Iron-induced changes in rat liver isoferritins

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The effects of single and of multiple iron injection on the distribution of isoferritins was studied in rat liver with the aid of ^{14}C -labelling either after or before iron treatment. Several effects of iron can be seen. Analysis of protein and labelling patterns show that it not only produces a disproportionate increase in the more-basic isoferritins, but may, in sufficient dose, actually lead to a decrease in the more-acidic isoferritins. Use of iron injection after radioactivity shows that it must give rise to post-assembly changes causing acidic isoferritins to become more basic. With a moderate iron dose this change is relatively slow, taking several hours, and seems to occur in addition to a differential stimulation of the synthesis of the more-basic isoferritins. With higher iron dosage the post-assembly changes may be so rapid that it is difficult to distinguish them from a switch in the pattern of synthesis.

The iron-storage protein, ferritin, consists of 24 protein subunits assembled round an iron-core of ferrihydrite (Clegg *et al.*, 1980; Theil, 1983). When subjected to isoelectric focusing, ferritin focuses over a relatively wide pH range, giving several bands, or isoferritins (Arosio *et al.*, 1978; Lavoie *et al.*, 1978; Russell & Harrison, 1978; Wagstaff *et al.*, 1978). On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis most ferritins separate into two subunits bands, known as H and L (heavy and light respectively). The relative proportions of H and L subunits vary with tissue of origin and across the pH profile (Arosio *et al.*, 1978; Bomford *et al.*, 1981). Two-dimensional separation, combining isoelectric focusing with subsequent sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (O'Farrell, 1975), indicates further sub-division of the H and L subunits (Arosio *et al.*, 1978; Lavoie *et al.*, 1978; Watanabe & Drysdale, 1981). Although the observed subunit heterogeneity might be explained by differences in primary structure (Adelman *et al.*, 1975), it is only relatively recently that evidence has been found for the presence of more than one amino acid sequence in ferritin preparations from human (Wustefeld & Crichton, 1982; Addison *et al.*, 1983; Costanzo *et al.*, 1984), horse (Heusterspreute & Crichton, 1981) and rat (H. N. Munro, personal communication). However, formation of heteropolymers from various combinations of subunits of differing primary structure may not be the sole explanation of the observed heterogeneity in ferritin. Evidence has been found for post-translational effects giving rise

to changes in isoelectric point (Treffry & Harrison, 1980) and to differences in structure related to iron uptake and release (Russell & Harrison, 1978; Mertz & Theil, 1983).

Ferritin biosynthesis is stimulated by iron. This phenomenon has been studied most extensively in rat liver (Drysdale & Munro, 1966). Here it has also been shown that the more-basic isoferritins (which in rat liver are also the more L-rich) are increased relatively to the acidic (H-rich) species as a result of iron-loading (Kohgo *et al.*, 1980; Bomford *et al.*, 1981). It has been proposed on the basis of this observation and as a result of double-labelling experiments that H-rich isoferritins are synthesized and degraded more rapidly than are L-rich ones and that iron preferentially stimulates the latter (Kohgo *et al.*, 1980; Bomford *et al.*, 1981). In a separate study we have provided evidence (Treffry *et al.*, 1984a) that the rate of degradation of rat liver isoferritins are independent of their isoelectric points (and hence of subunit composition).

In the present paper we show that iron has two effects. We confirm the preferential stimulation by iron of the more-basic isoferritins and show that after iron-loading the rate of synthesis *de novo* of the major basic isoferritins overtakes that of the more-acidic species. However, we also show that this is not its sole effect, since iron administered after ^{14}C -labelling of ferritin produces a marked decrease in the radioactivity in the more-acidic species and a marked increase in that of the more-basic isoferritins, implying a post-synthetic change. In our experiments we have used injection

of $\text{NaH}^{14}\text{CO}_3$ as a means of introducing radioactivity into ferritin, since this means of labelling minimizes the effects of re-utilization (Swick & Ip, 1974; Waterlow *et al.*, 1978).

Experimental

Animals and injections

Locally bred female laboratory rats in the weight range 193 ± 10 g were used throughout and were given access to food and water. Each group consisted of four animals. A number of different regimes of iron and $\text{NaH}^{14}\text{CO}_3$ injections were followed. For iron administration ferric ammonium citrate (green form; BDH Chemicals, Atherstone, Warwicks. U.K.) adjusted to pH 7.2 in 0.9% NaCl was used, each rat receiving $400 \mu\text{g}$ of Fe/100 g body wt. at each injection. When radioactive label was administered, each rat received $800 \mu\text{Ci}$ of $\text{NaH}^{14}\text{CO}_3$ (specific radioactivity 58 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). Iron and radioactive label were given by intraperitoneal injection.

Regime I: short-term labelling with or without prior iron administration

One group of rats was injected with iron and a second with 0.9% NaCl 2 h before administration of label. A third group received iron at 0, 24, 36, and 48 h and label at 50 h. All three groups were killed at 1 h after $\text{NaH}^{14}\text{CO}_3$ injection.

Regime II: effect of iron on ferritin prelabelled with $\text{NaH}^{14}\text{CO}_3$

Two groups of rats received $\text{NaH}^{14}\text{CO}_3$. One group received iron 6 h after the radioactive label, and both groups were killed at 22 h after labelling.

Purification of ferritin

Pooled livers of four rats were homogenized in 4 vol. of distilled water plus 4 vol. of 0.03 M-NaCl. A 1 ml portion of the homogenate was frozen for subsequent ferritin determination. The remainder was heated at $70\text{--}75^\circ\text{C}$ for 10 min, cooled and centrifuged at $15000g$ for 30 min. The supernatant was concentrated in an Amicon 8MC ultrafiltration cell of 400 ml capacity with a PM-10 membrane (Amicon, High Wycombe, Bucks., U.K.). The crude ferritin solutions were passed once through a Sepharose 6B (Pharmacia, Uppsala, Sweden) column ($2.5 \text{ cm} \times 80 \text{ cm}$). Ferritin samples for use as standard were first purified by passing them twice through the Sepharose 6B column and then by high-speed centrifugation ($100000g$ for 2 h).

Antiserum preparation

Antiserum to horse spleen ferritin was obtained

from the blood of New Zealand White rabbits. Horse spleen ferritin (twice crystallized, Cd-free; Miles Laboratories, Stoke Poges, Slough, Bucks., U.K.) was first purified by high-speed centrifugation $100000g$ for 2 h. A 2 mg portion of this ferritin in 50% Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) was injected subcutaneously, and this was repeated twice at weekly intervals. Blood was taken 1 week later. Booster injections were given, and blood was withdrawn after 1 week. The antiserum was calibrated against standard rat liver ferritin (previously assayed by amino acid analysis), against which it had been shown to have a large degree of cross-reactivity.

Preparative isoelectric focusing

The LKB Multiphor system was used with the Ampholine electrofocusing kit for granulated gels (LKB, South Croydon, Surrey, U.K.). Samples containing 7–20 mg of protein were dialysed in an ultrafiltration cell against the Ampholine solution to be used for electrofocusing (a mixture of equal volumes of pH 4–6 and pH 5–7 LKB Ampholines). Electrofocusing was carried out overnight at 10°C and at a constant power of 8 W. Up to ten red-brown ferritin bands could be seen. These were collected and eluted with 3% (w/v) NaCl. The pH of each isoferritin solution was measured. Solutions were then dialysed for at least 5 days against 0.45% NaCl in 0.01 M-sodium phosphate buffer, pH 7.2, to remove Ampholine.

Ferritin analysis

Ferritin protein was determined by radial immunodiffusion against rabbit anti-(horse spleen ferritin) serum (Mancini *et al.*, 1965). The standard used was a purified solution of rat liver ferritin, the concentration of which has been determined by amino acid analysis (Huebers *et al.*, 1976).

Radioactivity counting

The radioactivities of all ferritin samples were counted after precipitation of $50 \mu\text{g}$ with rabbit anti-(horse spleen ferritin) serum. Counting was carried out in Triton/toluene scintillation fluid in a Philips model PW 4540 liquid-scintillation analyser. C.p.m. were corrected to d.p.m. by using a ferritin quench curve.

Calculation of radioactivities

The homogenates were treated with 1% deoxycholate/1% Triton X-100, and ferritin was measured by radial immunodiffusion (Mancini *et al.*, 1965) to give total ferritin/liver. Ferritin ($50 \mu\text{g}$) was precipitated with antiserum, washed and centrifuged through a sucrose gradient to

remove any adhering non-specific precipitate (Zahringer *et al.*, 1975).

Radioactivity was measured in the precipitated ferritin, and specific and total radioactivities were calculated.

The total radioactivity of each isoferritin was calculated from its specific radioactivity and the total isoferritin recovered. Samples were then corrected to the total isoferritin radioactivity/liver by using the amount of ferritin recovered after electrofocusing and the total ferritin/liver for each preparation.

Results

Short-term labelling with and without iron

In Table 1 we show the distribution of protein among rat liver isoferritins under three conditions of iron-loading as well as the percentage incorporation ¹⁴C into these isoferritins. The control rats were maintained on a normal diet without iron supplementation and the other two groups received either a single iron injection 2h before the administration of radioactive label or four iron injections at 50, 26, 14 and 2h before injection of NaH¹⁴CO₃. All groups were killed 1h after administration of the label. The results show that most of the protein is accounted for by three major bands, 4, 5 and 6, in all three groups of rats. A single iron injection only 3h before killing of the animals has little effect in either the total ferritin/liver or on the distribution of protein among the isoferritins. However, after four iron injections at least 50% of the protein is found in band 6.

More than 90% of the radioactivity appears in bands 4–6 after the 1h pulse in all three groups of rats. However, comparison of labelling patterns of animals receiving a single iron injection with those

of controls indicates a relative decrease of radioactivity in band 4 and a relative increase in bands 5 and 6. This trend is still more marked in rats that had received multiple iron injections before the labelling, as shown again as total radioactivities in Fig. 1. Thus, in the rats that had received four iron injections, band 6 has the highest rate of synthesis, whereas in the control rats it is band 4. In rats receiving a single iron injection before the labelling the highest rates of synthesis are shown by bands 5 and 4, which show almost equal rates. However, when these rates of synthesis are compared with the percentage protein in the isoferritin bands it is clear that they are not in proportion to their pool sizes. Thus in control rats 50% of the radioactivity appears in band 4 (more than in proportion to its 33% protein) and only 8% in band 6 (although it accounts for 20% of the protein). In rats receiving a single iron dose, an immediate shift in the distribution of radioactivity is seen away from band 4 and towards bands 5 and 6, even though the percentage protein in these bands has changed little. Thus a single iron injection produces an immediate change in the pattern of isoferritins being synthesized. In rats that had had four iron injections before the labelling the trend towards the synthesis of basic isoferritins is continued, although the distribution of radioactivity is skewed less markedly towards the basic than is the protein.

A comparison of the overall levels of radioactivity is also revealing. Fig. 1 and Table 1 show that a single iron dose causes a markedly greater incorporation of radioactivity into the combined isoferritins than did four injections (respectively 3-fold and 2-fold approx.) as compared with the untreated rats. Thus the same dose of iron gives a greater boost to ferritin biosynthesis in rats previously maintained on a control diet than when

Table 1. Distribution of protein and ¹⁴C radioactivity 1h after intraperitoneal injection of NaH¹⁴CO₃ (800 μCi/rat) among isoferritin bands in three groups of rats (regime I)

For full experimental details see the text. Control rats received saline 2h before the NaH¹⁴CO₃. Total ferritin/liver, 3.13 mg; total d.p.m. in ferritin/liver, 9296. For the single iron injection, rats received 400 μg of Fe/100g body wt. 2h before the NaH¹⁴CO₃. Total ferritin/liver, 3.46 mg; total d.p.m. in ferritin/liver, 26949. For the multiple iron injections, rats received 400 μg of Fe/100g body wt. 2, 14, 26 and 50h before the NaH¹⁴CO₃. Total ferritin/liver, 5.15 mg; total d.p.m. in ferritin/liver, 18209. Traces of a more-acidic band than those shown (band 1) could sometimes be seen, but since it did not contribute significantly to the total protein or total d.p.m. it has therefore been omitted.

Isoferritin band	pH	Control rats		Single Fe injection		Multiple Fe injections	
		(% of protein)	(% of d.p.m.)	(% of protein)	(% of d.p.m.)	(% of protein)	(% of d.p.m.)
2	4.83	1.1	1.1	3.9	1.0	1.0	0.3
3	4.95	3.1	4.3	6.0	3.8	2.2	1.3
4	5.04	33.2	50.0	28.7	38.0	8.5	12.2
5	5.14	33.0	33.6	26.8	40.7	18.7	35.7
6	5.25	20.8	8.4	20.4	14.0	51.3	48.2
7	5.32	5.9	1.0	8.7	1.4	12.3	1.8
8	5.44	1.2	0.3	1.9	2.8	3.9	0.5

given to rats that had previously received three iron injections. There appears, therefore, to be a limit to the stimulation by iron of ferritin biosynthesis, and this is also indicated by the observation that ferritin loads (mg/liver) are not increased proportionately to the dose in these and other experiments.

Fig. 1. ^{14}C radioactivity in rat liver iso-ferritins 1h after $\text{NaH}^{14}\text{CO}_3$ administration

The iso-ferritins were obtained by preparative isoelectric focusing and the ^{14}C radioactivity was calculated as described in the text. $\square$, Control rats; $\blacksquare$, rats that had received a single iron injection ($400\text{ }\mu\text{g}$ of Fe/100g body wt., ferric ammonium citrate) 2h before the labelling; $\hatched$, rats that had received four iron injections at 2, 14, 26 and 50h before labelling (regime I). For full experimental details see the text. The centre of the middle bar for each iso-ferritin corresponds to the isoelectric point for that iso-ferritin. The numbers on top of the bars correspond to the iso-ferritin bands in Table 1.

Effect of iron administered after the radioactive label

Consideration of the differences between the labelling patterns and protein distribution among iso-ferritins shown in Table 1 and Fig. 1 suggests that the large increase in protein mass in basic iso-ferritins after iron administration cannot be fully explained by changes in their rates of synthesis. Discrepancies between protein mass and radioactivity are even more pronounced 16–24h after a single iron injection. Thus by this time the protein distribution has altered to one resembling that shown in Table 1 for multiple iron doses, with band 6 being the most prominent iso-ferritin (several experiments, e.g. data in Table 3). Hence the slightly increased rates of synthesis of the more basic iso-ferritins indicated by their labelling pattern at 1h (Table 1, single Fe) are insufficient to account alone for their increase in mass some 15–23h later. One possible explanation for this phenomenon is that iron stabilizes ferritin against degradation, as suggested by Kohgo *et al.* (1980) and Bomford *et al.* (1981). However, we have provided evidence elsewhere (Treffry *et al.*, 1984a) that such stabilization is most unlikely. Another possibility, that iron causes a gradual change in the pattern of iso-ferritins being synthesized (see Table VII in Bomford *et al.*, 1981), is also insufficient explanation in view of the results for multiple iron injections shown in Table 1.

Previous work (Treffry & Harrison, 1980) has shown that, over a time span of 16–24h and longer, gradual redistribution of label from acidic to basic occurs even in rats that were given no additional iron. Accentuation by iron treatment of such redistribution in protein may account for the change in protein pattern observed 16–24h after iron injection.

In order to test this possibility, two groups of rats were studied. Both were given $\text{NaH}^{14}\text{CO}_3$. One group received no iron and the second group received one iron injection 6h after the label. Recoveries of activity and total protein are given in Table 2. Distributions of protein among the iso-ferritin bands are shown in Table 3 for both groups of rats. Fig. 2 shows the changes in ^{14}C total and specific radioactivities due to the iron injections.

Table 2. Recovery of ^{14}C radioactivity 22h after injection of $800\text{ }\mu\text{Ci}$ of $\text{NaH}^{14}\text{CO}_3$. For full experimental details see the text. Group II received $400\text{ }\mu\text{g}$ of Fe/100g body wt. 6h after the $\text{NaH}^{14}\text{CO}_3$ (regime II).

	Group I (normal)	Group II (Fe-treated)
^{14}C d.p.m. in total liver protein	1.20×10^6	1.26×10^6
^{14}C d.p.m./ μg of liver protein	1.08 ± 0.05	1.13 ± 0.07
^{14}C d.p.m. in ferritin/liver	9680 ± 260	9000 ± 860
^{14}C d.p.m./ μg of ferritin	2.93 ± 0.08	2.10 ± 0.20
mg of ferritin/liver	3.30 ± 0.20	4.29 ± 0.18

Fig. 2. Effects of iron injection 6 h after NaH¹⁴CO₃ administration on specific and total radioactivities in rat liver isoferritins (a) Specific radioactivity; (b) total radioactivity. □, Control rats; ■, rats receiving iron after the label. Both groups of rats were killed at 22h after NaH¹⁴CO₃ injection (regime II). For full experimental details see the text.

Table 3. Effect of administration of 400 μg of Fe/100 g body wt. on rat liver isoferritin distribution
Rats were killed 16h after iron administration. For full experimental details see the text.

Isoferritin band	Ferritin/liver (μg)	
	Control	Fe-treated
2	101	62
3	239	121
4	1290	872
5	835	1215
6	622	1666
7	169	275
8	43	80

Table 2 shows that ferritin/liver had increased by 30% 16h after the iron injection. Table 3 shows that this increase is more than accounted for by increases in bands 6 and 5, whereas band 4 has actually decreased. It can be seen from Table 2 that, although total and specific radioactivities of total liver protein are unaffected by iron, the specific radioactivity in ferritin in the iron-treated rats has decreased. This must be due to an increase in synthesis of unlabelled ferritin in response to iron, since other experiments have shown that labelling of the amino acid pool is insignificant at 6h after injection of NaH¹⁴CO₃ and that re-utilization of amino acids labelled by this method is minimized (Swick & Ip, 1974; Waterlow *et al.*,

1978). The effect of iron on the distribution of total and specific radioactivities among the isoferritins (Fig. 2) is striking. Although there is a decrease in specific radioactivities in bands 2–6, which can be explained by synthesis of unlabelled protein, in bands 7 and 8 radioactivities have markedly increased. Total radioactivities have increased dramatically in bands 6 and 7, whereas in bands 3 and 4 there is a dramatic decrease. Since the increase in label in the basic bands cannot be due to new synthesis, these data can only be explained by a redistribution of radioactivity from acid to basic isoferritins caused by iron after synthesis. This pattern of decrease in total radioactivity in the acidic bands and increase in the basic bands was reproduced in repeated experiments.

Discussion

It is clear from the results that we have presented here that iron has several effects on isoferritin synthesis and distribution. On the one hand it increases their overall rate of synthesis but produces a disproportionate increase in the synthesis of the more basic isoferritins (especially band 6) and, after repeated dosage, may actually lead to a decrease in the rate of synthesis of the more-acidic isoferritins (band 4). Its second effect, also leading to an augmentation of the basic species, is to increase the transfer of molecular species from acidic to basic bands, implying that it causes a change in

the isoelectric properties of ferritin molecules. The response to a single iron injection shows this in a particularly striking manner. Thus although at 1 h the rate of synthesis of bands 4 and 5 is nearly 3 times that of band 6, at 16–24 h a net loss from band 4 and a large gain in band 6 is observed. Taking into account the labelling experiment of Fig. 2, it is difficult to draw any other conclusion than that this is due to transfer of material from acidic to basic bands as a direct result of iron injection. We must then ask what causes the very large increase in the rate of synthesis of band 6 after four injections as compared with a single injection. One possibility is that a relative increase in the L-subunit pool size could give rise to faster assembly of L-rich molecules. However, it seems unlikely that this is the sole explanation for the switch in favour of synthesis of band 6 after four injections (as compared with bands 4 and 5 in rats receiving a single iron treatment), when one considers the small differences in subunit composition between these bands [subunit analysis (Treffry *et al.*, 1984b) indicates that band 6 has a composition $H_{8-9}L_{15-16}$, band 5 $H_{9-10}L_{14-15}$ and band 4 $H_{10-11}L_{13-14}$, and similar analyses have been given by Bomford *et al.* (1981) and Kohgo *et al.* (1980)]. Thus iron treatment may favour the assembly of band 6 molecules either by causing a switch to a different gene product or by processing the subunits after synthesis and before assembly. Alternatively (or additionally), it could accelerate the changes that occur after assembly, so that the shift from acidic to basic, which in the absence of iron treatment takes about 48 h (Treffry & Harrison, 1980), with one iron treatment takes about 16 h, and after multiple iron injections has largely occurred within 1 h after the initiation of synthesis.

Although our present experiments and those of Treffry & Harrison (1980) show that rat liver ferritin molecules undergo post-assembly processing, and that this processing may be accelerated by iron, they provide no information on what actual chemical or structural changes are taking place. However, such changes may not be unique to rat liver. Thus ferritins isolated from Chang cells cultured in iron-enriched and control media focus over different pH ranges, and individual iso-ferritins of the same pI have differences in immunoreactivity (Hoy & Jacobs, 1981). Mertz & Theil (1983) have found changes in subunit association within assembled ferritin shells from several animal species that relate to molecular iron content and hence, probably, to molecular age. Whether such changes can explain the observation we discuss here remains to be determined.

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