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DEMONSTRATION THAT BOVINE ERYTHROCYTE CYTOCHROME b_5 IS THE HYDROPHILIC SEGMENT OF LIVER MICROSOMAL CYTOCHROME b_5

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A structural comparison has been made between bovine erythrocyte cytochrome b_5 and solubilized forms of bovine hepatic microsomal cytochrome b_5. Two soluble forms of microsomal cytochrome b_5 (designated Forms A and B) were generated by digestion of microsomes with a crude hepatic lysosomal cathepsin preparation and purified by successive chromatography on DEAE-cellulose, Bio-Gel P-60 and DEAE-Sephadex A-50. Amino acid analyses and terminal residue analyses identified Form A as the segment corresponding to residues 1–95 of the native microsomal protein and Form B as the segment corresponding to residues 1–107. Erythrocyte cytochrome b_5 I was shown to be a protein which corresponds to a segment of the hepatic microsomal molecule containing residues 1–97, whereas erythrocyte cytochrome b_5 II is a protein corresponding to residues 1–95. Like the native microsomal cytochrome and the cathepsin-solubilized forms of the cytochrome, no amino terminal residue could be detected in the erythrocyte cytochrome. Carboxypeptidases A and B released from erythrocyte Form I a residue eluting at the position of serine, but released no residue from Form II. The results are consistent with serine being the residue at position 97 of the native microsomal protein, and proline and serine being the residues in positions 94 and 95, respectively. The maps of the tryptic peptides derived from the apoprotein forms of erythrocyte cytochrome b_5 I and II and cathepsin-solubilized microsomal Forms A and B were very similar, with eight of the expected twelve peptides displaying the same mobility on every map. Amino acid analyses of the isolated tryptic peptides from erythrocyte Form I and hepatic Form B confirmed the structural assignments of these proteins. These data demonstrate that the soluble forms of erythrocyte cytochrome b_5 correspond to hydrophilic segments of the native membrane-bound microsomal cytochrome b_5 and suggest that the hepatic lysosomal proteases serve as a good model for the putative erythroid proteases which solubilize microsomal cytochrome b_5 during erythroid maturation.

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Abbreviations: BANA, α-*N*-benzoyl-L-arginine naphthylamide; DFP, diisopropyl phosphorofluoridate; LNA, L-leucine β-naphthylamide; SDS, sodium dodecyl sulfate; TPCK, L-1-tosylamido-2-phenylethylchloromethylketone.

Data supplementary to this article are deposited with, and can be obtained from, Elsevier Biomedical Press, B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to BBA/DD/220/31240/705 (1982) 228. The supplementary information includes: details of the preparation of lysosomes, microsomes, of a cathepsin fraction; and solubilized liver microsomal cytochrome b_5 chromatography profiles, peptide maps and amino acid compositions of tryptic peptides from apocytochromes; expected tryptic peptides (structure and theoretical net charge).

Introduction

Erythrocyte cytochrome b_5 is a water-soluble protein which has been isolated from the supernatant fraction of human [1], rabbit [1], bovine [2], mouse [3] and pig [4] red blood cells. The role of cytochrome b_5 in the erythrocyte is to reduce methemoglobin to the physiologically active ferrous form [5,6].

Erythrocyte cytochrome b_5 is indistinguishable from protease-solubilized hepatic microsomal cytochrome b_5 on the basis of spectral properties [1] and ability to react with other redox proteins [6,7]. The erythrocyte protein is intermediate in size between detergent-solubilized and trypsin-solubilized forms of hepatic microsomal cytochrome b_5 [2,8].

Two forms of bovine erythrocyte cytochrome b_5 have been isolated and studied [2]. The forms differ very little in molecular weight and amino acid composition. Trypsin degrades these forms to core hemepeptides that are electrophoretically indistinguishable from each other and from trypsin-solubilized bovine hepatic microsomal cytochrome b_5. The purified tryptic hemepeptides derived from erythrocyte and hepatic cytochromes are also identical in terms of amino acid composition. The composition of erythrocyte cytochrome b_5 I agrees closely with residues 1–97 of hepatic microsomal cytochrome b_5. Similarly, the composition of erythrocyte cytochrome b_5 II agrees with the composition of the segment containing residues 1–95.

We have shown that cytoplasmic cytochrome b_5 is not present in an immature erythroid cell, but, instead, a membranous form of the cytochrome is present [3]. Moreover, electron microscopy has shown that the endoplasmic reticulum disappears during erythroid maturation. These findings, together with the similarities between erythrocyte and microsomal cytochrome b_5, suggested that microsomal cytochrome b_5 might be a precursor of the soluble erythrocyte protein.

This paper compares the structures of the forms of bovine erythrocyte cytochrome b_5 with those of two forms of solubilized bovine hepatic microsomal cytochrome b_5 prepared by digestion of the microsomal protein with a cathepsin fraction from hepatic lysosomes. We present chemical evidence that the forms of erythrocyte cytochrome b_5 indeed correspond to hydrophilic segments of microsomal cytochrome b_5 and report that the hepatic lysosomal proteases are capable of converting microsomal cytochrome b_5 to the erythrocyte form of the protein. Some of these findings have been presented previously in abstract form [9].

Experimental procedure

Details of these procedures have been placed in the BBA Data Bank (see footnote on p. 228).

Materials

Fresh bovine blood and liver were purchased from Kappler Packing Co., Ann Arbor, MI. Ultrafiltration membranes were obtained from Amicon; Bio-Gel P-4, P-30 and P-50 (100–200 mesh) from Bio-Rad; polyamide sheets from Gallard-Schlesinger; Whatman 3MM chromatography paper from A.H. Thomas; Fluoram (fluorescamine) from Hoffman-LaRoche; sulfanilic acid from Allied Chemical Co.; sodium dithionite, *N,N'*-methylenebisacrylamide, acrylamide, *N,N,N',N'*-tetramethylethylenediamine from Eastman; ammonium persulfate, DEAE-cellulose and sodium lauryl sulfate from Fisher; Coomassie brilliant blue R-250 from Mann Research; hydrochloric acid (constant boiling) and ninhydrin from Pierce Chemical Co.; BANA, dansyl chloride, DEAE-Sephadex (A 50–120), DFP-carboxypeptidases A and B, *N*-ethylmorpholine, LNA, *N*-(1-naphthyl)ethylenediamine dihydrochloride, *p*-dimethylaminobenzaldehyde, 8-hydroxyquinoline, 1-nitroso-2-naphthol, Sephadex G-10, Sephadex G-25, Sephadex G-100 and trypsin from Sigma; and dansyl amino acids standards from Schwarz-Mann. All other reagents were of analytical grade and were used without further purification. Deionized-distilled water was used throughout.

Analytical methods

Protein concentrations in subcellular fractions were determined by the method of Lowry et al. [10] using bovine serum albumin as a standard.

Cytochrome b_5 was identified by the absorbance spectra of its oxidized and dithionite-reduced forms and was quantitated on the basis of absorbance of the oxidized form at 413 nm using an ϵ_{mM} of 117 [11]. Purity of the protein was

assessed by the ratio of absorbance at 413 to that at 280 nm and by polyacrylamide gel electrophoresis by a modification of the method of Brewer and Ashworth [12].

Amino acid analyses were performed with a Durham model D-500 analyzer by AAA Laboratory of Seattle, Washington. Protein samples, containing a crystal of phenol, were hydrolyzed with 6 M HCl at 110°C. Hemin was not removed before hydrolysis. The amino acid composition data were normalized individually to a specific number of aspartic acid, alanine, glutamic acid, glycine, leucine and lysine residues. The results based on these six reliable residues were averaged.

Amino terminal residues were determined using dansyl chloride [13]. Proteins were reacted in 0.2 M $NaHCO_3$ or in 0.5 M $NaHCO_3$/8 M urea. The dansylated protein was purified by Sephadex G-25 chromatography or by a centrifugation method [14]. After hydrolysis, the dansyl amino acids were identified by thin-layer chromatography [15].

Carboxyl-terminal residue analyses were performed using carboxypeptidases A plus B [16]. Cytochrome (40–140 nmol) in 0.2 M *N*-ethylmorpholine acetate buffer, pH 8.5, containing 0.056 M SDS, was heated to 100°C and then incubated at 37°C with DFP-treated carboxypeptidase A (at an enzyme to protein molar ratio of 1:20) plus DFP-treated carboxypeptidase B (0.04 mg/50 nmol cytochrome).

Preparation of erythrocyte cytochrome b_5 I and II

Cytochrome b_5 I and II were isolated from 3.5 liters of bovine erythrocytes and purified by chromatography on DEAE-cellulose, Bio-Gel P-60 and DEAE-Sephadex A-50, as described previously [2]. Approx. 200 nmol pure Form I and pure Form II were prepared. The isolated forms of erythrocyte cytochrome b_5 appear to be more than 95% pure as assessed by polyacrylamide gel electrophoresis. Other forms of erythrocyte cytochrome b_5 are the only detectable contaminants.

Preparation of cathepsin-solubilized liver microsomal cytochrome b_5 A and B

Two solubilized forms of bovine liver microsomal cytochrome b_5 were prepared by incubation of 'acid-precipitated microsomes' with a crude preparation of lysosomal cathepsins. The cathepsin fraction was prepared by extraction of a lysosomal-mitochondrial pellet obtained by differential centrifugation of a homogenate of fresh bovine liver. Gel filtration of this extract gave a fraction which contained cathepsin B and presumably cathepsin D and catheptic carboxypeptidases.

Incubation of 'acid-precipitated microsomes' with this cathepsin fraction solubilized cytochrome b_5. Microsomal protein (11 g) was incubated with the cathepsin fraction (5.5 BANA-hydrolyzing units) for 21 h at 37°C in 0.05 M Tris-acetate buffer, pH 6.0, containing 10 mM cysteine and 2 mM EDTA. After adjusting the pH to 7.2, particulate material was removed by centrifugation. A crude fraction of soluble cytochrome b_5 was isolated from the supernatant fraction by DEAE-cellulose chromatography. Gel filtration separated the solubilized cytochrome b_5 into two fractions on the basis of molecular weight. Chromatography of the lower molecular weight fraction from DEAE-Sephadex gave a major peak which appeared homogeneous on polyacrylamide gel electrophoresis. This form of the solubilized protein has been designated Form A. Chromatography on DEAE-Sephadex of the larger molecular weight fraction from the gel filtration column gave one major peak. Analysis of this peak by polyacrylamide gel electrophoresis indicated that more than 95% of the protein in the pooled fractions migrated in a sharp band; this form has been designated Form B. A minor impurity in this preparation migrated as Form A.

Preparation and analysis of the tryptic peptides derived from various forms of apocytochrome b_5

Apocytochrome b_5 was prepared from 100–300 nmol of the various forms of cytochrome b_5 by acid-acetone treatment at 0°C [17]. The apocytochrome b_5 was chromatographed on Sephadex G-10 to remove residual HCl and then lyophilized to a small volume.

The apoproteins were degraded with TPCK-trypsin (2% by weight of the apoprotein) at pH 8.0 and 37°C. The tryptic digest of apocytochrome b_5 (25–100 nmol) was mapped on Whatman 3MM chromatography paper. Chromatography was carried out for 20 h in butanol/acetic acid/water/pyridine (15:3:12:10, v/v) and electrophoresis for 1 h at 3000 V in pyridine/acetic/

acid/water (100:4:896, v/v) at pH 6.5. Peptides were identified with fluorescamine reagent [18] and ninhydrin-cadmium reagent [19]. Peptides containing tryptophan, histidine, arginine or tyrosine were detected with Erlich, Pauly, Sakaguchi or 1-nitroso-2-naphthol reagents, respectively [20]. The electrostatic charge on the peptides was determined from the Offord plot [21] of log (electrophoretic mobility) versus log (molecular weight).

When tryptic peptides were to be isolated and analyzed, the peptide spots were detected with fluorescamine, cut from the paper maps and eluted with 10% acetic acid. The peptides were brought to dryness, acid-hydrolyzed and analyzed for amino acids. Recoveries of amino acids indicated an average peptide recovery of 24%. Whenever possible, one or more of six stable amino acids (alanine, aspartic acid, glutamic acid, glycine, leucine and lysine) were used to normalize the results. Since complete purity was not achieved in cutting the spots, a spot suspected of being contaminated by another spot was corrected by subtracting the contaminating peptide at a level determined by averaging values for amino acids unique to the contaminant. Uncorrected and corrected data for the amino acid compositions of the tryptic peptides have been placed in the BBA Data Bank.

TABLE I

AMINO ACID COMPOSITIONS OF BOVINE ERYTHROCYTE CYTOCHROME b_5 AND CATHEPSIN-SOLUBILIZED BOVINE HEPATIC MICROSOMAL CYTOCHROME b_5

For Form A values are normalized for the stable amino acids in residues 1–95 of bovine hepatic microsomal cytochrome b_5. Values are the means of single determinations after hydrolysis for 24 and 48 h. Serine and threonine were obtained by extrapolating to zero time from the normalized values at the two time-points. The value for phenylalanine is from a 24 h hydrolysis and the value for histidine is from a 48 h hydrolysis. For Form B values are normalized on the basis of the stable amino acids in residues 1–107. Values are the means of single determinations after hydrolysis for 24 and 48 h. Serine, threonine, phenylalanine and histidine were determined as above. For Form I the data of Douglas and Hultquist [2] were normalized for the stable amino acids in residues 1–97. Values are the means of duplicate determinations made at 25 and 47 h. Serine and threonine values were obtained by extrapolating to zero time from the normalized values at the two time-points of hydrolysis. The isoleucine value is based on only the 48 h determination. For Form II the data of Douglas and Hultquist [2] were normalized for the stable amino acids in residues 1–95. Values are the means of 24-h duplicate determinations. Values for serine and threonine have been corrected for degradation using the observed rates of degradation for these residues in Form I. The data for the segments of hepatic microsomal cytochrome b_5 are from the laboratories of Ozols and Strittmatter [22–26].

Amino acid	Residues/heme						
	Cathepsin-solubilized hepatic cytochrome b_5		Erythrocyte cytochrome b_5		Segments of hepatic microsomal cytochrome b_5		
	Form A	Form B	I	II	1–95	1–97	1–107
Alanine	4.6	4.5	5.2	5.1	5	5	5
Arginine	3.0	2.9	2.9	3.1	3	3	3
Aspartic acid	9.5	11.8	9.0	9.1	9	9	11
Glutamic acid	14.6	15.7	16.3	14.7	15	16	16
Glycine	6.5	6.6	6.6	7.5	6	6	6
Histidine	4.5	4.3	4.5	4.2	5	5	5
Isoleucine	4.5	6.8	4.5	4.4	5	5	8
Leucine	7.8	7.9	7.5	7.5	8	8	8
Lysine	8.8	8.9	8.3	7.8	9	9	9
Phenylalanine	2.9	3.0	2.8	2.8	3	3	3
Proline	3.4	4.1	3.3	3.3	3	3	4
Serine	7.6	10.4	9.2	8.0	8	9	11
Threonine	6.8	8.6	6.6	6.4	7	7	9
Tyrosine	3.8	3.7	3.6	2.9	4	4	4
Valine	4.2	4.2	3.6	4.5	4	4	4

Results

Cathepsin-solubilized microsomal cytochrome b_5 A and B

The amino acid compositions of the purified cathepsin-solubilized forms of microsomal cytochrome b_5 are given in Table I. Form A, the lower molecular weight form, has an amino acid composition which is very similar to the composition of residues 1–95 of the microsomal protein. Form B, the higher molecular weight form, has increased amounts of aspartic acid, glutamic acid, isoleucine, proline, serine and threonine. The composition of Form B is very similar to the composition of residues 1–107 of liver microsomal cytochrome b_5.

Amino-terminal analyses of both Form A and Form B were negative. Carboxy-terminal analyses of Form A and Form B were also negative; carboxypeptidase incubation with Form B for 5 and 10 h and with Form A for 14 h released no amino acid at a level above 0.1 nmol/nmol protein (Table II).

The map of the tryptic digest of cathepsin-solubilized liver microsomal apocytochrome b_5 B shows 12 spots (Fig. 1a). Staining of the map indicates that peptides 7, 8 and 10 contain arginine, peptides 4, 7, 8 and 10 contain histidine, and peptides 3 and 6 contain tyrosine. No tryptophan-containing peptide is dectected. The amino acid compositions of the 12 tryptic peptides agree well with the compositions of the tryptic peptides which would be derived from residues 1–107 of bovine microsomal cytochrome b_5.

The map of the tryptic digest of apocytochrome b_5 Form A is similar to that of Form B. The 12 spots stained the same as the 12 spots of Form B. The maps differed only in the location of one of their spots.

Erythrocyte cytochrome b_5 I and II

Carboxyl-terminal analysis of erythrocyte cytochrome b_5 I using carboxypeptidases A and B shows that a residue eluting at the position of serine is the terminal residue (Table II). A distinction was not made between serine, glutamine and asparagine, all of which co-elute. T he analysis suggests that the penultimate residue is a glycine residue. No residue was released upon digestion of

TABLE II

CARBOXYL-TERMINAL RESIDUE ANALYSIS OF CYTOCHROME b_5

For details see Experimental procedure. Only those residues detected in yields greater than 0.1 nmol/nmol cytochrome are listed.

Preparation	Incubation time (h)	Amino acid	nmol/nmol cytochrome
Erythrocyte cytochrome b_5 I	3	serine [c]	0.25
		glycine	0.16
	5 [a]	serine [c]	0.43
		glycine	0.28
	10 [a]	serine [c]	0.88
		glycine	0.55
Erythrocyte cytochrome b_5 II	3, 6, 14	none	
Cathepsin-solubilized microsomal cytochrome b_5 Form A	14	none	
Cathepsin-solubilized microsomal cytochrome b_5 Form B	5, 10	none	
Microsomal cytochrome b_5 (residues 5–97) [b]	3	serine	0.43
		glutamic acid	0.28
	5	serine	0.57
		glutamic acid	0.55
	24	serine	0.91
		glutamic acid	0.82
Microsomal cytochrome b_5 (residues 5–95) [b]	4, 7	none	

[a] An internal standard was not included and values could not be corrected for handling losses.

[b] Values for 'lipase'—solubilized bovine liver microsomal cytochrome b_5 from the studies of Ozols and Strittmatter [27].

[c] Asparagine and glutamine also elute at the position of serine and thus this technique did not distinguish among these amino acids.

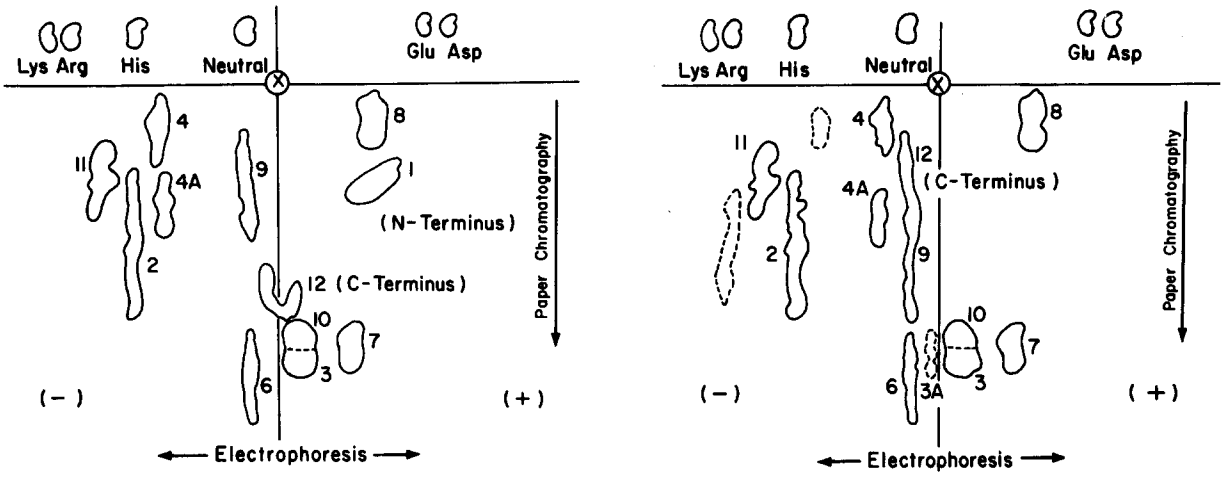

Fig. 1. Peptide maps of the tryptic digests of the apo forms of erythrocyte cytochrome b_5 I and cathepsin-solubilized cytochrome b_5 B. The maps were stained with fluorescamine and ninhydrin-cadmium acetate reagents. The spots above the line at the top of the maps show the migration of standard amino acids upon electrophoresis under the same conditions. Minor spots are shown with dashed lines. a, (left-hand figure) Peptide map of cathepsin-solubilized microsomal cytochrome b_5 B; a composite of 14 maps is depicted. Peptides were subsequently identified by amino acid analysis. b, (right-hand figure) Peptide map of erythrocyte cytochrome b_5 I; a composite of six maps is depicted.

erythrocyte cytochrome b_5 II with carboxypeptidases A and B. Amino-terminal residue analyses of Forms I and II using dansyl chloride were negative.

The map of the tryptic digest of bovine erythrocyte apocytochrome b_5 I shows 11 major spots (Fig. 1b). Spots 7, 8 and 10 stain positively for arginine, spots 4, 7, 8 and 10 stain positively for histidine, and spots 3 and 6 stain positively for tyrosine. The amino acid compositions of the tryptic peptides derived from erythrocyte cytochrome b_5 I are shown in Table III.

The map of the tryptic digest of apocytochrome b_5 II is similar to that of apocytochrome b_5 I. Ten major spots are seen. The staining of these spots is the same as that seen with Form I.

Discussion

The data presented in this paper provide evidence that the forms of erythrocyte cytochrome b_5 corresponds to segments of the microsomal cytochrome b_5 molecule. The amino acid sequence of bovine hepatic microsomal cytochrome b_5, deduced by the laboratories of Ozols and Strittmatter [17,22–28], is shown in Fig. 2. The protein is a single chain containing 133 residues. The molecule is comprised of a hydrophilic domain which contains the catalytic site and a hydrophobic domain which anchors the protein in the endoplasmic reticulum membrane [29–32].

Like the intact microsomal cytochrome b_5, the two solubilized forms of cytochrome b_5 generated by incubation with a cathepsin fraction from hepatic lysosomes and the two forms of cytochrome b_5 present in the cytoplasmic fraction of bovine erythrocytes have blocked amino termini. These proteins failed to show an amino-terminal residue upon analysis with dansyl chloride and the amino-terminal tryptic peptides derived from these proteins were difficult to detect with fluorescamine and ninhydrin-cadmium reagents. The complete structure and sequence of this amino-terminal peptide of bovine microsomal cytochrome b_5 has not been published. However, our observation that peptide 1 migrates rapidly as an anion at pH 6.5 indicates that at least one of the Glx residues of this peptide is a glutamic acid residue with a free carboxylate group.

In our studies of tryptic peptides from the solubilized microsomal and erythrocyte cytochromes, peptide 5 was not detected on maps from either cytochrome. Earlier studies of the sequence of bovine liver microsomal cytochrome b_5 failed to detect this peptide in column eluants of tryptic digests [23]. In the present study, values for iso-

TABLE III

AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES DERIVED FROM BOVINE ERYTHROCYTE APOCYTOCHROME b_5 I

Values were determined, normalized and corrected. Peptides were eluted from two maps, each of which had been spotted with the equivalent of 25 nmol erythrocyte cytochrome b_5 I. Corresponding peptides from the two maps were pooled and analyzed.

Peptide	Cytochrome b_5	Residues													
		Ala	Arg	Asp	Glu	Gly	His	Ile	Leu	Lys	Phe	Pro	Ser	Thr	Tyr	Val
1	Theory	1			2					1			2			
2	Erythrocyte I	0.9		0.2		0.7				1.1						1.1
	Theory	1								1						1
3	Erythrocyte I			0.4	2.8	0.7		0.9	1.1	1.0				0.9	0.8	
	Theory				3			1	1	1				1	2	
3A	Erythrocyte I			0.1	2.5	0.4		0.4	1.1	1.1	0.1			0.7	1.1	
	Theory				3			1	1	1				1	2	
4	Erythrocyte I			2.0	0.5	0.9	1.0			1.0			1.1			
	Theory			2			1			1			1			
4A	Erythrocyte I			1.9		0.9	0.3			1.1			1.1			
5	Theory						1	1	2	1			1	1	1	
6	Erythrocyte I			1.0	0.2	0.2			1.0	0.9				0.9	0.8	0.9
	Theory			1					1	1				1	1	1
7	Erythrocyte I		1.0	0.5	4.0	2.2	0.9		2.0	0.1	0.9	1.1				1.2
	Theory		1		4	2	1		2		1	1				1
8	Erythrocyte I	3.1	1.2	4.1	3.8	3.9	1.1			0.8	1.6		1.3	1.9		1.4
	Theory	3	1	4	4	3	1				1		1	2		1
9	Erythrocyte I			0.3	1.1	0.4		0.4	0.9	1.1			1.2	0.5		
	Theory				1				1	1			1			
10	Erythrocyte I		1.0	2.1	0.5	1.5	0.8	1.1	0.7		1.1	1.0		0.6		
	Theory		1	2	1	1	1	2	1		1	1		1		
11	Erythrocyte I			0.1		0.4				1.0			0.8			
	Theory									1			1			
12	Erythrocyte I			0.4	0.7	0.5		0.8		1.0		1.2	1.6	1.0		
	Theory (91–97)				1			1		1		1	2	1		
Totals for Erythrocyte I																
Sum of peptides [a]		5.0	3.2	9.7	15.4	10.4	4.8	3.8	7.7	9.9	3.6	3.3	9.0	6.8	2.6	4.6
Protein analysis [b]		5.2	2.9	9.0	16.3	6.6	4.5	4.5	7.5	8.3	2.8	3.3	9.2	6.6	3.6	3.6
Theory (1–97) [c]		5	3	9	16	6	5	5	8	9	3	3	9	7	4	4

[a] The sum of the residue values for the tryptic peptides derived from erythrocyte cytochrome b_5 I. The residues from peptides 3A and 4A were not included. Values for the expected compositions of peptides 1 and 5 were included.

[b] See Ref. 2.

[c] Based on data from the laboratories of Ozols and Strittmatter [17,22–28].

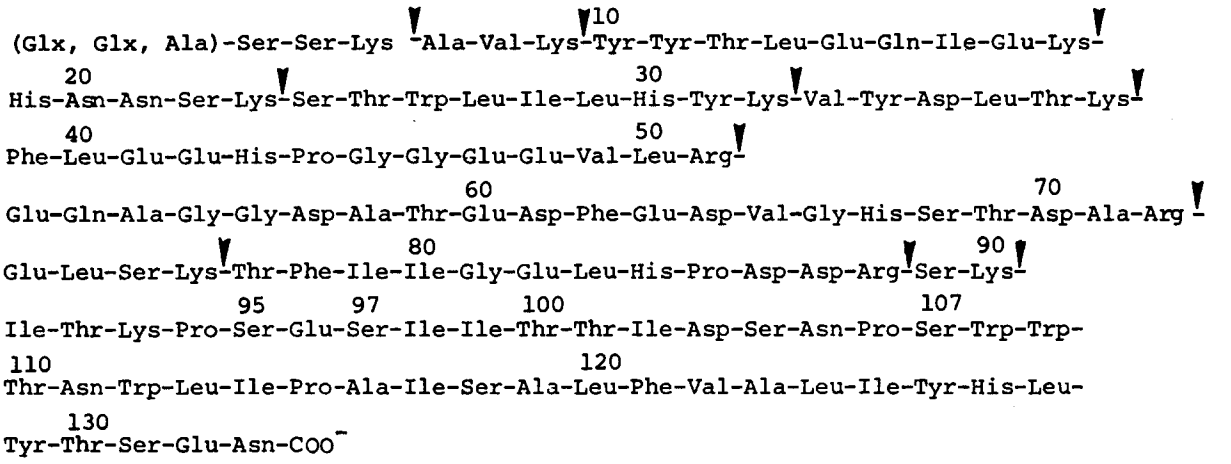

Fig. 2. The amino acid sequence of bovine hepatic microsomal cytochrome b_5 derived from data of the laboratories of Ozols and Strittmatter [17,22–28]. The arrows indicate the sites at which trypsin cleaves the apoprotein. The peptides are numbered from the amino terminus.

leucine were low in analyses of the cytochrome and in tryptic peptides 10 and 12 derived from the proteins. A possible explanation for this finding would be incomplete hydrolysis of the Ile-Ile bonds in these structures; such bonds have been reported to be very resistant to acid hydrolysis [33,34].

Identification of cathepsin-solubilized microsomal cytochrome b_5 Form B as residues 1–107 and Form A as residues 1–95

Cathepsin-solubilized cytochrome b_5 B was identified as residues 1–107 on the basis of amino acid analysis, terminal residue analysis and peptide mapping of the protein, and of amino acid analysis of the isolated tryptic peptides. The electrophoretic mobilities, staining patterns and amino acid compositions of the tryptic peptides 1, 2, 3, 4, 6, 7, 8, 9, 10, 11 and 12 correspond to the peptides predicted to arise from tryptic degradation of apocytochrome b_5 containing residues 1–107. The sum of the compositions of the tryptic peptides (including the composition of the undetected peptide 5), agrees very well with the analysis of an acid hydrolysate of the Form B molecule and with the composition of residues 1–107 of microsomal cytochrome b_5. The single spot (spot 12) on the map of Form B which did not appear on the map of Form A shows a composition corresponding to that of residues 91–107 of the microsomal molecule and appears to be the carboxyl-terminal peptide of Form B. The presence of proline in a penultimate position (residue 106) explains the inability of carboxypeptidases A and B [27] to release a residue from Form B.

Cathepsin-solubilized microsomal cytochrome b_5 A was tentatively identified as residues 1–95 on the basis of amino acid analysis, terminal residue analyses and peptide mapping. As expected for a peptide with a penultimate proline residue, no residue was released by carboxypeptidases A and B. In further support of the designated structure, the carboxyl-terminal tryptic peptide of Form A (i.e., the peptide not present on the map of Form B) showed an electrophoretic migration expected for a pentapeptide with a positive net charge of one.

Identification of erythrocyte cytochrome b_5 Form I as residues 1–97 and Form II as residues 1–95

Terminal residue analyses, amino acid analysis and peptide mapping of erythrocyte cytochrome b_5 I and amino acid analysis of its tryptic peptides establish that erythrocyte cytochrome b_5 I is a segment of microsomal cytochrome b_5 corresponding to residues 1–97. A residue eluting from analyzer columns at the position of serine was identified as the carboxyl-terminal residue. The carboxyl-terminal peptide (peptide 12) was shown

to have a composition corresponding to residues 91–97.

Except for the positions of peptides 12 and 4, the peptide map of Form I has great similarity to the maps of cathepsin-solubilized Forms A and B. Spots 2, 3, 6, 7, 8, 9, 10 and 11 from erythrocyte Form I correspond to spots (of the same number) of the hepatic proteins in terms of position on the maps, intensity of fluorescence following reaction with fluorescamine, shade of color and rate of color development with ninhydrin-cadmium acetate, and selectivity of staining for histidine, arginine and tyrosine.

The amino acid compositions of these peptides agree very well with the compositions of the corresponding peptides from cathepsin-solubilized Form B and with the theoretical composition of their proposed structures (Table III). Likewise, the sum of the compositions of the tryptic peptides agrees with the composition of residues 1–97. A discrepancy noted for both Forms I and B is a low value of tyrosine in peptide 3.

Peptides 4 and 4A of Form I differed from the corresponding peptides of the solubilized liver cytochromes only with regard to electrophoretic mobility. The corresponding peptides showed the same amino acid compositions, the same chromatographic mobilities and the same staining patterns. Since the peptides from Form I appeared to have a less-positive charge than the hepatic proteins, it is possible that an asparagine is present as an aspartic residue in erythrocyte cytochrome b_5 I. The compositions, mobilities and staining indicate that for both the erythrocyte and hepatic proteins, peptide 4A corresponds to peptide 4 without the histidine residue. Peptide 4A might be the result of secondary chymotryptic-like cleavage occurring between residues 19 and 20 during tryptic incubation.

Peptide mapping, terminal residue analyses and amino acid analysis provide evidence that erythrocyte cytochrome b_5 II is a segment of microsomal cytochrome b_5 corresponding to residues 1–95. Form II, like Form A, not only shows an amino acid composition characteristic of residues 1–95 but fails to liberate a residue upon incubation with carboxypeptidases A and B. Cytochrome b_5 II differs from Form I only in that it is missing two residues (Glu-Ser) at its carboxyl terminus and has retained ammonia in an asparagine residue at position 20 or 21. Our studies provide no suggestion that release of carboxyl-terminal dipeptide or release of ammonia occurs during the isolation of the erythrocyte cytochromes.

Significance

Chemical [6–8,35] and immunological [36] studies of human and bovine erythrocyte cytochrome b_5 had previously led to the recognition of the similarities between the erythrocyte and microsomal cytochromes. Likewise, the similarities between erythrocyte and microsomal cytochrome b_5 reductase had been recognized on the basis of enzymatic [7], immunological [36–39], and degradative studies [40]. These similarities, and our findings that immature erythroid cells possess only microsomal forms of cytochrome b_5 and cytochrome b_5 reductase whereas erythrocytes contain only cytoplasmic forms of these proteins, led to the suggestion that the erythrocyte proteins were generated by proteolysis of the microsomal proteins [3,6,7].

The present chemical study of the forms of bovine erythrocyte cytochrome b_5 firmly establishes that these proteins correspond to hydrophilic segments of hepatic microsomal cytochrome b_5. The study also demonstrates that a hepatic lysosomal fraction, containing cathepsin B and probably cathepsin D, is capable of solubilizing microsomal cytochrome b_5 and generating, as a major product, one of the forms of erythrocyte cytochrome b_5. We postulate that, during erythroid maturation, proteases of the immature erythroid cell release the hydrophobic tails of the microsomal proteins and generate the hydrophilic forms of these proteins which then function in methemoglobin reduction. The present study suggests that the hepatic cathepsins serve as an excellent model for the putative erythroid proteases which catalyze this processing.

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