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ELECTRON TRANSFER REACTIONS OF CYTOCHROME *c* CONFINED WITHIN ERYTHROCYTE GHOSTS

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Summary

We have prepared and characterized resealed erythrocyte ghosts in which the only discernible pigment is cytochrome *c*. The resealed ghosts have the normal orientation and are free of 'leaky' species; they are stable and can be maintained at 4°C for many days without lysis.

The internal cytochrome *c* participates in redox reactions with both soluble and insolubilized cytochrome *c* present externally, and with external cytochrome b_5. No reaction was observed with plastocyanin, cytochrome *c* oxidase or NADPH-cytochrome *c* reductase.

A study has been made of the reaction of the internal cytochrome *c* with the low molecular weight reductants, ascorbate and glutathione. Complex kinetics are observed with both reagents: with ascorbate the results are best explained by assuming the existence, in the membrane, of a redox-active species able to undergo dedimerization. A protein bound disulfide bond would satisfy the requirement.

Introduction

The mechanisms whereby oxidation-reduction proteins undergo electron transfer is a topic of considerable interest and a substantial amount of information is now available on the reactions of small, well-characterized redox-active proteins such as cytochrome *c* with a variety of redox-active reagents including proteins and unphysiological but nonetheless informative reagents such as dithionite and potassium ferricyanide [1]. Cytochrome *c*, in particular, has

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Abbreviations: CM-52, microgranular carboxymethyl cellulose; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

been studied in a variety of ways and the quantitative aspects of its reaction with suitable redox active species is well documented [2]. Despite such intensive effort, however, the microscopic details of the process of electron transfer in this and other proteins are unclear; there are frequent suggestions for a special role of the protein in effecting electron transfer though there does not appear to be any unequivocal evidence for this viewpoint [3].

The situation becomes even more complicated when one considers that the natural habitat for many of these proteins is lipophilic, either embedded in or attached to a membrane. There has been relatively little attention paid to the properties of a physically well-defined system in which oxidant and reductant are separated by a lipophilic barrier, in contrast to the intensive spectroscopic and kinetic characterization of physiologically meaningful systems, in particular mitochondria and chloroplasts, for which the structural parameters are lacking.

In initiating an approach to this problem we looked for a system whereby an oxidation-reduction couple could be separated by a barrier of defined physiological dimensions. We originally planned to employ synthetic membranes made from phospholipids, but discovered objections to such systems, and eventually resorted to exploiting the property of erythrocyte membranes, the resealing phenomenon. When red blood cell ghosts are placed in a medium of appropriate tonicity they reform osmotically competent vesicles which contain as their internal volume the components of the resealing fluid. By introducing cytochrome *c* into the resealing fluid we have been able to prepare red blood cells in which the only discernible pigment is the cytochrome and in this paper we describe the preparation and characterization of these artificial cells and the reactivity of the entrained cytochrome *c* with a variety of redox active systems.

Materials and Methods

Materials

Type A+ human blood was obtained from the Institute for Hemotherapy, University of Texas Medical School, Houston, TX. Reduced nicotinamide-adenine dinucleotide (NADH), reduced nicotinamide-adenine dinucleotide phosphate (NADPH), cytochrome *c* (Type VI), serum albumin (bovine Fraction V), glutathione reductase, 5,5'dithiobis-(2-nitrobenzoic acid) (DTNB), dithiothreitol, Triton X-100, cholic acid, reduced (GSH) and oxidized (GSSG) glutathione, and cysteine · HCl were obtained from Sigma. The following were obtained as indicated: egg lecithin (Supelco), 8-anilino-1-naphthalene sulphonic acid and cyanogen bromide (Eastman), sodium ascorbate (Calbiochem), azolectin (Associated Concentrates), [1-^{14}C]ascorbic acid (New England Nuclear), and carboxymethyl-cellulose (Whatman, Inc.). All other chemicals were of the highest purity commercially available. Cholic acid was recrystallized from 50% aqueous ethanol and used as a 20% solution of its potassium salt. 8-Anilino-1-naphthalene sulphonic acid was recrystallized by the method of Azzi et al. [4]. Cobalt tris(*o*-phenanthroline) chloride was prepared according to Pfeiffer and Werdelmann [5].

Cytochrome b_5 and NADPH-cytochrome *c* reductase were prepared by trypsin extraction of calf liver microsomes by the method of Omura and Takesu

[6]. Plastocyanin was isolated by the method of Sato et al. [7].

Reduced cytochrome *c* was prepared by treatment of the protein with excess ascorbate followed by chromatography on Bio-Gel P-6; reduced cytochrome b_5 was made the same way, substituting dithionite for ascorbate and performing the chromatography anaerobically. This reduced protein was kept under an atmosphere of argon.

Methods

1. Quantitation

Protein was assayed according to Lowery et al. [8]. GSH was determined with DTNB [9] using an extinction coefficient of 13.6 $mM^{-1} \cdot cm^{-1}$. Both free cytochrome *c* and protein contained in resealed ghosts were determined spectrophotometrically from the difference in absorbance between the reduced and oxidized forms at 550 nm [10]. The hemoglobin content of the erythrocyte ghosts was determined from the optical difference spectrum generated from the dithionite-reduced form by the addition of CO using an extinction coefficient of 210 $mM^{-1} \cdot cm^{-1}$ for the peak at 420 nm. The NADH-cytochrome *c* reductase of erythrocyte membranes and the NADPH-cytochrome *c* reductase activity of microsomes were assayed by the methods of Steck and Kant [11] and Masters et al. [12], respectively. Oxidized glutathione (GSSG) was determined using GSSG reductase monitoring the oxidation of NADPH spectrophotometrically at 340 nm [13]. Cytochrome oxidase activity was assayed by the method of Smith and Conrad [14], superoxide dismutase by the method of Fridovitch and McCord [15], and catalase as described by Beers and Sizer [16]. All enzymatic activities were measured at 25°C and corrected for the rate in the absence of enzyme.

Cytochrome *c* was bound to Sepharose 6B by the method of Cuatracasas and Anfinsen [19]. The final product contained approx. 5 nmol of cytochrome *c* per ml of gel. The immobilized cytochrome *c* was reduced at 4°C in 0.1 M Tris-HCl, pH 7.6 (at room temperature), 0.1 mM EDTA, by exposure to a 60 watt incandescent light source (filtered through Pyrex heat absorbing glass) for 12 h. As judged by optical spectra of the gel suspended in 50% glycerol, reduction of the cytochrome *c* was at least 90% complete.

Anaerobic spectrophotometry was carried out as described by Palmer [17] and Lambeth and Palmer [18].

The fluorescence enhancement of 8-anilino-1-naphthalene sulphonic acid upon binding to membranes was measured by the method of Azzi et al. [4] using a Hitachi-Perkin Elmer MPF-2A spectrofluorimeter. Optical spectra were measured on a Cary 17 spectrophotometer and EPR spectra of the ghost preparation were recorded on a Varian E-6 spectrometer under the following conditions: 0.2 mwatt power at 21 K with 20 gauss modulation.

Stopped-flow spectrometry was carried out on a home-made apparatus. The instrument had a dead time of 2—3. 0 ms and was interfaced to a minicomputer for data acquisition and manipulation.

2. Preparation of erythrocyte ghosts

The following solutions were used in the preparation: 310 ideal milliosmolar sodium phosphate buffer pH 7.4 (buffer I); ideal milliosmolar sodium phosphate buffer pH 7.4 (buffer II); 2 parts of 0.9% NaCl plus 1 part of buffer I (medium I); 0.1 mM $CaCl_2$ in buffer II (medium II).

The isolation of the erythrocyte ghosts is a modification of the method of Dodge et al. [20]. All steps are carried out at 6—8°C. 15 ml of whole blood is mixed with 15 ml of 0.9% NaCl and centrifuged in a Beckmann J-20 rotor at 6000 rev./min ($2800 \times g$ at average radius) for 15 min. The supernatant is decanted carefully and the floating layer of white cells is removed by aspiration. The pelletted cells are then swirled with 30 ml of 0.9% saline and decanted into a clean tube, leaving behind any coagulated cells that may have formed. This step is repeated twice resuspending the cells in medium II in the first wash and buffer I in the second. Finally the cells are lysed by suspension in 30 ml of buffer II. After incubation on ice for 30 min the lysate is centrifuged at 17 000 rev./min ($23\,000 \times g$) for 30 min and the sediment resuspended by swirling in 30 ml of buffer II and recentrifuged at the same speed for 17 min. Resuspension and centrifugation are repeated at least 4 times or until the membranes are completely white. After each centrifugation it is essential that the resuspended material be transferred to a clean tube in order to avoid contamination of the ghost preparation with proteases shown by Steck and Kant [11] to be present in the hard, white, leukocyte pellet.

Erythrocyte ghosts prepared by this procedure are colorless and translucent. Hemoglobin cannot be detected by difference spectroscopy (deoxyhemoglobin ± CO at heme/mg protein. EPR spectra (X-band) of the oxidized or dithionite-reduced ghosts at 21 K are featureless indicative of a very low contamination with paramagnetic impurities such as iron(III) and copper(II). A signal at $g = 6$ in the oxidized state for high-spin ferric heme could only be observed after the ghosts were concentrated 30-fold by lyophilization and resuspension in a minimal amount of water. Altogether, these physical methods imply a very low contamination of the preparation by hemoglobin, its degradation products and other paramagnetic species.

Ghost protein at 10 mg/ml had no superoxide dismutase activity (less than 1% inhibition of the rate of cytochrome *c* reduction), and possessed no detectable catalase or cytochrome oxidase activity.

Resealing of erythrocyte ghosts

Resealing of the erythrocyte ghosts was performed according to the method of Bodemann and Passow [21].

The quality of the resealing was determined using the criteria and methods of these workers [21].

The rate of spontaneous lysis of the preparation was estimated by incubating a known amount of cells (0.5 mg ghost protein/ml, 12.5 μM cytochrome *c*) in medium II at 4°C. Samples were removed at various times, centrifuged and the supernatants analyzed for cytochrome *c* content spectrophotometrically. The maximum amount of lysis observed was less than 5% per week.

Preparation of cytochrome c: lipid dispersions

0.1 g of azolectin was homogenized in a teflon-and-glass homogenizer with 14 mg of cytochrome *c* in 1.6 ml of 20 mM sodium phosphate 1 mM EDTA pH

7.4 and the suspension transferred to a 15-ml glass centrifuge tube and capped with a serum stopper. The stopper was pierced with a syringe needle connected to a vacuum/argon manifold and the contents of the tube made anaerobic as already described. The anaerobic suspension was placed in 5 cm of water in a Branson Sonifer cleaning bath and sonicated at 4°C for 150 min. Exogenous cytochrome *c* was then removed by passage of the sample through a 1-ml syringe column of CM-52 cellulose equilibrated with the same buffer.

Egg-lecithin liposomes were prepared and sized on Sepharose 4B in medium II according to Huang [22]. The phospholipid concentration (organic phosphorus) was determined by the method of Bartlett [23].

Radioisotope counting

[1-^{14}C]Ascorbic acid was dissolved in medium II and standardized spectrophotometrically from the reduction of potassium ferricyanide (A_{420nm} = 1.02 $mM^{-1} \cdot cm^{-1}$ [24]). Aliquots were diluted to 5 mM, sealed under argon and stored at −20°C. Before an experiment, the [1-^{14}C]ascorbic acid solution was diluted with unlabelled sodium ascorbate and added to the assay.

Up to 0.1 ml samples of whole resealed erythrocytes or ghosts were added to 10 ml of scintillant (toluene solution: 5.5 g PPO/liter, 0.1 g POPOP/liter and 333 ml Triton X-114/liter) and automatically counted to 0.2% error.

Results

Erythrocyte ghosts resealed in the presence of cytochrome *c* are red and possess the normal discoid shape when viewed by phase contrast microscopy. To determine the fraction of cells with normal right-side-out orientation, the preparation was assayed for a marker enzyme of the inner half of the bilayer, NADH-cytochrome *c* reductase [2], before and after lysis with 0.1% potassium cholate. In the absence of detergent, less than 0.01% of the total activity could be detected whereas unresealed ghost protein had the same specific activity as the detergent lysed preparation. These data indicate that greater than 99.9% of the resealed cells have normal orientation.

To test whether cytochrome *c* is bound to the exterior of the ghost membrane to any significant degree, resealed cells (0.5 mg ghost protein/ml) were incubated for 12 h at 4°C in the presence of 1 mM cytochrome *c*, centrifuged, the pellet washed three times with a 10-fold excess of buffer and then analyzed for cytochrome *c* content by difference spectroscopy; less than 0.5% of the cytochrome entrapped by the ghosts during resealing could be detected in the washed cells.

The possibility that the cytochrome *c* might be inserted into the membrane, was studied using the method developed by Azzi et al. [4].

Preparations of unresealed ghosts, albumin-sealed ghosts and ferricytochrome *c*-sealed ghosts were incubated with 10 μM 8-anilino-1-naphthalene sulphonic acid and fluorescence emission spectra recorded before and after the addition of ascorbate. The emission maximum in the presence of ghosts, 470 nm, was unaffected by the presence or absence of reduced or oxidized cytochrome *c*. Whereas this evidence is not conclusive, it implies that an 8-anilino-1-

naphthalene sulphonic acid-detectable protein : lipid interaction does not occur between cytochrome *c* and the red cell membrane.

Washing the lysed cells with buffer II can remove cytochrome *c* from the membranes until it is below the limit of detection (0.025 nmol cytochrome *c*/mg protein). It thus seems that cytochrome *c* has been entrapped in a relatively stable system, behind a membrane of known characteristics, free from unwanted contaminants.

Reactions of the entrained cytochrome c with redox proteins

The reactivity of the cytochrome was probed with various impermeable oxidants and reductants, the first class of which were proteins. In the following experiments, the standard concentration of cytochrome *c*-sealed ghost preparation used was 0.5—0.8 mg ghost protein/ml, giving an average concentration of 12 μM cytochrome *c* in the final solution. At 25°C the internal protein is reduced by the exogenous ferrocytochrome with kinetics that are reasonably first order (Fig. 1); however, at 4°C, the kinetics deviate from first order as indicated by the nonlinear semi-logarithmic plot which exhibits the convex curvature of a second-order reaction (Fig. 1). The half-life for the reduction at 25°C varies from 30 to 60 min depending on the batch of erythrocytes; this corresponds to a second-order rate constant of from 0.38 to 0.76 $M^{-1} \cdot s^{-1}$. The reaction is insensitive to the presence of a CO atmosphere (up to 2 mM CO in solution), and is unaffected by pretreatment of the cells (at 5 mg ghost protein/ml, 125 μM cytochrome *c*) with 1 mM *N*-ethylmaleimide, followed by multiple washes to remove the excess reagent. This implies that neither the residual high spin ferric-heme nor membrane sulphydryl groups can account for the reduction. If high spin ferric-heme were an intermediate, it would bind CO as soon as it became reduced and become non-reactive. Likewise, alkylation by *N*-ethylmaleimide should remove active sulphydryls if indeed they are involved. This

TABLE I

THE OXIDATION REDUCTION OF CYTOCHROME *c* CONFINED WITHIN RED BLOOD CELL GHOSTS WITH EXTERNAL OXIDO-REDUCTANTS

cyt, cytochrome; NEM, *N*-ethylmaleimide.

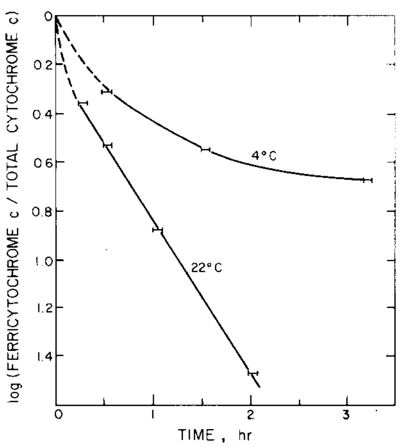
Outside		Inside	Half-time for reaction ($t_{1/2}$)
1 cyt c^{2+}	(argon)	cyt c^{3+}	30—60 min
	(CO)		30—60 min
	(NEM pretreatment)		30—60 min
2 cyt c^{3+}		cyt c^{2+}	30—60 min
3 cyt c^{2+}	Sepharose *	cyt c^{3+}	30—60 min
4 cyt b_5^{2+}		cyt c^{3+}	300 min
5 plastocyanin^{2+}		cyt c^{2+}	no detectable reaction
6 cyt *c* oxidase		cyt c^{2+}	no detectable reaction
7 NADH		cyt c^{3+}	no detectable reaction
8 NADH-cyt *c* reductase		cyt c^{3+}	no detectable reaction

* One ml of ferricytochrome *c*-sealed ghosts (average 20 μM cytochrome (cyt) *c* in final solution) was applied to an 0.8 × 10 cm Sepharose 6B column to which cytochrome *c* had been covalently coupled, reduced, and equilibrated with medium II. The column was slowly eluted with medium II, the resealed cells appearing after 30 min of exposure to the Sepharose. The amount of reduction of the cytochrome, quantitated by difference spectroscopy, was found to be approx. 60%.

redox reaction appears to be quite reversible and external, oxidized cytochrome *c* could be reduced by the internal ferrocytochrome with approximately the same half-time as observed for the reaction in the reverse direction.

In addition to the reaction of the entrapped protein with external soluble cytochrome, reduction by external insoluble reductant has also been observed. Using a column technique ferricytochrome *c*-sealed ghosts were reacted briefly with ferrocytochrome *c* covalently attached to Sepharose (Table I).

After 30 min of exposure of the ferricytochrome *c*-sealed ghosts to the insolubilized reductant approx. 60% of the soluble protein was reduced. The reduced, soluble cytochrome *c* was still confined to the erythrocyte ghosts as the degree of cell lysis was established to be very much less than 1%. This was concluded from two observations. Firstly the partially reduced cells were sedimented and the supernatant examined for soluble protein; less than 0.1% was detected. Secondly, all of the cytochrome *c* eluted with the intact cells in the void volume of the column and there was no detectable color migrating more slowly than the cells. Due to technical difficulties, quantitation of the kinetics with the insoluble cytochrome *c* has not been attempted; however the similarity of the degree of reduction of the entrapped cytochrome to that observed over a similar time in the reaction of the cells with soluble ferrocytochrome *c* suggests that both reactions may have a similar rate determining pathway. In the reaction with the Sepharose-cytochrome *c*, the external reductant is unambiguously impermeable; leakage of the cytochrome from the gel could not be detected during control runs using an equal volume of either medium II or albumin-sealed cells in place of the cytochrome *c*-sealed ghosts.



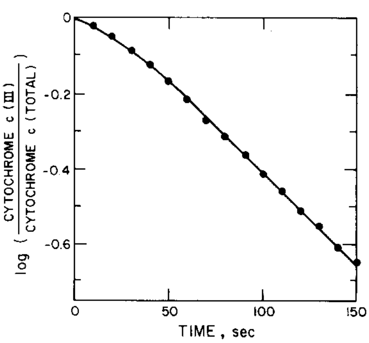


Fig. 1. The reduction of entrapped cytochrome *c* (12 nmol cytochrome *c*/ml and 0.5 mg ghost protein/ml) by 0.5 mM exogenous ferrocytochrome *c*: 4°C (upper trace) and 25°C (lower trace). Cytochrome *c*-sealed ghosts were incubated anaerobically with 0.5 mM reduced cytochrome *c* for various times and temperatures. The reaction was stopped by centrifugation of the material followed by washing the cells 3 times with a 10-fold excess of medium II. The final pellets were resuspended in a known volume of medium II and the degree of reduction of the entrapped cytochrome *c* determined spectrophotometrically.

Fig. 2. First-order plot of the reaction of ascorbate (10 mM) with 12 μM internal cytochrome *c* as determined spectrophotometrically. The concentration of ghost protein was 0.5 mg/ml. The reaction was carried out at 25°C in medium II.

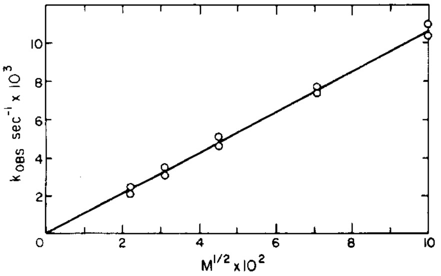
There is a limited specificity associated with the reaction as indicated by the varying degrees of oxidation or reduction obtainable with other macromolecules (Table I). In each case, the concentration of the external protein and the conditions of the reaction were identical to those used for the reduction of the entrapped protein by soluble ferrocytochrome *c*. Of all the other proteins tested only ferrocytochrome b_5 was able to reduce the entrapped cytochrome *c*. Again the kinetics were first order; however the half-time for the reaction was 10 times longer than that observed with a comparable concentration of ferrocytochrome *c*.

Neither cytochrome oxidase nor oxidized plastocyanin were able to accept electrons from cells which had been resealed in the presence of ferrocytochrome *c*. With cytochrome oxidase, the reaction was carried out under both aerobic and anaerobic conditions without any difference observed. In both cases the reaction is not limited thermodynamically as there is a difference of 100 mV in the midpoint potentials between cytochrome *c* and plastocyanin, while in the case of cytochrome oxidase oxygen is the ultimate acceptor. The microsomal enzyme, NADPH-cytochrome *c* reductase, in the presence of 10 mM NADPH, was unable to reduce the heme-protein in 14 h (<0.1% reduction of cytochrome *c*). However, upon lysis with detergent (0.1% potassium cholate), the internal cytochrome was completely reduced within seconds.

Reactions of internal cytochrome c with ascorbate and glutathione

Ascorbate

The reaction of cytochrome *c*-sealed cells with excess ascorbate is pseudo-first order for five half-lives over the range of ascorbate concentrations from 0.5 mM to 10 mM (Fig. 2). Each first order reaction is preceded by a small, finite lag. The observed rate constant (k_{obs}), is an order of magnitude slower than for the corresponding solution reaction and is a nonlinear function of the ascorbate concentration (Fig. 3); a double reciprocal plot of the data is also nonlinear. Thus the reaction of cytochrome *c* in the erythrocyte with the exogenous ascorbate does not conform to the solution reaction. However, k_{obs} is seen to be a linear function of the square root of the ascorbate concentration,



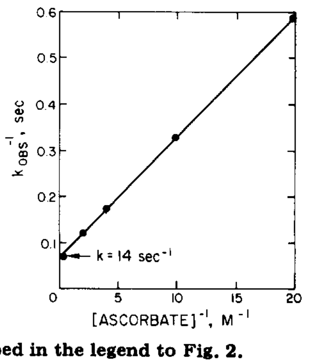


Fig. 3. The dependence of k_{obs} on [ascorbate]$^{1/2}$ for the reaction described in the legend to Fig. 2.

Fig. 4. Hyperbolic dependence of the reaction of ascorbate with 12 μM free cytochrome *c* at 25°C in medium II. The data are plotted as $1/k_{obs}$ vs. $1/$[ascorbate].

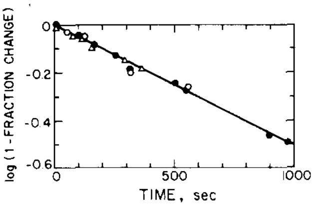
suggesting that a de-dimerization reaction is initially rate limiting to the reduction of the entrained cytochrome (Fig. 3). As is observed with the reductant ferrocytochrome *c*, reaction of ascorbate with the entrapped protein is insensitive to CO or to pretreatment of the cells with *N*-ethylmaleimide.

The solution reaction of cytochrome *c* with excess ascorbate was studied in medium II over the range of 47 μM to 500 mM ascorbate; k_{obs} depends hyperbolically on the ascorbate concentration (Fig. 4), with a second-order rate constant of 40 $M^{-1} \cdot s^{-1}$, and a limiting first order rate of 14 s^{-1}.

There are two mechanisms for the reduction of cytochrome *c* by the external ascorbate: (1) diffusion of the ascorbate into the red cell ghost or (2) interaction of ascorbate with a compound in the membrane that in turn reacts with the internal cytochrome. In order to quantify the uptake of ascorbate from the external fluid, the efflux of ascorbate from the ghosts into the medium, and the dependence of the equilibrium, $[\text{ascorbate}]_{in}/[\text{ascorbate}]_{out}$ on ascorbate concentration was studied using [1-^{14}C]ascorbic acid. The specific activity of the ascorbate used in each experiment was adjusted with unlabelled ascorbate in order to obtain a difference of at least 5000 cpm between the lowest detected ascorbate concentration and the highest.

The uptake of radioisotope from the solution into the cells was assayed by mixing resealed ghosts at 1 mg ghost protein/ml (25 μM cytochrome *c* average in solution) with a known concentration of ascorbate. Samples were removed at various times, centrifuged and a portion of the supernatant counted. The initial point was obtained by counting an aliquot of the mixture prior to centrifugation correcting for the volume occupied by the cells. This volume could be derived by adding a known amount of the impermeant, cytochrome *c*, to a suspension of cells followed by centrifugation and quantitation of the concentration of cytochrome *c* in the supernatant, and a parallel sample run in the absence of cells.

Efflux of ascorbate from the cells into the medium was studied by an analogous method. The resealed ghosts were incubated as just described for 6 h in order to achieve equilibrium with respect to the ascorbate. The cells were then



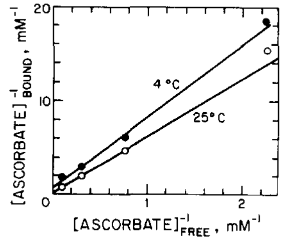


Fig. 5. The first-order behavior of the influx and efflux of ascorbate to and from cytochrome *c*-sealed erythrocyte ghosts. (●) 0.5 mM ascorbate, efflux: (○) 1 mM ascorbate, influx; (△) 1 mM ascorbate, efflux. Cytochrome *c* was present at an average concentration of 25 μM (1.0 mg ghost protein/ml) and the reactions performed at 25°C in medium II.

Fig. 6. Saturation of the binding of ascorbate to cytochrome *c* resealed ghosts at two temperatures. 1 mg/ml of resealed ghosts were equilibrated for six hours with increasing concentration of [1-^{14}C]-ascorbate in medium II at either 4 or 25°C. Total radioactivity was determined on a sample withdrawn at the end of the incubation period. The reaction mixture was then centrifuged and the radioactivity of the supernatant measured.

centrifuged and resuspended in a known volume of medium II. At various times, samples were removed, centrifuged, and the supernatants analysed for radioactivity. The influx and efflux measurements both yield a k_{obs} of $1.2 \pm 0.1 \cdot 10^{-3}$ s^{-1} at 0.5 mM and 1 mM ascorbate (Fig. 5). Although this is good evidence in favor of an equilibration of ascorbate across the erythrocyte membrane, it does not rule out a slow binding phenomenon.

The equilibrium for ascorbate 'binding' was assayed by incubating resealed ghosts (1 mg ghost protein/ml) with different concentrations of ascorbate for 6 h, the time necessary to achieve equilibrium. The samples were centrifuged the supernatants counted and the concentration of ascorbate in the supernatant was corrected for volume lost to the cells. The total ascorbate concentration in the solution before removal of the cells was determined by counting known amounts of the samples before centrifugation. The data obtained from the distribution experiments indicate that the process is saturable. A plot of $1/[\text{ascorbate}]_{\text{bound}}$ vs. $1/[\text{ascorbate}]_{\text{free}}$ gives a straight line implying a hyperbolic dependence of these two parameters at the two temperatures studied (Fig. 6). This saturation behavior indicates that the equilibrium of ascorbate across the membrane, if it does indeed occur, must be carrier-mediated. The data are also consistent with a slow binding of ascorbate to the resealed vesicles.

The data obtained from the ascorbate reduction of cytochrome *c*-sealed vesicles have been simulated using a digital computer by numerical integration of the appropriate differential equations. No simple mechanism explains the experimental result.

However the data is well described by a mechanism that requires the existence of the membrane of species capable of accepting 2 electrons in the oxidized state and dedimerizing to two, independent, one-electron donors when reduced. One possible candidate is an inter-peptide disulphide bond. Ascorbate reduction would produce two independent reactive sulphydryls, each capable of reducing cytochrome *c*. The data would be consistent with the results from pretreatment of the cells with *N*-ethylmaleimide since this reagent cannot alkylate the disulphide, and hence cannot inhibit the reaction.

Glutathione

Three thiol compounds were tested for their reactivity toward the entrained cytochrome; these were cysteine, glutathione and dithiothreitol. In each case, 100 μM thiol reduced cytochrome *c* in a first-order process with a half-time of approx. 20 min. The glutathione reaction was subjected to further characterization because the erythrocyte membrane is known to be impermeable to GSSG [24,25] and assumed to be impermeable to GSH. In addition, the disulphide (GSSG) is actively extruded into the surrounding medium by the intact erythrocyte [25]. The permeability of the ghost membrane to oxidized glutathione was tested. Oxidized glutathione was incorporated into the cells by resealing. After the incubation at 37°C and subsequent centrifugation, the ghosts were resuspended in a known volume of medium II, aliquots removed, centrifuged and the supernatants assayed for oxidized glutathione using glutathione reductase. The amount of the disulphide in the external medium reaches a stable level which was 30% lower than that obtained upon lysis indicating

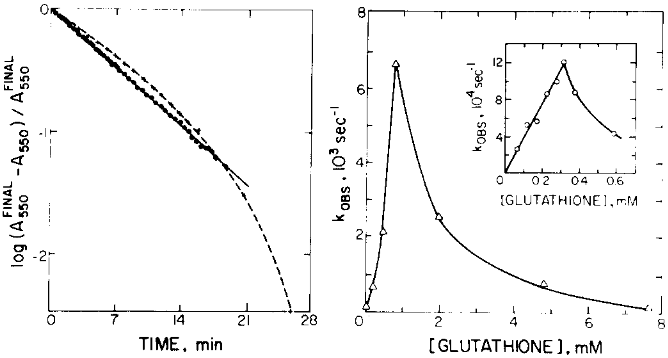


Fig. 7. (Left) First-order plots of the reaction of glutathione with cytochrome *c*: (•———•) 0.3 mM glutathione with 12 μM cytochrome *c*, sealed in erythrocyte ghosts and (+------+) 0.1 mM glutathione with 12 μM cytochrome *c*. (Right) Concentration dependence of k_{obs} on glutathione for soluble cytochrome *c* and for cytochrome *c*-sealed cells (inset). The reactions were performed in medium II at 25°C.

that the preparation has become impermeable to the GSSG upon resealing. In solution, glutathione reduction of cytochrome *c* is autocatalytic with a continuous downward curvature of the semilogarithmic plot (Fig. 7). By contrast the reaction of reduced glutathione with cytochrome *c* in resealed ghosts is quite exponential with linear first-order plots proceeding over four half-lives (Fig. 7). However the observed rate is somewhat slower than that observed with both reagents in solution. The observed first-order rate constant exhibits an extremely nonlinear dependence on glutathione concentration with the rate reaching a maximum at 1 mM, decreasing at higher concentrations of reductant. A qualitatively similar concentration dependence was observed with soluble cytochrome *c*. However, the quantitation of these effects varies with the preparation of glutathione and has thus not been pursued further. Neither reaction exhibits more than a 2-fold sensitivity to EDTA suggesting that contamination by Cu^{2+} is minimal in our reagents (cf. Ref. 26).

Low molecular weight oxidants

Both potassium ferricyanide and cobalt tris(*o*-phenanthroline) chloride will oxidize the reduced entrained cytochrome. When solid oxidants are added, the half-lives of the reactions are typically 20 to 30 min. These slow kinetics are only slightly faster than the rate of ferricyanide oxidation of hemoglobin in intact cells (Olson, J.S., unpublished data). Because of the lack of permeability data on the oxidants, detailed kinetic characterization was not attempted.

Reactions of cytochrome c trapped between phospholipid bilayers

To test the hypothesis that the anomalous behavior of the entrained cytochrome may result from a property common to all lipid vesicles it was necessary to trap large amounts of cytochrome *c* within a lipid matrix.

Initial experiments involved attempts to trap cytochrome *c* inside single bilayer lecithin vesicles prepared by the sonication method of Huang [22]. Unfortunately, chromatography on Sepharose 4B [22] revealed that the liposomes (more accurately lipid phosphorus) migrated within the fractionation range of the gel whereas the cytochrome *c* emerged much later as a low molecular weight peak. Despite variations in the buffers, lipid : protein ratio, and sonication conditions, cytochrome *c* could not be entrapped by this procedure.

A successful preparation was achieved using the method Yamashita et al. [27,28] through sonication of a buffered solution of cytochrome *c* and azolectin. The formation of superoxide and its subsequent reduction of cytochrome *c* was avoided by carrying out the sonication under an argon atmosphere. Yamashita et al. [27] have noted that this dispersion produces concentric lamallae that trap cytochrome *c* between the bilayers. This was also suggested by the results of chromatography of the vesicles on Sepharose 4B [22]. The cytochrome and the lipid eluted at the void consistent with the aggregated nature and size expected for lamellar material.

The lipid dispersion, 0.1 mM in ferricytochrome *c*, was mixed with 1 mM ferrocytochrome *c* and allowed to incubate overnight (14 h) at 22°C. After passage of the reaction mixture over a column of CM cellulose to remove the extent of the reduction of the internal cytochrome *c* was quantitated by difference spectroscopy. Less than 8% of the total cytochrome *c* of the dispersion became reduced: the same amount of cytochrome *c* was found to be accessible to ascorbate. In contrast to the resealed ghosts, the vast majority of the cytochrome cannot be reduced by external reductants, implying that there is a particular activity associated with the red cell membrane which gives it its unusual characteristics with respect to the reduction of cytochrome *c*.

Discussion

The reactivity of redox active proteins, particularly cytochromes, the blue (Type 1) copper proteins, and ferredoxins with a variety of oxidants and reductants has been examined by a number of laboratories in the past several years. The data accumulated so far allows a number of useful conclusions. The most significant of these is that the requirements of the Marcus Theory for outer sphere electron transfer are not met by most biological redox systems and that there is a substantial measure of reactivity which emanates from the specificity of interaction between oxidant and reductant. Furthermore, there are a number of systems in which the redox active center is completely insulated from the environment by the protein and in these cases one most ask whether or not 'long range' electron transfer can occur, and whether or not the protein per se can have any role in mediating the transfer process.

The tactic of compartmentalizing the partners of a redox reaction using a phospholipid membrane offers possibilities of examining aspects of electron transfer processes not feasible by experiments carried out in homogenous solution.

Our original objective was to encapsulate cytochrome *c* in lecithin liposomes, however it became apparent that the internal volume of these micelles was too small to entrap appreciable amounts of protein. Based on Huang's parameters [23] and the concentration of cytochrome *c* in solution, one could only hope to obtain a maximum of 0.4 mol of cytochrome *c* in each vesicle.

Our solution was to exploit a property of the red blood cell which had been thoroughly documented from the work of Dodge et al. [20], Morrison et al. [30], Steck and Kant [11] and Bodeman and Passow [21]. When the stroma (or red blood cell ghosts), obtained from the lysis of red blood cells are returned to an isotonic medium containing appropriate additional ions corpuscles are reformed with an internal fluid having the composition of the medium in which this process is effected. This phenomenon is called 'resealing' and the reformed corpuscles are called resealed ghosts. Using established principles it is possible to obtain in high yield vesicles with the membrane preserving its original sidedness and exhibiting full osmotic competence: These are called Type 1 ghosts in the nomenclature of Bodemann and Passow.

The ghosts that we prepare have no detectable color either visually or spectrophotometrically indicating minimal contamination by hemoglobin, S-protein (a *P*-420 homolog?) and erythrocyte catalase. From measurements of enzyme activity we find only negligible amounts of catalase, superoxide dismutase and cytochrome oxidase. Exceptionally concentrated samples exhibit a $g = 6$ EPR signal consistent with the presence of a high-spin ferrihemoprotein, possibly methemoglobin.

Resealed ghosts prepared in the presence of cytochrome *c* appear to have the normal orientation and are free of the 'leaky' Type III species as no NADH-cytochrome *c* reductase activity is observed until the resealed ghosts are destroyed by detergent. This observation was made using both internal and external cytochrome *c* indicating that NADH does not cross the red blood cell membranes. The cytochrome *c* containing resealed ghosts, are quite stable and can be maintained many days at 4°C without lysis.

Cytochrome *c* has thus been trapped behind a membrane of defined composition and probed for reactivity using both protein and low molecular weight oxidants and reductants. The reaction of the internal cytochrome with external ferrocytochrome *c* is a first order process at 22°C, however the reaction is markedly inhibited at lower temperatures. This may indicate that diffusion of a mediating compound within the membrane is the rate-limiting step. Lowering the temperature would tend to inhibit such movement. An example of this type of temperature behavior is found in the transfer of electrons between cytochrome b_5 and cytochrome b_5 reductase across the surface of the microsomal membrane [29].

A similar reduction of the entrapped cytochrome by the impermeable agarose-bound ferrocytochrome has also been observed. The limited specificity of the reaction has been demonstrated using other proteins as reductants and oxidants.

The reaction of the entrained cytochrome with ascorbate has been extensively characterized and a preliminary simulation undertaken. It appears that over the course of the experiment, ascorbate either binds weakly or is transported by a carrier into the erythrocyte. The data can be accurately fitted by assuming that the external ascorbate is in rapid equilibrium with a species in the membrane that is capable of de-dimerization into the monomer reductant of the internal cytochrome. A likely candidate for this membrane species is a disulphide linkage between two membrane proteins.

The reaction of glutathione with the entrapped cytochrome is not so easily explained. The plot of k_{obs} vs. glutathione concentration indicates that it has a similar substrate dependence to the reaction observed in solution, however the lack of autocatalysis indicates that the rate-limiting steps are different. Whether or not this is due to an interaction with a membrane component cannot be decided at this time.

It would appear that there are undefined species present in the membrane that are capable of transferring reducing equivalents across the bilayer. These components react with certain metalloenzymes such as cytochrome *c* and cytochrome b_5, and not at all with others (plastocyanin). Enzymes that use cytochrome *c* as a substrate do not reduce the entrained protein at all (cytochrome oxidase, NADPH-cytochrome *c* reductase).

In the case of ascorbate reduction of the entrained protein, evidence is presented that a candidate for the intermediate is a disulphide. However in the case of the redox-protein oxidants and reductants, the nature of the intermediate remains unclear. If this is a natural function of the red cell membrane, the kinetics with natural substrates may be much faster than observed here.

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