Sealed Reticulocyte Ghosts

AN EXPERIMENTAL MODEL FOR THE STUDY OF Fe** TRANSPORT*

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We found that sealed right-side-out reticulocyte ghosts transported and accumulated Fe** in a process that was concentration and time-dependent and presented all the characteristics of being membrane-mediated.

We estimate that sealed reticulocyte ghosts are a very good model to determine the basic characteristics of membrane iron transport.

EXPERIMENTAL PROCEDURES

Reticulocyte Sealed Ghosts—Reticulocyte-rich rabbit red cells (30-80% reticulocytes) were lysed with 20 volumes of 5 mM sodium phosphate (pH 8.0) and sedimented for 20 min at 35,000 × g (Steck and Kant, 1974). The stromal pellet was washed three times with 20 volumes of lysate buffer and the plasma membrane fraction was purified by sucrose density centrifugation as described (Lodish and Small, 1975; Núñez and Glass, 1983). The material banding in the 20-40% sucrose interface was collected, suspended in distilled water, washed three times by centrifugation and stored at -20° C. Purified ghosts were pelleted and sealed by incubation for 30 min at 37° C with 50 mM NaCl, 50 mM KCl, 1 mM MgSO4, 20 mM MOPS* (pH 7.0). The sealed ghosts were repeatedly washed with saline buffer: 50 mM NaCl, 50 mM KCl, 20 mM MOPS* (pH 7.0).

Microscopy—Sealed or unsealed ghosts were fixed for 60 min at room temperature in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After fixation, the ghosts were rinsed with phosphate buffer, mounted under 30% (v/v) acetone on aluminum plaquettes covered with 1% gelatine, gradually dehydrated with acetone, and dried to critical point with liquid CO2. The samples were then coated with a 20-40% sulfo-sulfonic acid)-1,2,4-triazine.

The abbreviations used are: MOPS, 3-(N-morpholino)propanesulfonic acid; ferrozine, 3-(2-pyridyl)-1,2,4-bis(phenylsulfonic acid)-1,2,4-triazine.
with 10 μM 59Fe, 200 μM ascorbate in saline buffer (pH 7.0). The 59Fe-loaded ghosts were divided into aliquots and centrifuged for 10 min at 25,000 × g. 59Fe efflux was initiated by resuspending aliquots of the ghosts in saline buffer containing 200 μM ascorbate (pH 7.0). At specified times, the reaction was stopped by filtration as described above.

Reagents—Solutions were prepared with distilled deionized water further treated with Chelex-100 (Bio-Rad) to remove traces of divalent metals. Similarly, stock solutions of monovalent salts and buffers were routinely filtered through Chelex-100. 59Fe, as FeCl3 in 0.5 M HCl, was from Du Pont.

RESULTS

Morphology of Sealed Reticulocyte Ghosts—Sealed ghosts presented a distinctive morphology when compared with unsealed ghosts (Fig. 1). In scanning electron microscopy and in light microscopy, the sealed ghost showed the typical biconcave morphology of red cells with a diameter of 1–2 μm, whereas the unsealed ghost showed the appearance of empty bags. Substrate accessibility to glyceraldehyde-3-phosphate dehydrogenase and cytochrome c oxidoreductase indicated enzyme latencies of 89 and 98%, respectively. These values are in good agreement with published values for sealed erythrocyte ghosts (Steck and Kant, 1974).

59Fe Uptake by Ghosts—Reticulocyte ghosts incorporated Fe2+ as a function of incubation time with pseudo-first order kinetics (Fig. 2). In agreement with whole cell experiments (Egyed, 1988; Morgan, 1988), ghosts derived from erythrocytes did not incorporate iron (Fig. 2). The sealed reticulocyte ghosts effectively concentrated iron; considering an internal volume of 12.5 μl/mg of protein (see “Experimental Procedures”), the concentration of iron in the ghosts at equilibrium was 40–150-fold higher than the iron concentration in the incubation medium. The accumulation capacity of the sealed reticulocyte ghosts, expressed as moles of iron/mg of protein, was proportional to the reticulocyte content of the original blood.

Since a significant iron accumulation was observed, at least two processes have to be considered: the transport of iron into the intravesicular space and the sequestration of the transported iron in order to maintain the concentration gradient necessary for further transport. The observed kinetic behavior will be primarily a reflection of the slower process. To determine if the observed kinetics reflect the transmembrane iron transport process, 59Fe uptake was measured in the presence of the ionophore A23187, an effective membrane transporter of Fe2+ (Young and Gomperts, 1977; Núñez and Glass, 1983). If the membrane transport process is rate-limiting, the addition of extra transporters should increase the rate of 59Fe accumulation by the ghosts. A23187 (0.3 μM) increased by 1.7-fold the rate constant for 59Fe accumulation by reticulocyte ghosts, with a slight increase (6.7%) in the maximal accumulation (Fig. 2). Larger increases in the rate

FIG. 1. Microscopy of reticulocyte ghosts. Shown is the morphology of sealed (A and C) and unsealed (B and D) reticulocyte ghost when examined by scanning electron microscopy (A and B) or light microscopy (C and D) as described under “Experimental Procedures.”
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External salt

FIG. 4. Anion selectivity in supporting Fe\textsuperscript{2+} uptake. Ghosts were sealed with 100 mM KCl, 20 mM MOPS-Tris (pH 7.0) and pelleted. The time course of \textsuperscript{59}Fe\textsuperscript{2+} uptake was measured immediately after resuspension of the ghosts with buffers composed of 20 mM MOPS-Tris (pH 7.0) and: (a) 100 mM KCl, (b) 100 mM KNO\textsubscript{3}, (c) 100 mM KBr, (d) 80.7 mM K\textsubscript{2}SO\textsubscript{4}, or (e) 100 mM KI. Shown is the maximal amount of \textsuperscript{59}Fe accumulated by the ghosts for each external salt used.

FIG. 5. Effect of external iron on \textsuperscript{59}Fe efflux. Ghosts were loaded with \textsuperscript{59}Fe as described under "Experimental Procedures." Aliquots of the loaded ghosts were suspended in 1 ml of saline (pH 7.0) prewarmed at 25 °C and containing 200 \mu M ascorbate and FeCl\textsubscript{3} at: 10 \mu M (C), 25 \mu M (O), 50 \mu M (Δ), or none (▲). The amount of \textsuperscript{59}Fe remaining in the ghosts was measured as a function of the incubation time, and the resulting curves were fitted to a single exponential decay. In the experiment shown, the rate constants were 1.31 \times 10^{-3} s\textsuperscript{-1} in the absence of iron and 2.56 \times 10^{-3} s\textsuperscript{-1} with 25 or 50 \mu M iron in the external solution.

constant were observed at higher A23187 concentrations. In contrast, sealed erythrocyte ghosts did not transport iron, although in the presence of A23187 they showed a considerable uptake of \textsuperscript{59}Fe\textsuperscript{2+} (Fig. 2). These results indicate (a) that erythrocyte ghosts have lost the membrane iron transport system, (b) that the increase in the maximal amount of iron accumulated by reticulocyte ghosts produced by A23187 can be ascribed to the recruitment of erythrocyte ghosts, and (c) that although reticulocyte ghosts accumulate iron and reach internal concentrations well above the external concentration, the measured time courses reflect the kinetics of the transmembrane iron transport.

The \( K_a \) for the incorporation of Fe\textsuperscript{2+} (Fe\textsuperscript{2+}-ascorbate), determined from initial rates (Jacquez, 1980), was 1.1 \times 10^{-6} M (data not shown).

Effect of Temperature on the Rate Constants of Influx and Efflux—Transport processes through biological membranes often present break points in their rates values as a function of temperature. This criterium is used to differentiate trans-

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TABLE I

Effect of metals in the incorporation of Fe\textsuperscript{2+}

| Metal added | \textsuperscript{59}Fe uptake | % of control |
|-------------|-----------------------------|-------------|
| 10 \mu M Cu\textsuperscript{2+} | 58.60 ± 1.27 | % of control |
| 50 \mu M Cu\textsuperscript{2+} | 25.70 ± 0.57 | % of control |
| 100 \mu M Cu\textsuperscript{2+} | 16.15 ± 5.47 | % of control |
| 100 \mu M Mn\textsuperscript{2+} | 87.45 ± 3.85 | % of control |
| 1 mM Mn\textsuperscript{2+} | 58.72 ± 3.03 | % of control |
| 100 \mu M Cd\textsuperscript{2+} | 90.15 ± 0.50 | % of control |
| 1 mM Cd\textsuperscript{2+} | 60.24 ± 3.47 | % of control |
| 100 \mu M Zn\textsuperscript{2+} | 70.00 ± 7.78 | % of control |
| 1 mM Zn\textsuperscript{2+} | 32.91 ± 4.09 | % of control |
| 100 \mu M Co\textsuperscript{2+} | 83.85 ± 0.64 | % of control |
| 1 mM Co\textsuperscript{2+} | 34.11 ± 2.12 | % of control |

Fig. 6. Ghosts \textsuperscript{59}Fe uptake is sensitive to osmotic challenge. Ghosts were incubated in saline buffer and the osmolarity was increased by addition of mannitol. Maximal \textsuperscript{59}Fe accumulation was determined from kinetic curves such as shown in Fig. 2. From the composition of the sealing buffer, the osmolarity of the internal medium was estimated as 220 mosM.

Fig. 7. Effect of osmolarity on iron efflux rates. Ghosts were preloaded with \textsuperscript{59}Fe\textsuperscript{2+}, and the time course of \textsuperscript{59}Fe efflux at 25 °C was determined as described under "Experimental Procedures." External buffers were: 50 mM NaCl, 50 mM KCl, 200 \mu M ascorbate, 20 mM MOPS (pH 7.0) (O), and 50 mM NaCl, 50 mM KCl, 200 \mu M ascorbate, 300 mM mannitol, 20 mM MOPS (pH 7.0) (▲). Data, fitted by a single exponential decay equation, gave efflux rate constants of 0.97 \times 10^{-3} s\textsuperscript{-1} and 2.94 \times 10^{-3} s\textsuperscript{-1}, respectively.
port processes from simple binding of the substrate. Fig. 3 shows the dependence on temperature of the $^{56}\text{Fe}^{2+}$ influx and efflux rate constants. Break points at approximately 15°C were found for both iron influx and efflux. The rate constants for influx were considerably larger than those for efflux (Fig. 3), and, remarkably, iron efflux at temperatures below 15°C was negligible. Therefore, iron flux data obtained below this temperature are a measure of the influx components, without significant contributions from the efflux components.

**Effect of Anions on Iron Uptake**—The observed accumulation of iron indicates that the ghosts have systems to dissipate the electrical gradient generated by the iron influx. One way in which this may be accomplished is by the concurrent influx of an anion. The anion composition of the external buffer affected the amount of accumulated iron (Fig. 4). The preference in supporting maximal accumulation was: $\text{Cl}^- > \text{NO}_3^- > \text{Br}^- = \text{SO}_4^{2-} > \Gamma$. Glucuronate, even at low concentrations, inhibited the incorporation of iron, probably through chelation (not shown).

$^{56}\text{Fe}$ Efflux from the Ghosts—In agreement with kinetic models for carrier-mediated systems (Devés and Krupka, 1979; Devés and Boyd, 1989), the rate constant for $^{56}\text{Fe}$ efflux from the ghosts almost doubled when $\text{Fe}^{2+}$ was added to the external solution (Fig. 5).

$^{56}\text{Fe}$ Uptake Is Sensitive to Osmotic Challenge—An approach to differentiate between transport and binding of a substrate is the sensitivity of the putative transport system to variations of the medium osmolarity. The uptake of $^{56}\text{Fe}$ by ghosts was affected by changes in the osmotic pressure: increased osmolarity in the external solution resulted in a marked decrease of the maximal amount of iron incorporated by the ghosts (Fig. 6). The above experiment was followed by determining the effect of osmolarity on iron efflux. A 3-fold increase in the efflux rate constant was obtained when the osmolarity of the external solution was approximately doubled (Fig. 7).

**Disulat Transition Metals Inhibit Iron Uptake by Reticulocyte Ghosts**—Several authors have reported that transition metals inhibit iron uptake in whole cells (Morgan, 1988; Wright et al., 1988; Sturrock et al., 1990; Kaplan et al., 1991). Incorporation of $\text{Fe}^{2+}$ by reticulocyte ghosts was strongly inhibited by $\text{Cu}^{2+}$. Variable degrees of inhibition were also observed with $\text{Mn}^{2+}$, $\text{Cd}^{2+}$, $\text{Zn}^{2+}$, and $\text{Co}^{2+}$ (Table 1). $\text{Al}^{3+}$ concentrations up to 1 mM did not inhibit $\text{Fe}^{2+}$ uptake by the ghosts.

**DISCUSSION**

Reticulocyte-derived sealed ghosts accumulate $\text{Fe}^{2+}$ in a concentration and time-dependent fashion. In agreement with whole cell studies (Egged, 1988; Morgan, 1988), sealed ghosts derived from erythrocytes were unable to accumulate iron, but this property was generated when an artificial membrane iron transporter was added, indicating that erythrocyte ghosts, whereas lacking the membrane iron transport system, do have an operative accumulation system.

Because the concentration of intravesicular iron at equilibrium is severalfold higher than the extravesicular concentration, the question arises as to whether the observed kinetics were a reflection of the transport process or a subsequent process related to the drainage of the transported iron. Since the overall uptake rate should be determined by the rate-limiting step, the introduction of an artificial iron carrier should increase the observed rate only if the transport rate was limiting. The observed increase in the uptake rate caused by A23187 is therefore an indication that the observed rates are a reflection of the transport rate.

In studying putative transport processes, a fundamental problem is to differentiate between actual transport of the species and binding to the external surface. The effect of osmolarity in both the influx and the efflux of iron, as well as the effect of external iron on the efflux of previously internalized $^{56}\text{Fe}^{2+}$, indicate that we were determining a true transport process. Moreover, the doubling of the efflux rate constant in the presence of extravesicular iron indicates that the iron binding sites of the transporters are evenly oriented towards the extravesicular and the intravesicular environment (Devés and Krupka, 1979; Devés and Boyd, 1989).

An unexpected observation was that the ghosts accumulated iron severalfold over the external concentration. It is possible that the driving force for accumulation reside in iron binding sites present in the internal face of the membrane, although more experiments are needed to investigate this problem.

Accumulation of iron by the ghost implies the neutralization of the positive charges carried into the ghosts by $\text{Fe}^{2+}$. The capacity of $\text{Cl}^-$ and $\text{NO}_3^-$ to support maximal accumulation was similar to that found by Wright et al. (1988) in perfused liver and may reflect the capacity of these anions to neutralize the positive charges of the transported iron.

$\text{Fe}^{2+}$ transport was competed by several $2+$ transition metals, being $\text{Cu}^{2+}$ the most effective. The observed selectivity in competition, $\text{Cu}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+} > \text{Cd}^{2+}$, is somehow different to that reported by Morgan (1988) for reticulocytes, but similar to that reported by Kaplan et al. (1991) for HeLa cells when assayed in saline buffer. Since in this study metabolic poisoning can be discarded, it is likely that the above sequence better expresses the possible selectivity of the $\text{Fe}^{2+}$ transport system.

In summary, we have determined that sealed right-side out reticulocyte ghosts transport and accumulate iron offered as $\text{Fe}^{2+}$-ascorbate. The overall characteristics of the system make reticulocyte sealed ghosts an excellent model in determining the basic mechanisms of membrane iron transport.

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