Failure of a Cell-Free System from Rabbit Reticulocytes to Remove Iron from Transferrin

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1. Cell-free solutions prepared from rabbit reticulocytes were not able to release iron from rabbit transferrin. 2. The results, which differ from those obtained with *Rana catesbeiana* immature erythrocytes, indicate that cellular integrity is a requirement for this process in the rabbit reticulocyte system.

James & Frieden (1975) reported that a cell-free solution prepared from *Rana catesbeiana* immature erythrocytes was able to release iron from transferrin. Hence they concluded that the integrity of the cell was not necessary for this process. Their results and conclusions differ from those obtained with rabbit reticulocytes, in which it was found that a cell lysate could not remove iron from transferrin (Morgan, 1971). James & Frieden (1975) used gel filtration to separate iron released from transferrin from that bound to bipyridine, which was used as an iron acceptor. Morgan (1971) had previously separated the two forms of iron by dialysis. The suggestion was therefore made that the difference in results obtained with *Rana* and rabbit reticulocyte lysates was a result of loss of an active component during the dialysis procedure, but not during gel filtration (James & Frieden, 1975). However, it is possible that the difference was due to species difference rather than differences in experimental technique. The present experiments were undertaken to investigate this possibility.

Experimental

Iron removal from transferrin was measured by the bipyridine method (Morgan, 1971), modified so that transferrin and bipyridine were separated by gel filtration (James & Frieden, 1975) or by precipitation with ethanol. Transferrin-⁵⁹Fe was incubated at 37°C with the material to be tested, in the presence of 2 mM-bipyridine. This concentration of bipyridine was chosen on the basis of previous results, which showed that optimum iron transfer occurred with bipyridine concentrations between 1.0 and 10.0 mM. When cell-free preparations were used, samples of the whole incubation mixture were subjected to the separation procedures. However, when the incubations were performed with intact reticulocytes, samples of the mixture were first centrifuged and the supernatant solution was then used. Gel filtration was performed

in a cold-room at 4°C, by using a column (1.5 cm × 30 cm) of Sephadex G-25 eluted with 10 mM-Tris/HCl/140 mM-NaCl, pH 7.4. Fractions (1.5 ml) were collected and counted for radioactivity. Excellent separation of transferrin-⁵⁹Fe from bipyridine-⁵⁹Fe was achieved (Fig. 1). The elution profile of transferrin was demonstrated in some analyses by adding a trace amount of Fe₂-transferrin labelled with ¹²⁵I to the solution to be fractionated before application to the column, and by measuring ¹²⁵I as well as ⁵⁹Fe in the eluted fractions.

In the ethanol precipitation procedure the solutions containing transferrin and bipyridine were cooled in an ice bath, then 10 vol. of ice-cold ethanol was added, and the mixture was agitated on a vortex mixer before centrifugation for 10 min at 1000g at 4°C. A sample of the supernatant solution was counted for radioactivity. Preliminary experiments with bipyridine-⁵⁹Fe, transferrin-⁵⁹Fe and transferrin-⁵⁹Fe-¹²⁵I showed that this procedure resulted in complete precipitation of the transferrin and its bound ⁵⁹Fe, but did not precipitate any bipyridine-bound ⁵⁹Fe. A procedure similar to that described above, but with acetone instead of ethanol, was used by Egyed (1975).

Incubations of transferrin-⁵⁹Fe and bipyridine were performed in the presence of intact reticulocytes, whole lysates prepared from reticulocytes, the supernatant solutions from the lysates, and the solutions in which the cells were lysed. Control incubations in which the bipyridine was omitted were also performed. The lysates were prepared by freeze-thawing cells suspended in 110 mM-NaCl/2 mM-KCl/20 mM-Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid], pH 7.5 (James & Frieden, 1975), or in 0.15 M-KCl. The cells were freeze-thawed in an ethanol bath at -50°C. Lysate supernatant solutions were obtained by centrifugation at 45000g for 2 h.

The methods that were used for the purification of rabbit transferrin, preparation of apotransferrin, labelling of apotransferrin with ⁵⁹Fe, labelling of

iron-saturated transferrin with ^{125}I , counting of radioactivity and washing and incubation of reticulocyte-rich blood cells from rabbits with haemorrhagic anaemia have been described previously (Morgan, 1971; Hemmaphard & Morgan, 1974). Except where indicated, the degree of saturation of transferrin with iron was between 30 and 50%. The reticulocyte count of the blood samples was 25–32%.

Results and Discussion

The ethanol precipitation method was evaluated in several experiments using incubations with intact reticulocytes. The distribution of ^{59}Fe between the precipitate and supernatant fractions (obtained by the addition of ethanol to samples of the incubation solution after different times of incubation of reticulocytes in rabbit plasma containing transferrin- ^{59}Fe and bipyridine) was measured. The concentration of ^{59}Fe in the supernatant increased progressively, whereas that in the precipitate fell at the same rate, so that the total amount of ^{59}Fe in the incubation solution remained unchanged. Hence no ^{59}Fe was accumulated by the cells. It has been shown previously that 2 mM-bipyridine completely blocks iron uptake by reticulocytes (Morgan, 1971). The rate of iron exchange between the precipitate and supernatant fractions was shown to be directly proportional to the reticulocyte concentration of the blood, and to increase as the bipyridine concentration was raised from 0.1 to 1.0 mM, but to show no further increase as the concentration was increased further to 10 mM. No measurable exchange occurred during 1 h incubation if either reticulocytes or bipyridine were omitted from the incubation solution.

In another experiment, iron release from transferrin was measured in a series of incubations in which the reticulocyte count (28%), bipyridine concentration (2 mM) and transferrin concentration (3.7 mg/ml) were maintained constant while the iron concentration was increased from 1.5 to 4.9 $\mu\text{g}/\text{ml}$. The rate of iron exchange between transferrin and bipyridine remained constant at $0.27 \pm 0.02 \mu\text{g}/\text{h}$ per ml of reticulocytes (mean $\pm$ s.d. of eight measurements). Bipyridine was omitted from a second series of similar incubations, and the rate of iron uptake by the cells was measured; this was also unaffected by iron concentration, and the rate was $0.28 \pm 0.01 \mu\text{g}/\text{h}$ per ml of reticulocytes.

The experiments described above show that the ethanol precipitation method is a suitable one for measuring iron release from transferrin, and that essentially no release occurs during 1 h incubation in the absence of reticulocytes. The release process is dependent on the presence of reticulocytes and occurs at the same rate as the cells normally accumulate iron from transferrin. Similar conclusions were drawn from earlier experiments in which dialysis was

used to separate transferrin and bipyridine (Morgan, 1971). However, the ethanol method does not suffer from the theoretical disadvantage of the dialysis method, that during dialysis low-molecular-weight materials, possibly required for iron release from transferrin, may be lost.

Both the ethanol precipitation method and gel filtration were used to test whether cell-free preparations from rabbit reticulocytes could release iron from transferrin. The results of one experiment are summarized in Fig. 2. The results obtained with the two methods used to separate transferrin and bipyridine were very similar. Neither the whole reticulocyte lysate nor the supernatant fraction of the lysate was able to release iron from transferrin. Similar results were obtained in three experiments, two using lysates of reticulocytes in KCl and one using a lysate prepared in the same solution as that used by James & Frieden (1975).

Fig. 1. Gel filtration of incubation medium from intact cells or reticulocyte haemolysate

Intact rabbit reticulocytes or the cell-free supernatant fraction from the reticulocytes were incubated with ^{59}Fe -labelled transferrin at 37°C for 60 min in the presence of 2 mM-bipyridine. Samples of the medium were then fractionated by gel filtration on a column (1.5 cm × 30 cm) of Sephadex G-25. The reticulocyte count was 37% and the transferrin and iron concentrations in the incubation medium were 1.5 mg/ml and 1.1 $\mu\text{g}/\text{ml}$ respectively. The cell-free solution was prepared by freeze-thawing the cells in 110 mM-NaCl/2 mM-KCl/20 mM-Hepes. ●, Incubation medium from intact cells; ○, cell-free incubation medium.

Fig. 2. Iron uptake by intact reticulocytes (a), and iron exchange between transferrin and bipyridine as determined by the ethanol precipitation method (b) or by gel filtration (c)

Rabbit transferrin labelled with ^{59}Fe was incubated with intact reticulocytes in the absence (○) and in the presence (●) of bipyridine (2mM), and with the total haemolysate of the cells (□), the supernatant fraction of the haemolysate (△) or the haemolysis solution, 0.15M-KCl (△), all in the presence of 2mM-bipyridine. The Figure shows the results obtained after different periods of incubation at 37°C for the radioactivity in (a) the washed reticulocytes, (b) the supernatant solution after precipitation of the incubation medium with ethanol, and (c) the second peak of radioactivity obtained by gel filtration on Sephadex G-25. The results are expressed in µg/ml of reticulocytes or haemolysis solution (KCl). In the case of haemolysate and haemolysate supernatant the results are given per ml of reticulocytes from which the haemolysate was obtained. The reticulocyte count was 31%, and the transferrin and iron concentrations in the incubation medium were 2.7 mg/ml and 1.5 µg/ml respectively.

The results of the experiments with cell-free preparations of reticulocytes confirm those obtained earlier by using the dialysis method to measure iron release from transferrin (Morgan, 1971). Hence it appears that these results were not a consequence of the loss of an active component during dialysis, as suggested by James & Frieden (1975). Instead, it must be concluded that, after disruption by freezing and thawing or by osmotic lysis, the rabbit reticulocyte is unable to release iron from transferrin. The reason for this is uncertain, but it does imply that cell-membrane integrity is a requisite for this activity. The difference in results obtained with immature erythrocytes from *Rana catesbeiana*

(James & Frieden, 1975) and those from the rabbit are difficult to explain, except on the basis of a difference in cell function between the two species.

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