PURIFICATION AND STRUCTURAL STUDIES OF RABBIT ERYTHROCYTE CYTOCHROME b_5

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Summary: A single form of cytochrome b_5 has been isolated in highly purified
form from the cytosolic fraction of rabbit erythrocytes by sequential
chromatography on DE-52 cellulose, Sephadex G-75, and DEAE-Sephadex A50. The
cytochrome is structurally similar to the N-terminal, heme-binding domain of
rabbit liver microsomal cytochrome b_5. Like the liver protein, it is blocked at
the amino terminus. Its amino acid composition is similar to that of residues 1-
97 of the microsomal protein. With one exception, tryptic peptides derived from
apo-cytochrome b_5 of rabbit erythrocytes co-elute with the tryptic peptides
obtained from a soluble hemepeptide fragment of microsomal cytochrome b_5. These
findings, together with amino acid sequence analysis of the carboxyl terminal
tryptic peptides, identify the erythrocyte cytochrome b_5 as a 97-residue peptide.

Cytochrome b_5, the soluble hemeprotein component of the methemoglobin

reduction system of erythrocytes has been detected in human (1,2), bovine (3),

mouse (4), pig (5) and rabbit erythrocytes (1) and in rabbit reticulocytes (1).

The two predominant forms of bovine erythrocyte cytochrome b_5 have been purified

to homogeneity (3) and structural studies (6) have demonstrated that these

hemeproteins have the same amino acid sequence as the N-terminal, hydrophilic,

heme-binding segment of the membrane-bound cytochrome b_5 present in bovine liver

microsomes. Form I is composed of residues 1-97 of bovine microsomal cytochrome

b_5 and Form II is composed of residues 1-95 (6). Similarly, the single

predominant form of human erythrocyte cytochrome b_5 has been purified and

structural studies suggest that its structure corresponds to the heme-binding

domain of human liver microsomal cytochrome b_5 (2,7).

    We now report the purification of rabbit erythrocyte cytochrome b_5 and

present evidence demonstrating that the amino acid sequence of soluble

Abbreviations used: TFA, trifluoroacetic acid; ACD, acid-citrate-dextrose; TPCK,
L-1-tosylamide-2-phenylethylchloromethylketone.

erythrocyte hemeprotein is closely related to the sequence of the N-terminal,

heme-binding domain of rabbit liver microsomal cytochrome b_5.

EXPERIMENTAL METHODS

Materials. DE-52 cellulose was obtained from Whatman; Sephadex G-75-120, DEAE-Sephadex A50-120 and trypsin (TPCK-treated) from Sigma Chemical Co., and ultrafiltration membranes from Amicon. Whole blood from New Zealand and/or Californian white rabbits was purchased from Pelfreez Biologicals. Microsomal cytochrome b_5, partially purified from New Zealand white rabbit livers, was a gift from the laboratory of Dr. M. J. Coon at The University of Michigan and was further purified according to the procedure of Strittmatter, et al. (8). A protease-solubilized fragment of microsomal cytochrome b_5 was obtained after digestion with a cathepsin D-like protease from rabbit reticulocytes (9, and manuscript in preparation).

General methods. Cytochrome b_5 was quantitated spectrophotometrically at 413 nm using an extinction coefficient of 117 cm^{-1} mM^{-1} (10). Visible absorption spectra of oxidized and dithionite-reduced protein samples were used for identification of cytochrome b_5 during the initial stages of the purification procedure.

Electrophoresis in the absence of detergents was carried out in 11% polyacrylamide tube gels as described by Brewer and Ashworth (11). SDS-PAGE was carried out in 16.3% polyacrylamide slab gels by a modification of the general procedure of Laemmli (12); both the stacking and separating gels contained 6 M urea in addition to 0.1% SDS.

Preparation of apocytochrome b_5. Apocytochrome b_5 was prepared by reverse phase HPLC (using an IBM model LC/9533 HPLC) under conditions in which heme readily dissociates from the cytochrome. Ten to twenty nmol of cytochrome b_5 was injected into a Vydac TP201 ODS column (4.6 x 225 mm) equilibrated in aqueous 0.1% TFA (Buffer A). The column was developed at a flow rate of 1 ml/min with a series of linear gradients from Buffer A to CH_3CN containing 0.1% TFA (Buffer B) as follows: 0 to 50% Buffer B in 50 min, 50% to 80% Buffer B in 5 min and 80% to 100% Buffer B in 5 min. The effluent was monitored at 215 nm. Apocytochrome b_5 eluted with approximately 40% CH_3CN while free heme eluted with greater than 50% CH_3CN.

Tryptic peptide mapping by HPLC. Tryptic hydrolysis of 12-15 nmol of apocytochrome b_5 was carried out in 0.02 M sodium phosphate, pH 7.5, for 6 h at 25°C. Trypsin (as a freshly prepared 0.1% solution in 0.02 M sodium phosphate, pH 7.5, containing 1 mM $CaCl_2$) was added to give a final b_5:trypsin (mol:mol) ratio of 50. The reaction mixture was acidified with 1% TFA (v/v) to a final concentration of 0.1% TFA before peptide separation by HPLC.

Tryptic peptide separation was achieved as described above for the isolation of apocytochrome b_5 except that the composition of Buffer B was CH_3CN:2-propanol (3:1, v/v) containing 0.1% TFA. The effluent was monitored at 215 nm. The column was regenerated by flushing with 100% Buffer B for 10 min followed by re-equilibration with Buffer A for 20 min.

Purification of rabbit erythrocyte cytochrome b_5. Cytochrome b_5 was isolated from rabbit erythrocytes by a modification of the procedure developed for the purification of cytochrome b_5 from bovine erythrocytes (3). All chromatographic procedures were carried out at 4°C.

Erythrocytes were obtained from one liter of rabbit blood (containing ACD as anticoagulant) by centrifugation at 1000 x g for 15 min. The red cells were washed 3 times with 2 volumes of phosphate-buffered saline and lysed with three volumes of cold water. The pH of the lysate was adjusted to pH 6.0 with 1 M HCl and the supernatant fraction was obtained by centrifugation at 17,000 x g for 30 min.

Cytochrome b_5 was isolated from the hemolysate by anion exchange chromatography on DE-52 cellulose. The supernatant fraction was adjusted to pH 7.2 with 2 $\underline{M}$ KOH and applied to a DE-52 cellulose column (4.8 x 39 cm) which had been equilibrated in 3 m$\underline{M}$ potassium phosphate, pH 7.2. The column was washed with approximately two liters of 20 m$\underline{M}$ KH_2PO_4 containing 10 m$\underline{M}$ KCl until the effluent was colorless and cytochrome b_5 was visible as a reddish band at the top of the column. Resin containing bound cytochrome b_5 was removed from the top of the column and repacked into a 3 x 20 cm glass column. Cytochrome b_5 was eluted with 400 ml of 0.2 $\underline{M}$ KH_2PO_4 and the pH of the effluent was adjusted to pH 7.2 with 2 $\underline{M}$ KOH. The sample was concentrated by ultrafiltration on a UM-10 membrane, diluted with 50 m$\underline{M}$ potassium phosphate, pH 7.2, and concentrated to a final volume of 20 ml.

Cytochrome b_5 was further purified by gel filtration chromatography on Sephadex G-75-120 (3 x 80 cm) which had been equilibrated in 50 m$\underline{M}$ potassium phosphate, pH 7.2. A 10-ml aliquot of the sample was applied to the column; 5-ml fractions were collected and monitored for protein at 280 nm and for heme-containing proteins at 413 nm. The last hemeprotein to be eluted from the column was identified as cytochrome b_5 by the characteristic visible absorption spectrum of its oxidized and reduced forms.

Further purification was achieved by chromatography on DEAE-Sephadex A50-120 (1 x 18 cm) which had been equilibrated in 0.05 $\underline{M}$ Tris Cl, pH 7.7, containing 0.09 $\underline{M}$ sodium chloride. Cytochrome b_5 was eluted from the column as a single species using a linear gradient from 0.09 $\underline{M}$ to 0.25 $\underline{M}$ sodium chloride in 0.05 $\underline{M}$ Tris-Cl, pH 7.7. Fractions having an $A_{413\ nm}/A_{280\ nm}$ ratio greater than 5.0 were pooled and used for structural studies.

RESULTS AND DISCUSSION

The purification of rabbit erythrocyte cytochrome b_5 yielded a single

electrophoretic form of the hemeprotein that was nearly homogeneous. No other

hemeproteins were observed on the spectrophotometric scan of an 11%

polyacrylamide gel run under nondenaturing conditions (Fig. 1A). Staining of the

gel with Coomassie Blue revealed only minor protein contaminants which were

estimated to represent less than 2% of the total protein (Fig. 1B).

Electrophoresis in the presence of SDS and urea demonstrated a single protein

component (Fig. 1C).

Like rabbit liver microsomal cytochrome b_5 (13), the amino terminus of

rabbit erythrocyte cytochrome b_5 is blocked. Three cycles of Edman degradation

failed to release any PTH-amino acid derivatives from the purified cytochrome

b_5 (data not shown). Ozols and Heinemann recently determined that the N-

terminal residue of rat liver microsomal cytochrome b_5 is N-acetylalanine (14).

The amino acid composition of rabbit erythrocyte cytochrome b_5 was compared

to that of the N-terminal, hemepeptide segment of rabbit liver microsomal

cytochrome b_5 (Table I). The composition of the erythrocyte protein agreed with

the composition of residues 1-97 of the microsomal protein except that the
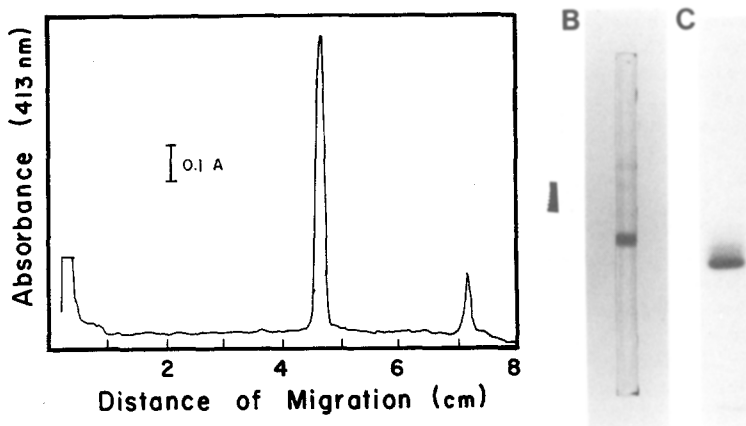
Fig. 1. Electrophoresis of rabbit erythrocyte cytochrome b_5. A, Spectrophotometric scan after electrophoresis of 1 nmol of cytochrome in an 11% polyacrylamide gel under nondenaturing conditions. The fastest migrating band is the tracking dye. B, The same gel shown in A after staining with Coomassie Blue. Electrophoretic migration is from top to bottom. C, Electrophoresis of 0.25 nmol of cytochrome in a 16.3% polyacrylamide gel in the presence of SDS and urea. The protein was visualized after staining with Coomassie Blue. Electrophoretic migration is from top to bottom.

erythrocyte protein contained more proline and aspartic acid, and less

isoleucine. We have observed previously that the various forms of bovine

cytochrome b_5 consistently give low analyses for isoleucine (6), probably as a

Table I. Amino Acid Composition of Rabbit Erythrocyte Cytochrome b_5

Amino Acid	Erythrocyte Cytochrome b_5[a]	Composition of Residues 1-97 of Rabbit Liver Microsomal Cytochrome b_5[b]
Asp	16.0	10
Thr	6.3	7
Ser	6.9	7
Glu	14.6	14
Pro	5.3	3
Gly	6.3	6
Ala	5.1	5
Val	3.5	4
Met	1.1	1
Ile	2.5	4
Leu	9.2	9
Tyr	3.0	3
Phe	3.3	3
His	6.9	7
Lys	9.9	10
Arg	3.2	3
Trp	n.d.	1

[a]Approximately 4 nmol of rabbit erythrocyte cytochrome b_5 was subjected to acid hydrolysis in constant boiling HCl at 108°C for 22 h. Values were normalized to valine (4 residues), alanine (5 residues), glutamic acid (14 residues), leucine (9 residues), lysine (10 residues), and arginine (3 residues), and are expressed as nmol/nmol of cytochrome. n.d., not determined.
[b]Amino acid composition taken from reference 13.

result of incomplete acid hydrolysis of the Ile–Ile bond. This bond, present in

rabbit microsomal cytochrome b_5 (13) and presumably in the erythrocyte protein,

could account for the low amount of isoleucine.

The erythrocyte and liver hemeproteins yielded, with one exception, tryptic

peptides having identical elution times on HPLC, indicating structural similarity

between erythrocyte protein and the hydrophilic, heme–binding domain of rabbit

microsomal cytochrome b_5. The two peptides that differed between the two tryptic

maps (see arrows in Fig. 2) were suspected of being the carboxyl terminal

peptides from each protein. The anomalous peptide from the protease–solubilized

form of microsomal cytochrome b_5 had a composition corresponding to residues 91–

98 of microsomal cytochrome b_5 (Table II). Sequence analysis of this peptide

established the sequence Leu–Ser–Lys–Pro–Met–Glu–Thr–Leu, confirming that this

peptide corresponds to the segment 91–98 of the microsomal protein (13).
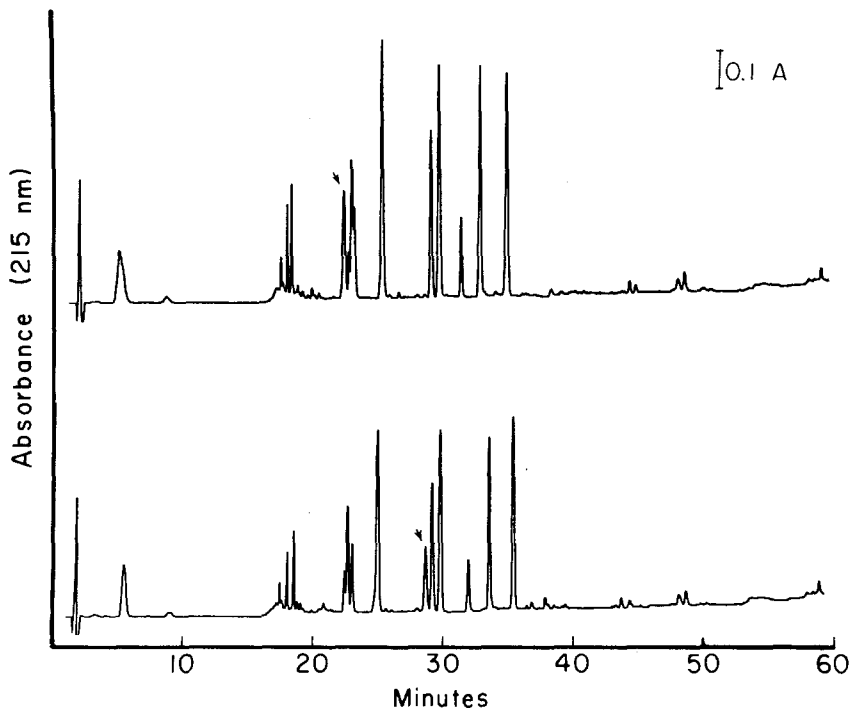

Fig. 2. Separation by HPLC of the tryptic peptides of erythrocyte cytochrome b_5 and of protease–solubilized microsomal cytochrome b_5. Tryptic peptides were derived from approximately 12 nmol of apo–cytochrome b_5. Upper chromatogram depicts the tryptic digest of rabbit erythrocyte cytochrome b_5. Lower chromatogram depicts the tryptic digest of the protease–solubilized microsomal cytochrome b_5. Arrows identify the C–terminal peptides that were subsequently subjected to sequence analysis. See Experimental Methods for details.

Table II. Amino Acid Compositions of the Carboxyl Terminal Tryptic Peptides of Cytochrome b_5[a]

Amino Acid	C-Terminal Peptide from Erythrocyte Cytochrome b_5	C-Terminal Peptide from Protease-solubilized Microsomal Cytochrome b_5	Residues 91-98 of Microsomal Cytochrome b_5[b]
Asp	0.21	0.52	0
Thr	0	0.83	1
Ser	0.64	0.97	1
Glu	1.23	1.38	1
Pro	3.78	1.39	1
Gly	0.37	0.86	0
Ala	0.09	0.38	0
Val	0	0	0
Met	0.68	0.84	1
Ile	0	0.12	0
Leu	1.04	1.67	2
Tyr	0.05	0	0
Phe	0.07	0	0
His	0.09	0.20	0
Lys	1.10	0.81	1
Arg	0.23	0.19	0

[a]Peptides were purified by HPLC (see Fig. 2). The anomalous peptide from the erythrocyte cytochrome b_5 (2 nmol) and the anomalous peptide from the solubilized microsomal protein (1 nmol) were subjected to acid hydrolysis in vacuo in constantly boiling HCl at 108°C for 24 h. Values were normalized to the amount of leucine, serine, lysine, and glutamic acid expected if the peptides corresponded to segments of microsomal cytochrome b_5. Compositions are expressed as nmol amino acid/nmol of peptide.
[b]Amino acid composition taken from reference 13.

The anomalous peptide from the erythrocyte cytochrome b_5 showed a composition with less threonine and leucine and more proline that segment 91-98. Edman sequence analysis of this peptide established the sequence Leu-Ser-Lys-Pro-Met-Glu-Pro. No additional residues were released from the peptide sample in two additional cycles. The peptide corresponds to the segment 91-97 of microsomal cytochrome b_5, except that a proline residue is present in the carboxyl terminal position, whereas residue 97 is threonine in the rabbit microsomal cytochrome. Preliminary studies using carboxypeptidase A and Y have provided additional evidence that proline is the carboxyl terminal residue of erythrocyte cytochrome b_5. Carboxypeptidase A (which is known to be unable to release terminal proline residues) released no amino acids from the erythrocyte protein after one hour. Subsequent addition of carboxypeptidase Y (which can release terminal proline residues (15)) to the same reaction mixture catalyzed the release of proline and other amino acids.

Rabbit erythrocyte cytochrome b_5 can now be added to those forms of cytochrome b_5 that have been purified and characterized. Like bovine and human erythrocyte cytochrome b_5, the soluble protein of rabbit erythrocytes is structurally closely related to the hydrophilic segment of the liver microsomal protein. The one difference detected between the erythrocyte and liver proteins remains to be explained. We have not eliminated the possibility that it might be ascribed to a difference between New Zealand white rabbits (which were the source of the liver) and California white rabbits (which, together with New Zealand white rabbits, were identified by the supplier as the source of the blood). It is possible that isoenzymes differing at residue 97 are present in rabbit tissue. The structural difference could result from the presence of distinct genes for the erythrocyte and liver proteins or from different processing mechanisms during the production of the mature mRNA for cytochrome b_5. Studies are now underway to discriminate among these possibilities.

ACKNOWLEDGEMENTS

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