel P-6. Homogeneous fluoresceincarboxamidomethylated peptide was prepared from a tryptic digest of fluoresceincarboxamidomethylated enzyme by specific adsorption–desorption from Sephadex G-25. The sequence of either peptide determined by the manual Edman dansyl procedure is:

Ala-Ser-Val-Gly-Phe-Gly-Gly-Ser-Cys-Phe-Glx-Glx-Gly-Lys

Each of the six apparently identical subunits of bovine liver UDP-glucose dehydrogenase contains 10–12 thiol groups, of which one is essential for enzyme activity (Gainey *et al.*, 1972; Uram *et al.*, 1972). At pH 8.0 iodoacetate treatment of the enzyme carboxymethylates only these six catalytic-site thiol groups and irreversibly abrogates enzyme activity (Franzen *et al.*, 1976). The catalytic-site thiol group is considered to form a thiohemiacetal with the first reaction intermediate, uridine 5'-(α -D-glucopyranosyldialdehyde pyrophosphate), which subsequently is oxidized to yield a thioester of the reaction product, UDP-glucuronate. The latter is released by irreversible hydrolysis of the thioester, thereby freeing the enzyme for another round of catalysis (Ordman & Kirkwood, 1977). This mechanism closely resembles the conversion into a thioester of the thiohemiacetal formed between glyceraldehyde 3-phosphate and the catalytic-site thiol group of glyceraldehyde 3-phosphate dehydrogenase (Harris & Waters, 1976), suggesting that the enzymes might also display homologies in amino acid sequence. However, determination for the se-

quence around the catalytic-site thiol group of UDP-glucose dehydrogenase

Ala-Ser-Val-Gly-Phe-Gly-Gly-Ser-

Cys-Phe-Glx-Glx-Gly-Lys

and comparison with the corresponding sequence of glyceraldehyde 3-phosphate dehydrogenase failed to reveal homology around the catalytic-site thiol groups of the two enzymes. Details are given in the present paper.

Experimental

Materials

Bovine liver UDP-glucose dehydrogenase [specific activity 3.5 units (μ mol/min)/mg of protein; purity 95%] was prepared and assayed as described previously (Franzen *et al.*, 1976). Trypsin treated with L-1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK') was purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); carboxypeptidase B was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). All reagents and solvents used for peptide isolation and sequencing were obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.) and were of Sequenal grade. Pyridine was redistilled over solid ninhydrin

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before use. 5-Iodoacetamidofluorescein was purchased from Molecular Probes (Plano, TX, U.S.A.). Biofluor, Protosol and sodium iodo[2- 14 C]acetate (specific radioactivity 15 mCi/mmol) were from New England Nuclear Corp. (Boston, MA, U.S.A.). All other chemicals were of the highest commercial quality available.

Chromatographic media

Chromatographic separations were done on 2 mm-thick layers of silica gel 60 without fluorescent indicator (Analtech, Newark, DE, U.S.A.), 0.25 mm layers of silica gel (Analtech), thin layers of micropolyamide sheets on a polyester support base (Schleicher and Schuell, Keene, NH, U.S.A.), 46 mm $\times$ 57 mm sheets of Whatman 3MM filter paper (Fisher Scientific Co., Pittsburgh, PA, U.S.A.), Bio-Gel P-6 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and Sephadex G-25 (Pharmacia, Piscataway, NJ, U.S.A.).

Amino acid analysis

Peptides were hydrolysed in 6 M-HCl in evacuated sealed tubes for 24 h at 110°C. Amino acid analysis was done on a Dionics D500 amino acid analyser (Durrum Instrument Corp., Palo Alto, CA, U.S.A.). Digestion with carboxypeptidase B was done in 80 mM-Tris/HCl buffer, pH 7.8, containing 0.1 M-CaCl₂. The amino acids released were identified as their dansyl (5-dimethylaminonaphthalene-1-sulphonyl) derivatives.

Radioactivity measurement

Radioactivity was measured in a Tri-Carb model 3375 liquid-scintillation spectrometer (Packard Instrument Co., Downers Grove, IL, U.S.A.). Radioactive substances were detected on chromatograms by radioautography on Kodak No-Screen X-ray film.

Amino acid sequence determination

(Unless otherwise noted, all evaporations were done at 0.01 mmHg pressure and 65°C.) The amino acid sequence of the purified peptide (about 30 nmol) was determined by using a manual dansyl-Edman procedure (Gray, 1972). Dansyl-amino acids were identified by three-dimensional chromatography on micro polyamide sheets as described by Woods & Wang (1967).

Results and discussion

Carboxymethylation and tryptic digestion of UDP-glucose dehydrogenase

The catalytic-site thiol groups of UDP-glucose dehydrogenase were carboxymethylated essentially as described by Franzen *et al.* (1976), on a preparative scale. All carboxymethylations were

done at 30°C in the dark. To 34.7 mg of enzyme in 4.5 ml of 0.15 M-Tris/HCl buffer, pH 8.0, containing 2 mM-EDTA, was added 2.75 mg of iodo[2- 14 C]-acetic acid (3.3 mCi/mmol), in 22-fold excess over the total catalytic-site thiol-group content. After 0.5 h, enzymic activity was decreased by 50% and 0.5 equiv. of reagent per enzyme subunit had been incorporated. The reaction was terminated by rapid chilling in ice, and excess of reagents was removed by exhaustive dialysis against 500 ml of 0.14 M-Tris/HCl buffer, pH 8.5, containing 2 mM-EDTA. Iodoacetic acid (286 mg, a slight excess over the total thiol-group content) was added to a solution of the [14 C]carboxymethylated enzyme that was 8 M with respect to urea and 0.10 M with respect to dithiothreitol, and after 0.5 h the 14 C-labelled enzyme was isolated by chromatography on a column (2.5 cm $\times$ 40 cm) of Sephadex G-25 (coarse grade), equilibrated and eluted with 50% (v/v) acetic acid. The isolated radioactive protein was freeze-dried, dissolved in a minimal volume of 0.1 M-ammonium, bicarbonate buffer, pH 8.5, dialysed for 24 h against two 1-litre volumes of water, and then digested with trypsin as described in the Experimental section.

Peptide purification (carboxymethylated enzyme)

Peptide 'mapping' of the tryptic digest on 0.25 mm layers of silica gel G showed the presence of 55–57 peptides, consistent with the arginine + lysine content per enzyme subunit (Gainey *et al.*, 1972; Uram *et al.*, 1972). One peptide contained approx. 95% of the total radioactivity.

Fractionation of the tryptic digest by chromatography on 20 cm $\times$ 20 cm plates of 2 mm-thick silica gel 60 in chloroform/methanol/14.8 M-NH₃ (2:2:1, by vol.), yielded a major radioactive band (approx. 95% of the total radioactivity). The radioactive band was eluted and chromatographed on a 46 mm $\times$ 57 mm sheet of Whatman 3MM paper in butan-1-ol/17 M-acetic acid/water (72:21:107, by vol.; upper phase) followed by electrophoresis in the second dimension in pyridine/17 M-acetic acid/water (1:10:89, by vol.), pH 3.5, for 90 min at 2000 V. The radioactive peptide was eluted with chloroform/methanol/14.8 M-NH₃ (2:2:1, by vol.), dried and dissolved in water. It was then chromatographed on a column of Bio-Gel P-6 equilibrated and eluted with 40 mM-NH₄HCO₃ at 37°C. Peptide-containing (fluram spray on silica gel) radioactive fractions were pooled and shown to contain a single peptide by two-dimensional 'mapping' on a thin layer of silica gel G; homogeneity was confirmed by N-terminal analysis.

Fluorescein carboxamidomethylation and tryptic digestion of UDP-glucose dehydrogenase

(All operations were done in the dark.) To 21.9 mg of UDP-glucose dehydrogenase (0.42 μ mol

of enzyme monomer) (7.1 mg/ml in 0.1 M-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA) was added 0.17 mg of 5-iodoacetamidofluorescein (approximately equimolar ratio of reagent to protein subunits). After 15 min at 25°C, at which time the enzymic activity had decreased by 50%, the reaction mixture was cooled in ice and chromatographed on a column (14 cm × 1.7 cm) of Sephadex G-25 (fine grade), equilibrated and eluted with 0.1 M-Tris/HCl buffer, pH 8.0. Labelled protein recovered by freeze-drying of the pooled fluorescent fractions was dissolved in 0.36 M-Tris/HCl buffer, pH 8.6, containing 8 M-urea, 3 mM-EDTA and 0.12 M-2-mercaptoethanol, and after 4 h at 25°C (Crestfield *et al.*, 1963) a freshly prepared solution of 0.268 g of recrystallized iodoacetic acid (slight excess over the total thiol-group content) in 1.0 ml of 1 M-NaOH was added. After 30 min the reaction mixture was fractionated by chromatography on a 2.4 cm × 40 cm column of Sephadex G-25 (coarse grade), equilibrated and eluted with 8.5 M-acetic acid. Pooled fluorescent fractions were dried, dissolved in buffer and digested with trypsin as described in the Experimental section.

Peptide purification (fluoresceincarboxamidomethylated enzyme)

The tryptic-digestion product was recovered by freeze-drying and redissolved in 0.5 ml of 50 mM-ammonium bicarbonate buffer, pH 8.0. Peptide 'mapping' on paper showed the presence of 56 separate peptides, only one of which was fluorescent when viewed under u.v. light. The peptide was chromatographed on a column (17 cm × 1.5 cm) of Sephadex G-25 (fine grade), equilibrated and eluted with 50 mM-ammonium bicarbonate buffer, pH 8.0. Fractions were assayed, after dilution of a sample with 5 vol. of water, by spectrophotofluorimetry with

an Aminco-Bowman fluorimeter (Amicon Instruments, Silver Springs, MD, U.S.A.) with excitation at 429 nm and emission observed at 520 nm. The fluorescent fractions, which emerged in a symmetrical peak, were pooled and rechromatographed on a column (17 cm × 1.5 cm) of Sephadex G-25 (fine grade), equilibrated and eluted with 0.1 M-ammonium bicarbonate buffer, pH 8.0. The purified fluoresceincarboxamidomethylated peptide was isolated by repeated freeze-drying from water, and its purity was confirmed by two-dimensional 'mapping' on silica gel G and by N-terminal analysis. In a separate experiment in which the residual thiol groups of the fluorescent protein were carboxymethylated with iodo[2-¹⁴C]acetate, the fluorescent tryptic peptide was devoid of radioactivity, confirming the ability of the Sephadex G-25 column to separate fluoresceincarboxamidomethyl-labelled and unlabelled peptides (Brandt, 1977) and showing that the fluorescent tryptic peptide is devoid of non-catalytic-site thiol groups.

The amino acid composition of the ¹⁴C-carboxymethylated peptide as well as of the fluoresceincarboxamidomethylated peptide is given in Table 1; the values obtained from sequence analysis are also presented. These data, as well as the other criteria presented, attest to the purity of each labelled peptide and to the identity of their amino acid composition.

Sequence analysis by the manual dansyl-Edman procedure showed that the sequence of the ¹⁴C-carboxymethylated peptide and of the fluoresceincarboxamidomethylated peptide was:

The persistence of fluorescence until the substituted

Table 1. Amino acid composition of labelled tryptic peptides from UDP-glucose dehydrogenase
For experimental details see the text.

Amino acid	Amino acid composition (mol/mol of peptide)		
	From amino acid analysis		From sequencing
	[¹⁴ C]Carboxymethylated peptide	Fluoresceincarboxamidomethylated peptide	
Ser	1.97	2.13	2
Glx	1.38	1.59	2
Gly	3.53	4.10	4
Ala	1.22	1.36	1
Cys	—	1.05	1
Val	1.25	1.11	1
Phe	2.12	2.02	2
Lys	1.0	1.0	1
N-Terminal	Ala	Ala	

cysteinyl residue was cleaved after nine cycles of Edman degradation provided a visual indication of the progress of the sequencing procedure. As expected, degradation of either peptide with carboxypeptidase B did not proceed beyond release of lysine, which is consistent with the penultimate position of glycine at the C-terminal demonstrated by the Edman procedure (see Zisapel *et al.*, 1973).

Glyceraldehyde 3-phosphate dehydrogenase and UDP-glucose dehydrogenase resemble each other with regard to certain aspects of structure, thiol-group activity and reaction mechanism. Both catalyse conversion of a thiohemiacetal into a thioester with transfer of a hydride ion to the *si* face of NAD⁺ (Krakow *et al.*, 1963; Schiller *et al.*, 1972; Harris & Waters, 1976), and both display apparent half-of-the-sites reactivity of the catalytic-site thiol group (Conway & Koshland, 1968; Franzen *et al.*, 1976, 1978). Despite these similarities, there is no obvious amino acid sequence homology around the catalytic-site cysteine residues of UDP-glucose dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase [Asn-Ala-Ser-Cys-Thr-Thr-Asn-Cys-Leu-Ala-Pro (Harris, 1964)]. Neither is there homology with the sequence [Leu-Cys-Gly-Val-Glu-Asp-Val-Phe (Bitar *et al.*, 1977)] around the reactive thiol group of histidinol dehydrogenase from *Escherichia coli*, an enzyme that, like UDP-glucose dehydrogenase, catalyses the NAD⁺-linked oxidation of a primary alcohol to a carboxylic acid.

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