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Amino Acid Sequences containing Cysteine or Half-cystine Residues in β-Glucuronidase

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Abstract

Amino acid analysis of oxidized or reduced and carboxymethylated β-glucuronidase have shown the presence of 24 cysteic acid or *S*-carboxymethylcysteine residues respectively per mole of the tetrameric enzyme. Titration of sulfhydryl groups gave eight cysteine residues, and by difference 16 half-cystine residues per mole.

Six peptides containing radiolabelled cysteine residues were isolated from pepsin and chymotrypsin digests of reduced and *S*-carboxymethylated β-glucuronidase by ion-exchange chromatography or gel filtration, followed by paper ionophoresis and paper chromatography. The peptides were analysed for amino acids and sequenced by the dansyl–Edman procedure. Peptides containing cysteic acid were selectively recovered from thermolysin digests of performic acid-oxidized glucuronidase. The amino acid sequences confirmed that there were only six different peptide sequences containing either cysteine or half-cystine residues in the tetrameric enzyme, supporting the presence of four identical subunits. These sequences were:

(A) -Val-Asx-Val-Ile-Cys-Val-Asx-Ser-Tyr-

(B) -Gly-Asx-Leu-Cys-Ser-Gly-

(C) -Phe-Val-Val-Ile-Asx-Glx-Cys-Pro-Gly-Val-Gly-

(D) -Val-Val-Cys-Leu-

(E) -Gln-Ser-Gly-Cys-Leu-Val-Lys-Gly-Tyr-

(F) -Cys-Asp-Arg-Tyr-Gly-Ile-Val-Val-

Introduction

The enzyme β-glucuronidase is present in most mammalian tissues and body fluids, with the preputial gland of the rat the richest known source. Purification (Snaith and Levvy 1967) and crystallization (Hawley and Marsh 1970; Dickson *et al.* 1979) of the enzyme from rat preputial gland enabled characterization of the enzyme as a tetrameric glycoprotein with leucine as the sole amino terminal residue and subunits of molecular weight of approximately 75 000 (Hawley 1973; Tulsiani *et al.* 1975; Dickson *et al.* 1979) that appear to be identical.

Amino acid analyses of the purified enzyme (Ohtsuka and Wakabayashi 1970; Hawley 1973; Himeno *et al.* 1975; Tulsiani *et al.* 1975) are in reasonable accord, with least agreement on the content of the amino acids in lesser amounts, namely cysteine and cystine, methionine and tryptophan.

The present study is concerned with quantitation of the content of the sulfur-containing amino acids, both by amino acid analysis and amino acid sequence studies. The amino acid sequences around the cysteine and half-cystine residues in the β-glucuronidase were investigated by analysing peptides isolated from enzyme digests

of β-glucuronidase reduced and labelled with [2-^{14}C]iodoacetate, in conjunction with analyses of cysteic acid peptides isolated from thermolytic digests of the performic acid-oxidized enzyme.

Materials and Methods

β-Glucuronidase was isolated from the preputial glands of both male and female albino Wistar and Sprague Dawley rats by the method of Snaith and Levvy (1967) as described by Hawley (1973).

Enzyme activity was measured at pH 4·5 and 37°C using *p*-nitrophenyl-β-D-glucuronide (Kato *et al.* 1960) as substrate, the released *p*-nitrophenol being measured at 400 nm in 0·2 M glycine–NaOH buffer, pH 10·7.

Titration of Sulfhydryl Groups

The method of Ellman (1959) in 6 M guanidine hydrochloride and the procedure of Fisher and Thompson (1979) was used to estimate sulfhydryl groups. To estimate the total number of sulfhydryl groups arising from both free cysteine residues and reduction of cystine residues (Habeeb 1972) the following procedure was used. To a 10-ml graduated tube containing 1·71 g guanidine hydrochloride was added 0·1 ml 0·1 M EDTA (disodium salt) and 1 ml protein solution containing 5 mg β-glucuronidase. The solution was reduced with 1 ml 2·5% (w/v) sodium borohydride at 37°C for 16 h under nitrogen. Excess borohydride was destroyed with 0·5 ml 1 M KH_2PO_4 containing 0·2 M HCl followed after 5 min with 2 ml acetone. After bubbling nitrogen through the solution for 5 min 0·5 ml 0·1 M 5,5′-dithiobis-(2-nitrobenzoic acid) was added, the volume brought to 6 ml with water, and the absorbance measured at 412 nm.

Preparation of S-*Carboxymethyl* (*SCM*)-*glucuronidase*

A sample (2–10 nmoles) was dissolved in 1 ml 0·25 M Tris–5 mM EDTA–6 M guanidine hydrochloride buffer with a 10-fold molar excess of dithiothreitol (DTT) over the approximate number of cysteine plus cystine residues, and taken to pH 10·5 under N_2. After standing over night at 37°C the pH was lowered to 8·5 and the protein alkylated with iodoacetate (equivalent to the DTT sulfhydryls) plus Tris to maintain the pH. After dialysis against water the solution was freeze-dried.

Preparation of Labelled SCM-glucuronidase

Labelled [2-^{14}C]iodoacetic acid (57 mCi/mmole) was supplied by the Radiochemical Centre, Amersham, England. The β-glucuronidase (150 mg) for labelling was freeze-dried from the dialysed solution from Sephadex columns in the presence of an amount of guanidine hydrochloride to facilitate redissolving the freeze-dried material and give 15 ml 6 M guanidine hydrochloride solution with added water.

Reduction with DTT (15·4 mg) at pH 8·5, 37°C for 2 h was followed by alkylation first with 250 μCi (0·5 ml) labelled iodoacetate for 15 min, then with a solution of unlabelled iodoacetate (39 mg) equivalent to the total thiol groups in the mixture and a weight of Tris to keep the pH at 8·5. After 15 min the solution was dialysed against water and freeze-dried. Incorporation of label amounted to 2×10^6 cpm/mg.

Separation of Labelled SCM-Cysteine Peptides

The fractionation of a peptic digest of reduced and labelled SCM-glucuronidase was done on a sulfonated polystyrene column (150 by 0·9 cm) equilibrated with 0·2 M pyridine–acetate (pH 3·1) with a linear gradient of 500 ml each chamber, to 2 M pyridine–acetate (pH 5). The radioactive peaks, detected with the scintillation mixture used by Butler (1961), were dried, desalted on Sephadex G10–0·05 M NH_4OH and purified by paper ionophoresis at pH 1·8 followed by paper chromatography in butanol–acetic acid–pyridine–water (15:3:10:12 v/v). After radioautography the zones were cut out, sewn on paper and fractionated by ionophoresis at pH 6·4. Radioactive zones detected by radioautography were analysed for amino acids after hydrolysis, and sequenced by the dansyl–Edman procedure. Partial oxidation of *S*-carboxymethyl cysteine residues during purification resulted in multiple spots for the same peptide, with the sulfoxide form predominating.

A chymotryptic digest of SCM-glucuronidase was fractionated by gel filtration on a column of Sephadex G25 in 0·05 M NH_4OH. Each fraction containing labelled material was freeze-dried and peptide mapped by ionophoresis at pH 6·4 and chromatography as described above. Radioactive zones were analysed and sequenced.

Isolation of Cysteic Acid Peptides

Performic acid oxidation, thermolytic digestion of oxidized β-glucuronidase and isolation of cysteic acid peptides were effected as described by Thompson and Fisher (1978).

Peptide Fractionation and Amino Acid Sequence Determination

Peptide fractionations by gel filtration, paper ionophoresis, paper chromatography, amino acid analysis, and sequence determinations by the dansyl–Edman procedure were substantially the same as previously described (Air and Thompson 1969, 1971; Beard and Thompson 1971).

Results

Sulfur-Amino Acid Content of β-Glucuronidase

β-Glucuronidase isolated by the method of Hawley (1973) gave a single peak on Sephadex G100, a single band on polyacrylamide gel electrophoresis using the discontinuous buffer system of Davis (1964) and a single band in the presence of sodium dodecyl sulfate (Weber and Osborn 1969) corresponding to a subunit moleular weight of approximately 76 000. Recovery of enzyme was 55–65% with specific activities in the range 650 000–750 000 Fishman (1967) units.

The amino acid compositions of β-glucuronidase isolated from both Wistar and Sprague–Dawley rats were reported by Hawley (1973) and were in close agreement. Our values with enzyme isolated from preputial glands from a mixture of these strains were in good agreement with the earlier results. Particular attention was paid to thiol, disulfide and methionine contents since the amino acid compositions reported in the literature for β-glucuronidase isolated from rat preputial glands (Ohtsuka and Wakabayashi 1970; Hawley 1973; Himeno *et al.* 1975; Tulsiani *et al.* 1975) show considerable variation in the estimates of half-cystine and methionine residues.

The number of sulfhydryl groups titrated in 6 M guanidine hydrochloride with Ellman's reagent was 7·9 moles per mole of tetrameric enzyme (mol wt 310 000), titration after reduction of cystine residues, 22·7 moles per mole.

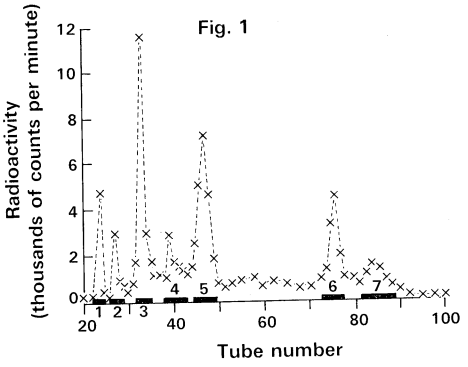
Amino acid analyses for oxidized β-glucuronidase gave a mean value of 23·6 moles cysteic acid per mole when compared with the phenylalanine content (108 mole), while the *S*-carboxymethylated enzyme gave 23·2 moles *S*-carboxymethylcysteine. These results indicate that there are two residues of cysteine and two residues of cystine per subunit.

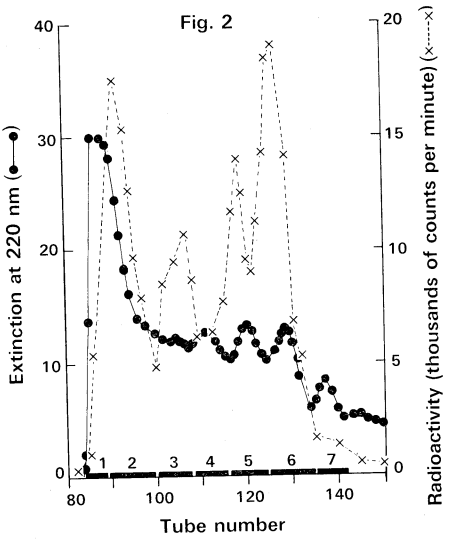
The performic acid-oxidized enzyme gave a mean of 14 residues methionine sulfone per subunit calculated on the same phenylalanine content.

Identification of Amino Acid Sequences containing Cyst(e)ine Residues

Because of the high subunit molecular weight of β-glucuronidase the peptides containing modified cysteine derivatives in enzymic digests were mixed with a large number of other peptides. The selective methods for their detection involved radio-labelling with [2-^{14}C]iodoacetate, chromatography, and identification of radioactive fractions on paper using radioautography. The initial resolution of peptic peptides on a column of sulfonated polystyrene with a pyridine–acetate buffer gradient is shown in Fig. 1. Each radioactive peptide fraction was subjected to multiple fractionations by paper ionophoresis and chromatography to ensure adequate purification from non-labelled peptides. Chymotryptic peptides were fractionated first by size as shown in Fig. 2 before further purification by similar techniques. The larger peptides proved difficult to handle by paper techniques.

Alternatively, from oxidized β-glucuronidase digests the cysteic acid peptides could be selectively removed because of their acidic nature at pH 3 as a result of the





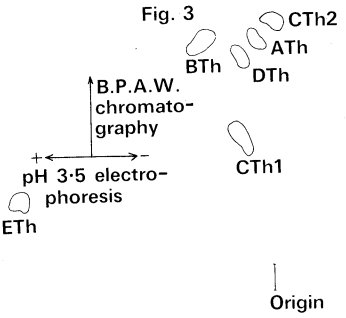


Fig. 1. Separation of peptic peptides from β-glucuronidase (150 mg) which had been reduced and carboxymethylated with [2-^{14}C]iodoacetate on a column (150 by 0·9 cm) of sulfonated polystyrene resin (X2, 200–400 mesh). The column was pumped at 32 ml/h with a linear gradient (500 ml each chamber) of pyridine–acetate buffer [0·2 M (pH 3·1) to 2 M (pH 5)]. Samples (10 μl) were mixed with 0·49 ml water and 5 ml scintillation mixture to count the radioactivity. Tubes containing fractions combined for labelled peptide recovery are shown as bars. No further radioactivity was eluted by 0·2 M NaOH.

Fig. 2. Gel filtration on a column (126 by 2·6 cm) of Sephadex G25 in 0·05 M NH_4OH of the chymotryptic digest of β-glucuronidase (70 mg) which had been reduced and carboxymethylated with [2-^{14}C]iodoacetate. Fraction size 3 ml. Extinction was measured at 220 nm using a light path of 2 mm. Samples (50 μl) of each tube were mixed with 0·45 ml water and 5 ml scintillation mixture to count the radioactivity. Tubes containing fractions combined for labelled peptide recovery are shown as bars. No further radioactivity was eluted up to tube 250.

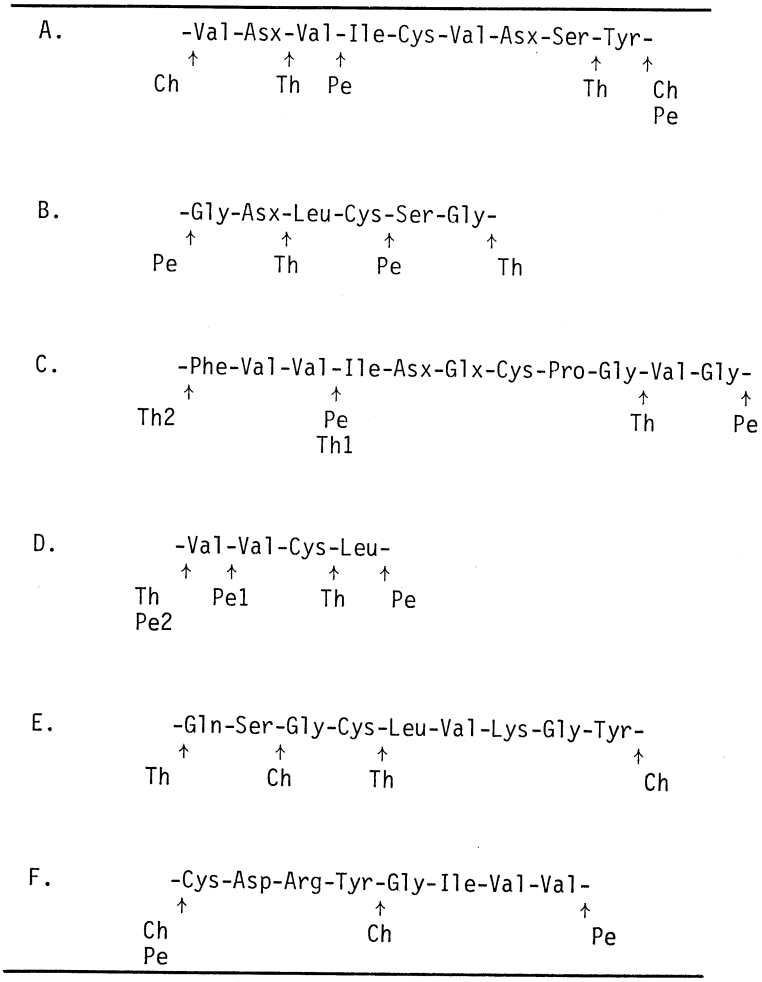
Fig. 3. Peptide map of thermolytic peptides of performic acid-oxidized β-glucuronidase not adsorbed on a column (23 by 1 cm) of sulfonated polystyrene resin (Bio Rad AG 50-X2, 100 mesh) equilibrated and eluted at pH 2·8 with 0·2 M acetic acid. The peptides were mapped by paper ionophoresis at pH 3·5 followed by chromatography in butanol–pyridine–acetic acid–water (B.P.A.W. 15:10:3:12 v/v), and detected with 0·02% (v/v) ninhydrin solution. The peptides are identified by the abbreviations ATh, BTh, CTh1, etc. as shown in Table 1.

strong ionization of the sulfonic acid group. The peptide map of thermolytic peptides isolated by pH 3·5 ionophoresis followed by chromatography is shown in Fig. 3.

The peptides identified by amino acid composition and sequence studies are shown in Table 1 placed in six sequences labelled A–F. Peptides containing Ile-Val or Val-Val sequences gave low recoveries of isoleucine or valine. Because of the small amounts of material available it was not usually possible to resolve the amide status of the

Table 1. Amino acid sequences containing modified cysteine residues in β-glucuronidase

The positions of enzyme cleavage and the enzymes responsible are indicated by arrows and the abbreviations Pe, Ch and Th for pepsin, chymotrypsin and thermolysin respectively. Pel and Th1 indicate bonds partially cleaved so that two peptides Pe1 and Pe2 or Th1 and Th2 were obtained. Peptides were sequenced by the dansyl–Edman procedure



glutamic or aspartic acid residues obtained on acid hydrolysis, or which of the peptides originally contained cysteine residues and which half-cystine residues. It also led in some cases (Table 2) to amino acid sequences being determined that were not fully supported by analyses of impure peptides, and the sequence data on this or related peptides was given greater weight.

Table 2. Peptides containing modified cysteine or cystine residues from enzyme digests of β-glucuronidase

Amino acid composition and chromatographic data of selected peptides (see Table 1 for sequences A-F and enzyme cleavage sites) from digest of *S*-carboxymethylated *β*-glucuronidase (Pe,Ch) and performic acid-oxidized glucuronidase (Th) are given. Hydrolysis was at 100°C for 24 h in unevacuated sealed tubes. Values are given as moles per mole of peptide with preferred values in parentheses, and are uncorrected for losses during hydrolysis or due to incomplete hydrolysis. *S*-Carboxymethylcysteine in peptides eluted from paper are mostly in the sulfoxide form which is destroyed during hydrolysis and is partially recovered as cysteic acid (cf. Webster and Thompson 1980)

Amino acid	APe	ACh	ATh	BPe	BTh	CPe	CTh1	CTh2	DPe1	DPe2	ECh	ETh	FPe	FCh
Lys											1·0 (1)			
His													0·9 (1)	1·0 (1)
Arg														
Asp	1·2 (1)	2·0 (2)	1·0 (1)	0·5 (1)		1·0 (1)	1·0 (1)	1·3 (1)			0·6 (0)		1·0 (1)	1·1 (1)
Thr		0·2 (0)						0·6 (0)						
Ser	1·2 (1)	0·5 (1)	1·0 (1)		1·0 (1)			0·6 (0)				1·0 (1)		
Glu		0·3 (0)		0·2 (0)		1·0 (1)	1·2 (1)	1·0 (1)				1·0 (1)		
Pro						1·0 (1)	0·7 (1)	0·9 (1)						
Gly		0·5 (0)		1·0 (1)	1·0 (1)	1·8 (2)	1·0 (1)	0·9 (1)			1·6 (2)	1·3 (1)	1·0 (1)	
Ala		0·7 (0)												
Val	1·1 (1)	1·8 (3)	1·6 (2)			0·9 (1)		1·9 (2)	0·9 (1)	1·2 (2)	1·2 (1)		1·3 (2)	
Ile	0·9 (1)	0·7 (1)	0·8 (1)			0·9 (1)	0·6 (1)	1·3 (1)					0·4 (1)	
Leu		0·5 (0)		1·0 (1)	0·9 (1)				1·0 (1)	1·0 (1)	1·2 (1)			
Tyr	1·0 (1)	0·2 (1)									0·6 (1)		1·0 (1)	0·4 (1)
Phe			0·3 (0)					0·6 (1)			0·3 (0)			
Cys	0·4 (1)	+ (1)	1·4 (1)	+ (1)	1·2 (1)	0·3 (1)	0·6 (1)	1·1 (1)	0·2 (1)	0·3 (1)	+ (1)	0·8 (1)	0·1 (1)	0·4 (1)
Total	6	9	6	4	4	8	6	9	3	4	7	4	8	4
Fraction[A]	5	3		5		3			5	5	6		6	6
Mobility														
Lys (pH 1·9)	0·3			0·5		0·35			0·5	0·35	0·4		0·5	
Asp (pH 6·4)	0·2	0·3		0·28		0·5			0·28	0·2	0		0·1	0·25
R_F (B.P.A.W.)[B]	0·45	0·5		0·4		0·2			0·6	0·5	0·4		0·45	0·25

[A] See Fig. 1 or Fig. 2. [B] Butanol–pyridine–acetic acid–water (15:10:3:12 v/v).

Peptide sequences were determined by the dansyl–Edman procedure, with the exception of the ETh sequence where the terminal residue gave no dansyl derivative and had an electrophoretic mobility suggesting a pyrrolidone carboxylyl residue had been formed from a glutaminyl residue. The sequence of this peptide was determined by partial acid hydrolysis (Sanger and Thompson 1953) to give tripeptides Glu(Ser, Gly) and Ser(Gly, $CySO_3H$).

Discussion

The peptides that contain modified cysteine residues in enzyme digests of β-glucuronidase or performic acid-oxidized β-glucuronidase could be accommodated in six sequences. Since two cysteine and two cystine residues per mole of protomer were found by titration with Ellman's reagent there are probably four identical subunits in the native enzyme of molecular weight 310 000.

In general, there was evidence from at least two enzyme digests for the sequences presented in Table 1. Because of the difficulties mentioned earlier in isolating pure peptides with stoichiometric ratios of amino acids and clear sequence data, it is impossible from these types of experiment to assert that no other peptides were present containing cyst(e)ine residues. Thus the presence of a basic amino acid near a cysteic acid residue as in sequence F will prevent peptides containing both these residues from being unadsorbed on sulfonated polystyrene at pH 3. Moreover, the partial oxidation of *S*-carboxymethylcysteine residues with paper techniques leads to weak zones of the same basic sequences which are not always amenable to purification and identification. With these assumptions in mind it seems probable that the four subunits of β-glucuronidase are identical or very closely related.

Further progress in amino acid sequence studies of β-glucuronidase, an enzyme with a large subunit molecular weight, will depend on the isolation of smaller fragments from cyanogen bromide digestion, or alternatively, by isolation of mRNA and nucleotide sequence analysis after cloning.

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