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SOLUBLE CYTOCHROME b_5 FROM HUMAN ERYTHROCYTES*

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SUMMARY

1. A hemeprotein with properties similar to microsomal cytochrome b_5 has been detected in the supernatant fraction of hemolysates of human, beef, and rabbit erythrocytes. A method has been developed for determining the amount of this soluble cytochrome in small volumes of blood. The amount of the protein decreases during cell storage at 4 °C. Blood cells rich in reticulocytes contain more of the protein than do mature cells.

2. The cytochrome has been purified from human erythrocytes by a procedure which employs chromatography on Amberlite CG-50 and DEAE-cellulose, ultrafiltration, and gel filtration. The purified protein sedimented in the ultracentrifuge as a single peak with an $s_{20,w}$ of 1.40. However, minor impurities were detected by polyacrylamide disc electrophoresis.

3. The molecular weight of the purified protein has been calculated to be 14600 from sedimentation and diffusion measurements and 18400 as determined by gel filtration. The prosthetic group has been identified as protoheme IX. The spectral properties of the hemeprotein are those of a low spin heme complex. The EPR spectrum of the oxidized form shows g values of 3.03, 2.21, and 1.39 and the visible spectrum has a Soret absorbance maximum at 413 nm. The protein is reducible by dithionite or NADH *plus* cytochrome b_5 reductase and the reduced form shows absorbance maxima at 423, 527, and 556 nm with a shoulder at 560 nm.

4. The cytochrome b_5 differs from the other B-type cytochrome of erythrocyte, S-protein (hemeprotein 559), and is not derived from this protein. The erythrocyte cytochrome b_5 is similar to the cytochrome b_5 solubilized from liver microsomes in terms of spectral properties, molecular weight, prosthetic group, and reactivity.

INTRODUCTION

Cytochrome b_5 has been detected in the microsomal fraction of many tissues from many different species[1], in the Golgi membranes[2], in the outer membrane of liver

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mitochondria[3,4], in neutrophilic granules of rabbit leucocytes[5], in the supernatant fraction of pig kidney homogenates[6], in the nuclear membrane of liver[7,8] and in promitochondria of yeast[9]. In the microsome, the cytochrome b_5 is a tightly bound component of the membranous system and is solubilized only after disruption of the microsome[10]. The small molecular weight hemepeptide resulting from disruption of liver microsomes with proteases has been purified and studied in great detail[11,12]. This solubilized form of the cytochrome is spectrally similar to the particulate form and serves as an electron acceptor for the solubilized form of microsomal cytochrome b_5 reductase[13].

The mammalian erythrocyte is believed to be devoid of endoplasmic reticulum[14], as well as mitochondria and nuclei, and thus microsomal redox proteins were assumed to be absent in these cells. However, the stromal fraction of human erythrocytes contains a hemeprotein, S-protein[15], which we have recently reported[16] to be similar to the degraded form of hemeprotein P-450, a hydroxylase found in the microsomes of many tissues[17]. The supernatant fraction of red cell hemolysates contains hemoglobin, catalase, a hemeprotein with a unique prosthetic group[18], and a hemeprotein with spectral properties similar to cytochrome b_5[19]. We have previously reported that this erythrocyte cytochrome b_5 stimulates the *in vitro* reduction of methemoglobin[20]. In this paper we report the purification and characterization of this soluble cytochrome b_5 from human erythrocytes and conclude that the protein is not derived from any of the known hemeproteins of the erythrocyte, but is similar to the cytochrome b_5 solubilized from microsomes and mitochondria of other tissues.

EXPERIMENTAL PROCEDURE

Materials

Amberlite CG-50, 200–400 mesh, was obtained from Mallinckrodt; DEAE-cellulose from Fisher; UM-2 and UM-10 ultrafiltration membranes from Amicon; blue dextran 2000 from Pharmacia; NADH and horse heart cytochrome *c* (Type III) from Sigma; ovalbumin, lysozyme, and chymotrypsinogen A from Worthington; and bovine serum albumin (Fraction V, B grade), myoglobin, trypsin, egg-white trypsin inhibitor (Grade B), human hemiglobin, and Bio-Gel P-30, P-60, and P-100, 100–200 mesh, from Calbiochem. Rat liver microsomal cytochrome b_5 reductase was a gift from Dr M. J. Coon. Erythrocyte cytochrome b_5 reductase was obtained as described in the accompanying paper[21]. S-protein was isolated as described previously[16].

Outdated human blood cells were graciously provided by St. Joseph's Mercy Hospital Blood Bank, Ann Arbor, Mich., The University of Michigan Medical Center Blood Bank, Ann Arbor, Mich., and the Bureau of Laboratories, Michigan Department of Public Health, Lansing, Mich. Fresh human blood was purchased from the University of Michigan Medical Center Blood Bank and was used within 2 h after drawing. Fresh beef blood was purchased from Kapler's Packing Co., Ann Arbor, Michigan. Normal rabbit blood cells were obtained by bleeding New Zealand white rabbits, and rabbit reticulocytes were obtained by bleeding the rabbits on the 10th day after injections of 3 % phenylhydrazine (0.5 ml/kg) on Days 1 and 5.

Methods

Protein concentrations were determined by the method of Lowry *et al.*[22] using serum albumin as standard. Disc-gel electrophoresis was run at pH 9.5 as described

by Davis[23] and subsequently stained for protein with Coomassie Brilliant Blue by the method of Chrambach *et al.*[24]. Sedimentation and diffusion were measured with a Spinco Model E ultracentrifuge equipped with schlieren optics and a temperature control system. A valve-type synthetic-boundary cell was used. Sedimentation coefficients were calculated according to Schachman[25] and diffusion coefficients according to Lamm[26]. Molecular weight was calculated from the sedimentation and diffusion constants[27], assuming a partial specific volume of 0.73 ml/g. Details for these procedures, together with the methods for the determination of molecular weight by Bio-Gel chromatography, for ultraviolet, visible, and electron paramagnetic resonance spectral characterization, and for the storage and washing of red blood cells, have been described previously[28].

The heme was removed from cytochrome b_5 by first precipitating and washing the protein with acetone and then extracting the protein with HCl–acetone (0.9 ml 37 % HCl in 10 ml acetone). The heme was converted to its porphyrin by the method of Morrison *et al.*[29]. The porphyrin was methylated in methanol–H_2SO_4 and the resulting ester was extracted into ether according to the method of Schwartz *et al.*[30].

Pyridine hemochromes of heme and hemeprotein were prepared in a total volume of 1.25 ml with the final concentration of NaOH and pyridine being 0.08 M and 2.1 M, respectively. The complex was reduced with a few crystals of sodium dithionite. The extinction coefficients reported by Falk[31] were used.

Hemes and porphyrins were chromatographed in the ascending direction on Whatman No. 1 paper at room temperature in a solvent composed of 2,4-lutidine–water (65:35, v/v) and saturated with NH_3[32]. Porphyrin methyl esters were chromatographed in the ascending direction on Whatman No. 1 paper at room temperature with a single development in the chloroform–kerosene or *n*-propanol–kerosene solvent systems of Chu *et al.*[33]. Porphyrins were detected on chromatograms as fluorescent spots under illumination with an ultraviolet lamp. Hemes were detected with the benzidine spray reagent of Connelly *et al.*[34].

Purification of erythrocyte cytochrome b_5

Cytochrome b_5 was isolated by modification of the methods used to isolate from red blood cell hemolysates a hemeprotein with a unique prosthetic group[18] and a pink copper protein[28].

Washed, outdated human red cells (2.6 l) were lysed by adding rapidly, with the aid of compressed air, 1.2 l cold water to 0.3 l aliquots of the packed cells. The lysate was adjusted to pH 6.0 with dilute HCl and centrifuged at 10 000 × *g* for 30 min to remove the stroma. The supernatant fraction (12.1 l) was readjusted to pH 6.0 and the hemoglobin removed by stirring this solution with 14.2 l of wet Amberlite CG-50 resin which had previously been washed once with water after equilibration with pH 6.0, 0.05 M potassium phophate buffer. After stirring for 45 min, the resin was allowed to settle. The supernatant fraction was removed by decantation and the resin was washed with 12 l water. The supernatant fraction and wash were pooled and adjusted to pH 7.2 using dilute KOH.

Further purification was achieved by chromatography on DEAE-cellulose at pH 7.2. The protein fraction was applied at a rate of 0.5 l/h to a 4.7 cm × 43 cm column of DEAE-cellulose previously equilibrated with 3 mM phosphate buffer, pH 7.2. The column was first washed at a rate of 0.12 l/h with 1.3 l of the same buffer

and then with a linear gradient formed with 4 l of 3 mM buffer in the mixer and 4 l of 0.2 M buffer in the reservoir. After 3.3 l of the gradient had passed through the column, the gradient was changed by making the remaining 0.2 M buffer in the reservoir 2 M in KCl. Continued chromatography eluted the cytochrome b_5 (Fig. 1). The cytochrome b_5 was identified by oxidized and reduced spectra, and by its ability to be enzymatically reduced with NADH[21].

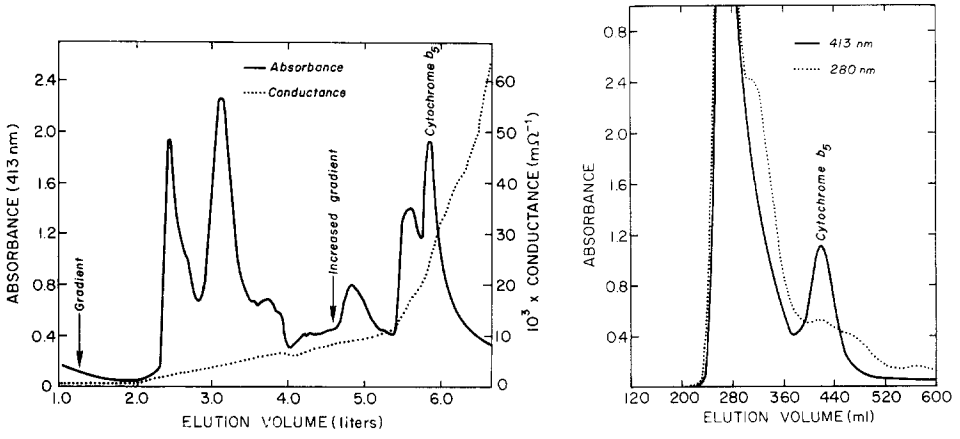


Fig. 1. Purification of erythrocyte cytochrome b_5 by chromatography on DEAE-cellulose. The supernatant fraction and the wash from the Amberlite CG-50 treatment were chromatographed at pH 7.2 as described in Experimental Procedure.

Fig. 2. Purification of erythrocyte cytochrome b_5 by chromatography on Bio-Gel P-60. The cytochrome b_5 fraction from the DEAE-cellulose column was first concentrated and then chromatographed as described in the Experimental Procedure.

Fractions containing the partially purified cytochrome b_5 were pooled and further purified by gel filtration on Bio-Gel P-60 and P-30 columns. The volume of the solution was reduced to about 50 ml by ultrafiltration on a UM-10 filter in a model 401 Diaflo cell and then to 6.8 ml on a UM-2 filter in a model 52 Diaflo cell. This sample was applied to a 3.4 cm × 107 cm Bio-Gel P-60 column previously equilibrated with 0.1 M Tris–HCl buffer, pH 8.1, containing $5 \cdot 10^{-4}$ M EDTA. The column was eluted at a rate of 16 ml/h with the same buffer (Fig. 2). Fractions containing cytochrome b_5 were pooled and the solution (44 ml) was reduced to 6.0 ml by ultrafiltration on a UM-2 membrane. This sample was then chromatographed on a 2.5 cm × 87 cm column of Bio-Gel P-30 with the Tris–HCl, EDTA buffer at 10 ml/h. Peak tubes were pooled and used without further treatment or after concentration by ultrafiltration.

The absorption peak at 413 nm served to detect the oxidized form of the cytochrome in fractions from chromatographic columns. In fractions devoid of other colored proteins the ratio, $A_{413\,nm}/A_{280\,nm}$, was used as a measure of purity and the quantity, $A_{413\,nm} \times$ ml, was used as a measure of the total cytochrome b_5.

Quantitation of cytochrome b_5 in small samples of red blood cells

The amount of cytochrome b_5 in small samples of red blood cells was determined by a procedure that involved partial purification of the cytochrome on DEAE-cellulose and enzymatic assay of the resulting sample.

Whole human blood was washed 3 times with 0.9 % NaCl at 2000 × *g*, being careful to remove the buffy coat. 5 ml of the resulting packed cells were lysed by the forceful addition of 20 ml of cold water. The pH of the hemolysate was adjusted to pH 6.0 with dilute HCl, and the stroma removed by centrifugation at 10000 × *g* for 30 min. The supernatant fraction was diluted with an equal volume of cold water, adjusted to pH 7.2 with dilute KOH, and applied to a 1 cm × 4 cm DEAE-cellulose column that had been equilibrated with 0.003 M potassium phosphate buffer, pH 7.2. The column was washed with 10 ml of 0.003 M buffer and then with 10 ml of 0.05 M buffer (or 0.02 M if rabbit blood was used instead of human blood). The cytochrome b_5 was then eluted from the column with 0.2 M potassium phosphate buffer containing 0.5 M KCl, pH 7.2.

Samples believed to contain cytochrome b_5 were assayed by recording the enzymatically-reduced *minus* oxidized difference spectrum. The sample cuvette contained in a total volume of 1.0 ml: 0.5 ml sample to be analyzed, 0.18 μmole NADH, 0.25 μmole EDTA, 25 μmole Tris–HCl, pH 8.1, and excess erythrocyte cytochrome b_5 reductase. The spectrum of the cuvette was recorded *vs* a cuvette containing the same constituents except for NADH and reductase. From the difference spectrum, the difference in absorbance at 424 nm and 450 nm (A_{424}–A_{450}) was calculated.

The amount of cytochrome was calculated from the following equation: Cytochrome b_5 (in nmoles) = (A_{424}–A_{450}) × 2.4 × 1/0.115. The factor 2.4 is the experimentally determined value for the ratio: A_{413}, oxidized/(A_{424}–A_{450}), reduced *minus* oxidized. The value, 0.115, is the μmolar extinction coefficient[35] of the oxidized form of microsomal cytochrome b_5 at 413 nm. The total amount of cytochrome b_5 in the sample applied to the DEAE-cellulose column was calculated by summing the values in each of the eluate fractions.

Determination of yield of cytochrome b_5 with increasing time of incubation of incubation of stroma

To determine whether the cytochrome b_5 was being solubilized from the stroma during isolation, the yields of cytochrome were determined for red cell hemolysates which had been stirred for varying lengths of time before the stromal fraction was removed. Washed red cells from human outdated blood were hemolyzed by the rapid addition of water, adjusted to pH 6.5, and stirred at 4 °C. At 0, 1, 2, and 5 h after hemolysis, aliquots were removed; after the pH was adjusted to 6.0, the stroma was removed by centrifugation. The amount of cytochrome b_5 in the supernatant fraction was determined as described in the previous section except that larger columns (1.8 cm × 44 cm) of DEAE-cellulose were employed.

RESULTS

The purification of erythrocyte cytochrome b_5 from 2.6 l of outdated human erythrocytes is summarized in Table I. The most concentrated fractions eluted from the Bio-Gel P-30 column were also the most pure, with an $A_{413\,nm}/A_{280\,nm}$ ratio of 3.17. After concentration, ultracentrifugation of the cytochrome b_5 showed a single symmetrical peak in the ultracentrifuge (Fig. 3). The same sample, however, showed

TABLE I

PURIFICATION OF ERYTHROCYTE CYTOCHROME b_5

Fraction	*Volume* (*ml*)	*Total* $A_{280\ nm}$ ($A_{280\ nm} \times ml$)	*Total* $A_{413\ nm}$ ($A_{413\ nm} \times ml$)	$\frac{A_{413\ nm}}{A_{280\ nm}}$
Hemolysate	12 080	$1.07 \cdot 10^6$	$3.97 \cdot 10^6$	
Amberlite CG-50 supernatant and wash	21 000	$2.73 \cdot 10^5$	$9.46 \cdot 10^5$	
DEAE-cellulose eluate	340	1670	421	0.25
DEAE-cellulose eluate after concentration	6.8	870	400	0.45
Bio-Gel P-60 eluate	44.0	22.4	40.3	1.80
Bio-Gel P-60 eluate after concentration	6.0	21.5	36.6	1.70
Bio-Gel P-30 eluate	12.0	7.7	24.5	3.17

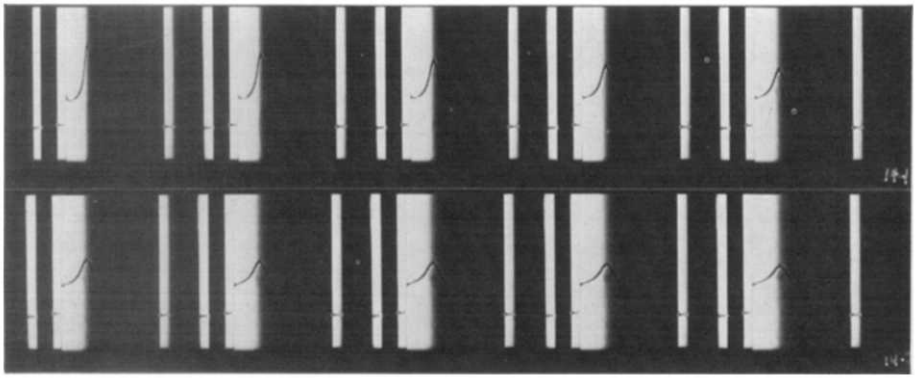
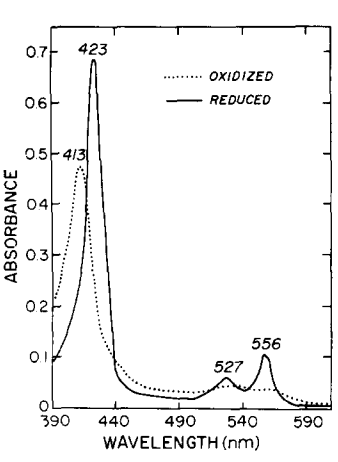


Fig. 3. Sedimentation pattern of erythrocyte cytochrome b_5. The protein (2.3 mg/ml) was subjected to ultracentrifugation at 20 °C and 59780 rev./min in 0.1 M Tris–HCl buffer containing 0.5 mM EDTA, pH 8.1. Photographs were taken at 4-min intervals after 2/3 speed was reached.

minor impurities by disc-gel electrophoresis. The major electrophoretic band was the only colored band and was estimated to account for at least 90 % of the total protein present on the gel.

A molecular weight of 18 400 ± 500 was calculated by averaging the values from two gel filtration determinations on a calibrated Bio-Gel P-30 column and three determinations on a calibrated P-60 column. From the ultracentrifugation data (Fig. 3), $s_{20,w}$ was calculated to be 1.40 and $D_{20,w}$ to be $8.67 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1}. Assuming a partial specific volume of 0.73 ml/g, a molecular weight of 14 600 was calculated from the sedimentation and diffusion data.

The visible absorbance spectrum of the isolated (oxidized) form and the dithionite-reduced form of erythrocyte cytochrome b_5 are presented in Fig. 4. The enzymatically reduced form of the cytochrome b_5, obtained by anaerobic incubation in the presence of NADH and cytochrome b_5 reductase (erythrocyte or liver microsomal), shows a spectrum identical to the dithionite-reduced cytochrome, with respect to both position and extinction of absorbance maxima. The reduced *minus* oxidized difference spectrum is presented in Fig. 5; the same spectrum is obtained when the reduction is carried out with dithionite as when enzymatic reduction is employed. In



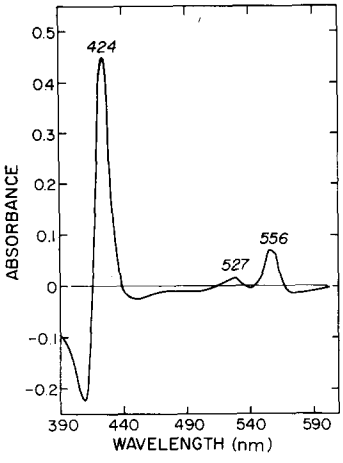


Fig. 4. Spectra of the oxidized and reduced forms of erythrocyte cytochrome b_5. The protein, 100 μg/ml, with a $A_{413\ nm}/A_{280\ nm}$ ratio of 3.33, was in 0.1 M Tris–HCl containing 0.5 mM EDTA, pH 8.1, at 25 °C. The sample was reduced by the addition of a few crystals of sodium dithionite.

Fig. 5. Reduced cytochrome b_5 *minus* oxidized cytochrome b_5 difference spectrum. The sample and conditions are those described in Fig. 4.

TABLE II

COMPARISON OF VISIBLE SPECTRAL PROPERTIES OF ERYTHROCYTE CYTOCHROME b_5, ERYTHROCYTE S-PROTEIN, AND LIVER MICROSOMAL CYTOCHROME b_5

Parentheses denote a shoulder rather than a distinct maximum.

Form	*Absorbance maxima (nm)*		
	Erythrocyte cytochrome b_5	*Erythrocyte S-protein*	*Liver microsomal cytochrome* b_5
Oxidized	413	412	413
Dithionite-reduced	423, 527, 556 (560)	426, 531, 559	423, 526, 556 (560)
Enzymatically-reduced *minus* oxidized	424, 527, 556 (560)	None	424, 527, 556 (560)
CO-reduced *minus* reduced	None	420, 541, 573	None

both the absolute spectrum of the reduced form and the reduced *minus* oxidized difference spectrum, the α peak is asymmetric with the maximum at 556 nm and a shoulder at 560 nm. The addition of CO did not alter the spectrum of either the oxidized or the reduced form. The positions of the absorbance maxima for erythrocyte cytochrome b_5 are summarized in Table II and compared with those of erythrocyte S-protein and solubilized liver microsomal cytochrome b_5. The α- and β-peaks of the visible absorbance spectra of the reduced erythrocyte cytochrome b_5 and erythrocyte S-protein are compared in Fig. 6.

EPR spectra of the oxidized cytochrome b_5 at 36 °K gave peaks with g values of 3.03, 2.21, and 1.39. There was also a peak at $g = 4.43$, presumably due to extraneous iron, and a peak at $g = 2.06$, which may be due to contaminating copper.

The pyridine hemochrome spectra of cytochrome b_5 and its isolated heme

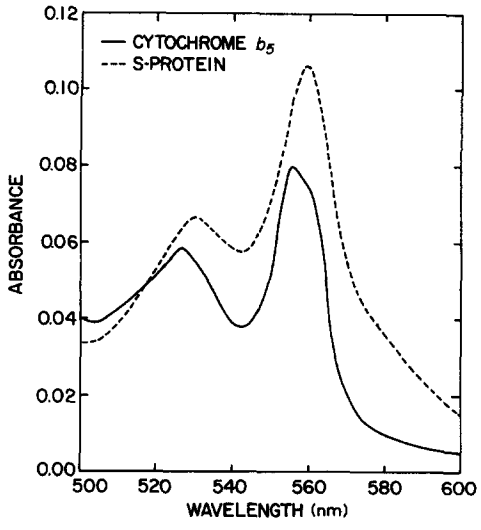


Fig. 6. Visible spectra of the reduced forms of erythrocyte cytochrome b_5 and erythrocyte S-protein. Conditions were similar to those described in Fig. 4 except that the cytochrome b_5 was in 0.04 M Tris–HCl, pH 7.5, and S-protein was in 0.03 M Tris–HCl *plus* 0.13 M potassium phosphate, pH 7.3.

prosthetic group are both indistinguishable from the pyridine hemochrome of ferroprotoporphyrin IX, with absorbance maxima at 419, 526, and 557 nm. The porphyrin and porphyrin methyl ester derived from the heme of cytochrome b_5 showed spectra in chloroform and diethyl ether which were essentially the same as the corresponding spectra of protoporphyrin IX. The heme and porphyrin derived from the protein co-chromatographed on paper in lutidine–water solvent with protoheme IX and protoporphyrin IX, respectively. The methyl ester of the porphyrin of cytochrome b_5 co-chromatographed with the methyl ester of protoporphyrin IX on paper in the chloroform–kerosene and *n*-propanol–kerosene solvent systems.

The yields of cytochrome b_5 from fresh and outdated human and rabbit erythrocytes and from rabbit reticulocytes are summarized in Table III. The yield of the cytochrome from outdated human erythrocytes did not change appreciably with duration of time between the hemolysis and removal of the stroma by centrifugation. The yields of cytochrome per 375 ml of cells for 0, 1, 2, and 5 h of incubation with

TABLE III

YIELDS OF CYTOCHROME b_5 FROM VARIOUS TYPES OF CELLS

Fresh cells had been drawn 1 day previously. Outdated cells had been drawn 4 weeks previously.

Source	*Cytochrome b_5 (nmoles/5 ml cells)*
Fresh human erythrocytes	4.06
Outdated human erythrocytes	1.22
Fresh rabbit reticulocytes	3.32
Fresh rabbit erythrocytes	2.32
Outdated rabbit erythrocytes	1.80

the stroma were 106, 94, 87, and 86 nmoles, respectively. When cells were washed and lysed in the presence of trypsin inhibitor (0.2 mg/ml), the usual yield of cytochrome b_5 was obtained.

Attempts to convert purified S-protein to cytochrome b_5 were unsuccessful. Digestion of S-protein with a 0.06 % solution of trypsin in 0.1 M Tris–HCl *plus* 1 mM EDTA, pH 8.1, for 22 h at 4 °C resulted in a disappearance of 40 % of the S-protein as judged by spectral analysis. During this period, no cytochrome b_5 could be detected in the incubation mixture by spectrophotometry after chemical or enzymatic reduction. When erythrocyte cytochrome b_5 was subjected to the same tryptic digestion, 98 % of the cytochrome appeared unaltered as judged from the dithionite-reduced spectrum and 90 % as judged by enzymatic reduction.

S-protein was likewise degraded without conversion to cytochrome b_5 by incubation in a solution[36] containing 0.5 % desoxycholate, 0.1 M citrate, 0.001 M dithiothreitol, 0.1 M KCl and 30 % glycerol, pH 7.2. In this solution, the spectral characteristics of S-protein had a half-life of about 40 min at 25 °C. After 60 h at 4 °C the spectral properties of the S-protein had completely disappeared. Whereas, no cytochrome b_5 was detected in these incubation mixtures, purified cytochrome b_5 was stable in this solution at both temperatures.

DISCUSSION

These studies establish that cytochrome b_5 is present in human, rabbit, and bovine erythrocytes. It is not likely that the protein arises from contaminating white cells since the amount of cytochrome recovered is 30 times greater than the amount of cytochrome b_5 reported to be present in all the white cells from this volume of blood[5]. Furthermore, exhaustive efforts to remove white cells from the erythrocyte preparations by repeatedly removing by aspiration the top layer of cells after centrifugation did not decrease the yield of cytochrome b_5. The observations that the amount of isolatable cytochrome b_5 decreases upon storage of the cells at 4 °C and is greater in a cell population high in reticulocytes than in mature red cells suggest that the cytochrome is not an artifact arising during storage or aging.

The spectral properties, molecular weight, and chromatographic behavior of erythrocyte cytochrome b_5 distinguish it from the other hemeproteins of the red cell. Of these other erythrocyte proteins, only S-protein[15,16,37] shows similarities to cytochrome b_5. Both proteins are B-type cytochromes with protoheme IX prosthetic groups. However, as shown in Table II, the S-protein differs from cytochrome b_5 in that it fails to serve as an electron acceptor for cytochrome b_5 reductase, it binds CO, its reduced form has absorbance maxima at slightly longer wavelengths, and the α-peak of this form is symmetrical. In contrast to cytochrome b_5, S-protein is a lipoprotein, has a molecular weight of 25 000, readily undergoes dimerization and polymerization reactions, precipitates at pH 5.5, and is unstable in the presence of aqueous desoxycholate or aqueous butanol. Furthermore, in cell hemolysates, S-protein is a stromal protein and cytochrome b_5 is a supernatant protein.

The relationship between these two proteins appeared quite analogous to the relationship between two forms of cytochrome b_5 solubilized from liver microsomes by incubation with trypsin and detergent, respectively[38]. It therefore seemed possible that erythrocyte cytochrome b_5 was derived from S-protein, perhaps by the action of

the stromal proteases which are activated after hemolysis[39,40]. However, the yield of cytochrome b_5 did not increase with increases in the time interval between hemolysis and removal of stroma and did not decrease when trypsin inhibitor was present in the hemolysate, as would have been expected if the cytochrome b_5 were being formed as a result of the proteolytic digestion of S-protein. Moreover, the S-protein is not converted to cytochrome b_5 by incubation with trypsin, desoxycholate, or butanol. Removal of the lipid component of S-protein had previously been shown to yield not a cytochrome b_5-type protein, but rather a highly polymerized product[37]. Thus, the studies reported here indicate that cytochrome b_5 does not arise from the degradation of S-protein.

The present study shows that the erythrocyte cytochrome is very similar to cytochrome b_5 solubilized from the microsomes and mitochondrial outer membranes of other tissues. The absorbance spectra of the oxidized and reduced forms, the g values of the EPR spectrum, the approximate molecular weight, and the protoheme IX prosthetic group of the erythrocyte cytochrome b_5 are indistinguishable from those reported for solubilized liver microsomal cytochrome b_5[35,41]. The reduced form of the erythrocyte cytochrome, like the liver protein[11,42], fails to bind CO but is oxidized by molecular oxygen, ferricytochrome c, and methemoglobin[21,50]. Both the erythrocyte and liver cytochrome b_5 serve as electron acceptors for cytochrome b_5 reductases isolated from liver and erythrocytes[21]. Thus, erythrocyte cytochrome b_5 appears similar to microsomal cytochrome b_5 except for the fact that freezing–thawing or hypotonic shocking yields a soluble protein. Whereas the cytochromes from the erythrocyte and from the pig kidney cytoplasm[6] are both soluble proteins following gentle disruption of cells, these proteins differ greatly as to molecular weight. It is possible that the erythrocyte protein is identical to the readily solubilized cytochrome b_5 from the outer membrane of mitochondria. In any event, it appears likely that soluble or readily solubilized forms of cytochrome b_5 are widely distributed among tissues and among subcellular fractions.

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