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Ascorbate-dependent electron transfer across the human erythrocyte membrane

James M. May *, Zhi-chao Qu

Departments of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232-6303, USA

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Abstract

Reduction of extracellular ferricyanide by intact cells reflects the activity of an as yet unidentified trans-plasma membrane oxidoreductase. In human erythrocytes, this activity was found to be limited by the ability of the cells to recycle intracellular ascorbic acid, its primary trans-membrane electron donor. Ascorbate-dependent ferricyanide reduction by erythrocytes was partially inhibited by reaction of one or more cell-surface sulfhydryls with *p*-chloromercuribenzene sulfonic acid, an effect that persisted in resealed ghosts prepared from such treated cells. However, treatment of intact cells with the sulfhydryl reagent had no effect on NADH-dependent ferricyanide or ferricytochrome *c* reductase activities of open ghosts prepared from treated cells. When cytosol-free ghosts were resealed to contain trypsin or pronase, ascorbate-dependent reduction of extravesicular ferricyanide was doubled, whereas NADH-dependent ferricyanide and ferricytochrome *c* reduction were decreased by proteolytic digestion. The trans-membrane ascorbate-dependent activity was also found to be inhibited by reaction of sulfhydryls on its cytoplasmic face. These results show that the trans-membrane ferricyanide oxidoreductase is limited by the ability of erythrocytes to recycle intracellular ascorbate, that it does not involve the endofacial NADH-dependent cytochrome b_5 reductase system, and that it is a trans-membrane protein that contains sensitive sulfhydryl groups on both membrane faces.

Keywords: Ascorbic acid; Ferricyanide; Ascorbate recycling; Trans-membrane oxidoreductase; Human erythrocyte

1. Introduction

Virtually all cells appear capable of reducing extracellular ferricyanide to ferrocyanide [1]. This activity has been linked to several important cellular functions, including stimulation of cell growth and differentiation [2,3], reduction and uptake of ferric iron [1], and protection against an extracellular oxidant stress [4]. Since ferricyanide does not penetrate the cell membrane [5], its reduction requires either that electrons be transferred from the cell interior across the plasma membrane, or that the cells release a substance capable of reducing ferricyanide. NADH

Abbreviations: AFR, ascorbate free radical; DABS, diazobenzene sulfonic acid; DHA, dehydroascorbic acid; DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PBS, phosphate-buffered saline; PCMBS, *p*-chloromercuribenzene sulfonic acid; Tempol, 2,2,6,6-tetramethyl-4-hydroxy-piperidine-*N*-oxyl; TNBS, trinitrobenzene sulfonic acid

* Corresponding author. 715 Medical Research Building II, Vanderbilt University School of Medicine, 2220 Pierce Ave., Nashville, TN 37232-6303, USA. Fax: +1-615-936-1667; E-mail: james.may@mcmail.vanderbilt.edu

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has long been considered a source of intracellular electrons for ferricyanide reduction [1,6]. Extracellular ferricyanide reduction is also enhanced in proportion to the cellular content of ascorbic acid [6,7]. Moreover, in cell types as diverse as human erythrocytes [8], pulmonary artery endothelial cells [9], and HL-60 cells [10], ascorbate at physiologic concentrations appears to be the major electron donor to the trans-membrane activity. Although ascorbate-induced ferricyanide reduction was initially considered to be due to direct reduction of ferricyanide by ascorbate that had leaked from the cells [6], subsequent work has shown that intracellular ascorbate donates electrons to an as yet unidentified trans-plasma membrane ferricyanide oxidoreductase activity [4,7,10].

Ascorbate-dependent ferricyanide oxidoreductase activity is thought to be mediated at least in part by a protein or enzyme, since reduction is saturable with increasing concentrations of extracellular ferricyanide or intracellular ascorbate [7,10], and since it is inhibited by poorly penetrant protein reagents such as diazobenzene sulfonic acid (DABS) [11] and *p*-chloromercuribenzene sulfonic acid (PCMBS) [7,10,11]. However, it is possible that other factors account for apparent saturation of ferricyanide reduction, and that these reagents penetrate the cell membrane or lack specificity for proteins. It is also unknown whether the oxidoreductase spans the membrane or involves the prominent NADH-dependent cytochrome b_5 reductase system on the cytoplasmic membrane face. Regarding the latter, Van Duijn et al. [10] recently reported that ferricyanide reduction by ascorbate-free HL-60 cells, which is presumably NADH-dependent, is more sensitive to inhibition by PCMBS than is ferricyanide reduction in ascorbate-loaded cells.

In contrast to NADH-dependent ferricyanide reduction, which can be assessed in open membranes and in purified preparations, study of ascorbate-dependent activity requires separation of the two reactants, because of the rapid direct reduction of ferricyanide by ascorbate. Therefore, the present studies were carried out in intact human erythrocytes and in resealed erythrocyte ghosts, with the goal of establishing the substrate dependence, kinetics, and susceptibility to protein reagents of both membrane faces of the trans-membrane ascorbate-dependent ferricyanide reductase. The major findings are that ascorbate-dependent ferricyanide reduction is limited more by the ability of cells to recycle ascorbate than by electron-transfer capacity, that the exposed cytoplasmic acceptor of ascorbate electrons involves a protein that is distinct from the cytochrome b_5 reductase complex, and that function of this protein requires one or more endofacial sulfhydryl groups.

2. Materials and methods

2.1. Materials

DHA, 2,2,6,6-tetramethyl-4-hydroxy-piperidine-*N*-oxyl (Tempol), and tridecylamine were from Aldrich (Milwaukee, WI). All other reagents were purchased from Sigma (St. Louis, MO). DABS was synthesized as described previously [11] and stored under nitrogen for up to a week before use without loss of activity.

2.2. Erythrocyte and erythrocyte ghost preparation

Human erythrocytes were prepared from heparinized blood that had been drawn from normal volunteers. Erythrocytes were obtained by three centrifugation washes in 10 vols. of phosphate-buffered saline (PBS), which consisted of 140 mM NaCl and 12.5 mM Na_2HPO_4 in deionized water, adjusted to pH 7.4. The buffy coat of white cells was removed with each wash.

Body leaky and 'pink' resealed erythrocyte ghosts were prepared as described by Steck and Kant [12]. Resealed hemoglobin-free or 'clear' ghosts were prepared according to a previously described method [13] with the following minor modifications. Open or leaky erythrocyte ghosts were prepared initially to be free of visible hemoglobin contamination. These were incubated with mixing for 5 min on ice in 5 mM sodium phosphate buffer, pH 8.0, that contained 1 mM $MgSO_4$ and agents as noted. While the ghosts were on ice, NaCl was added to a final concentration of 150 mM. The ghost suspension was warmed to 37°C and incubated for an additional hour. The ghosts were washed three times by centrifugation at $13\,000 \times g$ in ice-cold PBS and taken for assays as noted.

2.3. Measurement of ascorbate, GSH, and α-tocopherol in cells and in resealed ghosts

Ascorbate was measured in intact cells following cell lysis and ultrafiltration as previously described [14]. Prior to some experiments, endogenous erythrocyte ascorbate was depleted by three repetitive treatments with 1 mM Tempol [14]. For assay of intravesicular ascorbate in resealed ghosts, the ghosts were lysed with an equal volume of 0.4% (v/v) Triton-X100 prior to ultrafiltration and subsequent assay of ascorbate as described for erythrocytes. The GSH content of erythrocytes was measured with the fluorometric assay of Hissin and Hilf [15].

The α-tocopherol content of erythrocytes was measured as follows. Packed cells (0.2 ml) were mixed with an equal volume of 0.5% pyrogallol (w/v) in water. This was followed by 0.2 ml of reagent alcohol (95% ethanol:5% isopropanol, v/v) with vigorous mixing. Heptane (0.4 ml) was added, and the lysate was vortexed for at least 1 min. The lysate was microfuged and an aliquot of the clear upper heptane layer was removed and taken to dryness under a stream of nitrogen. The residue was dissolved in 0.2–0.4 ml of a 1:1 mixture of ethanol and methanol for assay by HPLC with electrochemical detection. Samples were chromatographed in the isocratic mode on a Waters DeltaPak C_{18} column (300 μm, 5 μ) that was preceded by a 4-mm guard column of the same packing material. The mobile phase was 95% methanol and 5% water containing 20 mM sodium perchlorate. Detection of α-tocopherol was accomplished by a modification of the reduction–oxidation method described by Takeda, et al. [16]. An ESA model 5020 guard cell, which was preceded by a graphite in-line filter, was placed just after the analytical column and set in the reducing mode at −0.5 V. This was followed by an ESA model 5011 analytical cell that was used for detection, with the first electrode in the analytical cell set at −0.5 V, and the second detecting electrode set at +0.6 V. At a flow rate of 1 ml/min, α-tocopherolquinone was detected at 4.3–4.6 min, γ-tocopherol at 5.6–5.9 min, and α-tocopherol at 6.1–6.4 min. The assay sensitivity for α-tocopherol was 2–5 pmol/sample.

Erythrocyte and ghost concentrations of ascorbate, α-tocopherol, and GSH are expressed relative to the water content of packed erythrocytes, or 70% of the packed cell volume [6].

2.4. Assay of extracellular oxidant reduction

Reduction of extracellular ferricyanide to ferrocyanide by erythrocytes was measured with the method of Avron and Shavit [17] as previously described [4], following incubation of 5% cells for the indicated time at 37°C in PBS that contained 5 mM D-glucose and 1 mM potassium ferricyanide. A correction was made for absorbance of medium from cells incubated in the absence of ferricyanide.

Reduction of oxidized cytochrome *c* (ferricytochrome *c*) was measured by incubating control and ascorbate-loaded cells at a 20% hematocrit in PBS that contained 5 mM D-glucose and 100 μM ferricytochrome *c* for 30 min at 37°C. The cells were pelleted in a microfuge and the absorbance of an aliquot of the medium was measured at 550 nm. Correction was made for interfering substances released from the cells, including ascorbate, by carrying out paired cell incubations that did not contain ferricytochrome *c*, incubating the medium from such cells for 10 min at 37°C with 100 μM ferricytochrome *c*, measuring the absorbance at 550 nm in an aliquot of that medium, and subtracting this reading from the value obtained when cells were incubated with ferricytochrome *c*.

Reduction of ferric citrate by control and ascorbate-loaded erythrocytes was measured by incubating cells at a 5% hematocrit in PBS that contained 5 mM D-glucose, 80 μM bathophenanthroline disulfonate (BPS), and the indicated concentration of ferric citrate for 30 min at 37°C. Soluble ferric citrate was prepared by dissolving ferric chloride to a 1 mM concentration in a 5-fold molar excess of trisodium citrate. Reduction of Fe^{3+} to Fe^{2+} was measured as the BPS chelate of Fe^{2+} at 535 nm in the supernatant from the cell incubations. Correction for release of ascorbate and other interfering substances by the cells was made as described for assay of ferricytochrome *c* reductase activity.

For assay of ferricyanide reduction by erythrocyte ghosts that had been resealed to contain ascorbate and other agents, aliquots of ghosts prepared from 0.1 or 0.2 ml of packed erythrocytes were incubated

with 1 mM ferricyanide at 37°C in a total volume of 1 ml. At 10 min, the ghosts were pelleted in a microfuge at (13 000 × *g* for 1 min) and duplicate 100 μl aliquots of the supernatant were taken for assay of ferrocyanide. Paired ghost samples were also incubated in the absence of ferricyanide for 10 min, the ghosts were pelleted in the microfuge, and the supernatant was then incubated with 1 mM ferricyanide for another 10 min at 37°C. Duplicate 100-μl aliquots of this sample were taken for assay of ferrocyanide and the readings in these samples were subtracted from those of the first sample to correct for background due to incubation with the ghosts and for any ascorbate that had leaked from the ghosts. For 'clear' or washed resealed ghosts this correction was typically 30–50% of the experimental reading, and for standard 'pink' resealed ghosts it was about 10%.

2.5. Data and statistical analysis

Data are shown as mean ± S.E. Curve-fitting was carried out by non-linear least-squares regression using the graphics program Origin 5.0 (Microcal Software, Northampton, MA). Statistical significance was assessed by one- or two-way analysis of variance using the statistics program Sigmastat 2.0 (Jandel Scientific, St. Louis, MO).

3. Results

3.1. Basal and ascorbate-dependent erythrocyte ferricyanide reduction

To assess differences in ascorbate-dependent and -independent ferricyanide reduction in intact erythrocytes, cells were loaded with increasing concentrations of DHA, and both the cellular ascorbate content and ferricyanide reduction were measured. As shown in Fig. 1A, DHA loading increased intracellular ascorbate concentrations, which in turn enhanced the initial rates of ferricyanide reduction in a saturable fashion. At the higher DHA concentrations ferricyanide reduction was linear for only about 5–10 min (results not shown), so a time point of 5 min was used in these experiments to obtain an estimate of the initial rate of reduction. The maximal ascorbate-stimulated ferricyanide reduction was almost 20-fold that of the basal rate (59 ± 8 nmol (ml cells)$^{-1}$ min^{-1} in basal cells compared to 1125 ± 100 nmol (ml cells)$^{-1}$ min^{-1} in cells incubated with 320 μM DHA). After subtraction of the basal rate, the data were fit to an hyperbolic model, which resulted in an apparent K_m of 1.8 ± 0.4 mM, and a predicted maximal rate of 1988 ± 290 nmol min^{-1} (ml cells)$^{-1}$. However, most of the observed saturation of ferricyanide reduction is due to a decrease in intracellular ascorbate induced by ferricyanide. As shown in the inset to Fig. 1A, intracellular ascorbate concentrations measured after 5 min of ferricyanide exposure had fallen to about 20% of the initial values for ascorbate concentrations of 140 μM or higher (resulting from DHA loading concentrations of ≥ 50 μM). On the other hand, the cells were able to maintain basal ascorbate concentrations despite the oxidant stress induced by ferricyanide. Based on the extent of ferricyanide reduction, and on the measured initial and final ascorbate contents, the cells recycled cellular ascorbate about 2–3 times over the 5-min period of ferricyanide reduction. These results show that the ascorbate recycling capacity of erythrocytes, although substantial, limits the activity of the trans-membrane oxidoreductase. This further suggests that the trans-membrane oxidoreductase activity operates well below its capacity at physiologic ascorbate concentrations within the cells (30–80 μM).

The marked stimulation of ferricyanide reduction by ascorbate-loading shown in Fig. 1A was observed in cells that contained endogenous ascorbate. To more directly compare rates of ascorbate-dependent and -independent ferricyanide reduction, cells were first depleted of endogenous ascorbate by several treatments with the cell-penetrant nitroxide, Tempol, which removes about 80% of erythrocyte ascorbate without affecting α-tocopherol or GSH [14]. As shown in Fig. 1B, ferricyanide reduction in ascorbate-depleted cells was very low compared to that in ascorbate-loaded cells, although both showed saturation with increasing amounts of ferricyanide. At least with regard to the ascorbate-loaded cells, the apparent saturation of ferricyanide reduction is likely due at least in part to exceeding the capacity of cellular ascorbate recycling. Also, despite the use of short incubation times (5 min), low concentrations of cells (5%) and 100 μM DHA, it can be calculated

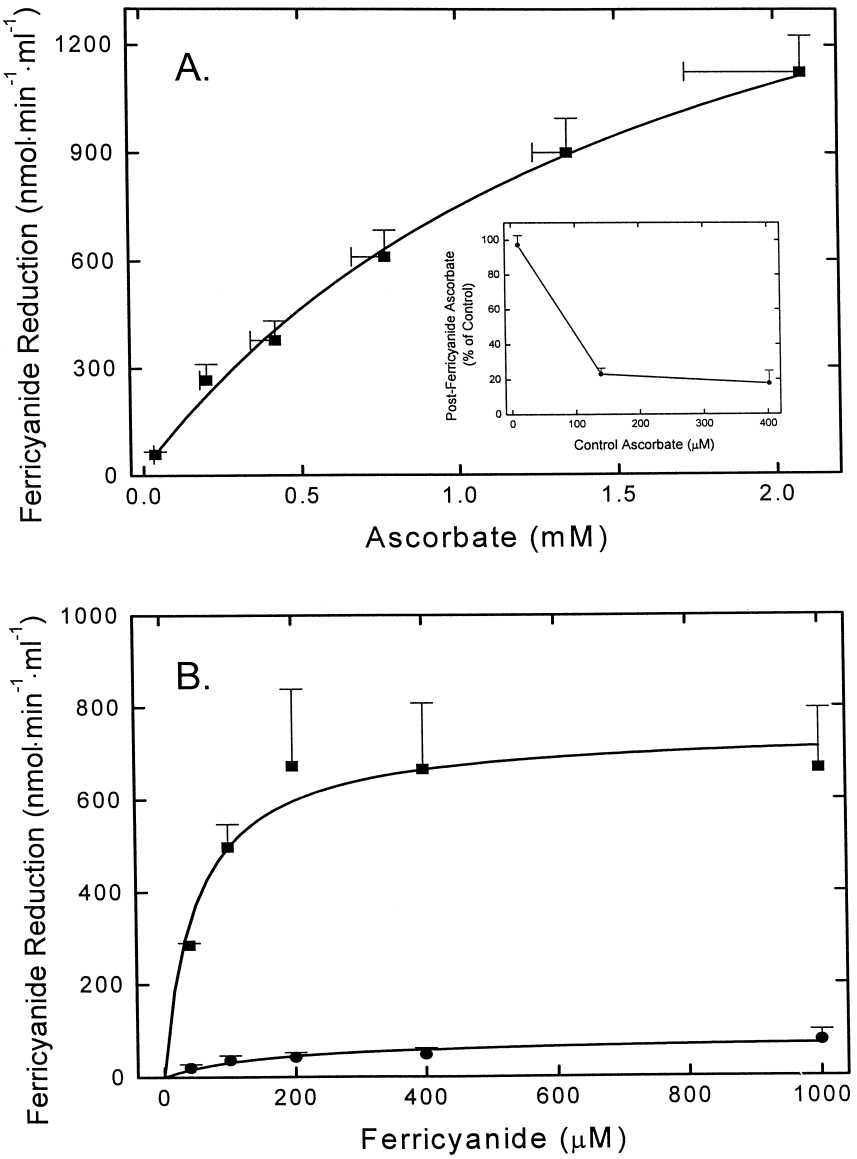

Fig. 1. Ascorbate-dependent reduction of ferricyanide in intact erythrocytes. (A) Erythrocytes at a 10% hematocrit were incubated for 15 min at 37°C with 5 mM D-glucose and freshly prepared DHA (0, 20, 40, 80, 160, 320 μM) in PBS. The cells were washed by centrifugation in 5 vols. of PBS, and aliquots were taken for assay of the intracellular ascorbate concentration and for measurement of ferricyanide reduction during a 5-min incubation. Data are shown from four experiments, with the solid line representing an hyperbolic fit to the data following subtraction of the basal rate. Inset: the cellular content of ascorbate at the end of the ferricyanide incubation was measured in three experiments, and expressed as a percent of the initial ascorbate concentration. (B) Erythrocytes that had been depleted of endogenous ascorbate with Tempol were incubated without (circles) or with 100 μM DHA (squares) as described for A, washed three times by centrifugation, and incubated for another 5 min at 37°C with the indicated initial concentration of ferricyanide. The cells were pelleted and aliquots of the supernatants were taken for assay of ferrocyanide during a 5-min incubation. Data are shown from three experiments, with the solid lines indicating fits to hyperbolic models.

that at the lowest ferricyanide concentrations 75–83% of extracellular ferricyanide was reduced by the DHA-treated cells. Thus, changes in the concentrations of both substrates for this reaction prevent any conclusions to be drawn regarding whether the saturation of the ascorbate-dependent reaction relates to saturation of the trans-membrane oxidoreductase. Nonetheless, these results highlight marked

differences in the kinetics of ferricyanide reduction in ascorbate-loaded compared to ascorbate-depleted cells.

3.2. Erythrocyte reduction of other electron acceptors

The ability of erythrocytes to transfer electrons to extracellular acceptors other than ferricyanide was evaluated with regard to ascorbate dependence, since such a comparison might indicate how many transfer systems are operative. As shown in Table 1, comparisons were made between reduction of ferricyanide, ferricytochrome *c*, and ferric iron. Reduction of ferricytochrome *c* was measured directly, and reduction of Fe^{3+} was detected by a change in UV absorbance on chelation of Fe^{2+} by bathophenanthroline disulfonate (BPS) outside the cells. The rates of both activities were found much lower than that of ferricyanide reduction (Table 1, first column), even though the latter was not an initial rate, since measurements were made at 30 min. Ascorbate that has leaked from cells will directly reduce each of the oxidants, so control incubations were performed to assess and correct for such a leak, as shown in the second and third columns of Table 1, respectively. Efflux of ascorbate could account for only 1.5% of the observed rate of ferricyanide reduction. In contrast, there was no significant cellular reduction of ferricytochrome *c* following correction of ascorbate leak (Table 1). Comparison of these results provides compelling evidence that ascorbate-dependent ferricyanide reduction is not due to leak of ascorbate from the cells.

In contrast to ferricytochrome *c*, there remained a small but significant effect of intracellular ascorbate to reduce extracellular Fe^{3+}, even after correction for ascorbate leak. The dose-dependence of this effect was evaluated in ascorbate-depleted cells, with the results shown in Fig. 2. Reduction of Fe^{3+} was saturable with increasing concentrations of ferric citrate. When fit to hyperbolic models, the apparent K_m values for basal (8 ± 3 μM) and ascorbate-stimulated (60 ± 11 μM) ferric iron reduction differed, as did the calculated maximal rate of ferric ion reduction (1.3 ± 0.1 for basal versus 13.9 ± 1.4 nmol min^{-1} (ml cells)$^{-1}$). Intracellular ascorbate concentrations at the highest ferric citrate concentration (80 μM) were unchanged (results not shown), indicating that the observed plateau in reduction is due to the transmembrane process and not to changes in ascorbate. Whereas ascorbate-dependent ferric iron reduction is markedly greater than reduction in ascorbate-depleted cells, it undergoes saturation in a manner that differs from that observed for ascorbate-dependent ferricyanide reduction.

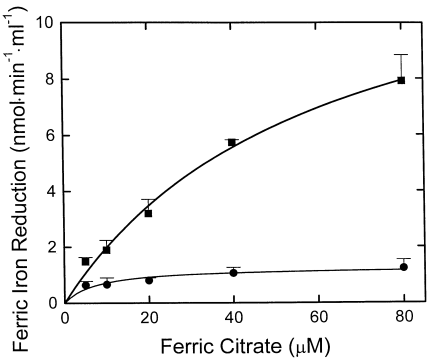

Fig. 2. Ascorbate-dependent reduction of ferric citrate. Erythrocytes that had been depleted of ascorbate by Tempol treatment were incubated at a 15% hematocrit for 15 min at 37°C in the absence (circles) or presence (squares) of 100 μM DHA, washed three times in PBS, and assayed for reduction of the indicated concentration of ferric citrate. Results from three experiments are shown, with fits indicated by the solid lines to hyperbolic models.

Table 1
Reduction of extracellular oxidants by ascorbate-loaded erythrocytes

Oxidant	Cells	Supernatant	Difference
Ferricyanide	336 ± 43[a]	5 ± 2	331 ± 43
Ferricytochrome *c*	7 ± 1	7 ± 1	0
Fe^{3+}-citrate	16 ± 2[a]	5 ± 1	11 ± 1

Cells at a 20% hematocrit were incubated with 100 μM DHA and 5 mM D-glucose for 15 min at 37°C, then washed three times by centrifugation. Reduction assays were carried out as described in Section 2, all for 30 min. All units are nmol min^{-1} (ml erythrocytes)$^{-1}$. Data are from at least four experiments for each treatment.

[a] $P < 0.05$ compared to the rate of reduction in the cell supernatant.

Table 2
Effects of sulfhydryl reagents on ascorbate-dependent ferricyanide reduction and cell antioxidants

Agent	Ferricyanide reduction		Ascorbate	α-Tocopherol	GSH
DIDS (25 μM):	(−)	(+)	(−)	(−)	(−)
PCMBS (200 μM)	0.47 ± 0.03[a]	0.66 ± 0.04[a]	0.89 ± 0.04[a]	0.96 ± 0.16	0.95 ± 0.05
TNBS (1 mM)	0.56 ± 0.07[a]	0.66 ± 0.04[a]	0.51 ± 0.07[a]	0.94 ± 0.23	0.9 ± 0.09
DABS (300 μM)	0.06 ± 0.002[a]	0.07 ± 0.01[a]	0.7 ± 0.09[a]	0.06 ± 0.01[a]	1.07 ± 0.08
Tetrathionate (50 mM)	0.68 ± 0.03[a]	0.87 ± 0.08	0.92 ± 0.07	0.99 ± 0.23	0.39 ± 0.03[a]
DTNB (3 mM)	1.06 ± 0.02	1.00 ± 0.01	1.09 ± 0.1	0.91 ± 0.26	0.96 ± 0.03

Cells at a 10% hematocrit were incubated with 200 μM DHA with or without DIDS as indicated in PBS containing 5 mM D-glucose. After 15 min at 37°C, the agents were added to the concentrations shown, and the incubations were continued for another 15 min. The cells were washed three times and ferricyanide reduction was determined over 30 min. Antioxidant concentrations were determined in basal cells treated with and without the agents under the same conditions. All results are shown as percent of the corresponding value in untreated control cells from 3–6 experiments. Control values were: ferricyanide reduction, 291 ± 31 nmol (ml erythrocytes)$^{-1}$ min^{-1}; ascorbate, 59 ± 10 μM; α-tocopherol, 3.9 ± 0.6 μM; and GSH, 2.0 ± 0.2 mM.
[a] $P < 0.05$ compared to the appropriate control.

3.3. Sensitivity of ferricyanide reduction to poorly permeant inhibitors

To assess the extent to which the trans-membrane activity is exposed on the cell surface, the sensitivity of ferricyanide reduction to poorly penetrant covalent inhibitors was examined. Agents likely to interact only with extracellular groups were chosen to increase selectivity for trans-plasma membrane activities. As shown in Table 2, ascorbate-stimulated ferricyanide reduction was inhibited 30–50% by PCMBS, TNBS, DABS, and tetrathionate, but was unaffected by DTNB. Each of the agents that showed inhibition also had some effect on cellular GSH, ascorbate, or α-tocopherol (Table 2), but none caused hemolysis. The loss of ascorbate and/or GSH in response to treatment of cells with the inhibitory agents could indicate that the agent did not remain outside the cells during incubation. That is, some of the decrease in ferricyanide reduction observed with these agents could be due to their reaction with intracellular proteins. Of these agents, tetrathionate and PCMBS are already known to enter erythrocytes slowly on the anion transporter [18,19]. If effects of the sulfhydryl reagents are due to intracellular reactions, blockade of their entry on the anion transporter with 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid (DIDS) should prevent the effect [18].

To determine whether an agent had entered the cells on the anion transporter, DIDS was included during loading of the cells with ascorbate, with the results shown in the second column of Table 2. Treatment with DIDS alone did not affect either basal or ascorbate-stimulated ferricyanide reduction (results not shown). DIDS largely prevented the inhibition by tetrathionate, decreased that due to PCMBS by about 30%, but had no effect on inhibition by DABS or TNBS. These results suggest that

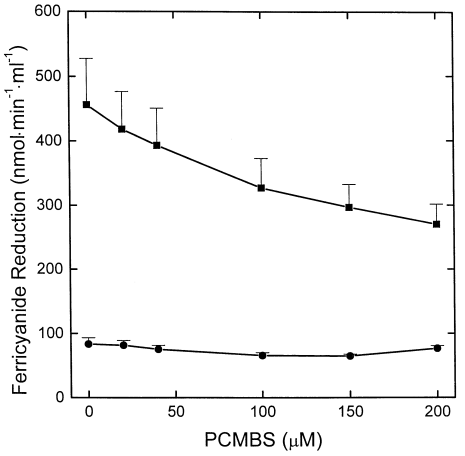

Fig. 3. Inhibition of ascorbate-dependent ferricyanide reduction by PCMBS. Cells that had been depleted of endogenous ascorbate by treatment with Tempol were incubated at 37°C in PBS at a 10% hematocrit with 25 μM DIDS, 5 mM D-glucose, and either without (circles) or with 100 μM DHA (squares). At 15 min, PCMBS was added to the indicated final concentration and the incubation was continued for another 15 min. Aliquots of mixed cells and buffer were removed, washed three times in PBS, and taken for measurement of ferricyanide reduction during a 30-min incubation. Data from 7 experiments are shown.

the effect of tetrathionate is due to reaction with an intracellular sulfhydryl necessary for function of the oxidoreductase, whereas most of the effect of PCMBS is due to interaction with a cell-surface protein sulfhydryl. Treatment with DIDS was without effect on the decreases in intracellular ascorbate induced by DABS, TNBS, or PCMBS (results not shown). This could indicate that these agents caused a trans-membrane oxidant stress that lowered intracellular ascorbate. Since TNBS and DABS substantially depleted ascorbate and/or α-tocopherol, further experiments were carried out with PCMBS, using pretreatment with DIDS to prevent uptake of PCMBS. The concentration-dependence of PCMBS inhibition of erythrocyte ferricyanide reduction was examined with the studies shown in Fig. 3.

Inhibition of ascorbate-dependent ferricyanide reduction by PCMBS in DIDS-pretreated cells occurred rapidly, and was essentially complete in about 15 min (results not shown). To assess the effects of PCMBS on ascorbate-independent ferricyanide reduction, cells were again largely depleted of endogenous ascorbate by repetitive treatments with Tempol. In ascorbate-depleted, DIDS-treated cells, increasing amounts of PCMBS caused only about a 20% decrease in ferricyanide reduction (Fig. 3). On the other hand, in ascorbate-loaded cells there was a progressive inhibition of ferricyanide reduction to about 40% of control over the range of PCMBS concentrations used. These differences in sensitivity to inhibition by PCMBS suggest that basal and ascorbate-mediated ferricyanide reduction are not due to the same activity.

Since ascorbate-dependent ferric citrate reduction by erythrocytes differed in several respects from ferricyanide reduction, the effects of PCMBS on ferric citrate reduction were tested. However, there was no effect of PCMBS up to concentrations of 0.2 mM on ferric citrate reduction. This also suggests that these two activities are not mediated by the same process.

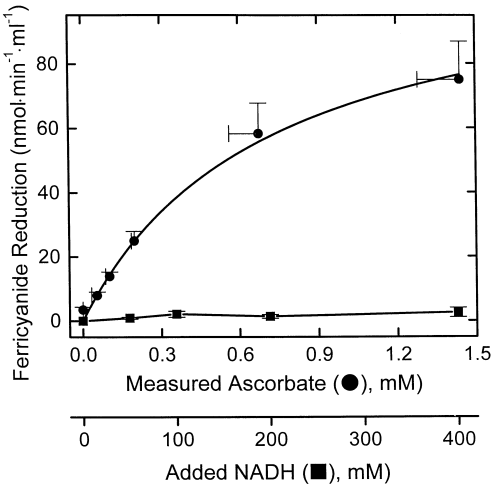

Fig. 4. Ferricyanide reduction in resealed erythrocyte ghosts loaded with increasing concentrations of ascorbate or NADH. Resealed ghosts were prepared to contain the measured ascorbate concentrations noted on the upper *x*-axis, and their ability to reduce ferricyanide in the presence of 2 U/ml ascorbate oxidase was determined. The results from four experiments are shown, with the fit to an hyperbolic model shown by the solid line. NADH-dependent reduction was measured in three experiments at the loading concentrations of NADH shown on the lower *x*-axis.

3.4. *Trans-membrane ferricyanide reduction in erythrocyte ghosts*

Resealed 'pink' erythrocyte ghosts were studied to determine whether inhibition of trans-plasma membrane reduction of ferricyanide by PCMBS persists after cell disruption or whether it requires cytosolic components. When present during the resealing phase, both ascorbate and NADH are trapped within these ghosts and can support reduction of extravesicular ferricyanide [20]. As shown in Fig. 4, in-

Table 3
Lack of effects of PCMBS treatment of intact cells on cytochrome b_5 reductase

Activity	Control	PCMBS	PCMBS+DIDS
Ferricyanide reductase	76 ± 15	80 ± 12	87 ± 6
Ferricytochrome *c* reductase	8.8 ± 1.4	6.6 ± 0.7	8.7 ± 1.5

Cells at a 10% hematocrit were incubated with or without 25 μM DIDS and 5 mM D-glucose for 15 min at 37°C, followed by addition of 200 μM PCMBS where noted for another 15 min. The cells were then washed three times by centrifugation and taken for preparation of leaky ghosts. Reduction assays were carried out as described in Section 2. Units are nmol min^{-1} (mg ghost protein)$^{-1}$. Data are from four experiments for each treatment.

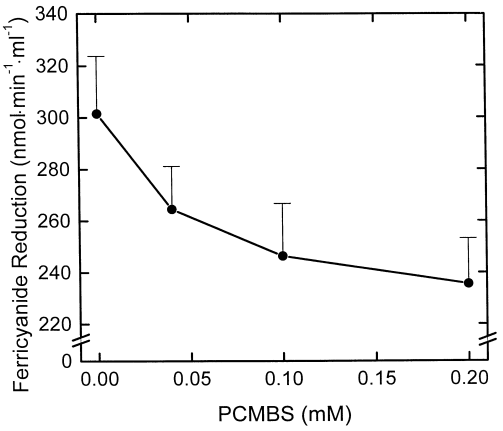

Fig. 5. Ferricyanide reduction in resealed erythrocyte ghosts prepared from PCMBS-treated cells. Erythrocytes at a 10% hematocrit were preincubated at 37°C in PBS that contained 25 μM DIDS and 5 mM D-glucose. At 15 min the indicated concentrations of PCMBS were added. After another 15 min, the cells were lysed and resealed ghosts were prepared to contain 4 mM ascorbate for incubation in the resealed ghost ferricyanide reduction assay. Data are shown from six experiments.

creasing amounts of trapped ascorbate enhanced ferricyanide reduction in a saturable manner. The initial intravesicular ascorbate concentration at which ferricyanide reduction was half-maximal was 0.65 ± 0.1 mM, and the predicted maximal effect was 110 ± 7 nmol (ml ghosts)$^{-1}$ min^{-1}. In comparison to the results of Fig. 1A, it is evident that the rate of ferricyanide reduction at the same initial intracellular ascorbate concentration is about 9-fold greater in the cells than in the ghosts. Contributing to this difference is the fact that these ghosts are unable to recycle ascorbate, so that intravesicular ascorbate concentrations will fall even during short incubations. In contrast to the results observed with ascorbate, increasing amounts of NADH caused little increase in ferricyanide reduction (Fig. 4). These results were not altered by inclusion of an NADH-regenerating system consisting of lactate and lactate dehydrogenase (results not shown).

To further compare the mechanism(s) of ascorbate- and NADH-dependent ferricyanide reduction, ghosts prepared from PCMBS-treated cells were loaded with ascorbate and assayed for changes in their ability to reduce extravesicular ferricyanide. As shown in Fig. 5, even with DIDS pretreatment, PCMBS inhibited ferricyanide reduction by the ghosts 22%. Although this less than the effect observed in intact cells (see Fig. 3), it is nonetheless significant. This shows that the effect of PCMBS persists after cell lysis, and that it involves a component of the ghost membrane rather than a cytosolic component.

In contrast to trans-membrane ascorbate-dependent ferricyanide reduction, there was no effect of PCMBS treatment of DIDS-treated cells on either NADH-dependent ferricyanide reductase or on ferricytochrome *c* reductase activities in leaky erythrocyte ghosts that were prepared from the cells (Table 3).

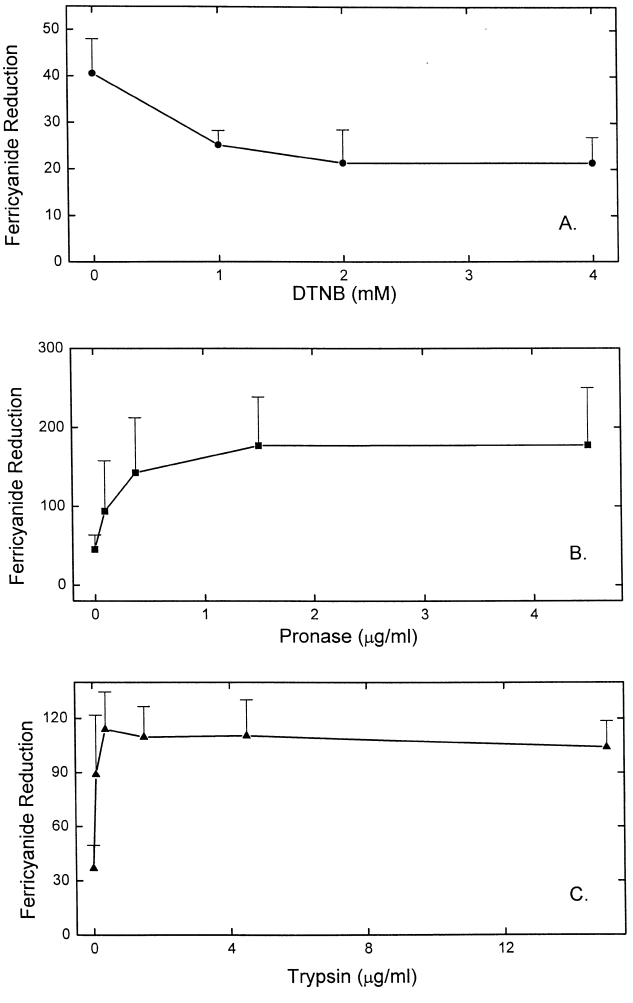

Fig. 6. Effects of proteolytic enzymes and DTNB on resealed clear erythrocyte ghost ascorbate-dependent ferricyanide reductase activity. Clear erythrocyte ghosts were prepared to contain 4 mM ascorbate and the indicated concentration of DTNB (A, $n=5$), pronase (B, $n=3$), or trypsin (C, $n=6$). Ferricyanide reduction by the ghosts was measured with correction for ascorbate leakage. Units are nmol min^{-1} (ml ghosts)$^{-1}$.

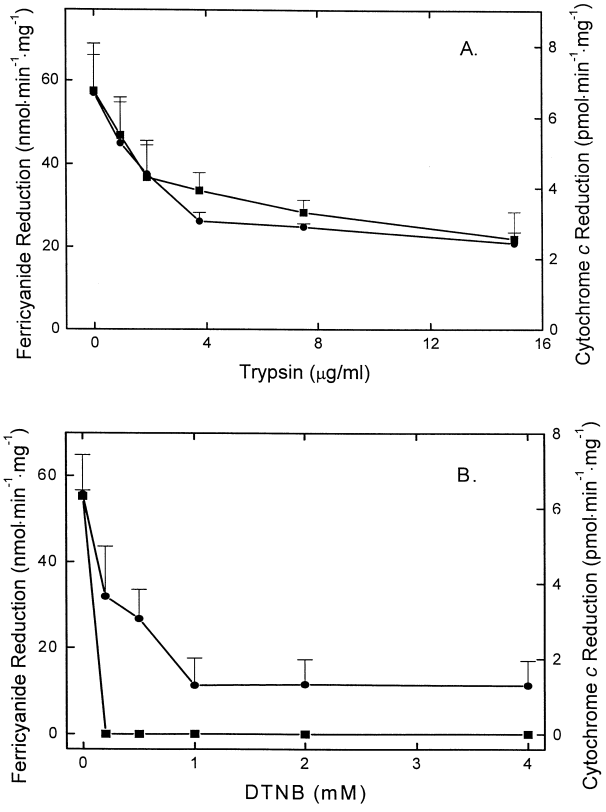

Fig. 7. Inhibition of ferricyanide and cytochrome *c* reductase activities by trypsin and DTNB in open ghosts. Leaky erythrocyte ghosts (0.2–0.4 mg/ml) were incubated with the indicated concentrations of trypsin (A) or DTNB (B) for 1 h at 37°C in 5 mM phosphate buffer, pH 8.0. The ghosts were washed three times by centrifugation and aliquots were taken for assay of ferricyanide (circles) and ferricytochrome *c* (squares) reductase activities. Data from three experiments with each agent are shown.

Ferricyanide reductase activity was about 10-fold greater than ferricytochrome *c* reductase activity. Whereas PCMBS inhibition of ascorbate-dependent ferricyanide reduction persists in resealed ghosts, no residual effect of external PCMBS reaction is evident in open ghost membranes using NADH as an electron donor. This suggests that the ascorbate-dependent trans-membrane activity and the bulk of the membrane-bound NADH-dependent reductase activities are different.

3.5. *Effects of reaction of endofacial sites with protein reagents*

To gain additional insight into the components of the trans-membrane ferricyanide reductase that are exposed on the cytosolic membrane face, the effects of proteolytic enzymes and DTNB were assessed in resealed and leaky erythrocyte ghosts. It was not possible to use standard resealed erythrocyte ghosts in these experiments, since the agents to be studied would react directly with cytosolic components and hemoglobin that are still present during the resealing process. Therefore, hemoglobin- and cytosol-free resealed ghosts were prepared to contain either DTNB, trypsin or pronase. In preliminary studies, it was determined that ferricyanide reduction in clear resealed ghosts loaded with 4 mM ascorbate was linear for at least 5 min (results not shown), and this time taken for subsequent studies. Although DTNB had no effect on ascorbate-dependent ferricyanide reduction in intact cells (Table 2), it decreased this activity in resealed clear ghosts by about 50% (Fig. 6A). On the other hand, both pronase (Fig. 6B) and trypsin (Fig. 6C) tripled ascorbate-dependent ferricyanide reduction by the ghosts. Neither enzyme had any effects on ascorbate-dependent ferricyanide reduction in intact cells (results not shown). Furthermore, the proteolytic enzymes did not increase ascorbate leak out of the ghosts (results not shown), and this leak was corrected for in a paired sample as described in Section 2. Together, the results of Fig. 6 suggest that the ascorbate-dependent ferricyanide reductase activity of the erythrocyte membrane has sensitive protein components exposed on the cytoplasmic face of the cell membrane.

Different results were observed when leaky erythrocyte ghosts were treated directly with DTNB or trypsin, washed by centrifugation, and assayed for NADH-dependent ferricyanide and ferricytochrome *c* reductase activities. As shown in Fig. 7, both activities were inhibited about 50% by the same range of trypsin concentrations that stimulated ascorbate-dependent ferricyanide reduction in resealed ghosts (Fig. 6C). Low concentrations of DTNB were more potent in inhibiting both activities than were the same DTNB concentrations in inhibiting extracellular ferricyanide reduction (see Fig. 6A). This dichotomy in the effects of trypsin and DTNB in resealed and open ghosts strongly suggests that the ascorbate-dependent trans-membrane activity does not correspond to the major cytosolic NADH-dependent reductase.

4. Discussion

Ferricyanide reduction by erythrocytes and probably by nucleated cells is mediated by an as yet unidentified trans-membrane oxidoreductase activity. The present studies provide additional support for the notions that ascorbate is the major intracellular electron donor for this activity in erythrocytes and that a reactive exofacial sulfhydryl is required for activity. New findings regarding the ascorbate-dependent trans-membrane oxidoreductase are that it differs in several respects from basal or ascorbate-independent activity, that its activity is limited by intracellular recycling of ascorbate, that it is not involved in reduction of ferric iron, that it does not correspond to the cytochrome b_5 reductase system present on the endofacial membrane surface, and that it has exposed protein on its cytoplasmic face that contains one or more reactive sulfhydryl groups.

Erythrocytes have very low rates of ascorbate-independent ferricyanide reductase activity (Fig. 1). In cultured pulmonary artery endothelial cells [9] and HL-60 tumor cells [10] that contain little or no endogenous ascorbate, basal activity is also 10% or less of maximal ascorbate-dependent activity. Furthermore, NADH supported ferricyanide reduction in resealed erythrocyte ghosts to only a fraction of the rate observed for ascorbate, even with correction for leak of ascorbate out of the ghosts (Fig. 4). These results, and the finding of different inhibitory curves by PCMBS in basal and ascorbate-loaded cells (Fig. 3) support the previous conclusion derived from study of HL-60 cells [10] that these two activities are mediated by different proteins.

Ascorbate-dependent ferricyanide reduction was both enhanced and limited by the ability of the cells to recycle ascorbate. Although erythrocytes were able to maintain physiologic intracellular ascorbate concentrations (30–60 μM) in the face of ferricyanide treatment, at initial intracellular ascorbate concentrations of 140 μM and greater, ascorbate fell back to basal levels following even a short 5-min ferricyanide treatment (Fig. 1A, insert). This suggests that the trans-membrane ferricyanide reductase does not limit ferricyanide reduction over a physiologic range of ascorbate concentrations, despite apparent saturation with increasing initial intracellular ascorbate concentrations. Such considerations probably also apply to previous demonstrations of the saturability of ferricyanide reduction in response to increasing intracellular ascorbate concentrations in nucleated cells [9,10]. In other words, the capacity for ferricyanide reduction may depend more on the ability of cells to recycle ascorbate than on the ability of the cells to transfer electrons across the plasma membrane.

Although ascorbate may be the intracellular substrate for the trans-membrane oxidoreductase, the natural extracellular electron acceptor is unknown. A trans-membrane oxidoreductase has been implicated in reduction of both ferricytochrome *c* [21] and methemoglobin [22]. However, when efflux of ascorbate is taken into consideration, neither erythrocytes (Table 1) nor endothelial cells [9] reduce ferricytochrome *c*. A similar explanation probably also accounts for reduction of methemoglobin by erythrocytes over several hours of incubation at 37°C [22]. Erythrocytes did reduce ferric iron in an ascorbate-dependent manner, even after correction for ascorbate that had leaked from the cells (Table 1 and Fig. 2). A role for the trans-membrane ferricyanide oxidoreductase in transferrin-independent reduction and uptake of iron has long been a consideration [6]. Nevertheless, the rate of ferric iron reduction by erythrocytes was both saturable and very low compared to that of ferricyanide, and it was not inhibited by PCMBS. This makes it unlikely that the same activity mediates both ferricyanide and ferric iron reduction. We were recently unable to show that intracellular ascorbate enhances extracellular ferric iron reduction with the same assay in human monocytic U-937 cells, despite the presence of ascorbate-dependent ferricyanide reduction in those cells [23].

Previous studies in erythrocytes [7] and in HL-60 cells [10] showed inhibition of the trans-membrane ascorbate-dependent oxidoreductase by PCMBS, leading to the suggestion that this activity is mediated by a protein with one or more reactive exofacial sulfhydryls. On the other hand, PCMBS enters erythrocytes at appreciable rates on the anion transporter [18,19], so that the observed inhibition by PCMBS could be due to interaction with internal sulfhydryls. Nevertheless, our results support the original contention, since even after blockade of anion transport with DIDS, ferricyanide reduction was still decreased by 30–40% in intact erythrocytes (Table 2 and Fig. 3). Other reagents were either ineffective (DTNB),

highly penetrant (tetrathionate), or less specific in their interactions with the cells (DABS and TNBS). DABS has been shown to inhibit basal ferricyanide reduction in intact erythrocytes by about 35% [11], and we found a similar effect on ascorbate-dependent reduction. However, we also found that DABS severely depleted membrane α-tocopherol (Table 2). It is possible that loss of α-tocopherol caused part of the effect of DABS to inhibit ferricyanide reduction. For example, α-tocopherol incorporated into liposomes has been shown to mediate ferricyanide reduction by ascorbate trapped within the liposomes [24,25], and we found that α-tocopherol added to erythrocyte ghosts enhances trans-membrane ferricyanide reduction by ascorbate [20]. Nevertheless, the results with PCMBS do suggest the presence of a sensitive exofacial sulfhydryl on the trans-membrane enzyme.

Several findings in this study indicate that the ascorbate-dependent trans-plasma membrane ferricyanide reductase does not involve the ubiquitous NADH:ferricyanide reductase of isolated plasma membranes [1]. Most of the ferricyanide reductase activity of open erythrocyte ghost membranes is mediated by cytochrome b_5 reductase [26]. Both this enzyme [27] and its associated cytochrome b_5 [28] are restricted to the cytoplasmic membrane face and thus are not accessible to cell-impermeant reagents. We found that pretreatment of intact cells with PCMBS resulted in persistent inhibition of ascorbate-dependent ferricyanide reduction in resealed ghosts (Fig. 5), but failed to inhibit either ferricyanide or ferricytochrome *c* reductase activities in open erythrocyte ghost membranes when intracellular effects of PCMBS were prevented by DIDS (Table 3). Second, since NADH is the preferred substrate for cytochrome b_5 reductase [26], the low rates of NADH- compared to ascorbate-dependent ferricyanide reduction in resealed erythrocyte ghosts (Fig. 4) argue against NADH as an intracellular electron donor. Third, incorporation of proteolytic enzymes within resealed ghosts enhanced the trans-membrane ferricyanide reductase activity (Fig. 6), whereas direct treatment of leaky ghosts with the same range of enzyme concentrations decreased both ferricyanide and ferricytochrome *c* reductase activities (Fig. 7). Finally, DTNB treatment of open ghosts was more effective in blocking NADH-dependent ferricyanide and ferricytochrome *c* reductase activities (Fig. 7) than DTNB within resealed ghosts was in preventing ascorbate-dependent trans-membrane ferricyanide reduction (Fig. 6A). These results provide compelling evidence that the trans-membrane ascorbate-dependent ferricyanide reductase does not have the cytochrome b_5 reductase system as its intracellular component.

There remains the possibility that cytochrome b_5 reductase may participate in preserving ascorbate as a substrate for the trans-membrane enzyme. Ferricyanide generates intracellular AFR in intact erythrocytes as a result of a one-electron reduction mediated by the trans-membrane enzyme [29]. Microsomal cytochrome b_5 reductase has been shown to reduce the AFR to ascorbate in an NADH-dependent manner [30]. By reducing the AFR to ascorbate, the erythrocyte cytochrome b_5 reductase could help to recycle ascorbate and augment the activity of the trans-membrane enzyme. In this regard, we have previously shown that the effects of ascorbate and NADH are additive in enhancing ferricyanide reduction by resealed ghosts [8].

The cytoplasmic portion of the trans-membrane ascorbate-dependent ferricyanide reductase has exposed protein with a reactive sulfhydryl. Thus, proteolytic enzymes trapped within resealed ghosts enhanced extravesicular ferricyanide reduction (Fig. 6), but trypsin was without effect on this activity in intact cells. Similarly, when resealed within ghosts, DTNB caused up to a 50% inhibition of ascorbate-dependent ferricyanide reduction (Fig. 6A), but did not inhibit ferricyanide reduction by intact cells (Table 1). DTNB, which undergoes disulfide interchange reactions, is highly specific for protein sulfhydryls. These results suggest that the involved protein spans the plasma membrane, and that it has a reactive endofacial sulfhydryl that is required for full activity.

Of possible candidates for the trans-membrane oxidoreductase, negative results with DIDS in this work and with cytochalasin B in a previous study [7] rule-out the anion and glucose transporters, respectively. A trans-membrane NADH:ferricyanide reductase has been purified from human erythrocyte membranes [31]. This enzyme was found to differ from the cytochrome b_5 reductase with regard to size, kinetics, prosthetic group, and in the presence of about 6% glycosylation by weight [31]. The latter

finding supports the contention that this protein is a trans-membrane ferricyanide reductase, although there is no evidence that it can use ascorbate as an intracellular electron donor. As recently reviewed [32], the present results suggest that ascorbate-dependent ferricyanide reduction is mediated by such a trans-membrane protein, and that sulfhydryls on both membrane faces may aid in its purification and identification.

Acknowledgements

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