Non-random distribution of iron entering rat liver ferritin *in vivo*

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Radioactive iron incorporated into rat liver ferritin *in vivo* and then released *in vitro* up to 24 h after injection follows 'last-in–first-out' behaviour. Thus, within this period at least, the added iron does not equilibrate with iron already present in the molecule's iron-core.

The iron-storage protein ferritin is made up of 24 protein subunits arranged as a hollow shell inside which variable amounts of iron up to a maximum of about 4500 Fe(III) atoms may be deposited (Clegg *et al.*, 1980). The stored iron is in the form of microcrystals of a mineral known as 'ferrihydrite' with which inorganic phosphate is associated, mainly adventitiously (Towe, 1981; Ford *et al.*, 1984). The size of the ferrihydrite crystals is limited to about 8 nm (80 Å) by the protein shell. In ferritin the complex kinetics of both deposition and release of iron appear to depend on the available surface area of the iron-cores (Harrison *et al.*, 1974). Accumulation of iron can be likened to the growth of a crystal within a confined space, the iron being deposited layer by layer on the iron-core particles. Similarly iron (or phosphate) is stripped off the iron-cores layer by layer, atoms on the outermost layer at any time being the most available for release. The 'last-in–first-out' behaviour predicted from this model (Harrison *et al.*, 1974) has been observed for iron (Hoy *et al.*, 1974b) and for phosphate (Treffry & Harrison, 1978) *in vitro*. Studies of iron metabolism often use ^{59}Fe , sometimes ^{59}Fe -labelled ferritin, as the iron source, and it is important to know whether the iron is deposited into ferritin non-randomly as *in vitro* and, if so, whether such non-random distribution is conserved for periods of several hours. In the present investigation we compare the release of ^{59}Fe with that of total Fe from ferritin isolated from rat liver at various times after injection of label under alternative regimes of iron loading, and find evidence for non-random behaviour.

Experimental

Iron injections

Thirty-two female rats (214 ± 7 g body wt.) each received an injection of $800\text{ }\mu\text{g}$ of Fe as ferric

ammonium citrate (green form; BDH Chemicals, Atherstone, Warwicks., U.K.) in 0.9% NaCl, pH 7.2, to boost ferritin synthesis (Drysdale & Munro, 1966). This was followed 12 h later by a further injection of $800\text{ }\mu\text{g}$ of Fe/rat labelled with $5\text{ }\mu\text{Ci}$ of $^{59}\text{FeCl}_3$ (Amersham International, Amersham, Bucks., U.K.). As shown previously (Hoy & Harrison, 1975, 1976), some of this radioactive iron will be taken up into preformed ferritin molecules. The rats were then divided into four groups of eight animals each. As shown in Scheme 1, three groups were killed at various times after the second injection without further treatment, and the fourth group received a third injection of unlabelled iron 1 h before being killed.

Ferritin preparation

Ferritin was isolated from the livers by the procedure of Linder & Munro (1972), and each preparation was passed three times through a

Scheme 1. Injection plan

column of Sepharose 6B. To avoid problems due to differential rates of iron release from ferritins of different iron contents (Hoy *et al.*, 1974a), fractional sedimentation was carried out so that fractions of similar iron contents could be used for the release studies. Each preparation was centrifuged in a Beckman L2-65 centrifuge (10 × 10 ml swing-out rotor). Centrifugation was carried out in four steps, the centrifugal force being diminished at each step, and after each step 2 ml fractions were taken successively from the top of the tube, leaving the sedimented ferritin behind. This ferritin was resuspended in 0.02M-sodium phosphate buffer, pH 7.2, containing 0.9% NaCl and centrifuged again. The four centrifugation steps were: 90 min at 250 000g, 40 min at 200 000g, 20 min at 110 000g and 10 min at 110 000g. Fractions were analysed for iron and protein. Ferritin with an average of 3000 Fe atoms/molecule was obtained from the supernatant of the third centrifugation, and these fractions were used in all the release experiments.

Iron and protein measurements

Iron was measured as described by Drysdale & Munro (1965), and protein by the method of Lowry *et al.* (1951), with horse apoferritin as standard.

Iron release

This was performed as described in Treffry & Harrison (1978) in an ultrafiltration cell (Amicon,

High Wycombe, Bucks., U.K.) with a constant volume of 5 ml of ferritin solution (0.5 mg of protein/ml) and 10 mM-thioglycolic acid/10 mM-sodium acetate buffer, pH 4.3, as the reducing agent. Total iron was assayed as its 2,2'-bipyridine complex. ^{59}Fe was measured in a Philips PW4540 liquid-scintillation analyser with a 400–800 window, and radioactivity counts were corrected by means of a ferritin quench curve.

Results and discussion

The results of the iron-release experiments are expressed (Fig. 1a) as percentage of ^{59}Fe released plotted versus percentage of total Fe released. As previously discussed (Hoy *et al.*, 1974b; Treffry & Harrison, 1978), if, during the course of the experiment, the added ^{59}Fe completely equilibrates with iron already present in ferritin (or deposited afterwards), then the points should fall on the 45° line (i.e. no difference in rates of release of ^{59}Fe or total Fe). If, however, the ^{59}Fe has been deposited and retained in surface layers of the iron-cores, then it should be released, on reduction, ahead of total Fe. Fig. 1(a) shows that ^{59}Fe found in ferritin isolated at 1 h, at 5 h and even at 24 h after injection is released faster than the previously accumulated iron. The 24 h points are closer to the 45° line than those at 1 h or 5 h, presumably because during this longer period more non-radioactive

Fig. 1. Release of ^{56}Fe and ^{59}Fe from ferritin

Release was carried out in an ultrafiltration cell with 10 mM-thioglycolic acid/10 mM-sodium acetate buffer, pH 4.3. The volume of the ferritin solutions was kept constant at 5 ml. The eluate was collected in 1 ml fractions and assayed for total iron with bipyridine and for ^{59}Fe by radioactivity counting. The rats were killed at different times after administration of ^{59}Fe : Δ , 1 h; $\circ$, 5 h; $\square$, 24 h; $\bullet$, 5 h after ^{59}Fe and 1 h after a further 800 μg of ^{56}Fe was administered.

iron has been deposited over the ^{59}Fe . In the fourth group of rats the injection of ^{59}Fe was followed 4 h later by an injection of ^{56}Fe . The release points are now below any of those with ^{59}Fe alone and fall close to the 45° line. If all the ^{59}Fe had been deposited and it was covered by ^{56}Fe , then the points would have been expected to fall below the line. The fact that they do not is unlikely to mean that almost complete equilibration has taken place within the core. There may still be a relatively large amount of ^{59}Fe available for deposition with the newly injected non-radioactive iron. Alternatively, the added ^{56}Fe may not have completely covered the ^{59}Fe already deposited.

In Fig. 1(b) the same data are plotted to show the percentage excess of ^{59}Fe released over that expected if the two types of iron ions were randomly distributed (the 45° line of Fig. 1a). This Figure indicates that the degree of error introduced by the assumption of random distribution is not constant, but varies depending on the amount of iron released from the ferritin. It may be noted that, if iron were released from a single core made up of distinct layers, then at 1 h and 5 h after labelling the newly deposited ^{59}Fe should be released ahead of the bulk of the previously deposited ^{56}Fe , and excess ^{59}Fe released over that expected would be highest for the lowest amount of total iron released. Instead, we observe that the excess ^{59}Fe released over that expected is low at low total iron released, rising to a maximum at 7% and 15% for 1 h and 5 h respectively. This suggests heterogeneity in the distribution of iron within the ferritin molecules, and that some of the iron is probably not bound within a large core and therefore available for faster release. Another explanation for this behaviour, that it is due to the presence of molecules with variable iron content, is excluded by the fact that the release studies were carried out with molecules of well-defined iron content (3000 ± 300 Fe atoms/molecule). The effect of the administration of non-radioactive iron 4 h after ^{59}Fe is clearly shown in Fig. 1(b), where the excess of ^{59}Fe released over that expected has been greatly diminished. The release studies shown here clearly indicate that within the time span of the experiments equilibration of the iron has not occurred. Although this time span was relatively

short, samples that were stored (*in vitro*) for a month showed the same non-random release behaviour. [This result departs from the previously reported finding (Hoy *et al.*, 1974b) that ferritin iron exchanges within the core within 72 h *in vitro*. It now seems that the previous finding may have been due to experimental error.] Other experiments (Treffry *et al.*, 1984) suggest that equilibration of ferritin iron *in vivo* occurs within 72–122 h after ^{59}Fe injection, a time span similar to the turnover time of 2–3 days of ferritin molecules (Linder & Munro, 1973). However, in the short term it is clear that ferritin iron must not be regarded as a random pool.

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