HOMOGENEOUS CYTOCHROME b_5 FROM HUMAN ERYTHROCYTES*

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Summary: Homogeneous cytochrome b_5 has been isolated from large volumes of human erythrocytes by sequential chromatography on DEAE-cellulose, Amberlite CG-50, Bio-Gel P-60, and DEAE-Sephadex A-50. A molecular weight of 15,300 was determined by SDS disc gel electrophoresis. Trypsin converted the protein to a smaller hemepeptide which was indistinguishable from trypsin-cytochrome b_5 of human liver microsomes by disc gel electrophoresis. The data suggest that erythrocyte cytochrome b_5 has the same structure as a segment of liver microsomal cytochrome b_5 and is intermediate in size between the trypsin- and detergent-solubilized forms of the liver protein.

We have previously shown that a soluble form of cytochrome b_5, isolated from human, beef, and rabbit erythrocytes (1,2), is a component of the methemoglobin reduction system (3,4). Erythrocyte cytochrome b_5 is similar to cytochrome b_5 solubilized from liver microsomes in terms of its protoheme 9 prosthetic group, visible and EPR spectral properties, ability to serve as a substrate for microsomal and erythrocyte cytochrome b_5 reductase, and ability to stimulate the catalytic reduction of methemoglobin (2). The isolation procedure was long and costly as a consequence of the cation exchange step to remove hemoglobin. In addition, the preparation was shown to contain minor impurities by disc gel electrophoretic analysis. This paper describes a greatly simplified scheme for the isolation of homogeneous cytochrome b_5 from human erythrocytes and presents evidence that this hemepeptide is a segment of liver microsomal cytochrome b_5.

EXPERIMENTAL

Materials. DEAE-cellulose was obtained from Fisher; Amberlite CG-50, 200 to 400 mesh, from Mallinckrodt; Bio-Gel P-60 from Bio-Rad; DEAE-Sephadex

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A-50-120 from Pharmacia; UM-2 ultrafiltration membranes from Amicon; Ampholine from LKB; N,N,N',N'-tetramethylethylenediamine, acrylamide, and N,N'-methylenebisacrylamide from Eastman; and trypsin, 3 x crystallized, from Worthington. Cytochrome b_5 solubilized from human liver microsomes by lipase and trypsin (5) was a gift from Dr. J. Ozols. Human blood cells that had been stored 3 to 4 weeks were graciously provided by the Bureau of Laboratories, Michigan Department of Public Health, Lansing, Michigan.

Isolation of Cytochrome b_5. Cytochrome b_5 was isolated from large volumes of out-dated human erythrocytes by combining a modification of our previous procedure (2) with DEAE-Sephadex chromatography as has been used to isolate liver microsomal cytochrome b_5 (6). Cells were stored, washed, and hypotonically lysed, and the stroma was removed as described previously (7), except that the hemolysate was stored at -60° before the stroma was removed. The purification was carried out at 4° using potassium phosphate buffers. Protein fractions were concentrated and dialyzed using UM-2 ultrafiltration membranes. The ratio of absorbances at 413 nm and 280 nm (A_{413}/A_{280}) was used throughout the purification as an index of purity.

In a typical isolation, the supernatant fraction from 4.4 l of cells was adjusted to pH 7.2 with dilute KOH and charged onto a 6.4 x 56 cm DEAE-cellulose column equilibrated with 0.003 *M* phosphate buffer, pH 7.2. The column was washed free of hemoglobin with 0.003 *M* buffer, pH 7.2, and then eluted with 16 l of an ionic strength gradient which was linear from 0.003 *M* buffer, pH 7.2, to 0.2 *M* buffer, pH 7.2, containing 0.5 *M* KCl. Cytochrome b_5 was identified by its oxidized and reduced spectra. The pooled fraction containing cytochrome b_5 was adjusted to pH 6.0, concentrated, diluted with 10 volumes of water, and passed rapidly through a column containing an excess of Amberlite CG-50, equilibrated with 0.05 *M* phosphate buffer, pH 6.0. The eluate was concentrated and dialyzed against 0.001 *M* buffer, pH 7.2, and then rechromatographed on a 4.4 x 53 cm DEAE-cellulose column. The column was eluted with 8 l of a gradient which was linear from 0.003 *M* buffer to 0.2 *M* buffer con-

taining 0.5 *M* KCl. The cytochrome b_5-containing samples were pooled to give 161 ml of solution with an absorbance at 413 nm of 0.305 and an A_{413}/A_{280} ratio of 0.43. After concentration, this sample was purified by gel filtration on a 3.4 x 103 cm column of Bio-Gel P-60, 100-200 mesh. The eluted fraction from this column contained 0.20 nmole of cytochrome b_5 [based on an assumed ε at 413 nm of 115 $mM^{-1}cm^{-1}$(8)] and had an A_{413}/A_{280} ratio of 2.72.

This preparation was pooled with other preparations of similar history to give a sample containing 0.47 nmole of cytochrome b_5 with an A_{413}/A_{280} ratio of 3.32, and the combined fraction was purified by chromatography on DEAE-Sephadex A-50 at pH 7.7. As shown in Fig. 1, a major hemeprotein peak

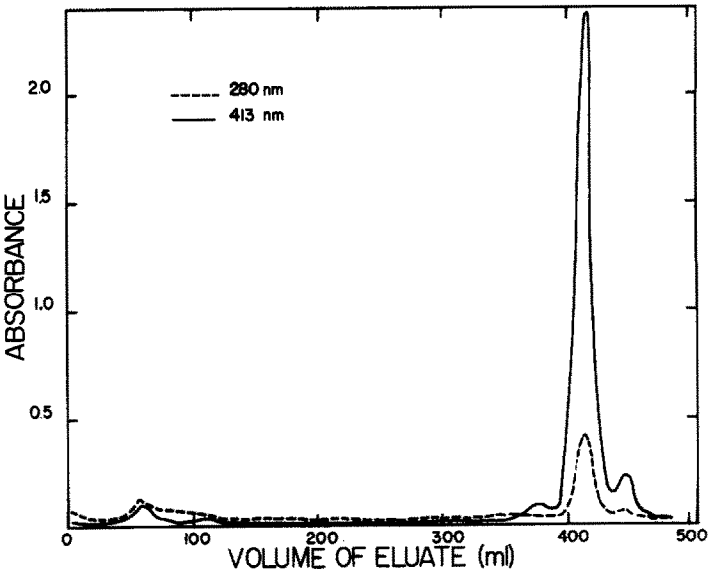

Fig. 1. Chromatography of erythrocyte cytochrome b_5 on DEAE Sephadex A-50. The sample, dialysed extensively against 0.05 *M* Tris-HCl buffer, pH 7.7, containing 0.09 *M* NaCl, was applied to a 1 x 60 cm DEAE Sephadex column equilibrated with the same buffer. The column was developed with a shallow concave gradient prepared by using 250 ml of the above buffer in a round bottom flask as a mixer and 250 ml of the pH 7.7 Tris-HCl buffer, containing 0.25 *M* NaCl, in a 250 ml Erlenmeyer flask as the reservoir.

and two minor hemeprotein peaks were eluted from this column. All three proteins showed the oxidized, reduced, and pyridine hemochrome spectra typical of cytochrome b_5. In order of elution, they accounted for 0.02, 0.41 and 0.04 nmoles, and showed A_{413}/A_{280} ratios of 2.4, 6.4, and 3.8, respectively. The

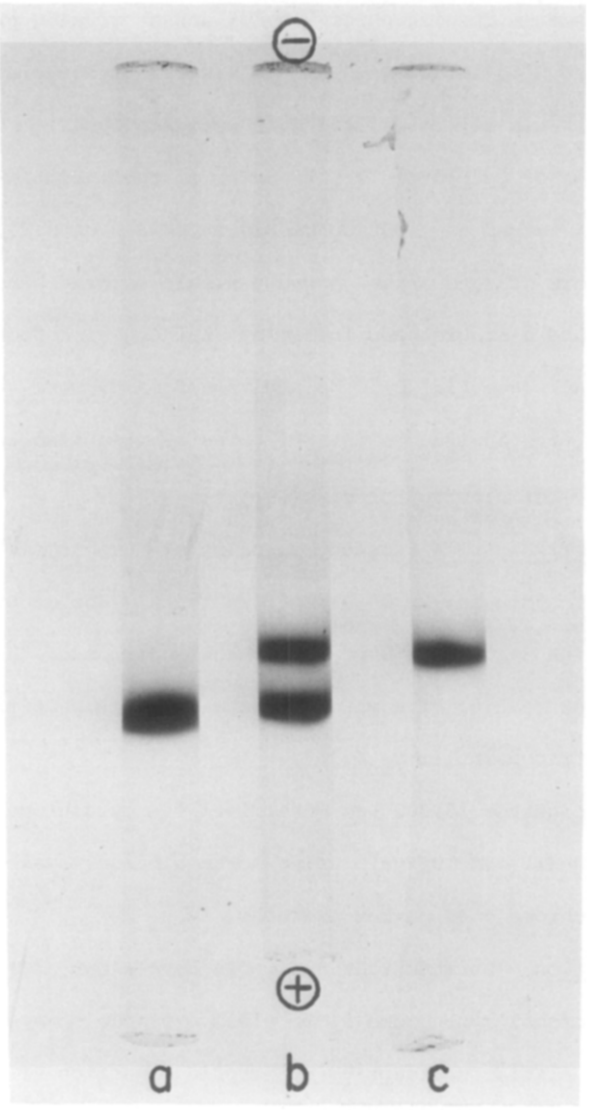

Fig. 2. Disc gel electrophoresis of human erythrocyte cytochrome b_5 and human liver microsomal cytochrome b_5-trypsin at pH 9.5. Gels were stained with Buffalo Black. a) 16 µg of liver cytochrome b_5-trypsin; b) 16 µg of liver cytochrome b_5-trypsin plus 17 µg of erythrocyte cytochrome b_5; c) 20 µg of erythrocyte cytochrome b_5.

major form of cytochrome b_5 showed an A_{556} (reduced)/A_{280} (oxidized) ratio of 1.7. In some cases the cytochrome b_5 obtained by the above procedure still contained a minor impurity, in which case homogeneity was achieved by chromatography on Bio-Gel P-60, minus 400.

Demonstration of Homogeneity. Purity of proteins was assessed by disc

gel electrophoresis at a running pH of 9.5 (9) and at running pH values of 8.4 and 7.5 obtained with imidazole-N,N,N',N'-tetramethylethylenediamine-HCl buffer systems (10). In all cases, 15% gels were employed.

The three forms of cytochrome b_5, in order of their elution from DEAE-Sephadex, showed R_f values on electrophoresis at pH 8.4 of 0.77, 0.79, and 0.82. The major form of cytochrome showed a single colored band in each of the three systems and a single band following staining with Coomassie Brilliant Blue or Buffalo Black (see Fig. 2). No additional bands were seen when large amounts of protein were applied to the gel. The protein also appeared homogeneous on SDS disc gel electrophoresis (11).

Physical Properties. The isoelectric point of cytochrome b_5 was determined by isoelectric focusing at 0° using 3 mm thick slabs of Bio-Gel P-60 with 1% Ampholine (pH 3-10) at 500 v. After the position of the colored band was stabilized, a pH reading of a water suspension of this portion of the gel showed the isoelectric point to be 4.9.

A molecular weight of 15,300 was determined for cytochrome b_5 by SDS disc gel electrophoresis (11) in 10% gels using serum albumin, ovalbumin, myoglobin, lysozyme, and cytochrome *c* as marker proteins.

Trypsin Digestion. Strittmatter and Ozols have shown (12) that trypsin cleaves liver microsomal cytochrome b_5 to yield a stable hemepeptide core. We have obtained a similar hemepeptide by trypsin digestion of erythrocyte cytochrome b_5. To 13.4 µg of erythrocyte cytochrome b_5 in 0.20 ml of 0.05 *M* Tris-HCl buffer, pH 7.7, containing 0.25 *M* NaCl, was added 0.4 µg of trypsin in 0.05 *M* Tris-HCl buffer, pH 7.7, containing 0.09 *M* NaCl and 0.001 *M* $CaCl_2$. After incubation at 30° for 4 hours, an additional 0.4 µg of trypsin was added and the incubation continued for a total of 12 hours.

The resulting tryptic reaction mixture was compared by disc gel electrophoresis with the native protein and with the tryptic-hemepeptide from human liver microsomes. As shown in Fig. 3, trypsin completely converted erythrocyte cytochrome b_5 (R_f of 0.68) to a core hemepeptide (R_f of 0.78) which co-

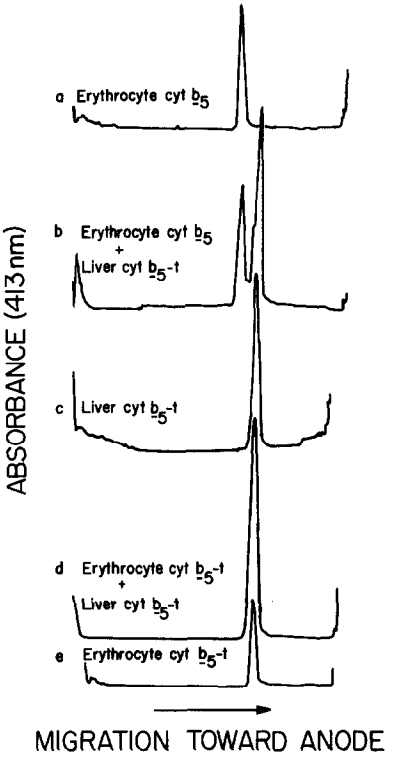

Fig. 3. Comparison by disc gel electrophoresis of tryptic hemepeptides derived from erythrocyte and liver microsomal cytochrome b_5. Unstained gels were immediately scanned for hemeproteins at 413 nm using a Gilford Spectrophotometer equipped with a linear transport. Tracings are aligned by the tracking dye. a) 13 µg of erythrocyte cytochrome b_5; b) 13 µg of erythrocyte cytochrome b_5 plus 16 µg of liver cytochrome b_5-t (trypsin degraded); c) 16 µg of liver cytochrome b_5-trypsin; d) 13 µg of erythrocyte cytochrome b_5-trypsin plus 16 µg of liver cytochrome b_5-trypsin; e) 13 µg of erythrocyte cytochrome b_5-trypsin.

migrated with the core hemepeptide from liver microsomal cytochrome b_5.

DISCUSSION

The isolation of homogeneous cytochrome b_5 from very large volumes of human erythrocytes was possible only because direct removal of the cytochrome from hemolysates by anion exchange eliminated the need to bind the massive amounts of hemoglobin by cation exchange, as was required in the previous procedure (2). This modification allowed larger preparations to be carried out more rapidly, with less effort, and at less cost.

The availability of milligram-quantities of the homogeneous protein has allowed us to initiate structural studies of the protein. The results reported here suggest that the structure of erythrocyte cytochrome b_5 corresponds to a segment of liver microsomal cytochrome b_5. Trypsin cleaves the erythrocyte protein to give a hemepeptide which is electrophoretically indistinguishable from the core hemepeptide (12) obtained by tryptic digestion of liver microsomal cytochrome b_5. The molecular weight of 15,300 places the erythrocyte cytochrome as intermediate in size between detergent-solubilized and trypsin-solubilized cytochrome b_5 from liver microsomes. This value for molecular weight obtained by SDS disc gel electrophoresis agrees with the value of 14,600 previously calculated from sedimentation and diffusion measurements (2). It is not known whether the two minor forms seen in Fig. 1 are naturally occurring or a consequence of the isolation procedure, but minor forms of cytochrome b_5 have been isolated from other cells (13).

The similarity of the core hemepeptides from erythrocyte and liver microsomal cytochrome b_5 provides further evidence for our contention (14) that many of the soluble hemeproteins, flavoproteins, and copper-proteins of mature red cells, including cytochrome b_5, cytochrome b_5 reductase, and hemeprotein P-420 (7), have been derived from the membranous fractions of immature erythroid cells during erythroid maturation.

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