Characterization of a Peptide Fragment Containing the Essential Half-Cystine Residue of a Microsomal Disulfide Interchange Enzyme*

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ABSTRACT: Tryptic and chymotryptic peptides containing the critical sulfhydryl group involved in the enzymatic activity of a microsomal disulfide interchange enzyme were isolated from preparations in which the reactive sulfhydryl group was labeled with [^{14}C]iodoacetic acid. Tryptic fragments were degraded with leucine aminopeptidase and carboxypeptidase. The results of these experiments, together with the compositions of radioactive peptides produced by chymotrypsin digestion, indicate that the functionally involved sulfhydryl group is present in the unique sequence, Ala-Tyr-Cys-(His,Gly)-Lys.

An enzyme from beef liver microsomes that catalyzes disulfide interchange in proteins has recently been purified and characterized (De Lorenzo *et al.*, 1966). This enzyme, found in rat and beef liver microsomes (Goldberger *et al.*, 1963) and in chicken, pigeon, and pig pancreas (Venetianer and Straub, 1963a,b), has been shown to catalyze the rearrangement of random pairs of half-cystine residues deliberately introduced in disulfide bonded proteins such as bovine pancreatic ribonuclease, egg white lysozyme (Goldberger *et al.*, 1964), soy bean trypsin inhibitor (Steiner *et al.*, 1965), and pepsinogen (F. De Lorenzo and C. B. Anfinsen, 1966, unpublished data). The purified disulfide interchange enzyme contained three half-cystine residues (De Lorenzo *et al.*, 1966).

Studies on the mechanism of the enzymic catalysis have shown that the enzyme contains a single reactive thiol group, essential for enzymic activity (Fuchs *et al.*, 1967). This sulfhydryl group, which appears to be partially masked in the freshly isolated enzyme, is fully converted to the SH form when the purified enzyme is treated with 2-mercaptoethanol. The remaining two half-cystine residues appear to be linked by a disulfide bond and do not react with sulfhydryl reagents in the native enzyme but only after reduction in the presence of denaturing agents. Carboxymethylation of the easily reduced half-cystine residue with [^{14}C]iodoacetic acid produced complete inactivation and peptide maps of trypsin digests of such labeled material showed the presence of a single major radioactive peptide together with a second minor component. The data in the present paper show that both of these peptide fragments are derived from the same portion of the amino acid sequence of the polypeptide chain. Studies on the structures of these two peptides and of several chymotryptic fragments derived from the same portion of the chain are described in this report.

Materials and Methods

The disulfide interchange enzyme was purified from beef liver microsomes as previously described (De Lorenzo *et al.*, 1966). The concentrations of protein solutions were determined by the method of Lowry *et al.* (1951).

Measurements of pH were made with a Radiometer, Model PHM-25, pH meter. [1-^{14}C]Iodoacetic acid was purchased from Nuclear Research Chemicals, Inc. Radioactive samples were counted in a Tri-Carb liquid scintillation spectrometer using Bray's (1960) solution as scintillator. Solutions of urea, recrystallized from 95% ethanol, were prepared immediately prior to use. Trypsin (DFP[1] treated (Potts *et al.*, 1962), twice crystallized), α-chymotrypsin (three times crystallized), leucine aminopeptidase (DFP treated), and carboxypeptidase B (DFP treated) were obtained from Worthington Biochemical Corp. Sephadex G-25 (fine beads) was obtained from Pharmacia (Uppsala).

Dowex AG50WX2 (200–400 mesh) was obtained from the California Corp. for Biochemical Research. The resin was washed with distilled water, 1 N NaOH, distilled water, 3 N HCl, and distilled water. Peptide maps were performed by the method of Katz *et al.* (1959). Samples of proteolytic digests were applied to sheets of Whatmann No. 3MM filter paper and chromatographed in butanol–acetic acid–water (4:1:5).

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[1] Abbreviations used: DFP, diisopropylphosphorofluoridate; CMC, carboxymethylcysteine; LAP, leucine aminopeptidase.

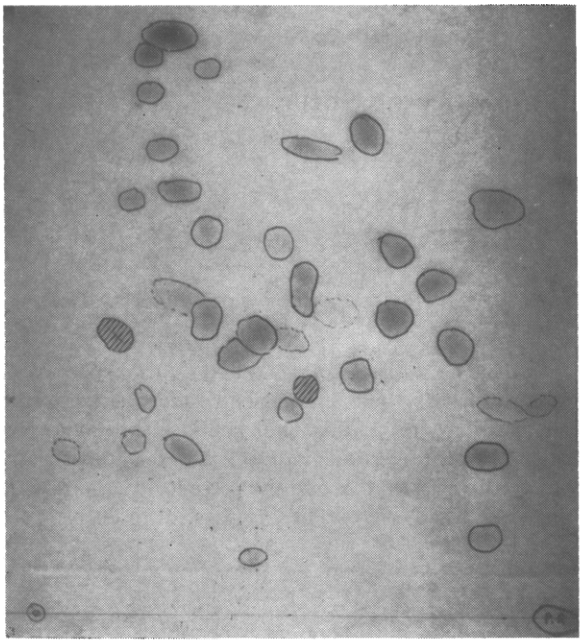

FIGURE 1: Peptide map of a trypsin digest of the [^{14}C]-carboxymethylated enzyme. The sample, containing 2 mg of peptides and 80,000 cpm, was applied to Whatman No. 3MM paper for two-dimensional separation. Phenol red and lysine were used as reference standards in the chromatographic and electrophoretic dimension, respectively.

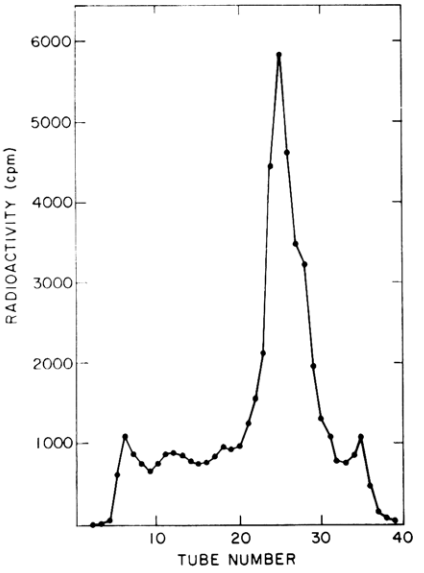

FIGURE 2: Gel filtration on Sephadex G-25 of a trypsin digest of [^{14}C]carboxymethylated enzyme. Fractions of 2.7 ml were collected. The column was equilibrated with 0.001 M ammonium bicarbonate and eluted with the same buffer. Tubes 21–31 were pooled and lyophilized.

Phenol red was added at the origin as a reference. After drying, the paper was submitted to electrophoresis in pyridinium acetate buffer, pH 3.5, for 80 min at 2500 v in a Gilson, Model D, electrophoresis apparatus. The peptide maps were submitted to radioautography using medical X-ray film (Blue Brand Kodak).

Samples for amino acid analysis were hydrolyzed in 0.5 ml of constant boiling HCl in sealed, evacuated tubes at 110° for 24 hr. The amino acid analyses were performed using a Spinco amino acid analyzer, Model 120B, equipped with a Minneapolis Honeywell recorder containing a no. 365928-999 resistor card for the range 4–5 mv. Values for residues per mole of peptide were calculated on the basis of integral numbers of lysine residues. The analytical results suggest that an unusually large fraction of *S*-carboxymethylcysteine has been converted to cystine during acid hydrolysis. We have observed a similar phenomenon in other experiments on the amino acid composition of peptides eluted from peptide maps. Since LAP digestion released as much as 88–90% of the radioactive counts as *S*-carboxymethylcysteine in some experiments, we have reported residues of this amino acid on the basis of the sum of the values for CMC and half-cystine.

Results

Alkylation of the Enzyme with [^{14}C]Iodoacetic Acid. As described elsewhere (Fuchs *et al.*, 1967), the half-cystine residue essential for the enzymic activity of the freshly isolated disulfide interchange enzyme is masked and does not react with alkylating agents. After treatment of the enzyme with β-mercaptoethanol, this thiol group becomes available and reacts stoichiometrically with iodoacetate. For the purpose of labeling, and subsequent isolation of a labeled peptide, samples of the enzyme (generally 100 mg) were dissolved in 5 ml of 0.1 M Tris–HCl buffer, pH 7.8, and treated with β-mercaptoethanol, 0.057 M, for 3.5 hr at room temperature. To this mixture were added 60 mg of [^{14}C]iodoacetic acid dissolved in 0.5 ml of 1 M Tris base and 2.5 ml of 1 M Tris–HCl, pH 8.5. The mixture was incubated for 10 min at room temperature and 25 μl of β-mercaptoethanol was added to stop the reaction. Removal of excess reagents was carried out by gel filtration on a Sephadex G-25 (fine beads) column, 2.5 × 50 cm. Only one radioactive peak, containing all the protein, emerged from the column. The radioactive fractions were pooled and concentrated by vacuum dialysis. Based on radioactivity measurements, the extent of labeling was 1.2 moles of [^{14}C]-iodoacetate/mole of protein, assuming a molecular weight of 42,000 (Fuchs *et al.*, 1967). Further reduction and alkylation of the enzyme was carried out as previously reported (Fuchs *et al.*, 1967).

Trypsin Digestion. The treated protein (50 mg) was dissolved in 15 ml of 0.2 M ammonium bicarbonate and incubated with trypsin (1% by weight of substrate) at 37°. After 2-hr digestion the mixture was lyophilized. Peptide map of the tryptic digest showed two radioactive spots (Figure 1) of different intensity. The rela-

TABLE I: Amino Acid Composition of Radioactive Peptides Obtained after Trypsin Digestion.

	Amino Acids (residue/mole)[a]							
			½-Cys					
Peptide	Ala	Tyr	CMC	½-Cys	Sum[b]	His	Gly	Lys[c]
T_1	—	—	0.49 (0.0045)	0.47 (0.0043)	0.96 (0.0088)	1.0 (0.0095)	1.1 (0.0105)	1.0 (0.0092)
T_2	1.0 (0.0175)	0.9 (0.0152)	0.33 (0.0056)	0.32 (0.0054)	0.65 (0.0110)	0.97 (0.0164)	1.1 (0.0190)	1.0 (0.0170)

[a] The values in parentheses are the results of amino acid analysis and are expressed as micromoles. [b] See text. [c] Values given for residues per mole of peptide are calculated on the basis of integral numbers of lysine residues.

tive amounts of those two components were variable in different digests.

Isolation of Tryptic Peptides. The lyophilized material was dissolved in 0.2 M ammonium bicarbonate and subjected to gel filtration on a Sephadex G-25 (fine beads) column, 1 × 95 cm. The chromatographic pattern is shown in Figure 2. The radioactive material that emerged at the effluent front of the column represents undigested [^{14}C]carboxymethylated enzyme. Fractions 21–31 were pooled. After lyophilizing, the material was applied on a 0.9 × 93 cm Dowex AGW 50-X2 column, following the procedures described by Canfield (1963). Gradient elution was performed at a flow rate of 24 ml/hr with a Sigma motor pump, Model T8 (Middleport, N. Y.). The buffer volume in each chamber of the Varigrad was 150 ml. As shown in Figure 3, two radioactive peaks (T_1 and T_2) were eluted from the column. The fractions comprising each peak were pooled and lyophilized. Because the radioactive peptides from both peaks were contaminated with trace amounts of other peptides, a further purification was achieved by paper chromatography and electrophoresis as described above.

Composition and Structure of Peptide T_1 and T_2. The peptide indicated as T_1 represented the major radioactive peptide. It was obtained in pure form by elution with 25% (v/v) pyridine–water from paper after chromatography and electrophoresis in a yield of 45% as based on the radioactivity measurements. The amino acid composition is given in Table I and corresponds to a tetrapeptide containing equivalent amounts of *S*-carboxymethylcysteine, glycine, histidine, and lysine. Digestion of 0.02 μM peptide with leucine amino-peptidase in 0.05 M ammonium bicarbonate and 0.1 M $MgCl_2$ for 2 hr released, in micromoles: CMC, 0.010; Gly, 0.005; His, 0.004; and Lys, 0.005. Hence *S*-carboxymethylcysteine appears to be the NH_2-terminal residue. Digestion with carboxypeptidase B in ammonium bicarbonate buffer, 0.2 M, at 37° for 6 hr led to the quantitative release of lysine as expected. Subsequent degradation with carboxypeptidase A failed to release further amino acids.

Peptide T_2 was also obtained in pure form by elution after two-dimensional peptide mapping but

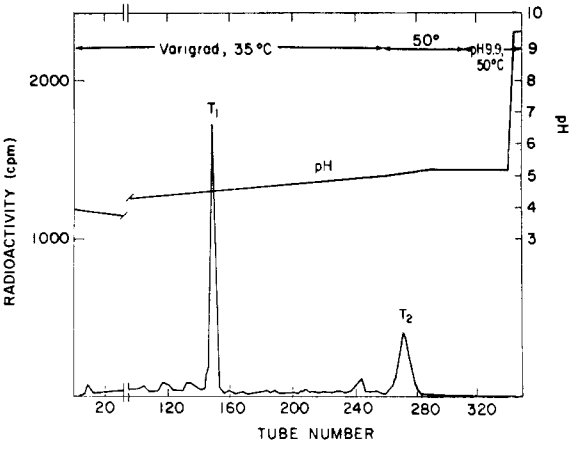

FIGURE 3: Elution pattern of tryptic peptides, pooled from the G-25 Sephadex column, on Dowex 50-X2 resin. Fractions of 4 ml were collected. Aliquots of the effluent fractions were used to measure the radioactivity. Fractions comprising the two radioactive peaks were pooled and lyophilized.

with a lower recovery (approximately 25%). The composition of T_2 was (Ala, Tyr, CMC, Gly, His, Lys) (Table I). Digestion with carboxypeptidase B again led to quantitative release of lysine. Hence, it appears that alanine and tyrosine are at the NH_2-terminal end of peptide T_2.

Chymotrypsin Digestion. For chymotrypsin digestion, 50 mg of the [^{14}C]carboxymethylated protein was used. The lyophilized protein was dissolved in 15 ml of 0.2 M ammonium bicarbonate and incubated with chymotrypsin (2% by weight of substrate) at 37° for 2 hr. A small aliquot from the chymotryptic digestion was used for peptide mapping. These maps showed, on radioautography, six radioactive components (Figure 4).

Isolation of Chymotryptic Peptides. The radioactive chymotryptic peptides were separated by gradient elution from Dowex 50-X2 resin using the same conditions described for the tryptic digest. The elution

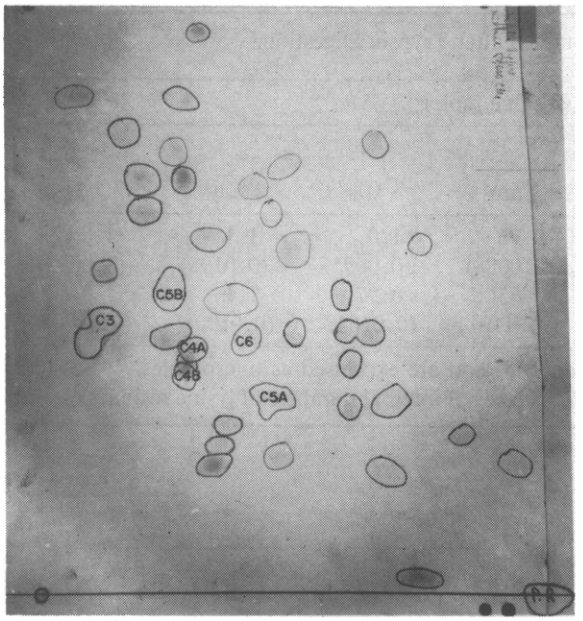

FIGURE 4: Peptide map prepared from a chymotrypsin digest of [^{14}C]carboxymethylated enzyme. Digested material (2 mg) was applied on Whatman No. 3MM paper. The numbers refer to the radioactive peptides, the compositions of which are given in the text.

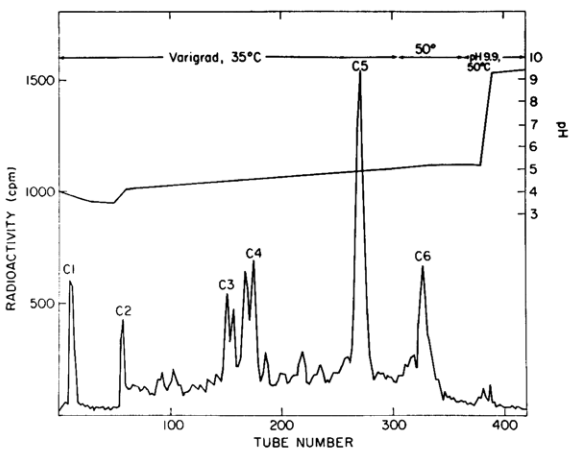

FIGURE 5: Chromatography of the chymotryptic digest on a Dowex 50-X2 column. Fractions of 4 ml were collected. Radioactivity was measured on aliquots of the effluent fractions.

pattern is shown in Figure 5. Fractions comprising each of the six radioactive peaks were pooled, lyophilized, and subjected to two-dimensional peptide mapping for further purification.

Amino Acid Composition of the Chymotryptic Peptides. The radioactive peptides obtained from Dowex 50-X2 were located on the peptide maps by radioautography, and eluted with pyridine (25% (v/v) in water). To examine the purity of the radioactive peptides from

TABLE II: Amino Acid Composition of Radioactive Peptides Obtained after Chymotrypsin Digestion.

Peptide	Amino Acids (residues/mole)[a]															
	Ala	Tyr	½-Cys CMC	½-Cys	Sum[b]	His	Gly	Lys[c]	Glu	Asp	Thr	Pro	Ile	Leu
C_3			0.4 (0.0044)	0.3 (0.0039)	0.7 (0.0083)	1.0 (0.0116)	1.2 (0.0143)	1.0 (0.0116)	1.2 (0.0143)					
C_{4B}	1.8 (0.0148)	0.7 (0.0053)	0.5 (0.0039)	0.7 (0.0057)	1.3 (0.0096)	0.8 (0.0063)	1.3 (0.0104)	1.0 (0.0085)	1.6 (0.0131)	0.8 (0.0065)		0.9 (0.0075)		1.0 (0.0079)
C_{6A}	1.2 (0.0140)	0.9 (0.0106)	0.3 (0.0040)	0.4 (0.0053)	0.7 (0.0093)	0.9 (0.0108)	2.1 (0.0254)	2.0 (0.0234)	2.4 (0.0287)	1.5 (0.0181)	1.2 (0.0137)	1.3 (0.0154)	1.0 (0.0119)	2.0 (0.0237)

[a] The values in parentheses are the results of amino acid analysis and are expressed as micromoles. [b] See text. [c] Values given for residues per mole of peptide are calculated on the basis of integral numbers of lysine residues.

peaks C_3 to C_6, peptide maps were performed in duplicate. One, with a smaller applied load, was stained with ninhydrin (0.25 mg/ml) while the other was used for elution of the radioactive spots. The material from peak C_1 showed no radioactive spots on the map except for some radioactive material that did not move from the origin. Peak C_2 yielded a single very faint radioactive spot that was insufficient for amino acid analysis. The amino acid compositions of the radioactive components eluted from peptide maps of peaks C_3–C_5 are given in Table II. Peaks C_4 and C_5 yielded two radioactive components on peptide maps, designated as A and B, respectively, on Figure 4. Peptides C_{4A}, C_{5B}, and C_6 were produced in small amounts and further purification was not possible. Therefore their amino acid analyses are not given, although they all contained carboxymethylcysteine, histidine, glycine, and lysine.

Discussion

Evidence for the involvement of a specific thiol group in the enzymic catalysis of disulfide interchange by the microsomal enzyme has been reported previously (Fuchs *et al.*, 1967). Labeling of this sulfhydryl group with [^{14}C]iodoacetic acid has provided a marker for identification and characterization of the amino acid sequence in the vicinity of this group. Two radioactive peptides have been isolated from tryptic digests. Peptide T_1 is the major radioactive peptide, representing 70% of the total radioactivity, while peptide T_2 contained six amino acids including tyrosine and accounted for the remaining 30% of the radioactivity. Because of the presence of tyrosine in peptide T_2, and because this peptide contains all the other four amino acids found in peptide T_1, it is reasonable to suppose that peptide T_2 was produced by trypsin digestion while peptide T_1 was formed by a subsequent cleavage of the tyrosyl bond of peptide T_2 either by contamination or intrinsic chymotrypsin activity in the trypsin employed. This suggestion is also supported by the isolation of peptide C_3 (CMC-(Gly,His)-Lys-Glu) and peptides C_{4B} and C_{5A} from chymotryptic digests. The limited amount of enzyme available and the low yield of purified peptides restricted the number and the kinds of techniques that could be applied in the elucidation of the amino acid sequence bearing the essential thiol group. Thus, no dependable information following removal of the COOH-terminal lysine residue with carboxypeptidase B could be obtained by the Edman degradation procedure or by hydrazinolysis. The results obtained by digestion with LAP and carboxypeptidase B, and from amino acid analysis of the chymotryptic fragments, suggest the following structure for peptide T_2: Ala-Tyr-CMC-(Gly,His)-Lys. The presence, in all the labeled peptide fragments, of *S*-carboxymethylcysteine, glycine, histidine, and lysine strongly suggests a unique sequence containing the single reactive thiol group.

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