Biochimica et Biophysica Acta, 522 (1978) 74—83

BBA 68319

HUMAN ERYTHROCYTE TRANSGLUTAMINASE

PURIFICATION AND PROPERTIES

STEPHEN C. BRENNER * and FINN WOLD

Department of Biochemistry, University of Minnesota, St. Paul, Minn. 55108 (U.S.A.)

(Received: May 16th, 1977)

Summary

Transglutaminase has been isolated from human erythrocytes, and some of its molecular and catalytic properties have been determined. An enzyme preparation of about 15% purity is readily obtained in about 25% yield after DEAE-cellulose fractionation and gel filtration. In order to achieve this yield of enzyme it is essential to add to the buffers a dialyzable stabilizing factor which is present in the early enzyme fractions. This natural factor can be partly replaced by chelating compounds and totally replaced by ATP, and in practice, the purification of the enzyme is best carried out with ATP present in the buffers. The role of ATP in stabilizing the enzyme is unknown. Complete purification of the erythrocyte transglutaminase can be accomplished by preparative acrylamide gel electrophoresis. The pure enzyme has a molecular weight of 82 000 ± 5000, as established by gel filtration and SDS gel electrophoresis, and its catalytic properties are essentially identical to those of guinea pig liver transglutaminase. The guinea pig liver and the erythrocyte enzymes have also been compared as catalysts for protein modification reactions, and have been found to have quite similar specificity requirements for protein substrates. Both enzymes catalyzed significant incorporation of amines into 4 of 20 soluble proteins tested and into proteins 1, 2 and 3 of the red cell membrane. The partially purified erythrocyte enzyme has been found to be completely satisfactory for protein modification experiments, and the ready availability of outdated human blood and the simple purification procedure should make this enzyme a convenient protein-modifying or crosslinking reagent.

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* Present address: Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235, U.S.A.

Abbreviations: Cbz, benzyloxycarbonyl; CPD, citrate/phosphate/dextrose.

Introduction

Transglutaminase catalyzes the substitution of the γ-amide nitrogen of specific peptide bound glutamine residues with a variety of primary amines, including lysine ε-amino groups of the same or adjacent polypeptide chains. In the absence of amine donors, the enzyme will also catalyze the hydrolysis of the amide bond. (For a review, see ref. 1). The enzyme has been isolated from a number of sources, and at present it is useful to consider three distincs types of transglutaminases, the tissue type (of which the guinea pig liver [2] and more recently the human epidermal [3] enzymes are examples), the platelet type (platelet and plasma clotting factor XIII, also referred to as fibrin stabilizing factor [4]) and the hair follicle type [5]. The latter two types of enzymes appear to have well defined biological functions in catalyzing the formation of N_ϵ-(γ-glutamyl)-lysine crosslinks in fibrin and keratin, respectively; while the function of the tissue type enzymes is yet unclear.

Dvilansky [6] and Chung and Folk [7] have reported that red cells contain significant amounts of transglutaminase, and preliminary studies of the partially purified enzyme from guinea pig erythrocytes and from human erythrocytes have been reported [7]. Lorand et al. [8] have recently observed that the activity of the erythrocyte enzyme is not expressed either in the cell or in lysates unless the Ca^{2+} ion concentration is increased to 0.5 mM. Under these conditions of elevated Ca^{2+} concentration extensive membrane protein crosslinking or incorporation of suitable transglutaminase amine substrates into membrane proteins was observed. Since the transglutaminases are Ca-activated enzymes, the observation is perhaps not very surprising, but it does raise questions about the biological role of the tissue type transglutaminases which warrant further studies.

In view of this interest in exploring the in vivo functions of the erythrocyte transglutaminase, and also because of the increasing interest in using transglutaminases as reagents for protein crosslinking and protein modification under mild conditions, we have endeavored to purify the human erythrocyte transglutaminase and to establish some of the properties of the enzyme.

Materials and Methods

Materials

Cbz-glutaminylglycine and guinea pig liver transglutaminase used for preliminary experiments were gifts from Dr. J.E. Folk. Cbz-glutaminylglycine was subsequently purchased from Cyclo Chemical Co. and guinea pig liver transglutaminase was purified according to the method of Connellan et al. [9]. Dansylcadaverine was purchased from Sigma, and [^{14}C]methylamine from ICN Pharmaceuticals Inc. β-Casein was prepared essentially according to the urea fractionation method of Hipp et al. [10], as outlined by McKenzie [11].

Assays

Routine assays. The routine determination of transglutaminase activity was based on the fluorescent assay of Lorand et al. [12] and Cooke and Holbrook [13] using β-casein, and dansylcadaverine as substrates. The fluorescence was

measured in a Hitachi-Perkin Elmer MPF-2A Fluorescence Spectrophotometer. The arbitrary unit of activity in the fluorescent assay was standardized against quantitative incorporation of methylamine into Cbz-glutaminylglycine using the paper strip assay technique [14]. For kinetic experiments the relative initial velocities of fluorescence increase were used to determine apparent K_m values from a direct linear plot [15]. Protein determinations were carried out by the standard biuret/phenol method.

Determination of amine-incorporation into protein substrates. Aldolase and lactate dehydrogenase, which were obtained as ammonium sulfate suspensions were dialyzed overnight against two changes of buffer, the other proteins were simply dissolved in buffer. The final 0.4 ml reaction mixtures contained 10 mg/ml protein in 0.1 M imidazole chloride pH 7.0, 10 mM calcium chloride, 10 mM ^{14}C-methylamine (0.65 mCi/mmol) and 55 units (see above) of either guinea pig liver or human erythrocyte transglutaminase (purified through the gel filtration step). After incubation at room temperature for 5 h, the reactions were stopped by addition of 10 vols. of cold 10% trichloroacetic acid. The precipitates were centrifuged and washed by redissolving in 0.5 ml of cold 0.2 M sodium hydroxide and reprecipitating as above. After three washing cycles, the precipitate was dissolved in 0.5 ml of 0.2 M sodium hydroxide, transferred to a hydrolysis vial and 0.5 ml of 12 M hydrochloric acid was added. After hydrolysis at 110° C for 18 h, the samples were dried and dissolved in 1 ml distilled water. Aliquots were finally taken for scintillation counting and quantitative amino acid determination with trinitrobenzenesulfonic acid [16]. Corrections were made throughout for background radioactivity using control samples of each protein which had been treated identically except for the absence of transglutaminase.

Labeling of erythrocyte membrane proteins [17]

Intact red cells were prepared from 10 ml of freshly drawn human blood and twice washed with Tris-buffered saline (TBS). The red cells were divided into two portions and incubated with 0.1 mM dansyl cadaverine, 0.15 M sodium chloride, 5 mM Tris chloride pH 7.5, 5 mM calcium chloride, one portion without and one with 20 units of transglutaminase. After incubation for one hour at room temperature, membranes were prepared from the cells after washing twice with isotonic Tris and lysine [18]. A second 10-ml sample of freshly drawn blood was used directly for preparation of membranes [18]. These membranes were twice frozen at −76° C and thawed. They were then suspended in 0.15 M sodium chloride, 5 mM Tris chloride pH 7.7, 0.1 mM dansyl cadaverine and 20 units of transglutaminase, one half of the sample with 5 mM calcium chloride and the other half with 5 mM EDTA. After one hour incubation at room temperature, the membranes were washed twice with 0.011 M Tris chloride pH 7.6. The membranes from all four portions were solubilized with 1 ml of 1% SDS and analyzed by SDS gel electrophoresis in the system of Fairbanks et al. [19].

Results

Purification procedures

General remarks. The starting material was human blood which had been

stored under several different conditions: fresh whole blood collected in CPD anticoagulant; recently outdated CPD whole bank blood or packed red cells; deglycerolized red cells. All gave equal qualitative and quantitative yields of enzyme unless the cells had begun to deteriorate as judged by hemolysis during washing. White cells and plasma contain no detectable transglutaminase activity (Table I). (Plasma contains coagulation Factor XIII which, upon conversion to XIIIa by plasmin, possesses transglutaminase activity [4].) Initial separation of hemoglobin is essential since transglutaminase connot readily be assayed in the presence of large amounts of hemoglobin. This initial separation was accomplished by adsorption of the red cell hemolysate to DEAE-cellulose, washing out the majority of the hemoglobin and subsequent elution of the transglutaminse (S.I. Chung, personal communication). Subsequent attempts to purify the enzyme by ion exchange chromatography on several adsorbents or by gel filtration in various buffers all gave very low recovery of activity. Extensive dialysis also resulted in loss of activity, even in the presence of calcium (which is required for activity) or reducing agents such as dithiothreitol. It was found, however, that if the enzyme was dialyzed against only a small volume of buffer, full activity was retained. Furthermore, if the dialysate from this step was subsequently included in the buffers, further purification could be achieved with good recovery of activity from a column of DEAE-cellulose or upon gel filtration. Several attempts to fractionate the dialysate and directly identify the factor responsible for this protection of activity were unsuccessful. Since the spectrum of the dialysate closely corresponded to that of adenine-osine derivatives and red cells are known to contain about 1 mM ATP, ATP and several other compounds were tested for their ability to protect the transglutaminase activity during gel filtration. Complete protection was achieved only with ATP; partial protection was observed with EDTA, tripolyphosphate and pyrophosphate, but other chelating compounds, nucleosides and nucleoside triphosphates had little or no effect. These observations provided the basis for including 0.1 mM ATP and 1 mM EDTA in the buffers used in the purification. Except as noted all steps were carried out at 4°C.

Step 1: Hemolysis. The cells from 900 ml of whole blood were suspended to a total volume of 1.5 l with a buffered saline solution containing 0.15 M NaCl, 5 mM Tris chloride, pH 7.5 and 1 mM EDTA. The cell suspension was cen-

TABLE I

DISTRIBUTION OF TRANSGLUTAMINASE IN BLOOD PLASMA AND CELLS

450 ml of blood were subjected to special cell fractionation techniques [20], and the composition of the resulting cell fractions were determined in a Coulter Counter. Each fraction was then lysed, adsorbed to DEAE-cellulose and eluted as described below (steps 1 and 2 in the purification).

Blood fraction	Number of cells in starting material		Transglutaminase activity (arbitrary units/ml)
	Red	White	
Washed erythrocytes	$46 \cdot 10^9$	$28.5 \cdot 10^6$	70
Dextran-sedimented and washed erythrocytes	$46 \cdot 10^9$	$3.3 \cdot 10^6$	70
Leucocytes	$<3 \cdot 10^9$	$2.9 \cdot 10^8$	non detectable
Plasma	$<10^7$	$3 \cdot 10^6$	non detectable

trifuged at 2500 × *g* for 5 min. The supernatant solution and the buffy coat were aspirated, sacrificing some red cells, and the wash with the buffered saline solution was repeated. The twice washed cells were lysed by suspending them in 540 ml of distilled water for one minute, whereupon 60 ml of 10 times concentrated buffered saline solution was added. Whether or not ghosts and resealed vesicles were removed at this point did not affect the preparation.

Step 2: Separation from hemoglobin by DEAE-cellulose adsorption. The hemolysate was stirred for 30 min with 30 g of damp DEAE-cellulose which had been pre-equilibrated with 50 mM Tris/chloride pH 7.5. The suspension was filtered through a sintered glass funnel and washed on the funnel with 200 ml of the buffered saline solution. The filtrate and wash contained the bulk of the hemoglobin, while the transglutaminase activity remained bound to the DEAE-cellulose. The activity was obtained by eluting the DEAE-cellulose with 100 ml of a solution containing 0.5 M NaCl, 5 mM Tris chloride, pH 7.5 and 1 mM EDTA.

Step 3: Chromatography on DEAE-cellulose. The batch eluted material from Step 2 was diluted at room temperature with 10 mM imidazole chloride pH 6.5 to a conductivity of 7.5 $m\Omega^{-1}$ (corresponding to about 0.1 M salt). This solution was fractionated on a 2.4 × 20 cm column of DEAE-cellulose at room temperature. The column had been equilibrated with 50 mM Tris chloride pH 7.5, 0.1 M sodium chloride, and the elution was carried out at room temperature with a 640 ml linear gradient of 0.1 to 0.5 M sodium chloride in a buffer containing 10 mM imidazole chloride pH 6.5, 1 mM EDTA and 0.1 mM ATP. The center tubes from the activity peak were pooled (approximately 50 ml) and concentrated by ultrafiltration to a volume of 4 ml or less.

Step 4: Gel filtration on Bio-Gel A 0.5 M. The concentrated, yellow solution from Step 3 was applied to a 1.8 × 130 cm column of Bio-Gel A 0.5 M, previously equilibrated with a buffer containing 10 mM imidazole chloride pH 6.5, 50 mM sodium chloride, 1 mM EDTA and 0.1 mM ATP. This column was eluted with the same buffer (minus ATP) at 30 ml per hour. The fractions containing the peak of activity (about 70% of the activity applied) were pooled and concentrated by ultrafiltration. This material served as the enzyme preparation for most subsequent studies. Transglutaminase constitutes approximately 15% of the protein at this point and is known to be contaminated with a labile phosphatase activity. The transglutaminase activity is stable in this form for over one month at 4°C (with 0.1 mg/ml sodium azide added). Most of the activity is lost when the preparation is lyophilized or frozen at −20°C.

Step 5: Preparative polyacrylamide gel electrophoresis. Final purification of transglutaminase was accomplished by two sequential applications of preparative polyacrylamide gel electrophoresis. Using an appratus similar in design to that of Jovin et al. [21], a separating gel was cast to a height of 3.5 cm with a total acrylamide concentration of 6.0% or 8.5% and a ratio of acrylamide to methylene bis-acrylamide of 38 : 1. The gel buffer was identical with the upper and lower buffers: 0.089 M Tris, 0.089 M boric acid, 2.5 mM disodium EDTA, pH 8.4. The gel was subjected to pre-electrophoresis until a tracking dye (Bromphenol Blue) had run completely through the gel. Following this, ascorbic acid at 1 mg per ml of gel, neutralized with sodium hydroxide, was layered on with tracking dye and electrophoresis continued until the tracking

dye had again moved through the gel. After these pre-electrophoresis treatments, the pooled, concentrated enzyme from the Bio-Gel A 0.5 M column was applied and electrophoresis was carried out at 70 V until the current stabilized and then at 100 V until the enzyme was eluted. The enzyme was first purified on a 6% gel. The active fractions from this run were pooled, concentrated and exchanged by ultrafiltration into 50 mM Tris chloride pH 7.5, 1 mM EDTA. This was then applied to an 8.5% gel (Fig. 1). The most active fractions were again pooled, concentrated and exchanged into buffer as above. The enzyme at this point was found to be unstable and we have concluded that the low specific activity observed after the second gel filtration step reflects this instability. When this product was stored at 4°C, a second, faster migrating band appeared upon electrophoresis in a discontinuous system [22], although no changes were seen upon electrophoresis in SDS. Fig. 1 demonstrates the final purification achieved, and a summary of a typical preparation is given in Table II.

Properties of human erythrocyte transglutaminase

Molecular weight. The molecular size of red cell transglutaminase was determined by gel filtration. The Bio-Gel A 0.5 M column which was routinely used during the purification was calibrated with known proteins, and the same column was also used during purification of guinea pig liver transglutaminase. Both the transglutaminases eluted at exactly the same volume, and in relation to the elution position of the reference proteins, their apparent molecular weight is 85 000. The apparent molecular weight of the red cell transglutaminase was also determined by SDS gel electrophoresis. The reference proteins covered the molecular weight range from 16 000 (hemoglobin monomer) to 150 000 (yeast alcohol dehydrogenase tetramer, crosslinked with dimethyl suberimidate [23]) and the red cell transglutaminase was found to have a molecular weight of 82 000 ± 5000. Chung [24] has reported 80 000 ± 4000 daltons for the guinea pig transglutaminases from liver and red cells.

Catalytic properties. Some kinetic properties of the human red cell trans-

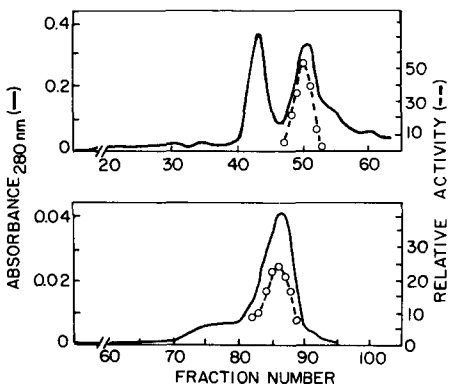

Fig. 1. Final purification of erythrocyte transglutaminase by preparative acrylamide gel electrophoresis. The active fraction obtained from the gel filtration step was subjected to electrophoresis on a 6% gel (A); the resulting peak of activity was pooled, concentrated and subjected to electrophoresis on an 8.5% gel (B). The experimental details are given under Results.

TABLE II

PURIFICATION OF HUMAN ERYTHROCYTE TRANSGLUTAMINASE.

Typical purification from the red cells from 900 ml of whole blood. The activity is given in arbitrary units of fluorescence incorporation. Because of the difficulties in obtaining good activity measurements in the original hemolysate, the eluate after the DEAE-cellulose adsorption in step 2 is considered as the starting material in the calculation of yields.

Purification step	Protein (mg)	Specific activity (units/mg)	Yield (%)
1. Hemolysate	55 000	—	—
2. DEAE batch elution	570	29	100
3. DEAE chromatography	106	111	71
4. Gel filtration	32	123	24
5. Preparative gel electrophoresis (6% gel)	2.3	860	12
6. Preparative gel electrophoresis (8.5% gel)	1.3	610	5

glutaminase and the guinea pig liver enzyme were compared. Their pH vs. activity profiles are essentially identical with an optimum near pH 7.0 The apparent K_m for various substrates was also determined for both enzymes, and these results are summarized in Table III.

Human erythrocyte transglutaminase was also tested for susceptibility to sulfhydryl reagents, since the guinea pig liver enzyme is known to be extremely sensitive to such reagents [25]. Iodoacetamide and *p*-hydroxymercuribenzoate at 1 mM concentration each completely inactivated the enzyme as rapidly as their effect could be tested. In contrast to guinea pig liver transglutaminase which requires the presence of Ca^{2+} for inactivation [25], the erythrocyte enzyme was completely inactivated both in the presence and in the absence of calcium. Acrylamide was also tested as a sulfhydryl reagent. With 1 mM acrylamide in the presence of calcium, the enzyme had a half-life of 100 min. In the absence of calcium, no detectable inactivation occurred during the same time period.

Application of transglutaminase as a protein reagent. The substrate specificity of erythrocyte and guinea pig liver transglutaminase was also compared by measuring the incorporation of radioactive methylamine into a number of native proteins under identical conditions. The results are given in Table IV, and differ from previous results [27] in showing a greater degree of discrimination in the utilization of different protein substrates. This difference is not

TABLE III

APPARENT Km DETERMINATIONS FOR TRANSGLUTAMINASE

Assay method	Substrate	K_m (apparent)	
		Erythrocyte enzyme	Liver enzyme
Paper strip	methylamine	6 mM	5 mM
Fluorescent	dansyl-cadaverine	23 μM	13 μM
	α-casein	82 μg/ml	31 μg/ml
	β-casein	32 μg/ml	16 μg/ml

TABLE IV

NATIVE PROTEINS AS TRANSGLUTAMINASE SUBSTRATES

Protein	mol methylamine/mole protein incorporated		
	Erythrocyte enzyme	Liver enzyme	Liver** enzyme
Albumin, bovine serum	0.16	0.28	—
Aldolase	0.82	1.14	
β-casein*	2.72	4.15	++++
Chymotrypsinogen	0.02	0.26	
oxidized	0.12	0.14	
β-glucosidase*	1.08	2.48	
β-glucuronidase*	0.42	0.77	
Hemoglobin, horse	0.14	0.32	
Hemoglobin, human	0.14	0.23	
Hyaluronidase*	0.26	0.42	
Insulin	0.01	0.02	3.0
oxidized	0.02	0.16	3.2
Lactic dehydrogenase	0.58	1.24	
Lysozyme	0.02	0.02	0.2
oxidized	0.05	0.07	3.0
Ovalbumin	0.13	0.20	—
Pepsin	1.13	2.02	++++
oxidized	0.38	0.68	
Ribonuclease A	0.06	0.15	1.1
Transferrin	0.09	0.12	

* Values for proteins based on moles of amine incorporated per 500 amino acid residues.

** Some literature values are included for comparison. Values reported as + or — are from Clarke et al. [26]. Numerical values are from Toda and Folk [27].

surprising. Toda and Folk [27] used 5.0 mM glutathione in their incubation mixture and incubated the samples (with glycine ester instead of with methylamine) for up to 24 h, while our incubation was carried out for only 5 h in the absence of any sulfhydryl reagents. Thus, the difference in the extent of amine incorporation is some of the substrates must be due to a more pronounced difference in rate when the enzyme is used in the absence of glutathione, and the shorter incubation time accentuates these rate differences to give a picture of what appears to be an all-or-none substrate utilization.

Human erythrocyte transglutaminase was also tested for its ability to label erythrocyte membrane proteins with dansyl cadaverine. When the membrane proteins, solubilized in SDS was subjected to gel electrophoresis (see Methods), it was found that only the samples containing the transglutaminase and calcium contained fluorescent label. The labeling pattern was consistent with that observed by Lorand et al. [28] in similar experiments using the guinea pig liver enzyme. Proteins 1, 2 and 3 contained fluorescent label, and in addition a band at the top of the gel, presumably a crosslinked protein band, also showed intense fluorescence.

Discussion

In most respects, human erythrocyte transglutaminase appears very similar to the guinea pig liver enzyme which has been described extensively by Folk

and co-workers [4,24]. The molecular weights of the two enzymes appear identical, and the very similar $K_{m_{app}}$ values for four substrates, the nearly identical specificity for various proteins as substrates, and the similar inactivation by sulfhydryl reagents all argue for close structural and mechanistic similarities between the two enzymes. A unique property of the human erythrocyte enzyme is the apparent requirement for ATP as a stabilizing agent during the purification of this enzyme. The basis for this requirement is still obscure. The possibility that the enzyme might be subject to phosphorylation-dephosphorylation regulation was tested by incubation of active enzyme with human erythrocyte acid and alkaline phosphatases (crude fractions obtained during the purification) and with *Escherichia coli* alkaline phosphatase. These treatments had no effect on the activity of the transglutaminase, nor did incubation of inactive enzyme with ATP and various soluble fractions obtained during the purification. The fact that the requirement for ATP becomes less pronounced as the enzyme is purified also suggests that there is no direct effect by ATP on the enzyme. Since chelating agents such as EDTA can partially substitute for ATP in stabilizing the enzyme, the most attractive explanation of the phenomenon appears to be that some enzyme, for which ATP is a negative effector and some metal ion is a positive effector, can modify and inactivate human erythrocyte transglutaminase, and further, that this enzyme is normally present in erythrocytes and is removed during the purification.

The instability of human erythrocyte transglutaminase during and after preparative gel electrophoresis is probably a reflection of different phenomena. During electrophoresis, the enzyme may well be inactivated through modification of an essential sulfhydryl by residual acrylamide monomer in the gel. Upon storage of the purified enzyme, it may catalyze its own deamidation by hydrolyzing a susceptible glutamine residue of another enzyme molecule. It is clear from our experiments that in the absence of added protein, transglutaminase will act as its own substrate (e.g., fluorescent label is incorporated into the tranglutaminase band on polyacrylamide gels which are stained for activity). The slow formation of a more rapidly migrating band in the purified enzyme is consistent with the acquisition of a more negative charge such as would occur upon hydrolysis of glutamine to a glutamic acid. Clotting factor XIIIa and guinea pig tranglutaminase are both known to act as their own substrates [17,29].

The potential utility of transglutaminase as a protein reagent has been discussed by several workers [17,27,28] but, to date, its application has been quite limited. A study of the mechanism of calcium transport in sarcoplasmic reticulum membranes [30], has been the only sample to date of an application of transglutaminase to a problem which is more than a model system chosen to demonstrate its potential as a protein reagent. Perhaps the availability of the enzyme has been a limiting factor in fully exploring transglutaminase as a protein-modifying reagent. The main source of the enzyme to date has been the guinea pig liver, and although the preparation of the enzyme is quite short, simple and reproducible, the starting material may not always be readily available. Since outdated human blood can be obtained from most blood banks and hospitals, the human erythrocyte enzyme may represent an attractive alternative to the liver enzyme in future studies.

Acknowledgements

The authors wish to thank Drs. J.E. Folk and S.I. Chung for their advice and generous help throughout the course of this work. We also wish to thank Ms. Terry Brown for her technical assistance and Dr. Michael Nesheim for loan of and guidance in the use of his preparative gel electrophoresis apparatus. We also wish to thank the staff of the Minneapolis Veterans Administration Hospital Blood Bank who provided the blood used in this study. This work supported in part by a U.S. Public Health Service Research Grant (GM 15053) and training Grant (GM 00157) from the National Institutes of Health. S.C.B. is a USPH Predoctoral Trainee.

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