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PURIFICATION AND PROPERTIES OF AN ERYTHROCYTE HEMOPROTEIN WITH A UNIQUE PROSTHETIC GROUP

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SUMMARY

1. A unique hemoprotein has been isolated from a hemolysate of normal human erythrocytes by a procedure which involves $(NH_4)_2SO_4$ precipitation and chromatography on Amberlite CG-50, DEAE-cellulose, Bio-Gel P-60, and Bio-Gel P-30.

2. The purified hemoprotein appeared homogeneous on ultracentrifugation although small amounts of impurities were detected by polyacrylamide gel electrophoresis. The molecular weight was estimated to be 21 000 by gel filtration. Absorption maxima were located at 416 mμ for the oxidized form, 430 mμ for the CN^- complex of the oxidized form, 434 mμ for the reduced form, and 425 mμ for the CO derivative of the reduced form. The reduced pyridine hemochrome of the isolated prosthetic group has maxima at 434, 540, and 579 mμ, clearly indicating that the protein possesses a previously unrecognized heme.

INTRODUCTION

The detection and characterization of hemoproteins of erythrocytes has proven difficult because of the very high concentration of hemoglobin in these cells. Studies have been directed toward the detection and purification of erythrocyte hemoproteins[1,2], but the only hemoproteins which have been isolated and characterized are catalase[3], S-protein[4], and the various forms of hemoglobin. The detection and partial characterization of a fourth hemoprotein was achieved after hemoglobin had been efficiently removed from human red cell hemolysates[5]. This paper reports the further purification and characterization of this unique hemoprotein.

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EXPERIMENTAL PROCEDURE

Materials

Crystalline ovalbumin was kindly supplied by Dr. M. J. Coon. Chemicals and materials were obtained from the following commercial sources: sodium dithionite (Fisher); Blue Dextran 2000 (Pharmacia); bovine serum albumin, Type V (Sigma); DEAE-cellulose (Fisher); Amberlite CG-50, 200–400 mesh (Mallinckrodt); Bio-Gel P-60 and P-30, 50–150 mesh (Calbiochem); and collodion bags, porosity less than 5 mμ (Schleicher and Schuell). All other chemicals were of reagent grade from commercial sources. Out-dated human blood cells were graciously supplied by Dr. James Sgouris of the Bureau of Laboratories, Michigan Department of Public Health, Lansing, Mich., The University of Michigan Medical Center Blood Bank, Ann Arbor, Mich., and St. Joseph's Mercy Hospital Blood Bank, Ann Arbor, Mich. Stored cells were used within 3 weeks of the expiration date. Fresh cells were purchased from The University of Michigan Medical Center Blood Bank. All blood was collected in units of 450 ml in the presence of 67.5 ml of acid–citrate–dextrose Formula A (2.45 g glucose, 2.20 g citrate (trisodium salt), 0.80 g citric acid in 100 ml water).

Methods

Protein concentrations were determined by the method of LOWRY *et al.*[6] using crystalline bovine serum albumin as standard. In fractions free of other colored proteins, the erythrocyte hemoprotein under study was quantitated by measuring the absorbance at 416 mμ and multiplying this value by the volume of the sample in ml ($A_{416\ m\mu} \times$ ml).

Absorption spectra of the protein in 0.05 M potassium phosphate buffer were observed at 25° with a Cary Model 14 spectrophotometer. Protein concentrations of approx. 0.8 mg/ml were employed except that dilution with the buffer was made before observing the Soret region of the spectrum. Anaerobic conditions were obtained by repeatedly evacuating and flushing the cuvettes with prepurified N_2 which had been bubbled through solution according to FIESER[7].

Polyacrylamide gel disc electrophoresis was run at pH 9.5 according to the method of DAVIS[8] and at pH 7.5 with the buffer systems of WILLIAMS AND REISFELD[9].

Sedimentation was carried out with a valve-type synthetic boundary cell using a Spinco Model E ultracentrifuge equipped with Schlieren optics and a temperature control system. Sedimentation was carried out at 8° and a velocity of 59 780 rev./min. The solution contained 3 mg of protein per ml of 0.05 M potassium phosphate buffer (pH 7.2) containing 0.1 M KCl.

The apparent molecular weight of the hemoprotein was determined by chromatography at 4° on a 3.4 cm × 95 cm Bio-Gel P-60 column equilibrated with 0.05 M potassium phosphate buffer (pH 7.2). The column was calibrated with Blue Dextran 2000, ovalbumin, chymotrypsinogen, and cytochrome *c*. Samples were applied in 2.0 ml of buffer and the flow rate was maintained at 6.0 ml/h. Blue Dextran was measured by the absorbance at 600 mμ, hemoproteins by the absorbance at their Soret maxima, and chymotrypsinogen by the absorbance at 280 mμ.

Hemolysis of red blood cells

Red cells (2300 ml) were washed 3 times at 4° by suspension in 1.5 vol. of 0.9%

NaCl solution followed by centrifugation for 10 min at 5000 × *g* and decantation of the supernatant and buffy coat. Water (750 ml) and toluene (5 ml) were added to 250-ml portions of the packed cells in a 5-l round-bottom flask. The suspension was rapidly shell-frozen in a dry ice–acetone bath and then thawed in a 65° water bath keeping the temperature of the lysate below 25°. The freeze–thaw procedure was repeated once. The suspension was then adjusted at 4° to pH 6.0 with HCl and the stroma removed by centrifugation for 20 min at 10 000 × *g*. The supernatant fluid (hemolysate) was readjusted to pH 6.0.

Hemoprotein purification

Potassium phosphate buffers, prepared in distilled, deionized water, were used throughout the purification. All operations were carried out between 0 and 5°. The new erythrocyte hemoprotein was identified by its pyridine hemochrome spectrum and the purification was followed by measuring the ratio of the absorbance at 416 mμ to the absorbance at 280 mμ.

Hemoglobin was removed from the hemolysate by passage through a cation exchange column at pH 6.0. The hemolysate was applied at 2.5 ml/min to each of two 8.0 cm × 115 cm Amberlite CG-50 columns that had been equilibrated with 0.05 M buffer (pH 6.0). When the hemoglobin band had spread to 65 cm the columns were eluted with the 0.05 M buffer at a rate 2.5 ml/min. Eluate fractions (25 ml) with an absorbance at 416 mμ of greater than 0.1 were combined. $(NH_4)_2SO_4$ was added slowly to 64% saturation (390 g/l of eluate) and stirring was continued for 30 min. The precipitate was removed by centrifugation for 40 min at 10 000 × *g* and discarded. $(NH_4)_2SO_4$ was then added to 96% saturation (an additional 349 g/l of original eluate) and the precipitate was recovered by centrifugation and dissolved in approx. 300 ml of 0.003 M buffer (pH 7.2). The solution was dialyzed for 24 h against three 5-l vol. of 0.003 M buffer (pH 7.2). Any precipitate which formed was removed by centrifugation and the dialyzed solution was adjusted to pH 7.2 with KOH.

The dialyzed fractions from each of the two Amberlite CG columns were then pooled, and chromatographed on DEAE-cellulose at pH 7.2. The dialyzed solution was applied at 1.2 ml/min to a 4.7 cm × 30 cm DEAE-cellulose column that had been equilibrated with the 0.003 M buffer (pH 7.2). The column was washed at the same rate with approx. 1 l of the 0.003 M buffer until the absorbance of the effluent at 280 mμ was less than 0.1. The column was eluted with a linear gradient formed with 4 l of 0.003 M buffer (pH 7.2) in the mixer and 4 l of 0.2 M buffer (pH 7.2) in the reservoir. The hemoprotein was eluted on the trailing edge of a colorless protein peak and fractions with an absorbance ratio of greater than 0.50 were combined. The combined fractions were concentrated to 5.0 ml by pressure dialysis in a collodion membrane using a water aspirator. Any precipitate present was removed by centrifugation.

Finally, the new hemoprotein was purified by gel filtration. The fraction obtained by chromatography on DEAE-cellulose was applied at 6 ml/h to a 3.4 cm × 95 cm Bio-Gel P-60 column that had been equilibrated with 0.05 M buffer (pH 7.2). The column was eluted at the same rate with the 0.05 M buffer and fractions (2 ml) with an absorbance ratio of greater than 1.3 were combined. The combined fractions were concentrated to 4.0 ml by pressure dialysis and applied at 6 ml/h to a 2.6 cm × 100 cm Bio-Gel P-30 column that had been equilibrated with 0.05 M buffer (pH 7.2). The column was eluted at the same rate with the 0.05 M buffer and 2.0-ml fractions were

TABLE I

PURIFICATION OF THE NEW ERYTHROCYTE HEMOPROTEIN

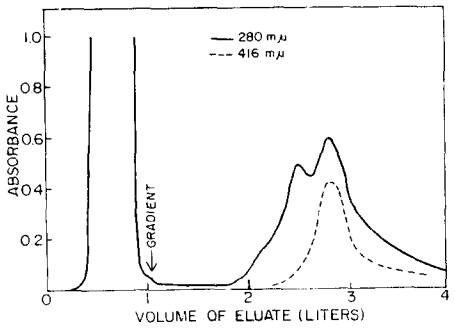
Fraction	$A_{280\ m\mu}$	$A_{416\ m\mu}$	$\frac{A_{416\ m\mu}}{A_{280\ m\mu}}$	*Fraction volume (ml)*	$A_{416\ m\mu} \times ml$
Hemolysate	131.0	441.0	3.37	8890	$3.92 \cdot 10^6$
Amberlite CG-50 eluate	2.49	0.198	0.076	8860	$1.67 \cdot 10^3$
$(NH_4)_2SO_4$ precipitate (64–96%)	30.5	1.68	0.055	320	537
DEAE-cellulose eluate	0.455	0.363	0.797	315	143
Bio-Gel P-60 eluate	1.20	1.75	1.46	31	54.4
Bio-Gel P-30 eluate	6.64	11.04	1.65	1.88	20.8

collected. Fractions with an absorbance ratio of greater than 1.62 were pooled and concentrated to 1.88 ml by pressure dialysis.

RESULTS

The elution profiles of the new erythrocyte hemoprotein on columns of DEAE-cellulose and Bio-Gel P-60 are depicted in Figs. 1 and 2. Table I summarizes the data for each step in the purification of the protein from 2300 ml of packed, stored cells. The most pure fraction of hemoprotein eluted from the Bio-Gel P-30 column showed 20.8 $A_{416\ m\mu} \times$ ml. A somewhat larger yield was obtained when the procedure was carried out on freshly drawn human red blood cells. The fraction obtained by precipitation at 64% saturation of $(NH_4)_2SO_4$ contained approx. 50% as much of the new hemoprotein as detected in the fraction precipitating between 64 and 96% saturation but this fraction was purified separately and is not included in Table I.

The most highly purified sample ($A_{416\ m\mu}/A_{280\ m\mu} = 1.65$) sedimented as a single symmetrical peak in the ultracentrifuge. However, impurities were detected by the technique of disc gel electrophoresis run at pH 7.5 and 9.5. Unsuccessful attempts were made to utilize these electrophoresis systems for further purification of the



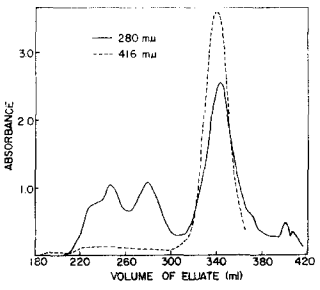


Fig. 1. Purification of a new erythrocyte hemoprotein by chromatography on DEAE-cellulose. Fractions obtained by $(NH_4)_2SO_4$ precipitation (64–96%) of the combined colored fractions from both Amberlite CG columns were subjected to chromatography at pH 7.2 as described in EXPERIMENTAL PROCEDURE.

Fig. 2. Purification of the new erythrocyte hemoprotein by chromatography on Bio-Gel P-60. The fraction eluted from the DEAE-cellulose column was passed through the Bio-Gel column as described in EXPERIMENTAL PROCEDURE.

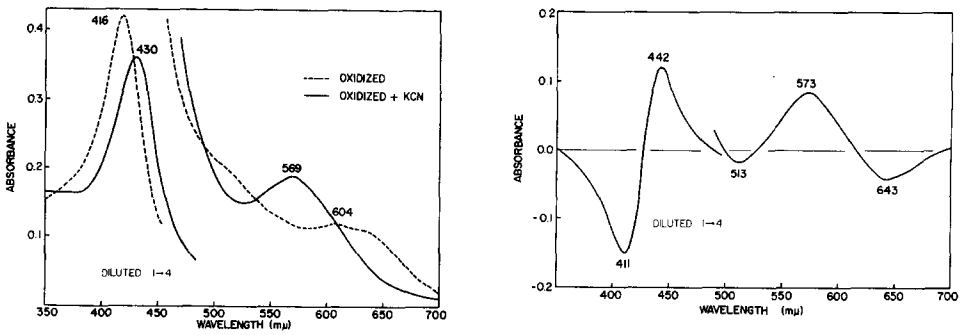


Fig. 3. Spectra of the oxidized form of the new erythrocyte hemoprotein and its CN^- complex. CN^- complex prepared by adding KCN to 0.03 M.

Fig. 4. Difference spectrum of the CN^- complex of the oxidized form *minus* the oxidized form. CN^- complex was prepared by adding KCN to 0.03 M.

protein. The colored band was cut out and placed in buffer maintained at pH 7.2. The recovered protein had a lower $A_{416\ m\mu}/A_{280\ m\mu}$ ratio than the fraction applied. Recrystallization of the acrylamide and bisacrylamide monomers or application of the protein in a sucrose solution did not alter the results.

The apparent molecular weight of the hemoprotein from its behavior on Bio-Gel P-60 was 21 000.

The absolute and difference spectra in the visible region are shown for the oxidized form and its CN^- complex in Figs. 3 and 4 and for the reduced form and its CO complex in Figs. 5 and 6. The reduced *minus* oxidized difference spectrum is shown in Fig. 7 and the pyridine hemochrome in Fig. 8.

DISCUSSION

As can be readily seen from Table I, the absorbance at 416 mμ serves as a measure of the amount of the new erythrocyte hemoprotein and the $A_{416\ m\mu}/A_{280\ m\mu}$ ratio as an index of purity only when all contaminating chromoproteins have been removed. In this purification scheme, the amount of the hemoprotein and the degree

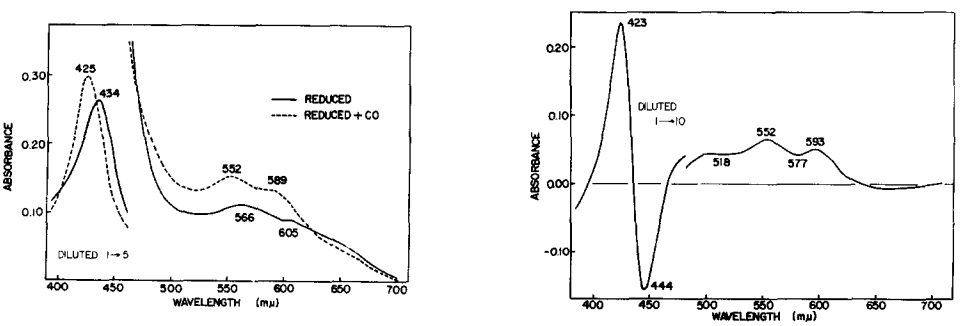
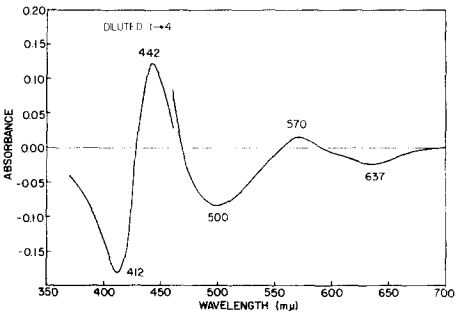


Fig. 5. Spectra of the reduced form of the new erythrocyte hemoprotein and its CO complex. The CO complex was formed anaerobically in the presence of 1 atm of CO.

Fig. 6. Difference spectrum of the CO complex of the reduced form *minus* the reduced form.



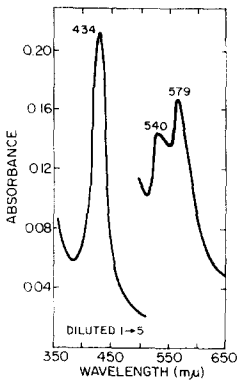


Fig. 7. Difference spectrum of reduced *minus* oxidized forms.

Fig. 8. Spectrum of the pyridine hemochrome of the new erythrocyte hemoprotein. A few crystals of sodium dithionite were added to a solution of the protein in 0.05 M NaOH-pyridine (5:1, v/v).

of purification can be approximated only after chromatography of the $(NH_4)_2SO_4$ precipitate on DEAE-cellulose. Larger yields could be obtained if the entire precipitate from the $(NH_4)_2SO_4$ fractionation (0–96% saturation) was carried through the purification, but a sample of higher purity was obtained by using the 64–96% saturation fraction.

The spectral properties of this erythrocyte protein are those of a low field heme complex with a capacity to undergo ligand exchange in both the oxidized and reduced forms. A Hill plot of the change of absorbance at 416 mμ, which results from increases in the concentration of CN^-, shows that the protein binds approx. 1 mole of CN^- with an apparent dissociation constant of 0.9 mM.

The isolation of somewhat larger amounts of the hemoprotein from fresh red cells than from stored cells demonstrates that this protein is not an artifact of storage. The approximate molecular weight of 21 000 establishes that this protein is a unique protein of the red blood cell and not simply a denatured or subunit form of hemoglobin, catalase, or S-protein. This is confirmed by the unique spectral and chromatographic properties of the protein.

Although the prosthetic group of hemoglobin has been established as protoheme 9, a number of reports have indicated that the heme extracted from red cell hemolyzates may not be homogeneous[10–16]. The isolation of this new hemoprotein with its unusual prosthetic group indicates that the observed heterogeneity of erythrocyte hemes was not completely attributable to artifacts of heme isolation. The position of the α peak of the pyridine hemochrome at 579 mμ demonstrates that the prosthetic group of this molecule differs not only from the prosthetic groups of all other erythrocyte proteins, but from all other hemes which have been found in nature.

Although a definite role for this protein has yet to be established, it probably is important in biological oxidation systems of the red cell. This unique hemoprotein has no peroxidase or catalase activity, but may be involved in an erythrocyte redox system[5].

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REFERENCES

1 M. A. HENNESSEY, A. M. WALTERSDORPH, F. M. HUENNEKENS AND B. W. GABRIO, *J. Clin. Invest.*, 41 (1962) 1257.
2 A. HAUT, G. R. TUDHOPE, G. E. CARTWRIGHT AND M. M. WINTROBE, *J. Clin. Invest.*, 41 (1962) 579.
3 M. J. STANSELL AND H. F. DEUTSCH, *J. Biol. Chem.*, 240 (1965) 4299.
4 W. J. PETERSON AND C. W. CARR, *J. Cell Biol.*, 23 (1964) 72 A.
5 M. MORRISON, *Nature*, 189 (1961) 765.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 L. F. FIESER, *J. Am. Chem. Soc.*, 46 (1924) 2639.
8 B. J. DAVIS, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404.
9 D. E. WILLIAMS AND R. A. REISFELD, *Ann. N. Y. Acad. Sci.*, 121 (1964) 373.
10 M. MORRISON AND J. L. COOK, *Federation Proc.*, 14 (1955) 258.
11 J. L. CONNELLY, M. MORRISON AND E. STOTZ, *J. Biol. Chem.*, 233 (1958) 743.
12 C. MIYAUCHI, *Proc. Japan Acad.*, 44 (1968) 743.
13 C. MIYAUCHI, *Proc. Japan Acad.*, 45 (1969) 69.
14 C. MIYAUCHI, *Proc. Japan Acad.*, 45 (1969) 72.
15 G. R. LEE, A. HAUT, T. H. CAINE, G. E. CARTWRIGHT AND M. M. WINTROBE, *Blood*, 23 (1964) 455.
16 L. D. TOALSON AND F. V. LUCAS, *Blood*, 20 (1962) 315.