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Terminal Sequences of Lysosome Solubilized Pig Liver Cytochrome b_5 Reductase

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Summary. Lysosome solubilized pig liver cytochrome b_5 reductase has the following twenty residues as NH_2-terminal sequence: Ser-Thr-Pro-Ala-Ile-Thr-Leu-Glu-Asn-Pro-Asp-Ile-Lys-Tyr-Pro-Leu-Arg-Leu-Ile-Asp. The fragment is predicted to exist largely in the random conformation with 2 β-bends at residues 9-12 and 14-17. The reductase fragment appears clean enough for complete sequence investigation and is very similar to cathepsin D-solubilized rabbit liver cytochrome b_5 reductase.

Our laboratory is currently investigating microsomal oxidases and associated redox proteins. We wish to report on the NH_2- and COOH-terminal sequences of the cytochrome b_5 reductase liberated from microsomes by lysosomal protease.

EXPERIMENTAL PROCEDURES

Materials. Cytochrome b_5 reductase fragment. Pig liver (about 500 g) was homogenized 5 min with 4 volumes of ice cold 0.15 M KCl, using a Matsushita Homogenizer, Model MX-140S. The homogenate was centrifuged at 10,000 g for 20 min, the supernatant solution adjusted to pH 5.3 with acetic acid and after 2 hours, centrifuged at 10,000 x g for 30 min at 4° C. The precipitate, "acid precipitated microsomes," was dissolved in 0.1 M potassium phosphate buffer (pH 7.7) containing 1 mM EDTA. The acid precipitated microsomes from pig liver (about 8 kg) was adjusted to pH 5.6 and incubated at 37° C for 3 hours and then were centrifuged at 10,000 g for 20 min. The cooled solubilized reductase was recovered from the supernatant fluid by fractionation with ammonium sulfate (45-75%). After dialysis was accomplished, Sephadex G-100, DEAE-52 and hydroxylapatite chromatography steps followed. The purified fragment was active

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in reducing cytochrome b_5 but did not bind to the microsomal membrane. The enzyme is very similar to the cathepsin D-solubilized rabbit enzyme isolated by Mihara et al.

Cpase A and B were purchased from Worthington Biochemical Corp. and other reagents used were of reagent quality or sequenal grade, purchased from standard chemical companies.

Methods. Protein Concentration was estimated by the method of Lowry et al. (2). Manual Edman sequencing as described by Tarr (3) required about 1 mg of protein. Amino acid analyses were performed on the Beckman Model 121 MB automatic amino acid analyzer as described by Spackman et al. (4). Pth-amino acids separated on an ultrasphere ODS column on the Beckman Model 110A HPLC and identified by thin layer chromatography according to Tarr (3).

RESULTS AND DISCUSSION

Purity and Molecular Weight. The purity of the pig liver cytochrome b_5 reductase, was checked by the Weber and Osborn (5) procedure. The SDS polyacrylamide gel pattern Fig. 1 shows only a small amount of a higher molecular component, which may or may not be a dimer. The estimated molecular weight shown in Fig. 2, is about 29,500, which is slightly higher than the 26,500 value reported for the rabbit liver fragment.

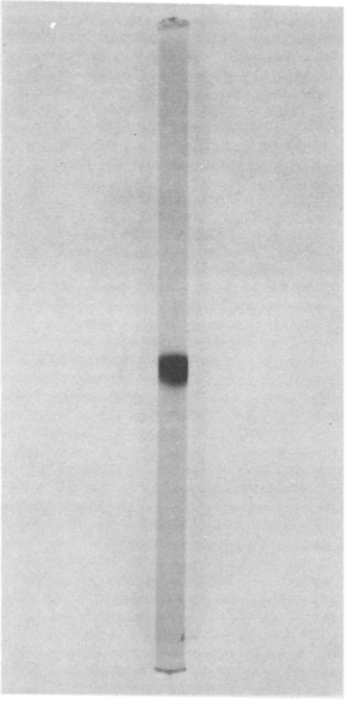
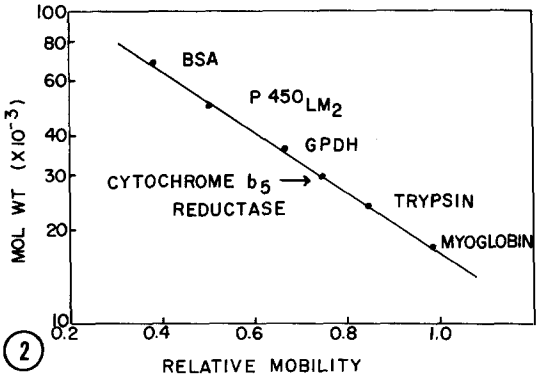


Figure 1. SDS polyacrylamide gel electrophoresis of cathepsin D treated Porcine cytochrome b_5 reductase (20 µg) on a 7.5% acylamide gel according to the method of Weber and Osborn.



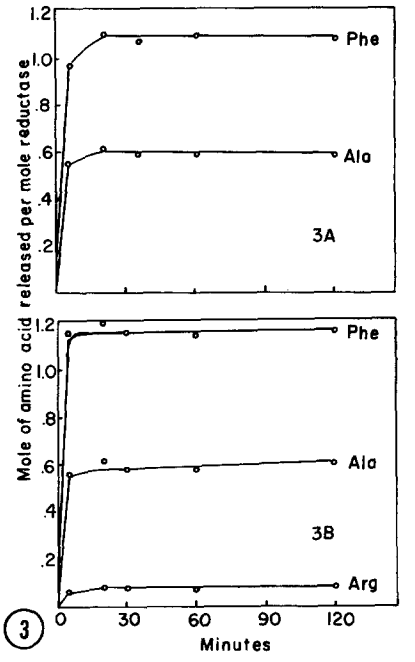


Figure 2. Molecular weight of cathepsin D treated porcine cytochrome b_5 reductase as determined by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn. The data are an average of three determinations. Proteins utilized as molecular weight standards are bovine serum albumin (BSA), rabbit liver cytochrome P450-LM_2 (P450-LM_2), rabbit muscle glyceraldehyde-3phosphate dehydrogenase (GPDH), trypsin, and myoglobin.

Figure 3. COOH-terminal amino acids released from lysosomal protease treated porcine cytochrome b_5 reductase by carboxypeptidase. Carboxypeptidase A (upper frame) and carboxypeptidase A plus B (lower frame) digestions of about 60 nmoles of the reductase were performed at 37° in 0.2 M N-ethylmorpholine-acetate pH 8.3, 1% SDS at molar ratios of 1:200 and 1:1:300, respectively. Approximately 10 nmole aliquots were periodically removed from each reaction then analyzed in a Beckman 121MB amino acid analyzer after being treated with AG 50-X8 resin (H^+ form) to remove undigested protein. All data was normalized to a norleucine internal standard.

Purity of Cytochrome b_5 Reductase and Molecular Weight. The amino acid composition of the pig liver fragment determined by us and the cathepsin D liberated soluble cytochrome b_5 reductase from rabbit liver as determined by Mihara et al. (1) can be found in Table I.

End Group Studies. A step of Edman degradation yielded Pth-serine with slight amounts of Asp, Lys and Gly. Carboxypeptidase A digestion (5) liberated Ala and Phe, Fig. 3A, Cpase A and B yield additional small amounts of arginine, Fig. 3B.

Table I. Amino acid composition of the cytochrome b_5 reductase fragments

Amino acid	Residues per mole	
	Rabbit liver	Porcine liver[a]
Lysine	16	16.31 (16)
Histidine	8	7.99 (8)
Arginine	14	13.95 (14)
Aspartic Acid	22	23.01 (23)
Threonine	9	10.25 (11)[b]
Serine	13	10.16 (10)[b]
Glutamic Acid	22	23.2 (23)
Proline	22	24.47 (24)
Glycine	17	17.44 (17)
Alanine	13	12.50 (13)
Cysteine	3	+
Valine	15	14.74 (15)[c]
Methionine	7	6.51 (7)
Isoleucine	13	14.94 (15)[c]
Leucine	21	21.0 (21)[c]
Tyrosine	7	6.53 (7)[b]
Phenylalanine	11	10.99 (11)
Tryptophan	3	+
Total residues	236	235 +
Mol. wt.	26,500	26,500[d]

[a]Average of 2-24 hr HCL hydrolyzates at 110°C unless otherwise indicated.

[b]Extropolated to zero hydrolysis time from 24 and 96 hrs hydrolyzates.

[c]Average of 24 and 96 hr HCL hydrolyzates.

[d]The Weber and Osborne procedure (5) yielded a molecular weight of 29,500, but amino acid composition data is thought to be more reliable.

NH_2-Terminal Sequence Results. Manual Edman degradation (3) on about 1 mg of the fragment yielded the results summarized in Table II. About an equal number of hydrophobic and hydrophilic amino acid residues are present in the NH_2-terminus of the fragment.

Table II. NH_2-Terminal Sequence of the Cytochrome b_5 Reductase Fragment

Step	Pth-amino acid	Hydrophobic residues	Hydrophillic residues
1	Ser	+	
2	Thr	+	
3	Pro		+
4	Ala		+
5	Ile		+
6	Thr	+	
7	Leu		+
8	Glu	+	
9	Asn	+	
10	Pro		+
11	Asp	+	
12	Ile		+
13	Lys	+	
14	Tyr	+	
15	Pro		+
16	Leu		+
17	Arg	+	
18	Leu		+
19	Ile		+
20	Asp	+	
Total Residues	20	10	10

Predicted Conformation of the NH_2-Terminal Region of Fragment. Assuming that the procedure of Chou-Fasman (7) is applicable to membrane proteins, the conformation of the NH_2-terminal region of the fragment was calculated to be largely in the random chain conformation with β-turns at residues 9-12 (Asn-Pro-Asp-Ile) and 14-17 (Tyr-Pro-Leu-Arg). It should be noted that Mihara et al. (1) predicted from far UV CD studies that the rabbit liver reductase fragment consisted of 33% α-helix, 3.7% β-sheet and 64% random conformations. Thus, the NH_2-terminal region would contribute towards a certain percentage of the random chain conformation of the fragment.

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