PURIFICATION AND PROPERTIES OF S-PROTEIN (HEMOPROTEIN 559) FROM HUMAN ERYTHROCYTES*

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SUMMARY

1. A procedure employing mild conditions has been developed for purifying S-protein (hemoprotein 559, erythrocyte), a protein present in the stromal fraction of human red blood cells. S-protein, solubilized by extracting the stroma of red blood cells with pH 8.2 buffer, was purified by chromatography on DEAE-cellulose and Bio-Gel P-60.

2. The oxidized and reduced forms of this protein show visible absorption spectra typical of b-type cytochromes. The oxidized form of the protein shows absorbance maxima at 412, 533, and 565 mμ, and the dithionite-reduced spectrum shows maxima at 426, 530, and 559 mμ. The prosthetic group has been isolated and identified as protoheme IX by spectral and chromatographic techniques. The spectral properties of this protein differ from those of cytochrome b_5.

3. The reduced form of the protein binds CO, and the resulting complex shows absorbance maxima at 421, 540, and 568 mμ. This spectrum, the CO-reduced *minus* reduced difference spectrum, and the spectra of the oxidized and reduced forms of the free protein are all indistinguishable from the corresponding spectra of hemoprotein P-420, the altered form of the microsomal hydroxylase, hemoprotein P-450. The spectral properties, together with other physical properties, suggest that S-protein is P-420 from erythrocytes.

INTRODUCTION

The insoluble or stromal fraction of red blood hemolysates contains, in addition to a number of colorless enzymes and structural proteins, a considerable amount of

colored protein. The amount of color associated with the stroma varies with the conditions of lysis and washing. This colored protein has often been referred to as "contaminating hemoglobin." In addition to the various forms of hemoglobin, however, the stroma contains a hemoprotein that has been called S-protein[1]. S-protein, solubilized by extracting stroma with slightly alkaline buffer, has been partially purified by repeated isoelectric precipitation at pH 5.5 and partially characterized[1-4]. A study of the solubility, sedimentation, and electrophoretic properties has been made on a partially purified preparation[5]. In a more detailed study[6,7], S-protein was shown to be a lipid-containing hemoprotein, of approx. 25000 mol. wt., which readily froms dimers. The spectral properties of the protein were similar to those of a *b*-type cytochrome. S-protein has been isolated from human, rabbit, and bovine red cells[7]. Attempts to further purify the protein by chromatographic techniques have been unsuccessful. The immunological properties of partially purified preparations of S-protein from a number of species have been reported[8,9], but a more pure sample of human S-protein has been shown to have no or extremely little antigenicity to the rabbit[7].

In our laboratory, we have been unsuccessful in demonstrating a biological activity for S-protein isolated by isoelectric precipitation. This preparation failed to activate succinate–coenzyme Q reductase activity in the reconstitution of mitochondrial electron transport[10] and failed to serve as an electron acceptor for the reaction catalyzed by microsomal cytochrome b_5 reductase.

Since it is possible that the low pH employed during the isolation of S-protein modified its properties, we sought a milder procedure for isolating the hemoprotein before initiating an extensive search for its biological activity. In this paper, we report the purification of S-protein from human red blood cells by chromatographic procedures carried out at pH 7.2, and draw attention to the similarities between the properties of this protein and P-420, the altered form of the microsomal hydroxylating enzyme, hemoprotein P-450. Parts of this work have been reported in abstract form[11].

MATERIALS AND METHODS

DEAE-cellulose was obtained from Fisher Chemical Co.; Bio-Gel P-60, 100–200 mesh, from Calbiochem; kerosene from Fisher Chemical Co.; and 2,6-lutidine (95%) from Eastman Organic Chemicals. Protoporphyrin dimethyl ester was prepared from red cells by the method of GRINSTEIN[12].

Out-dated human red blood cells were generously supplied 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. Cells were used and stored at 4° in acid–citrate–dextrose Formula A (2.45 g glucose, 2.20 g citrate (trisodium salt), and 0.80 g citric acid in 100 ml water) and were used within 3 weeks of their expiration date.

Disc electrophoresis was run at pH 9.5 according to the method of DAVIS[13]. The lower gel concentration was 7.5% and the protein was applied in 40% sucrose. The gel was stained for protein with Coomassie brilliant blue by the method of CHRAMBACH *et al.*[14].

Sedimentation velocity analysis was carried out with a Spinco Model E Ultracentrifuge equipped with schlieren optics and a temperature control system.

Visible absorption spectra were recorded at 25° with a Cary Model 14 Spectrophotometer. Aliquots of a single solution of S-protein in pH 7.2 potassium phosphate buffer (0.2 M) were employed for all spectral studies. S-protein was reduced by adding a small amount of solid sodium dithionite. The reduced form of the protein was converted to the CO complex by subjecting the protein solution to one atmosphere of CO for one min. The pyridine hemochromes were formed by adding a few crystals of sodium dithionite to solutions of S-protein or its prosthetic group in 0.05 M NaOH–pyridine (5:1, by vol.)

The heme prosthetic group was extracted from the S-protein by slowly adding a cold aqueous solution of the protein to 10 volumes of ethyl acetate–acetic acid (4:1, by vol.) at 4°. The colorless apoprotein was sedimented by centrifugation at 2000 × g and discarded. Evaporation of the supernatant fraction to dryness, *in vacuo* at 25°, left the heme as a residue. Iron was removed from the heme by the method of MORELL et al.[15], and the resulting porphyrins were converted to their methyl esters by treatment with methanol–sulfuric acid, according to the method of SCHWARTZ et al.[16]. Samples were chromatographed in the ascending direction on Whatman No. 1 paper at room temperature. A mixture of 2,6-lutidine–water (10:7, by vol.) in an atmosphere of NH_3[17] was employed for the chromatography of the heme. Chloroform–kerosene (13:20, by vol.) and n-propanol–kerosene (1:5, by vol.)[18] were employed for the chromatography of the porphyrin methyl esters.

Hemolysis of red blood cells

Out-dated human red cells were washed 3 times at 4° by centrifuging a suspension of cells in 0.9% NaCl solution at 5000 × g for 10 min and then decanting the buffy coat and supernatant fraction. Red cells were lysed by subjecting them to either hypotonic shock or to freezing and thawing. Lysis by freezing and thawing was accomplished by adding 3 volumes of water and 0.002 of a volume of toluene to the packed cells, and then rapidly freezing this suspension in a dry ice–acetone bath. The suspension was thawed and the procedure repeated. Hypotonic lysis was achieved by forcing (under air pressure) four volumes of cold water into the packed cells. The suspension obtained by either method of lysis was adjusted at 4° to pH 6.0 with HCl, and the stroma was removed by centrifugation for 20 min at 10000 × g.

Solubilization of S-protein

The stroma (150 ml) obtained from 375 ml of packed cells was washed by suspending the material in 1 l of cold water and, after adjusting the pH to 6.0, centrifuging at 17000 × g for 20 min. The stroma was then mixed with 250 ml of cold water. S-protein was released from the stroma by adjusting the suspension to pH 8.2 by the addition of 2.2 M potassium glycinate, and then stirring for 20 min while maintaining the pH at 8.2. The insoluble fraction was removed by adjusting the suspension to pH 7.2 with acetic acid and centrifuging at 17000 × g for 20 min. the extraction at pH 8.2 was repeated twice using 250-ml volumes of solution. The extracts were pooled and any additional precipitate was removed by centrifuging at 17900 × g for 20 min.

Purification of S-protein

All purification steps were carried out at 4° with potassium phosphate buffers

prepared with distilled-deionized water. Purification was followed by measuring the ratio of the absorbance at 412 mμ to the absorbance at 280 mμ. Once hemoglobin had been completely removed, the total absorbance at 412 mμ ($A_{412 \text{ m}\mu} \times$ ml) was used as a measure of S-protein.

The extract of the stroma was charged at a rate of 3 ml/min onto a 4.7 cm $\times$ 36 cm column of DEAE-cellulose previously equilibrated with 0.003 M buffer (pH 7.2). The column was washed with 2 l of the 0.003 M buffer at a flow rate of 3 ml/min. The column was then eluted at pH 7.2 with a linear gradient formed by placing 2.5 l of 0.003 M buffer in the mixing vessel and 2.5 l of 0.2 M buffer–1.0 M KCl in the reservoir. The flow rate during elution was 1.8 ml/min. The single colored fraction eluted from the column was identified as S-protein by its spectral properties.

The most concentrated part of this fraction (105 ml) was further purified. The solution was concentrated by lyophilization and then dialyzed for 1 h *versus* 2 l of water. The sample was finally lyophilized to 8 ml and placed on a 3.4 cm $\times$ 106 cm Bio-Gel P-60 column previously equilibrated with 0.2 M buffer (pH 7.2). The column was eluted with this buffer at a rate of 0.2 ml/min.

RESULTS

The purification of S-protein from stroma obtained from cells that had been lysed by freezing and thawing is summarized in Table I. Most of the purification was achieved in the first three steps of the procedure: the preparation of stroma, the extraction of S-protein from the stroma, and chromatography on DEAE-cellulose. However, it was not possible to assess the degree of purification or the yield in these steps, since the presence of contaminating hemoglobin made quantitation of S-protein impossible.

A single colored fraction was eluted from the DEAE-cellulose column by the linear salt gradient, and only one peak was eluted from the P-60 column. Using the total 412 mμ absorbance ($A_{412 \text{ m}\mu} \times$ ml) in the fraction eluted from the DEAE-cellulose column as a measure of S-protein, a yield of 1300 absorbance units was obtained from 375 ml of packed cells that had been lysed by the freezing and thawing method. In comparison, a yield of 836 absorbance units ($A_{412 \text{ m}\mu} \times$ ml) was obtained from the same volume of cells lysed by hypotonic shock. Assuming a molar extinction at 412 mμ of 124 cm$^2 \cdot$mmole^{-1} (see ref. 19) these yields correspond to 10.5 and 6.7 μmoles, respectively.

The most highly purified fraction of S-protein showed a $A_{412 \text{ m}\mu}/A_{280 \text{ m}\mu}$ ratio of 2.29. Electrophoresis of this sample on disc gel columns showed the presence of several contaminating proteins. The presence of a minor, more rapidly sedimenting component was demonstrated when this same sample was subjected to ultracentrifugation at pH 7.2 in 0.2 M potassium phosphate or 0.02 M Tris–HCl buffers, or at pH 8.2 in 1.0 M Tris–HCl buffer.

The visible absorbance spectrum of purified S-protein is shown in Fig. 1. The spectra of the reduced form of S-protein and the CO complex of the reduced form are shown in Fig. 2. The reduced *minus* oxidized difference spectrum is shown in Fig. 3 and the CO-reduced *minus* reduced difference spectrum in Fig. 4.

Both the heme prosthetic group, obtained by extracting S-protein with non-polar solvent under acidic conditions, and the intact protein formed pyridine hemo-
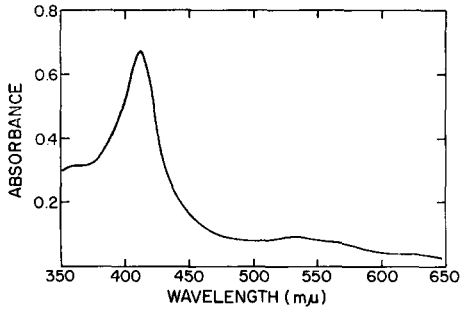
Fig. 1. Visible absorbance spectrum of the isolated form of S-protein. The spectrum of a diluted solution of the purified sample was observed in 0.2 M potassium phosphate buffer (pH 7.2).
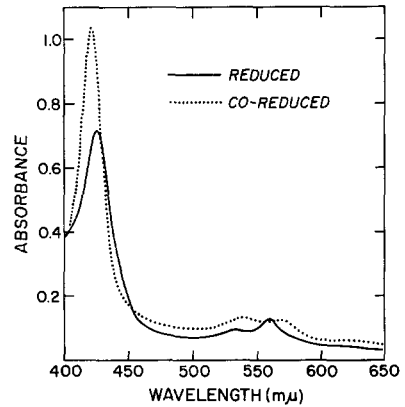
Fig. 2. Visible absorbance spectra of the reduced form of S-protein and the CO complex of the reduced form. Samples were reduced by the addition of a few crystals of sodium dithionite. The CO complex was formed anaerobically in the presence of 1 atm of CO. The sample and conditions are those described in Fig. 1.

chromes that were indistinguishable from the pyridine hemochrome of protoheme IX. These three complexes showed absorbance maxima at 418, 523, and 556 mμ. The heme of S-protein and protoheme IX migrated with the same R_F (0.73) when chromatographed on paper in the lutidine–water solvent.

The porphyrin derived from the prosthetic group of S-protein and the methyl ester of this porphyrin showed visible absorbance spectra in chloroform, methanol, and 90% formic acid that could not be distinguished from the corresponding spectra of protoporphyrin dimethyl ester. The methyl esters of the porphyrin derived from S-protein comigrated with the methyl ester of protoporphyrin when chromatographed in the chloroform–kerosene and n-propanol-kerosene systems.
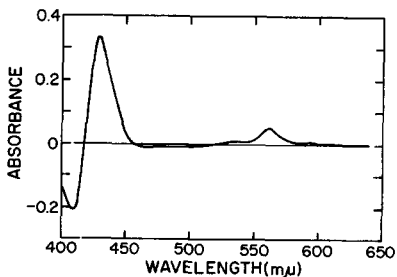

Fig. 3. Reduced S-protein *minus* oxidized S-protein difference spectrum. The sample and conditions are those described in Fig. 2.
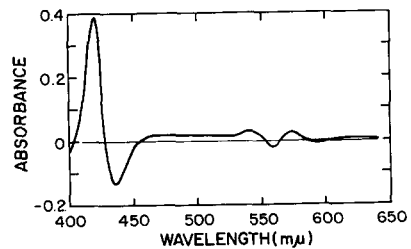
Fig. 4. CO complex of reduced S-protein *minus* reduced S-protein difference spectrum. The sample and conditions are those described in Fig. 2.

DISCUSSION

The isolation of S-protein from the insoluble stromal fraction of human red blood cells confirms the results of earlier studies that S-protein is a component of the membrane fraction of red cell hemolysates and, presumably, of intact cells. This conclusion is supported by our failure to detect S-protein in the supernatant fraction obtained from red cells by the procedure involving hypotonic lysis, adjustment of the pH to 6.0, and centrifugation. Whereas S-protein remains tightly bound to stroma in buffers of low ionic strength at pH 6.0, the protein is readily extracted from the stroma by buffers of pH 8.2 and to a smaller extent by buffers of pH 7.2. S-protein is effectively extracted at pH 7.2 by 1 M LiCl.

Large losses of S-protein occurred when ammonium sulfate precipitation and subsequent dialysis were used to concentrate the protein. Moreover, recovery of hemoprotein from Bio-Gel columns was poor when low ionic strength buffers were employed for the chromatography. These losses might have resulted from dissociation of the heme from the protein or from aggregation and subsequent precipitation of the protein. To avoid these losses, concentration was accomplished by lyophilization, and buffers of relatively high ionic strength were employed for the Bio-Gel chromatography.

In the purification procedure described in this paper, we have attempted to minimize the possibility of protein modification. In contrast to the previous isolations of S-protein, this procedure involves chromatographic methods rather than isoelectric precipitation at low pH. In the present procedure, the fractions were maintained at pH 7.2 throughout, except for a short period of time at pH 6.0 during the preparation of stroma and at pH 8.2 during the solubilization of the S-protein. However, there is no assurance that the protein isolated by this procedure is in its native form; in fact, as with other proteins that are bound to membranes, the solubilization might be expected to result in marked changes in biological activity as well as in physical properties. It is of interest that the spectral properties of this preparation are indistinguishable from the corresponding spectra of S-protein isolated in this laboratory by isoelectric precipitation.

S-protein has now been purified and characterized sufficiently to establish that it is distinct from all other proteins of the human red cell, and that it does not appear

TABLE I

PURIFICATION OF S-PROTEIN FROM HUMAN ERYTHROCYTES

The isolation was carried out on 150 ml of stroma obtained by freezing and thawing 375 ml of stored human red blood cells.

Fraction	Vol. (ml)	$A_{280\ m\mu}$	$A_{412\ m\mu}$	Total $A_{412\ m\mu}$ ($A_{412\ m\mu} \times ml$)
Stromal extract	605	10.2	19.5	11 800
DEAE-cellulose eluate	905	1.41	1.44	1300
Bio-Gel P-60 eluate	30	3.26	6.56	197[*]

[*] Only 21.5% of the S-protein fraction eluted from the DEAE-cellulose column was subsequently chromatographed on the Bio-Gel P-60 column.

to correspond to a more native or less native form of some previously characterized red cell protein. These studies establish that S-protein is a hemoprotein with protoheme IX as its prosthetic group and with spectral properties characteristic of b-type cytochromes. On the basis of spectral properties, S-protein should be named "hemoprotein 559, erythrocyte." The observed properties of S-protein distinguish it from an erythrocyte protein[20,21] which has been characterized as a low-field type hemoprotein with a unique, formyl-containing heme prosthetic group[22]. The spectral properties of the oxidized form, the reduced form, and the CO complex of the reduced form distinguish S-protein from the many forms of hemoglobin, from catalase, and from a cytochrome b_5-type protein that has been isolated from the supernatant fraction of red blood cell hemolysates[23]. A number of other properties also distinguish S-protein from all other proteins of the human red cell. Solubility properties, the presence of lipid, the molecular weight of approx. 25000 at pH 8 in buffers of low ionic strength, and the dimerization reaction that occurs at low pH and high ionic strength have all been reported previously for the protein purified by isoelectric precipitation[7]; these findings have been confirmed in our laboratory. Furthermore, we have shown that the S-protein prepared by isoelectric precipitation possesses an electron paramagnetic resonance spectrum which differs from that of the known erythrocyte hemoproteins.

TABLE II

SUMMARY OF VISIBLE SPECTRAL PROPERTIES OF S-PROTEIN AND P-420

For experimental details see Figs. 1 and 2.

Form	Absorbance maxima (mμ)						
	S-protein				P-420[19,24]		
Oxidized	(360)	412	533	(360)	414	535	
Reduced		426	531	559		426	530	559
CO-reduced		421	540	568		421	538	565
Reduced *minus* oxidized		429	530	559		427	530	560
CO-reduced *minus* reduced		420	541	573		420	540	575

The spectral properties of S-protein do correspond, however, to those of P-420[19,24], the altered and solubilized form of the hydroxylase, hemoprotein P-450, which has been detected in microsomes, adrenal mitochondria, and microorganisms. The spectra of the oxidized and reduced forms and the reduced *minus* oxidized difference spectra presented in Figs. 1–3 are indistinguishable from spectra reported for P-420. P-420, like S-protein, binds carbon monoxide in its reduced form to give a spectrum with a Soret peak at 421 mμ. The spectrum of the CO complex of the reduced form of P-420 and the CO-reduced *minus* reduced difference spectrum are very similar to the spectra of S-protein shown in Figs. 2 and 4. The similarity of the wavelengths of maximum absorbance for the various spectra of the two proteins are shown in Table II. The spectral changes resulting from changing the pH of the S-protein solution are similar to those that have been reported for liver microsomal P-420. In the case of each protein, increasing the pH of the solutions from 7.0 to 9.0 increases the extinctions and sharpens the absorbance maxima of the reduced form, and decreases the extinction and broadens the Soret peak of the oxidized form.

In addition to these spectral properties, S-protein has a number of other properties[7] similar to those reported for P-420[19,24,25]. Both proteins are present in cells in a membrane-bound form. In each case, solubilization yields a lipid-containing protein with a heme prosthetic group. Both are relatively low molecular weight proteins which aggregate, and finally precipitate, as the pH is gradually lowered from 8.0 to 5.5. Both proteins can be reduced with dithionite, and the resulting reduced forms are rapidly autoxidized. Each of the above findings has been confirmed for S-protein in our laboratory. The spectral and chromatographic studies of the present investigation establish that the prosthetic group of S-protein, like that of P-420, is protoheme IX. We have also shown that S-protein, like P-420[19], does not serve as an electron acceptor for liver microsomal cytochrome b_5 reductase.

The similarities between the properties of S-protein and P-420 suggest that S-protein may be the altered form of a red cell hydroxylase. Such an enzyme might be functional in the mature erythrocyte, or it might be functional only in the reticulocyte before maturation occurs. The fact that hemoprotein P-450 is found in the microsomal fraction of other tissues, suggests that a microsomal hydroxylase is present in reticulocytes. The loss of the endoplasmic reticulum during mammalian red blood cell maturation, might result in degradation of this hydroxylase to S-protein which could then become associated with the stromal fraction of the cell.

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