An Ascorbate-Mediated Transmembrane-Reducing System of the Human Erythrocyte

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ABSTRACT Actively metabolizing human erythrocytes catalyze the extracellular reduction of ferricyanide to ferrocyanide. Because neither of these anions can enter the cell, reducing equivalents generated in the course of glycolysis must in some manner be transferred across the cell membrane, thereby resulting in ferricyanide reduction. Work described in this paper suggests that the transmembrane reduction is effected by ascorbic acid. This compound in its oxidized form (dehydroascorbate) rapidly enters the cell. Here it obtains reducing equivalents which appear to come from NADH made available at the level of glyceraldehyde 3-phosphate dehydrogenase. Once reduced, it leaves the cell as ascorbic acid and accomplishes the nonenzymatic reduction of ferricyanide.

INTRODUCTION

Ferricyanide $[Fe(CN)_6]^{-3}$, an anionic species carrying three negative charges, can be reduced to ferrocyanide $[Fe(CN)_6]^{-4}$ by human erythrocytes (RBC)[1] (1, 2). If the RBC are deprived of metabolic substrate or exposed to an inhibitor such as iodoacetate, the rate of ferrocyanide generation is slowed (2). Because of their size and hydrophilic nature neither ferricyanide nor ferrocyanide can cross the cell membrane (3). It has therefore been presumed that reducing equivalents generated in the course of glycolysis are somehow transferred across the membrane.

We have examined the RBC-mediated ferricyanide reduction system with both intact RBC and resealed ghost preparations. Our results suggest that NADH generated at the glyceraldehyde 3-phosphate dehydrogenase (G3PD) reaction is the primary source of reducing equivalents, that ascorbate carries them across the cell membrane, and that ferricyanide is the final acceptor in the system.

METHODS

Venous blood was drawn into heparin-rinsed syringes from normal volunteers just before each study. Samples were obtained from chronic hemodialysis patients immediately before the dialysis procedure. Because the uptake of ascorbate by leukocytes is 40 times more rapid than by RBC (4), the RBC were washed in cold NaCl 165 mM (305 mosM) with the careful removal by aspiration of plasma, buffy coat, and uppermost RBC layers. The final ratio of RBC:leukocytes in the incubation flasks always exceeded 1,000.

Preincubations were carried out in plasma or in a "preincubation medium" which contained NaCl 140 mM, $NaHCO_3$ 25 mM, and glucose 10 mM. The flasks were gently swirled in a humidified atmosphere of 95% O_2 and 5% CO_2 for 10 min before the preincubation was begun. The pH of the cell suspensions was 7.30–7.35 at 37°C. After the preincubation the RBC were again washed in physiological saline.

All ferrocyanide generation experiments were performed in the "standard medium" which contained NaCl 100 mM, KCl 5 mM, Na_2HPO_4 20 mM, glycylglycine 10 mM, and glucose 10 mM. Dehydroascorbate (DHA) was added to the indicated flasks. The final pH was adjusted to 7.4 at 25°C., after which the RBC were included. The suspension was then warmed to 37°C. for 5 min, after which sufficient $K_3Fe(CN)_6$ was added to give a final concentration of 1 mM. Samples were taken at 0, 10, 20, and 30 min, placed immediately at 0°C in a refrigerated centrifuge, and spun for 5 min at 12,062 *g*. The supernate was carefully removed from the cell button by Pasteur pipette and assayed for ferrocyanide.

All incubations were performed in stoppered flasks at 37°C in a water bath shaker set at 100 oscillations/min over a 1-inch traverse. In both preincubation and ferrocyanide generation studies the cell:medium ratio was 1:10–1:20, and the total volume in each flask was 10 ml. RBC counts were performed using a model S Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

Ferrocyanide quantitation was performed using the phenanthroline complex as described by Avron and Shavit (5). For the assay, 100 μl of sample was diluted with distilled water to a 1.4-ml final volume. By this method a concentration of ferrocyanide as low as 1 μM can be accurately quantitated. Further, no effect of excess ferricyanide was observed on this assay system.

Resealed ghosts were prepared by a slight modification of

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[1] *Abbreviations used in this paper*: AA, ascorbic acid; DHA, Dehydroascorbate; G3PD, glyceraldehyde 3-phosphate dehydrogenase; Pi, inorganic phosphorous; RBC, human erythrocytes.

published methods (6, 7). Hypotonic lysis and reversal were carried out at 0°C in a thermostated beaker with a magnetic stirrer. Both temperature and pH were monitored continuously. Freshly drawn RBC were washed twice with cold unbuffered NaCl (165 mM), resuspended in this same medium at an hematocrit of 50%, and cooled on salted ice. 1 vol of cold RBC suspension was then added to 10 vol of hemolyzing medium which contained (millimolar): $MgSO_4$ 4, acetic acid 3.8, and ATP 0.5. Also added at this time were other metabolites which were to be incorporated into the ghost systems. Their concentrations are detailed in the appropriate figure legends. The pH of the lysate, measured at 0°C was 5.8–6.2. After 5 min at 0°C 1 vol (equal to the volume of RBC suspension originally added to the hemolyzing medium) of ice-cold reconstituting medium (KCl 1990 mM, Tris-OH 25 mM) was added, after which the pH of the hemolysate rose to 7.0–7.3. After a further 10 min at 0°C the entire hemolysate was removed from the 0°C beaker and transferred to a 37°C flask for resealing. After a 45-min incubation at 37°C, the resealed ghosts were removed and washed four times in a wash solution containing NaCl 165 mM and Tris-OH 2 mM, buffered to pH 7.4 at 25°C. The ghosts were now ready for use in the experimental protocols.

ATP was assayed fluorometrically as detailed by Lowry et al. (8). Neutralized perchloric acid extracts for this determination were prepared according to Orringer and Mattern (9). Total ascorbate in the plasma of dialysis and control subjects was measured according to Owen and Iggo (10).

Data presented in Table I and Fig. 6 are expressed as the mean±1 SD. The remainder of the figures are representative experiments, each of which has been repeated on multiple occasions.

RESULTS

The initial rate of ferricyanide reduction by fresh RBC is ≅5 mmol/liter RBC·h. After 30 min of incubation, however, this rate slows appreciably (1, 12). We noted that RBC which had spent 2 h at 37°C in the preincubation medium before exposure to ferricyanide also generated ferrocyanide slowly, while RBC which had undergone a similar 2 h preincubation in plasma reduced ferricyanide as well as fresh RBC (Fig. 1). This experiment suggested that a "plasma factor" might be of some importance in the reduction of ferricyanide.

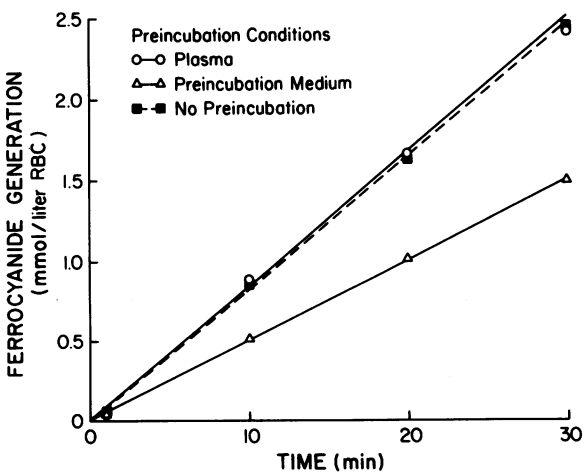

FIGURE 1 Ferrocyanide generation by RBC preincubated in plasma or preincubation medium for 2 h before ferricyanide exposure. The rate of ferrocyanide generation by fresh RBC is shown for comparison.

Ferrocyanide generation by RBC from normal donors was next compared to that demonstrated by RBC from chronic hemodialysis subjects. The cells from this latter group reduced ferricyanide at a significantly higher rate (Table I). To determine whether the accelerated ferrocyanide generation observed resulted from an intrinsic difference in the hemodialysis RBC or an increase in the plasma factor noted in the previous experiment, the following study was performed. RBC from a normal subject were separated from plasma, washed, and divided into two lots. These RBC were then preincubated in ABO-compatible plasma from either a hemodialysis or a normal donor. After a 2-h preincubation, the cells were washed free of plasma and exposed to ferricyanide. The hemodialysis plasma was able to confer the increased capacity for ferrocyanide generation onto the normal RBC (Table II).

The next group of studies were designed to characterize further the plasma factor. A careful review of our dialysis population revealed that each patient was given daily one Tabron tablet (Parke, Davis & Co., Detroit, Mich.), a multivitamin preparation which contains 500 mg of ascorbic acid (AA). Patients on chronic dialysis are supplemented with vitamin C because it is dialyzable, and AA depletion is known to occur (11, 12). When large oral doses (250–500 mg/d) are given, predialysis plasma AA levels have been invariably high (13). To confirm this observation, plasma ascorbate was measured in several of our dialysis and control subjects. In each case the plasma ascorbate level in the dialysis subjects exceeded that in the control population by close to an order of magnitude (dialysis subjects 100–250 μM; control 15–30 μM). Further those dialysis subjects with the highest AA levels also had the highest rate of ferrocyanide generation. This information coupled with the ability of ascorbate to enter the RBC led us to postulate that AA might be the plasma factor. The standard midpoint redox potentials for the couples ferri-/ferrocyanide and dehydroascorbate/ascorbate are respectively 0.36 and 0.058 V. Thus, the

TABLE I
Ferrocyanide Generation by RBC from Chronic Hemodialysis Patients and Normal Donors

	Normal	Hemodialysis
Ferrocyanide generation*	5.5±0.7‡	14.5±4.5‡
	n = 14	n = 12

* Expressed as mmol/liter RBC·h.
‡ Mean±1 SD.

TABLE II

Ferrocyanide Generation by RBC After Preincubation in Normal or Hemodialysis Plasma*

Preincubation plasma	Ferrocyanide generation‡
Normal	4.4
Hemodialysis	12.0

* Fresh RBC which were ABO-compatible with both the normal and the hemodialysis plasma, were obtained from a third, independent donor.

‡ Expressed as mmol/liter RBC·h.

reduction of ferricyanide by ascorbate is thermodynamically favored and has a standard free energy of −8,000 cal/mol (14). When mixed together, each mole of AA instantaneously generated 2 mol of ferrocyanide (Fig. 2). The reduction of ferricyanide was, therefore, accomplished by the oxidation of AA to DHA, a reaction that generated two reducing equivalents. Because the RBC contains a mechanism for DHA reduction (15, 16), a simple experiment was next designed in which DHA was added to the preincubation medium. If DHA at a physiological concentration (17) could prevent the decline in ferrocyanide generation otherwise observed after preincubation, one could make a strong case for the role of ascorbate as the plasma factor. As shown in Fig. 3, cells exposed to preincubation medium containing 50 μM DHA, reduced ferricyanide as well as cells preincubated in plasma.

Ferrocyanide generation was next examined in the presence of DHA. Two sets of flasks were prepared, each containing the standard medium and graded concentrations of DHA. The first group of flasks contained

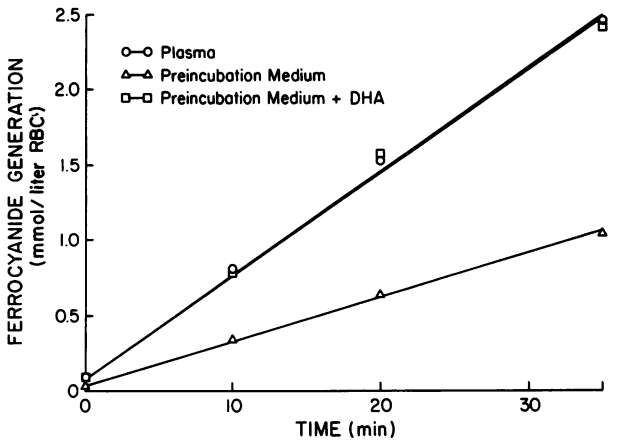

FIGURE 3 Ferricyanide reduction by RBC after a 2-h preincubation in: (*a*) plasma; (*b*) preincubation medium; or (*c*) preincubation medium plus DHA (50 μM).

10^{12} RBC/liter suspension whereas no RBC were present in the second set of flasks. The rate of ferrocyanide generation was then expressed as a function of the DHA concentration (Fig. 4). In the absence of RBC, little ferrocyanide appeared until the concentration of DHA exceeded 0.1 mM, and above this amount the rate of ferricyanide reduction appeared to be a linear function of the DHA concentration in the flask. In the presence of RBC, however, as little as 10 μM DHA exhibited a pronounced effect on the rate of ferricyanide reduction. Unlike the linear relationship observed with DHA alone, the ferricyanide reduction effected by the cells in the presence of DHA appeared to be a saturable function of the concentration of DHA. The additional ferricyanide reduction in this set of flasks at DHA concentrations exceeding 0.1 mM was accounted for almost

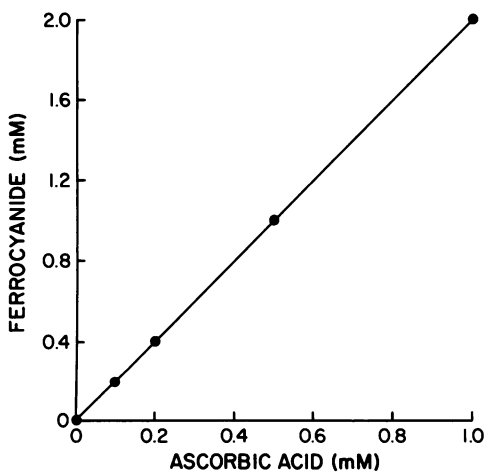

FIGURE 2 The instantaneous conversion of ferri → ferro by the addition of various amounts of AA. Each mole of AA reduced 2 mol of ferricyanide. (See text for details.)

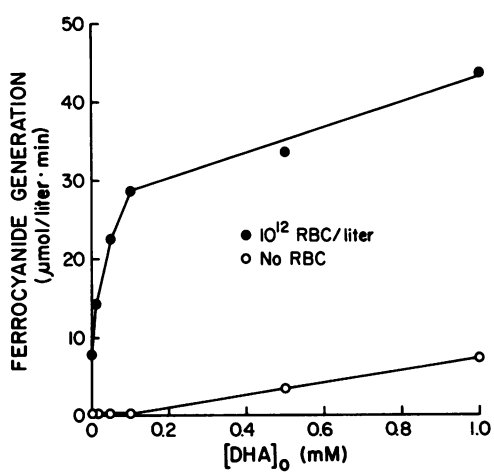

FIGURE 4 Ferrocyanide generation (μmol/liter·min) in the absence or presence of RBC (10^{12}/liter suspension) expressed as a function of the DHA concentration in the flask.

entirely by the direct interaction between DHA and ferricyanide.

The remaining experiments were designed to clarify the mechanism by which cellular DHA reduction (and ultimately ferrocyanide generation) occurred. Several lines of evidence suggested that the availability of NADH might be the critical determinant of DHA reduction. First, a direct relationship between the rate of ferricyanide reduction and the concentration of inorganic phosphorous (Pi) in the flask supported this hypothesis, since the rate of the G3PD reaction can be modulated by the concentration of Pi (18), and the availability of NADH is in turn dependent on the rate of the G3PD reaction. Second, the inclusion of pyruvate, an oxidant of NADH, slowed the rate of ferrocyanide generation. Third, G6PD-deficient subjects (both mild and severe variants) reduce ferricyanide as well as normal cells. In fact fresh RBC from a G6PD-deficient child with congenital nonspherocytic hemolysis exhibited an accelerated rate of ferrocyanide generation (8.5 mmol/liter RBC·h) vs. a simultaneous control subject (4.5 mmol/liter RBC·h).

The resealed ghost technique was used for the last studies. The absence of methemoglobin formation after ferricyanide exposure indicated that the ghosts were all tightly sealed to both hemoglobin and ferricyanide. The data presented in Fig. 5 represent the cumulative results of several experiments. All three ghost systems were prepared simultaneously with RBC obtained from the same donor. As is evident, the presence of an NADH-generating system increased the rate of ferrocyanide generation. Although resealed ghosts are not totally devoid of any component present in intact RBC, the next experiment was designed to demonstrate the importance of adequate amounts of NAD and Pi, two of the metabolites required for the G3PD reaction. In this study ghosts containing the entire NADH system were compared to those having all additives except NAD, and the rate of ferricyanide reduction by these two groups was examined in the presence and absence of Pi. The data indicated the importance of the complete system (Fig. 6). Finally, the dependence on both NADH and DHA in the ghost system is shown on Table III. The rate of ferricyanide reduction observed in the presence of the entire system (NADH plus DHA) is far greater than the sum of the rates seen individually.

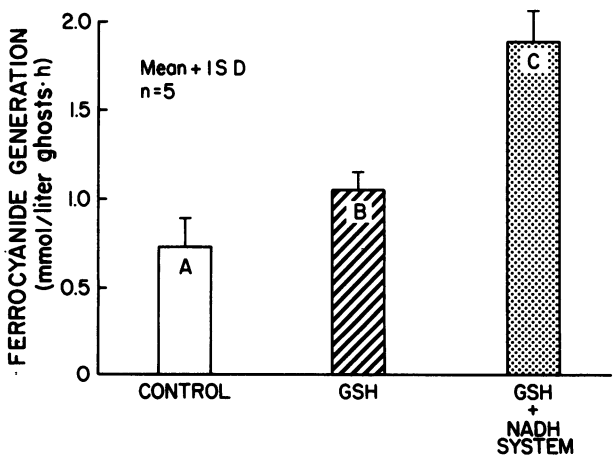

FIGURE 5 Ferrocyanide generation (mmol/liter ghosts·h) by resealed ghost preparations. Metabolic intermediates and enzymes within the ghosts included: (A) ATP 0.5 mM; (B) ATP 0.5 mM, GSH 1.0 mM; (C) ATP 0.5 mM, GSH 1.0 mM, NAD 0.25 mM, fructose diphosphate 0.5 mM, ADP 0.25 mM, nicotinamide 0.5 mM, aldolase 0.03 mg, G3PD 0.3 mg. All flasks contained DHA (50 μM).

DISCUSSION

The cell membrane presents an impenetrable barrier to the movement of ferricyanide (3). Therefore the demonstration that ferricyanide, when added to a RBC suspension, could induce cellular ATP synthesis, represented a most important observation (19). It suggested that an extracellular species could influence the rate of intracellular metabolic events. Confirmation and extension of these studies linking intracellular ATP synthesis with extracellular ferricyanide reduction were then published (2, 20). Arese et al. (21) compared the effect on RBC glycolysis of the impermeant species ferricyanide to that of pyruvate, an oxidant which enters the cell, increases the NAD:NADH ratio, and thereby activates the enzyme G3PD. They observed that although ferricyanide was unable to penetrate the cell membrane, it resulted in a pattern of phosphorylated intermediates identical to that seen with pyruvate. They concluded that ferricyanide was, like pyruvate, able to oxidize NADH and thereby increase the activity of G3PD. These experiments coupled with our data from both intact cells and resealed ghosts strongly support the hypothesis that NADH generated at the G3PD reaction is the source of reducing equivalents for extracellular ferrocyanide generation. If NADPH has a

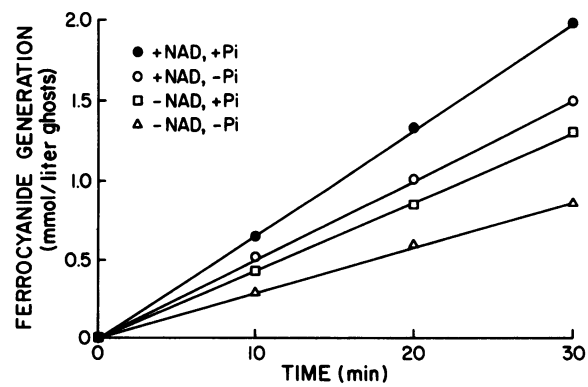

FIGURE 6 The effect of the omission of NAD and(or) Pi on the rate of ferrocyanide generation by resealed ghost preparations containing all other components of the NADH generating system. (DHA concentration 50 μM).

TABLE III

Ferrocyanide Generation by the Control‡ or NADH-Containing§ Ghost Systems in the Presence and Absence of DHA (50 μM)*

Ghost system	DHA	
	Minus	Plus
Control	0.29	0.82
NADH	0.48	3.81

* Expressed as mmol/liter ghosts·h.

‡ ATP 0.5 mM.

§ ATP 0.5 mM, GSH 1.0 mM, NAD 0.25 mM, fructose diphosphate 0.5 mM, ADP 0.25 mM, nicotinamide 0.5 mM, aldolase 0.03 mg, G3PD 0.3 mg.

role in ferricyanide reduction, it must quantitatively be quite small because O_2 consumption does not increase after ferricyanide exposure (2). Further, we have studied RBC from G6PD-deficient individuals (both mild and severe variants), all of whom generated ferrocyanide at normal rates.

Although the evidence implicating NADH as the primary source of reducing equivalents for ferrocyanide generation is rather convincing, the mechanism responsible for the transfer of electrons from NADH to ferricyanide is less certain. The demonstration of an NADH:ferricyanide oxidoreductase in RBC membrane preparation(s) initially suggested that an electron transfer apparatus existed as an integral part of the RBC membrane (22). Subsequent experiments have shown, however, that the oxidoreductase is localized to the inner face of the membrane (23). Its reducing equivalents were accessible to ferricyanide only when the membrane was disrupted, and its activity was inversely proportional to the physical integrity of the ghost preparation under study (23). Similar results were obtained when membrane preparations from *Escherichia coli* were examined (24, 25). In a more recent study with vesicles prepared from *Bacillus subtilis*, it could be demonstrated that although ferricyanide was inaccessible to the NADH dehydrogenase itself, it could receive electrons from other carriers of the respiratory chain (26). Of particular interest was the impaired electron transfer by vesicles prepared from mutants deficient in the vitamin K congener menaquinone. When these vesicles were supplied with the deficient vitamin, ferricyanide reduction was completely restored (26).

We have constructed a model to explain the mechanism of ferricyanide reduction as observed in human RBC (Fig. 7). DHA is known to penetrate the RBC membrane (4, 16, 17), and once within the cell it is reduced to ascorbic acid (4, 15, 27). Our data would suggest that the reduction involves the transfer of a hydrogen atom from NADH to DHA, a reaction also demonstrated by other investigators (27). From our experiments we cannot be certain whether the reducing equivalents pass directly from NADH to DHA or whether an additional carrier is involved. Although glutathione has been implicated in the reduction of DHA (15), its primary role could simply be to maintain the sulfhydryl groups of G3PD in the reduced form. Once formed, AA begins to leave the RBC at a constant rate (16), then becoming available for the nonenzymatic reduction of ferricyanide. The decline in the rate of ferricyanide reduction observed after the initial 20–30 min is also consistent with this hypothesis because DHA is an inherently unstable molecule. In an aqueous solution at neutral pH, 50% of the DHA present will decompose in 1 h (28). Its catabolism can be greatly accelerated by the presence of ferric iron (29). In fact, patients with iron overload demonstrate low AA levels (30) and an accelerated rate of AA catabolism (31).

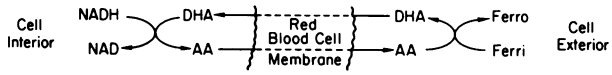

FIGURE 7 A model of the AA-mediated transmembrane-reducing system of the human RBC.

Many similarities suggest that this transmembrane reducing system has a physiological role in the process of cellular iron accumulation. The uptake of iron by RBC precursors involves the binding of transferrin, a β-globulin which carries two atoms of ferric iron per molecule of protein, the release from transferrin of iron, and the transmembrane movement of iron into the cell (32). At some point between the binding of transferrin and the cellular uptake of iron, a reduction from the ferric to the ferrous state must occur (33). Many features suggest that the AA-mediated transmembrane-reducing system described above is responsible for this vital step in cell iron uptake. First, unlike the binding of transferrin which rapidly saturates all available sites, in vitro cell iron accumulation occurs in a linear fashion over 30 min (32). Second, a serum factor is known to enhance the rate of iron uptake by reticulocytes (34). Third, ascorbic acid is known to increase gastrointestinal iron absorption (35). Last, AA is known to promote the release of iron from reticuloendothelial deposits (36). Animals placed on a scorbutic diet develop a progressive fall in such iron-containing cellular constituents as the cytochromes and hemoglobin and a concomitant increase in the nonheme iron content of spleen and muscle (37). Studies are presently underway in our laboratory to explore the potential relationship between the AA-mediated transmembrane-reducing system and cellular iron accumulation.

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