Iron Removal from Transferrin by a Cell-Free Amphibian System

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1. Transferrin-bound iron was released by a cell-free solution prepared from *Rana catesbeiana* immature erythrocytes, showing that cellular integrity is not necessarily a requirement for this process as previously thought.
2. The biological molecule(s) involved in the removal of iron from transferrin is relatively small.

Iron needed for haemoglobin synthesis is obtained by the immature erythroid cell from the protein transferrin (Jandl & Katz, 1963; Baker & Morgan, 1969). In haemoglobin, iron is in the Fe(II) state, whereas transferrin binds iron in the Fe(III) state. Morgan (1971) found that removal of iron from rabbit transferrin by rabbit reticulocytes is easily detected with Fe(II) chelators, which intercept iron normally incorporated into protoporphyrin to form haem. However, disrupted rabbit reticulocytes, i.e. 'ghosts' or whole lysates, apparently could not mediate removal of iron from rabbit transferrin. These findings led to the conclusion that metabolism of transferrin-Fe(III) is dependent on cellular integrity (Morgan, 1971; Egyed, 1973).

We reported (Osaki *et al.*, 1974) some developmental changes in iron metabolism during metamorphosis of the bullfrog tadpole; it was shown that a relatively large proportion of immature erythroid cells was present in the blood of animals at stages near metamorphic climax, and that these cells markedly took up iron from frog transferrin. Therefore this amphibian system is of interest in the study of iron removal from transferrin by erythrocytes. The present communication deals with the release of iron from transferrin by a cell-free solution obtained from lysed immature erythroid cells.

A brief abstract of this work has been presented (James & Frieden, 1974).

Experimental

Purification of *Rana catesbeiana* transferrin, preparation of apotransferrin, collection and washing of immature erythroid cells and counting of radioactivity were performed as described previously (Osaki *et al.*, 1974). Transferrin containing radioactive iron was also prepared as described by Osaki *et al.* (1974), except that citrate-iron was replaced by nitrilotriacetate-iron (Bates & Schlabach, 1973) as the iron donor. Lysates were obtained by freeze-thawing cells in a bath of solid CO₂-ethanol after

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suspending washed packed cells in an equal volume of 110mM-NaCl–2mM-KCl–20mM-Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)ethanesulphonic acid], pH 7.5. The lysate was centrifuged at 100000*g* for 90min (Penefsky & Tazgloff, 1971) and the resulting red supernatant (cell-free solution) was used for studies on iron-releasing activity as described below.

For detection of iron removal from transferrin, the bipyridine assay method (Morgan, 1971) was modified so that samples were submitted to gel filtration rather than to dialysis (see below). Transferrin-⁵⁹Fe was added to the cell-free solution containing bipyridine as the iron acceptor and carrier bipyridine-iron for prevention of non-specific adsorption or other losses of bipyridine-⁵⁹Fe formed in the system. Each sample was prepared as follows: 10 μ l of carrier bipyridine-iron was added to a 100 μ l portion of cell-free solution in a test tube such that the final concentrations were 1.6mM and 4 μ M respectively, then 25 μ l of transferrin-⁵⁹Fe (10mg/ml) was added to give a final concentration of 1.8mg of transferrin/ml. Transferrin was 50% saturated with iron; since the molecular weight of frog transferrin is about 80000 and it contains two iron-binding sites (Palmour & Sutton, 1971), the amount of iron initially bound to transferrin was 180ng/sample. The choice of 1.6mM-bipyridine was based on experiments showing that optimum iron transfer occurred within the range 0.5–4mM-bipyridine. The concentration of carrier bipyridine-iron was kept low to prevent inhibition of iron transfer. Transferrin at 1.8mg/ml and half-saturated with iron was chosen on the basis of data showing that these values are approximately physiological in tadpole plasma (G. T. James & E. Frieden, unpublished work). Incubation was carried out at 25°C and samples were either assayed immediately or frozen in a solid CO₂-ethanol bath. Little, if any, change in pH was found in the samples at the end of the incubation period. For detection of iron released from transferrin, samples were passed through a Sephadex G-25 column (0.9cm $\times$ 30cm) surrounded by a cold (7°C) jacket. Elution was carried out with 10mM-Tris-HCl–10mM-NaCl, pH 8.0, with a flow rate of 28ml/h. Excellent separation of bipyridine-iron from proteins

was obtained. Fractions (1.4ml) were counted directly for radioactivity in a Nuclear-Chicago γ -radiation counter. The amount of iron removed from transferrin- ^{59}Fe (and reduced by the system, then chelated with bipyridine) was calculated from the percentage of radioactivity found in carrier bipyridine-iron eluted from the Sephadex G-25 column.

Assay of intact cells by the modified method involved the following procedures. Cells were incubated essentially as described previously (Osaki *et al.*, 1974), but in addition bipyridine was added as described for the cell-free system; cells were then centrifuged and washed twice with buffer and the pool of combined supernatants was analysed by gel filtration.

Results and discussion

The release of iron from transferrin by intact cells was investigated. Radioactive iron in a low-molecular-weight form, presumably bipyridine-iron, was formed in the presence of intact tadpole immature erythroid cells, the Fe(II) chelator bipyridine (see Morgan, 1971) and frog transferrin- ^{59}Fe . Incorporation of iron into cellular haem (Thunell, 1965) was blocked when bipyridine was present. Relatively little bipyridine-iron was formed by mature erythrocytes compared with immature cells. These results are similar to those obtained with rabbit erythrocytes and rabbit transferrin (Morgan, 1971; Egyed, 1973).

Lysed tadpole immature erythroid cells removed iron from frog transferrin nearly as well as did an equivalent amount of intact tadpole immature

erythroid cells, in contrast with reports on the rabbit system (see above). All of the activity resided in the supernatant (cell-free solution) of a 90min centrifugation at 100000g of the immature-erythroid-cell lysate. Fig. 1 shows a typical plot of time versus iron removal from transferrin by the cell-free solution. Data forming the upper curve resulted from incubation of frog transferrin with a cell-free solution derived from immature erythroid cells. Usually up to one-half of the total iron present was found transferred at the plateau region of the curve, possibly indicating that the component(s) responsible for iron removal from transferrin is able to attack only one of the two iron-binding sites of transferrin. It has been suggested that *in vivo* the two sites of transferrin may have different physiological roles (Fletcher & Huehns, 1968). The lower line in Fig. 1 shows that very little iron was transferred when mature erythrocytes were the source of the cell-free solution; no iron was transferred when buffer replaced cellular components.

All iron-removal activity was lost when the cell-free extract was thoroughly dialysed in the cold against 110mM-NaCl-2mM-KCl-20mM-Hepes, pH7.5. Undialysed samples with the same buffer composition and kept in the cold retained full activity. To determine whether an active component had passed through the Visking tubing, a cell-free solution was dialysed in the cold against an equal volume of the above buffer. After 48h, the buffer outside the bag was assayed by our modified method as described above; this buffer removed 25ng of iron/h from transferrin, compared with 36ng of iron/h for an undialysed cell-free solution diluted 1:1 with buffer only. Therefore the component(s) responsible for removal of iron from transferrin is relatively small [ribonuclease has mol.wt. 14000 and dialyses very slowly through Visking tubing (McPhie, 1971)]. In our dialysed sample, leakage of larger molecules did not occur, since haemoglobin did not leak out (as judged by zero absorbance at 406-410nm and 540nm of buffer outside the bag before and after dialysis); the cell-free solution inside the bag was intensely red, owing to the presence of haemoglobin in the 100000g supernatant of lysed cells, and had an absorbance of about 800 at 410nm and 90 at 540nm.

Since we have shown that the active component(s) is of low molecular weight, lysed cells might appear to be much less active than intact cells when assayed by the following method. Cell lysate or whole cells plus transferrin were placed in a bag of Visking tubing and dialysed against buffer containing bipyridine; the appearance of bipyridine-iron outside of the bag was monitored (Morgan, 1971). This type of assay is not to be confused with our above experiments, in which samples were dialysed before incubation with transferrin. We tested the dialysis-based method (Morgan, 1971) with both tadpole intact immature

Fig. 1. Transfer of iron from transferrin to bipyridine plotted versus time of incubation

Heparinized blood was pooled from eight to ten animals and the cells were washed with buffer (Osaki *et al.*, 1974). The cell-free solution was then obtained and incubated with ^{59}Fe -transferrin as described in the text. Iron removal from transferrin is given in ng of iron per sample, i.e. per 100 μl of solution. ●, Cell-free solution from lysate of tadpole immature erythroid cells obtained from stage-XXII-XXIII animals (tadpoles near metamorphic climax; see Osaki *et al.*, 1974); ○, cell-free solution from lysate of tadpole mature erythrocytes obtained from stage-XII-XV animals; □, buffer control.

Table 1. *Iron removal from frog transferrin by intact immature erythroid cells or the cell-free solution, as assayed by two methods*

The data are in ng/h, obtained at the end of 2 h after the addition of transferrin- ^{59}Fe to each sample (see the text). Each value is the mean of three separate experiments $\pm$ S.D. from the mean. The amphibian cells and cell-free solution were assayed in our laboratory by the dialysis method as described by Morgan (1971); these experiments were run in parallel with those using our modified assay. For the modified assay method see the text.

Assay method	Iron transfer (ng/h)	
	Intact cells	Cell-free solution
Dialysis	30 $\pm$ 5	6 $\pm$ 1
Modified	42 $\pm$ 4	37 $\pm$ 4

erythroid cells and the cell-free solution; rates of iron transfer found were about 30 and 6 ng of iron/h respectively (Table 1). In contrast with these values, our method of assay detected about 42 and 37 ng of iron transferred/h (see Table 1). Apparently the dialysis-based assay is unsuitable for disrupted cells; this observation is undoubtedly explained by our finding that the component(s) responsible for removal of iron from transferrin is diffusible, and was lost from the dialysis bag during the assay. Thus it is possible that lysed rabbit reticulocytes were able to remove iron from rabbit transferrin but that detection of this process was obscured by the assay method used (Morgan, 1971; Egved, 1973). In related experiments Workman & Bates (1974) studied release of ^{59}Fe from rabbit reticulocyte membranes prepared after incubation of intact cells with ^{59}Fe -transferrin. Iron was apparently mobilized from the membranes by a small Fe(II)-binding protein found in erythrocyte lysates. This process might represent the next step in iron metabolism after release of the metal ion from transferrin.

Before incubation with transferrin, treatment of the cell-free system by several physical methods and chemical reagents yielded further data on the nature of the component(s) which removes iron from transferrin. Activity was undiminished during several weeks of storage at 4°C. After samples were boiled for 5 min, cooled and centrifuged to remove precipitated haemoglobin, activity was still present but was about one-third that of the unboiled samples. Preincubation with trypsin at 200 $\mu\text{g}/\text{ml}$ and for 2 h at 25°C did not inhibit iron-removal activity. The presence of 1 mM-*p*-chloromercuribenzenesulphonic

acid or 5 mM-*N*-ethylmaleimide (Riordan & Vallee, 1967) at 35°C for 30 min before the usual iron-transfer assay caused a decrease in activity to only 0.8–0.9 times the rate found from untreated samples, which probably means that the cell-free activity does not depend on the presence of a free thiol group. Filtration of the cell-free solution through Amicon membranes resulted in apparent binding of the active component(s) to PM but not XM membranes. This is interesting, since PM membranes bind certain types of molecules such as steroids (Publication I-101D, Amicon Co., Lexington, Mass. 02173, U.S.A.).

Since the active component(s) is relatively small, the process of iron removal from transferrin might be analogous to the case where a small molecule, reduced flavin mononucleotide (FMNH₂), itself causes release of iron from ferritin without the direct involvement of an enzyme (Sirivech *et al.*, 1974). We showed that FMNH₂ does not act on transferrin-bound iron. It is not known whether reduction of Fe(III) to Fe(II) is concomitant with the actual release of iron from transferrin.

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