Removal of iron from diferric rabbit serum transferrin by rabbit reticulocytes

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When radioiron-labelled transferrin with ^{55}Fe located predominantly in the *N*-terminal iron-binding site and ^{59}Fe predominantly in the *C*-terminal iron-binding site was incubated with rabbit reticulocytes, both radioisotopes of iron were removed at similar rates. Electrophoresis of transferrin samples taken during the course of an incubation, in polyacrylamide gels containing 6 M-urea, showed that iron was removed in a pairwise fashion, giving rise to iron-free transferrin.

About 15 years ago (Fletcher & Huehns, 1967, 1968) published the results of experiments that suggested that the iron-binding sites of transferrin displayed a physiologically important heterogeneity. This led to a considerable number of reports, some confirming and some refuting, the existence of such a heterogeneity (Harris & Aisen, 1975*a,b*; Okada *et al.*, 1979, and references cited therein). More recently, experiments with both human transferrin and rat transferrin have not found any evidence of a physiological heterogeneity in the iron-binding sites (Huebers *et al.*, 1981*a,b*; Van Der Heul *et al.*, 1981). The situation with rabbit transferrin is not so clear. van Baarlen *et al.* (1980) found that the iron-binding site located in the *N*-terminal domain of rabbit transferrin was 5 times as effective as its counterpart in donating iron to rabbit reticulocytes *in vitro*. A ferrokinetic study *in vivo* made in the same laboratory has not confirmed this (Groen *et al.*, 1982).

In the present paper the interaction of diferric rabbit transferrin with rabbit reticulocytes is re-examined.

antly in the *N*-terminal iron-binding site and ^{59}Fe located predominantly in the *C*-terminal iron-binding site were prepared as follows. A 300 mg portion of iron-free transferrin was dissolved in 30 ml of 0.1 M- NaHCO_3 and saturated with [^{55}Fe]ferric nitrilotriacetate. The transferrin solution was freeze-dried after passage down a Sephadex G-25 column (35 cm $\times$ 2 cm) equilibrated with 10 mM- NH_4HCO_3 . After incubation with desferrioxamine to remove iron from the *C*-terminal iron-binding site [as described in the preceding paper (Heaphy & Williams, 1982)], the transferrin solution was resaturated with iron by the addition of excess [^{59}Fe]ferric nitrilotriacetate. [This procedure can be expected to give an asymmetric distribution of the two isotopes of iron, since it was shown by Harris (1977) with human transferrin that no equilibration of the iron bound to transferrin occurs during the addition of the second radioisotope.] The transferrin solution was freeze-dried after passage down a Sephadex G-25 column as above. Before incubation with rabbit reticulocytes, the transferrin sample underwent a buffer-exchange step on a Sephadex G-25 column (35 cm $\times$ 2 cm) equilibrated with incubation buffer.

Materials and methods

Materials

^{59}Fe and ^{55}Fe were obtained from Amersham International. Ferric nitrilotriacetate was prepared as described by Williams *et al.* (1978).

Preparation of rabbit transferrin

Rabbit transferrin was prepared as described in the preceding paper (Heaphy & Williams, 1982). Diferric rabbit transferrin with ^{55}Fe located predom-

Collection of reticulocytes

Reticulocytosis was induced in New Zealand White rabbits by phenylhydrazine injection (as described by van Baarlen *et al.*, 1980). Reticulocyte-rich blood samples (>90%) were collected from a marginal ear vein.

Incubation procedure

Rabbit reticulocytes were prepared for incubation with rabbit transferrin as described by van Baarlen *et al.* (1980). Incubations took place in open-topped vessels at 37°C, with a haematocrit of 45% and a transferrin concentrations of 2 mg/ml. Samples were

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gently shaken. At intervals 0.5 ml portions of cells were taken and centrifuged at 12000 *g* for 1 min. Samples (100 μ l) of supernatant were taken for electrophoresis in polyacrylamide gels containing 6 M-urea or for liquid-scintillation counting of radioactivity.

6 M-Urea/polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in 6 M-urea was performed as described in the preceding paper (Heaphy & Williams, 1982). Gels were scanned at 560 nm in a Pye–Unicam SP.8100 spectrophotometer.

Liquid-scintillation counting of radioactivity

Samples (100 μ l) of supernatant were each added to 10 ml of Aquasol (New England Nuclear) and their radioactivities counted in a Nuclear–Chicago ISOCAP 300 instrument.

Results

Preparation of ^{55}Fe , ^{59}Fe -labelled rabbit transferrin

The procedure described in the Materials and methods section was designed to prepare a diferric transferrin sample with ^{55}Fe located largely in the *N*-terminal iron-binding site and ^{59}Fe largely in the *C*-terminal iron-binding site. A number of checks were made to ensure that this asymmetric distribution of iron had been achieved. Firstly, the transferrin samples were electrophoresed on a polyacrylamide gel containing 6 M-urea (see Fig. 1). This gel shows (1) that the transferrin sample was saturated with ^{55}Fe , (2) that incubation with desferrioxamine removed about half of the transferrin bound ^{55}Fe , mainly from the *C*-terminal iron-binding site, and (3) that addition of ^{59}Fe resaturated the transferrin. The A_{465}/A_{280} ratios of these samples are shown below the gel. These ratios also indicate that incubation with desferrioxamine removed about half of the transferrin bound ^{55}Fe and that addition of ^{59}Fe resaturated the transferrin.

Incubation of transferrin with rabbit reticulocytes

Table 1 shows the result of an experiment in which ^{55}Fe , ^{59}Fe -labelled transferrin was incubated with reticulocytes. The transferrin-bound iron was monitored as a function of time. The $^{55}\text{Fe}/^{59}\text{Fe}$ ratio was normalized to 1.00 at the beginning of the experiment and all other values are compared with this. If iron were selectively removed from the *N*-terminal iron-binding site of rabbit transferrin, then more ^{55}Fe would be removed from the transferrin-containing supernatant than ^{59}Fe , and the $^{55}\text{Fe}/^{59}\text{Fe}$ ratio would decrease. If iron were selectively removed from the *C*-terminal site, then the converse would apply. In this experiment the $^{55}\text{Fe}/^{59}\text{Fe}$ ratio did not deviate 1.00 during 120 min

Fig. 1. Electrophoresis of rabbit transferrin samples on a 6 M-urea/polyacrylamide gel

For experimental details see the text. Lane 1, ^{55}Fe -saturated transferrin; lane 2, ^{55}Fe -labelled transferrin after incubation with desferrioxamine; lane 3, ^{55}Fe , ^{59}Fe -saturated transferrin. The A_{465}/A_{280} ratios of these samples are shown. Key: Tf, iron-free transferrin; Fe-Tf, transferrin with the *N*-terminal iron-binding site occupied; Fe₂Tf, diferric transferrin. The arrow indicates the direction of electrophoresis.

Table 1. Removal of iron from ^{55}Fe , ^{59}Fe -labelled rabbit transferrin by rabbit reticulocytes

For experimental details see the text. The $^{55}\text{Fe}/^{59}\text{Fe}$ ratio at zero time was normalized to 1.00 and all other values are compared with this.

Time of incubation (min)	$^{55}\text{Fe}/^{59}\text{Fe}$	Fe taken up (%)
0	1.00	0
15	1.02	1
30	0.99	15
45	0.98	16
60	1.01	24
90	1.00	31
120	1.04	36

Fig. 2. Removal of iron from diferric rabbit transferrin by rabbit reticulocytes at 37°C

For experimental details see the text. Values were determined by cutting out and weighing the transferrin peaks from densitometer scans. ●, Diferric transferrin; ○, iron-free transferrin.

of incubation, during which 36% of the transferrin-bound iron was removed.

This experiment does not tell us if iron atoms are removed singly from transferrin or in a pairwise fashion. To determine this, samples of supernatant from another incubation were electrophoresed on a polyacrylamide gel containing 6M-urea and the site distribution of iron was examined (see Fig. 2). Only bands corresponding to diferric transferrin and iron-free transferrin were identified, indicating that iron removal from iron-saturated rabbit transferrin occurs in a pairwise fashion in the presence of rabbit reticulocytes. We say pairwise because no monoferric transferrin species are detected during the experiment. This could be for a number of reasons, the most probable of which is that transferrin is not released from the reticulocyte until both iron atoms have been removed.

Discussion

The results presented in this paper show that rabbit reticulocytes remove iron from both sites of rabbit transferrin at identical rates. Groen *et al.* (1982) in a ferrokinetic study *in vivo* arrived at a similar conclusion. In contrast, van Baarlen *et al.* (1980) found that the N-terminal iron-binding site of rabbit transferrin was 5 times more likely to donate iron to rabbit reticulocytes than was the C-terminal iron-binding site. The methods used in the study by van Baarlen *et al.* (1980) were similar to the ones described in the present paper except that quantification was by immunoelectrophoresis.

Other recent studies *in vitro* have shown that both human transferrin and hen ovotransferrin release iron in a pairwise fashion to human reticulocytes and immature chick erythroid cells respectively (Huebers *et al.*, 1981a; Heaphy, 1981). Human reticulocytes also remove iron with equal ease from either of the human monoferric transferrin species (Van der Heul *et al.*, 1981; Heubers *et al.*, 1981a).

Studies performed *in vivo* with the rat have shown that both atoms of iron are removed at the same time from diferric rat transferrin, and also that iron is removed with equal ease from either of the rat monoferric transferrin species (Heubers *et al.*, 1981b).

Experiments with a number of different species have therefore shown that there is unlikely to be a physiologically important difference between the two iron-binding sites of transferrin with respect to erythroid cells. The fact, however, that the N- and the C-terminal domains of the putative ancestral transferrin molecule have diverged from each other structurally and that these differences have been conserved for long periods of evolution suggest that the two domains do indeed have different biological roles (Williams *et al.*, 1982). However, these roles may not be concerned with delivery of iron to erythroid cells.

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