Heterogeneity in Horse Ferritins

A COMPARATIVE STUDY OF SURFACE CHARGE, IRON CONTENT AND KINETICS OF IRON UPTAKE

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Horse ferritins from different organs show heterogeneity on electrofocusing in Ampholine gradients. Both ferritin and apoferritin from liver and spleen could be fractionated with respect to surface charge by serial precipitation with $(\text{NH}_4)_2\text{SO}_4$. In the ferritin fractions, increasing iron content parallels increasing isoelectric point. After removal of their iron, those fractions which originally contained most iron accumulated added iron at the fastest rates. When unfractionated ferritins from different organs were compared the average isoelectric point increased in order spleen < liver < kidney < heart. The order of initial rates of iron uptake by the apoferritins was spleen > kidney > heart and initial average iron contents also followed this order. The relatively low rates of iron accumulation by iron-poor molecules may have been due to structural alteration, to degradation, to activation of the iron-rich molecules or to other factors.

Structural studies on the iron-storage protein ferritin have shown it to consist of 24 subunits symmetrically arranged around an iron-containing core (Harrison, 1977; Banyard *et al.*, 1978). Preparations of ferritin contain molecules with a range of iron contents from 0 to 4300 Fe atoms, and these can be fractionated by density-gradient centrifugation (Fischbach & Anderegg, 1965). Ferritins isolated from different organs of the same animal species may differ both in their iron content and in the rates at which this iron is turned over (Linder-Horowitz *et al.*, 1970). There are also inter-tissue differences in the electrophoretic mobilities of the protein (Alfrey *et al.*, 1967), and some tissues, e.g. heart (Linder-Horowitz *et al.*, 1970) or bone marrow (Alfrey *et al.*, 1967; Gabuzda & Gardner, 1967) may yield more than one ferritin, specific perhaps for different cell types within these tissues (Gabuzda & Pearson, 1968). Such electrophoretically different ferritins are usually called 'isoferritins' (Alfrey *et al.*, 1967) and evidence that these isoferritins have distinct primary structures has been presented (Linder *et al.*, 1972; Crichton *et al.*, 1973). Inter-species variation in surface charge and in amino acid composition is also commonplace in ferritins as in other proteins, although there is a considerable degree of conservation of amino acid content (Crichton *et al.*, 1977) and of three-dimensional structure, as judged by X-ray diffraction (Hoare *et al.*, 1975a) and electron microscopy (see Harrison *et al.*, 1974a).

Application of the technique of isoelectric focusing has demonstrated a further type of heterogeneity in ferritin, multiple band patterns (microheterogeneity) being observed in ferritins from several single organs

of humans (Powell *et al.*, 1975a,b), horse (Drysdale, 1977; Harrison *et al.*, 1977) and rat (Lee & Richter, 1971; Urushizaki *et al.*, 1973; Drysdale, 1977; S. M. Russell & P. M. Harrison, unpublished work). The individual bands in such patterns are also frequently referred to as 'isoferritins' (Powell *et al.*, 1975a). The cause of such microheterogeneity is, however, controversial. On the one hand it is proposed that ferritin molecules are hybrids of two or three genetically distinct subunits and that the proportions of these subunits vary systematically from band to band (Drysdale, 1977; Drysdale *et al.*, 1977). Others have considered the sharp bands to be artifacts arising from incomplete equilibrium focusing (Bryce & Crichton, 1973a) or from interaction of the protein with the Ampholines used in focusing (Lee & Richter, 1971).

Our involvement in the present study arose partly from a desire to study this very controversial area of ferritin biochemistry for ourselves and partly because of the relevance of the apparent heterogeneity to other studies on this laboratory on ferritin structure and function (Harrison *et al.*, 1977; Treffry *et al.*, 1977; Banyard *et al.*, 1978). We have shown (Macara *et al.*, 1972, 1973), as have others (Niederer, 1970; Bryce & Crichton, 1973b), that horse spleen apoferritin catalyses the oxidation of Fe(II) to Fe(III), retaining the latter as hydrous ferric oxide to give ferritin. We wished to discover whether individual 'isoferritins' (ferritins from different tissues or fractions of different isoelectric point from the same tissue) showed any functional differences as judged by their ability to oxidize and accumulate iron *in vitro* and to observe the effects of both removal and addi-

tion of iron on their focusing patterns. In previous studies (Macara *et al.*, 1972; Harrison *et al.*, 1974b) we have shown that rates of iron uptake by ferritin molecules depend on iron already present within them. We now examine the intrinsic ability of the different ferritin fractions to take up iron measured as initial rates of incorporation into molecules from which the original iron content has been completely removed. Most of our experiments were duplicated for both horse spleen and horse liver ferritin preparations.

A preliminary account of this work was given in Harrison *et al.* (1977).

Materials and Methods

All chemicals were AnalaR grade unless otherwise stated.

Ferritin preparation

Ferritin was purified from the tissues in one of two ways. Method I, which was adopted in most of the preparations, was a modification of the procedure of Linder & Munro (1972). It involved a heating step. Method II involved no heat treatment and was essentially that developed by S. Shinjo (personal communication). In method I the tissue was homogenized with 2 vol. of water, heated to 70°C for 5 min and then cooled rapidly to minimize heat denaturation of the ferritin. After centrifugation at 2000g for 15 min, 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ was added and the solution left at 4°C overnight. The precipitate was harvested by centrifugation at 2500g for 20 min, dissolved in water and dialysed against water at 4°C. After dialysis insoluble material was removed by centrifugation and the $(\text{NH}_4)_2\text{SO}_4$ -precipitation step repeated. The solution was then subjected to chromatography on Sepharose 6B in 0.01 M-sodium phosphate buffer, pH 7.0, in a 3.7 cm × 65 cm column. Purity was monitored by electrophoresis on 5% (w/v) polyacrylamide gels in 0.03 M-sodium borate buffer, pH 8.8, the gels being overloaded with protein. Duplicate gels were stained for either iron or protein. Further purification for some tissue ferritins, for example horse heart and kidney ferritins, was achieved by repeated chromatography on Sepharose 6B as above and preparative gel electrophoresis. In method II fresh horse spleen was cut into approx. 2 cm cubes and frozen overnight at -20°C. It was allowed to thaw slowly at room temperature (18–21°C) and was then minced and homogenized with 10 times its weight of water. The resulting homogenate was dialysed overnight against running tap water and for a further 24 h against repeated changes of distilled water. The homogenate was then spun at 10000 rev./min in a Mistral High-Speed 18 centrifuge for 20 min. The supernatant was made 40% (w/v) in $(\text{NH}_4)_2\text{SO}_4$ and a precipitate allowed

to form overnight. The precipitate was collected by centrifugation (50000g-min) and dialysed against distilled water. Any remaining precipitate was removed by centrifugation (12000g-min). The $(\text{NH}_4)_2\text{SO}_4$ -precipitation step was repeated. The solution was then purified by chromatography on Sephadex G-100 in 0.01 M-sodium phosphate buffer, pH 7.0, in a 3.7 cm × 45 cm column and further purified by chromatography on Sepharose 6B in the same buffer in a 3.7 cm × 65 cm column. Sample purity was checked, as before, by electrophoresis in polyacrylamide gels.

Apo ferritin preparation

Native apoferritin was prepared from ferritin solutions by differential centrifugation. The ferritin solution was either centrifuged in a 30% (w/v) sucrose solution in 0.05 M-sodium borate buffer, pH 8.6, in a Beckman L2-65 ultracentrifuge for 3 h at 100000g in the SW 41 rotor or for 2 h at 240000g in an MSE Superspeed 50 instrument (43114–119 rotor). The colourless solution at the top of the centrifuge tubes, containing native apoferritin, was removed with a syringe and the centrifugation repeated. Apoferritin was also prepared from pure ferritin samples by reduction with sodium dithionite, thioglycollic acid or reduced FMN. Ferritin was dialysed against either several changes of 4% (w/v) sodium dithionite in deaerated 0.1 M-sodium acetate buffer, pH 4.8, at 4°C or 0.1 M-thioglycollic acid in 0.1 M-sodium acetate solution, pH 4.25, to give dithionite- or thioglycollate-reduced apoferritin. Reduction times were kept as short as possible. Reduced FMN was prepared essentially by the method of Gibson *et al.* (1962). A stock solution of 32 mM-FMN in 30 mM-imidazole/HCl buffer, pH 6.5, was reduced by addition of 0.5 M-sodium dithionite (less than 1% of total volume). Ferritin solutions were de-aerated and a 10-fold molar excess of reduced FMN over ferritin iron was added. The containers were flushed with N_2 and sealed. Samples were left to reduce for 12 h at 4°C. Solutions were centrifuged at 12000g/min to remove iron-FMN complex and the supernatants fractionated on a Sephadex G-25 column (2 cm × 15 cm) eluted with 30 mM-imidazole/HCl buffer, pH 6.5. The apoferritin solutions obtained were concentrated by using an Amicon 8-MC ultrafiltration unit with a P10 membrane. Any remaining bound FMN was measured at 445 nm (ϵ_{445} 12500 litre · mol⁻¹ · cm⁻¹).

Fractionation with $(\text{NH}_4)_2\text{SO}_4$

Ferritin or apoferritin was fractionated either by serial precipitation with $(\text{NH}_4)_2\text{SO}_4$ or by serial solubilization. For serial precipitation the concentration of ferritin used was not greater than 1.33 mg/ml. The $(\text{NH}_4)_2\text{SO}_4$ was added in ten or more

increments within the range 14–70% (w/v) to the ferritin solution in 10mM-sodium phosphate buffer, pH 7.0, at 4°C. After each addition precipitate was collected by centrifugation (30000g/min). In a typical fractionation 1g of horse ferritin in 1 litre of 0.01M-sodium phosphate buffer was used as the starting solution.

For serial solubilization 1g of ferritin, precipitated from a 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ solution, was used. The precipitate was thoroughly homogenized first with 100ml of 30% (w/v) $(\text{NH}_4)_2\text{SO}_4$, centrifuged (30000g/min), and successively alternatively homogenized with $(\text{NH}_4)_2\text{SO}_4$ solutions of decreasing strength and centrifuged as above. Each supernatant fraction (about ten in all, in the range 30–0%, w/v) was raised to 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and the precipitate collected by centrifugation as above.

Fractionation by sucrose-density-gradient centrifugation

Portions (0.4ml) of ferritin solutions containing about 3–4mg of ferritin were layered on preformed linear sucrose gradients (5–30%, w/v) in 50mM-sodium borate buffer, pH 8.6, and centrifuged for 3h at 40000rev./min in a Beckman SW 41 rotor: 12 ml fractions were collected from the top of the gradient with a syringe.

Protein and iron determinations

Protein concentrations were determined by the method of Lowry *et al.* (1951) by using an apoferritin standard, and apoferritin was measured by its A_{280} ($A_{1\text{cm}}^{1\%} = 9.0$). Fe^{3+} in the micelle was measured by its A_{420} ($A_{1\text{cm}}^{1\%\text{Fe}} = 100$) or A_{310} ($A_{1\text{cm}}^{1\%\text{Fe}} = 450$) and Fe^{2+} by the A_{520} of its complex with $\alpha\alpha'$ -bipyridyl by using ferrous ammonium sulphate as the standard.

Isoelectric focusing

Isoelectric focusing was performed in thin slabs of 6% (w/v) polyacrylamide gels containing 2% (w/v) ampholytes. Narrow-range ampholytes (pH 4.0–6.0) were used, supplemented with pH 3.5–10.0 ampholytes in a 2:1 volume ratio (Ampholines; LKB Produkter, Bromma, Sweden). The LKB 2117 Multiphor apparatus was used for the focusing, with the LKB 3371 E.d.c. power supply unit. The gel was maintained at 4°C by using water circulated from an ice/water bath. The voltage, initially 100V, was increased during the first hour until 1000V was reached. This voltage, giving a maximum current of 50mA, was maintained for the rest of the electrophoresis. Protein samples were applied near the cathode on Whatman no. 1 filter paper (50–100 μg of protein per analysis). To ensure equilibrium focusing, the time taken for two samples applied at the anode and

cathode to coalesce was noted and a further 20% of this time was added, usually 3½h in all. (No change was observed in the patterns if electrophoresis was continued for a further 2–3h. After focusing, gels were stained for protein with Coomassie Blue or for iron with potassium ferrocyanide. A micro antimony pH electrode (Activion Glass Ltd., Halstead, Essex) was used to measure pH directly on the surface of some gels. Measurements were made at 4°C not more than 30min after electrophoresis (before staining).

Iron-uptake studies

Experiments were performed essentially as described by Macara *et al.* (1972, 1973) with the addition of ferrous ammonium sulphate to apoferritin in imidazole buffer, pH 7.45, except that in some cases the oxidant $\text{KIO}_3/\text{Na}_2\text{S}_2\text{O}_3$ was omitted and the Fe^{2+} was oxidized by dissolved oxygen. When making comparisons of rates of iron uptake of different apoferritins, we were careful to use standard conditions throughout and to vary the order in which samples were studied. The protein concentration used for each experiment was 0.33mg/ml and iron was added in the range 0–113 μM .

Results

Isoelectric-focusing patterns of ferritins from horse liver, spleen, kidney and heart

Isoelectric-focusing patterns of horse ferritins purified from these four tissues are shown in Fig. 1, gels (a)–(d). Each ferritin showed several bands in the system used, the patterns together extending from pH 4.1 to 5.1. Heart ferritin, which gave 15–18 bands, covered a wider range of pI than the other tissue ferritins. Liver ferritin was slightly, and heart and kidney ferritins were considerably, more basic than spleen ferritin. A similar trend has been observed in horse spleen, liver and heart ferritins by Drysdale (1977), although his band patterns differ from ours in detail.

Most of the results described subsequently in the present paper were obtained with horse spleen and liver ferritins, whose patterns usually exhibited 11–15 bands before fractionation.

Fractionation of horse spleen or horse liver ferritins

(a) *Sucrose-density-gradient fractionation.* Centrifugation through preformed sucrose density gradients fractionates ferritin into molecules continuously increasing in Fe content from 0 to about 4000 Fe atoms/molecule: 12 fractions were removed serially from the top of the gradient. Ten of these (omitting fractions 1 and 12) were dialysed against distilled

Fig. 1. Isoelectric focusing patterns of horse ferritin

Electrophoresis was performed in thin slabs of 6% (w/v) polyacrylamide gels containing LKB Ampholines, pH range 3.5–10 and 4–6 mixed in 1:2 (v/v) ratios. All samples were stained for protein with Coomassie Brilliant Blue. (a)–(d), Unfractionated ferritins from (a) liver, (b) spleen, (c) kidney, (d) heart. (e)–(n), Horse spleen ferritin fractions obtained by sucrose-density-gradient centrifugation. Fe atoms/molecule for these fractions are (e) 190, (f) 510, (g) 860, (h) 1550, (i) 1820, (j) 2150, (k) 2480, (l) 2750, (m) 3000, (n) 3020. Patterns in (a)–(d) correspond to a pH range from approx. 4.1 (lower end) to 5.1 (upper end).

Table 1. *Fractionation of horse spleen ferritin by serial precipitation with (NH₄)₂SO₄*
The starting solution to which solid (NH₄)₂SO₄ was added contained 1 g of ferritin in 1 litre of 10mM-phosphate buffer, pH7.0.

Fraction no.	(NH ₄) ₂ SO ₄ (% <i>, w/v</i>)	Protein (% of total)	Fe (mg/mg of protein)	Fe (atoms/molecule)
1	18	2.2	0.497	3910
2	20	4.8	0.467	3670
3	22	6.2	0.390	3070
4	24	13.0	0.370	2910
5	26	14.0	0.342	2690
6	28	15.2	0.306	2410
7	30	14.8	0.257	2020
8	32	12.3	0.224	1760
9	34	10.0	0.205	1610
10	40	4.5	0.095	750
11	55	3.0	0.025	200

Fig. 2. *Isoelectric focusing patterns of horse spleen ferritin or apoferritin*
Conditions were as for Fig. 1. (a)–(c), Ferritin fractions obtained by serial precipitation with (NH₄)₂SO₄ corresponding to upper, middle and lower ranges respectively of salt concentrations shown in Table 1. Fe atoms/molecule: (a) 500, (b) 1900, (c) 3060. (d) Unfractionated ferritin; (e), same sample as (d) after reduction to apoferritin with thioglycollate. (f), (g), Fractions obtained by serial (NH₄)₂SO₄ precipitation of apoferritin (derived by reduction of ferritin with sodium dithionite), (g) at 18% and (f) at 55% (w/v) (NH₄)₂SO₄. (h) Unfractionated ferritin; (i) native apoferritin separated from (h) by differential centrifugation.

water, concentrated and submitted to isoelectric focusing in polyacrylamide gels. The patterns for horse spleen ferritin in Fig. 1 gels (e)–(n), show that partial fractionation with respect to isoelectric point has accompanied fractionation with respect to molecular weight. Iron:protein ratios are indicated for each fraction in the legend. Fractions with lowest iron contents give the most acidic profiles and high-iron ferritins the more basic profiles. Similar results were obtained with horse liver ferritin.

(b) *Fractionation by serial precipitation with $(\text{NH}_4)_2\text{SO}_4$.* Table 1 shows the results of a typical serial precipitation experiment over the concentration range 18–55% (w/v) $(\text{NH}_4)_2\text{SO}_4$. Fractionation of horse spleen ferritin into small steps of decreasing Fe atoms/molecule has occurred as the $(\text{NH}_4)_2\text{SO}_4$ concentration is raised, in agreement with Mazur *et al.* (1950). Similar results were obtained with horse liver ferritin. After dialysis against water and concentration, the salt-free samples were applied to polyacrylamide-gel slabs for isoelectric focusing. Patterns forming a series very similar to that given by density-gradient centrifugation were obtained. An example showing samples from opposite ends and the middle of the range of $(\text{NH}_4)_2\text{SO}_4$ concentration is shown in Fig. 2, gels (a)–(c). As with density-gradient centrifugation, fractionation with respect to isoelectric point has accompanied fractionation with respect to Fe content. Since serial precipitation can be carried out with relatively large samples, it was used routinely to prepare samples for kinetic studies. Similar fractionation was obtained by serial solubilization of ferritin precipitated by 60% (w/v) $(\text{NH}_4)_2\text{SO}_4$.

Effects of addition or removal of iron on isoelectric focusing patterns

(a) *Removal of iron.* Apoferritins prepared from fractionated or unfractionated horse spleen or liver ferritins by reduction either with sodium dithionite or with reduced FMN exhibited isoelectric-focusing profiles almost identical with those of the original unreduced materials. In contrast, reduction with sodium thioglycollate of horse liver or spleen ferritins showed a simplification of the focused pattern to four or five bands (a loss of two to five basic bands). The effect can be seen dramatically in Fig. 2 (gels d and e) with unfractionated horse spleen ferritins and the corresponding apoferritin. With ferritin fractions the most pronounced change was with the fractions of high iron content. However, despite pattern simplification and a trend towards patterns like those for iron-poor ferritin, there still remained a small but progressive shift from acidic to basic in the iron-poor to iron-rich fractions after reduction.

(b) *Addition of iron.* Iron added by the method of Macara *et al.* (1972) to low-iron horse spleen ferritin

fractions had no effect on their isoelectric-focusing patterns. When iron was added to the dithionite- or FMN-reduced apoferritins, their focusing patterns again remained unchanged. However, thioglycollate-reduced apoferritins from iron-containing ferritins, whose isoelectric-focusing profile moved to a lower pH range after thioglycollate treatment, on re-addition of iron reverted to a pattern similar to that of the original fraction. The same result was achieved whether $\text{KIO}_3/\text{Na}_2\text{S}_2\text{O}_3$ or molecular O_2 was used as oxidant for iron uptake. However, we also found that $\text{KIO}_3/\text{Na}_2\text{S}_2\text{O}_3$ or $\text{Na}_2\text{S}_2\text{O}_3$ had the same effect, but not KIO_3 or O_2 .

Fractionation of apoferritin by serial $(\text{NH}_4)_2\text{SO}_4$ precipitation

Apoferritin could also be fractionated by serial $(\text{NH}_4)_2\text{SO}_4$ precipitation, whether reduction to apoferritin had been by dithionite or thioglycollate treatment, despite the narrower range of isoelectric points apparent on the thioglycollate-reduced protein. Identical focusing patterns were obtained with horse spleen apoferritins, whether fractionation preceded or followed reduction with either dithionite or thioglycollate. Fractions I and II of horse spleen dithionite-reduced apoferritin obtained by serial $(\text{NH}_4)_2\text{SO}_4$ precipitation (18% and 55%, w/v, respectively) are shown in Fig. 2 (gels f and g).

Separation of native apoferritin by differential centrifugation

The isoelectric-focusing pattern of natural horse spleen apoferritin, containing up to 26 Fe atoms/molecule, is shown in Fig. 2(i) and the original ferritin from which it was isolated in Fig. 2(h). Native apoferritin focuses at the acidic end of the ferritin profile. This method compares favourably with serial $(\text{NH}_4)_2\text{SO}_4$ precipitation (Figs. 2a–2c) as a means of separating natural apoferritin.

Kinetics of iron uptake by horse liver and spleen apoferritin at low concentrations of added iron

It has been shown by Macara *et al.* (1973) that, when ferrous ammonium sulphate is added to unfractionated apoferritin in imidazole buffer, pH 7.4, in the presence of excess $\text{KIO}_3/\text{Na}_2\text{S}_2\text{O}_3$, approximately hyperbolic iron-uptake progress curves are obtained (measured as increase in absorbance at 310 or 420 nm corresponding to the formation of the red-brown ferric complex inside the ferritin molecule), provided that not more than 100–150 Fe atoms/molecule of apoferritin are added. At higher Fe/protein ratios the uptake curves become S-shaped, i.e. there is an acceleration of the increase in absorbance after an initial slower phase. In the present study

Fig. 3. Initial rates of iron uptake by horse spleen or liver apoferritin molecules of different original iron contents removed by reduction

Ferritins (a)–(e) and apoferritin (f) were fractionated by serial $(\text{NH}_4)_2\text{SO}_4$ precipitation. Iron uptakes were recorded spectrophotometrically at 310 nm. Iron was added as $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ to apoferritin in imidazole buffer, pH 7.4, in the presence of excess $\text{KIO}_3/\text{Na}_2\text{S}_2\text{O}_3$ (a)–(d), (f), or with O_2 as the only oxidant (e). Initial velocities were measured for additions of up to 120 Fe atoms/molecule. Added iron concentrations indicated on abscissae. (a) Horse liver ferritin reduced with thioglycollate. Original Fe atoms/molecule: Δ , 200; $\square$, 650; $\circ$, 1000; $\blacktriangle$, 1600; $\blacksquare$, 1800; $\bullet$, 2000. (b) Horse spleen ferritin reduced with sodium dithionite. Original Fe atoms/molecule: Δ , 250; $\square$, 1050; $\circ$, 2000; $\blacktriangle$, 2400; $\blacksquare$, 2500; $\bullet$, 2780. (c) Horse spleen ferritin reduced by FMNH_2 . Original Fe atoms/molecule: Δ , 20; $\blacktriangle$, 2000; $\bullet$, 3000. (d) Horse spleen ferritin preparation made without a heating step and fractionated by serial $(\text{NH}_4)_2\text{SO}_4$ precipitation. 1 $\rightarrow$ 4 are fractions of increasing original Fe content (not determined but they were precipitated in reverse order, 4 $\rightarrow$ 1, as salt concentration increased). Fractions were reduced by thioglycollate. (e) Horse spleen ferritin reduced with sodium dithionite. Original Fe atoms/molecule: Δ , 400; $\square$, 850; $\circ$, 1350; $\blacktriangle$, 2000; $\blacksquare$, 2500; $\bullet$, 2700. (f) Horse spleen ferritin reduced with thioglycollate before fractionation. Original Fe atoms/molecule: $\square$, 800; $\circ$, 1850; $\blacktriangle$, 2450; $\blacksquare$, 2740; $\bullet$, 2900.

we usually used horse liver and spleen ferritin fractions obtained by serial $(\text{NH}_4)_2\text{SO}_4$ precipitation, and in different sets of experiments the fractions were

reduced variously by sodium dithionite, sodium thioglycollate or FMNH_2 . Initial rates of iron uptake by the different reduced fractions were compared at

Fig. 4. Double-reciprocal plot of initial rates of iron uptake of apoferritin fractions against Fe concentration. Conditions were as for Figs. 3(a)–3(d). Horse spleen ferritin, reduced with thioglycollate. Original Fe atoms/molecule: $\square$, 800; $\circ$, 1850; $\triangle$, 2450; $\blacksquare$, 2700; $\bullet$, 2900.

constant pH and temperature at the same concentrations of protein, oxidant and added iron. Uptake curves were recorded for each fraction at several values of added iron in the range 10–100 Fe atoms/molecule. We found that the fractions differed in their initial rates of iron uptake, and this was true whether the source of the original ferritin was horse liver or spleen and whichever reductant had been used to provide apoferritin. Essentially the same results were obtained in each case. Some typical examples are shown in Figs. 3(a)–3(f), in which initial rates are plotted against added iron concentration, and in the double-reciprocal plots of Fig. 4. In general it was found that the fractions which originally contained the most iron gave the fastest initial rates of iron oxidation. The relation between V_0 or V_{max} and original numbers of iron atoms/molecule was not a linear one. Each fraction gave linear double-reciprocal plots of initial velocity versus iron concentration. The apparent K_m values were approximately the same for all fractions, although V_{max} increased with iron content. Thus the plots suggest increasing non-competitive inhibition along the series towards

Fig. 5. Density-gradient fractionation of reconstituted horse spleen ferritin

Unfractionated ferritin was reduced with thioglycollate and reconstituted by addition of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ to an average of 2500 Fe atoms/molecule. The reconstituted ferritin was fractionated by centrifugation through a sucrose density gradient, and protein ($\blacksquare$) and Fe ($\bullet$) were measured for each fraction.

low-iron fractions. Similar systematic differences in rates of iron uptake were observed when O_2 alone was used as oxidant (Fig. 3e) and when fractionation followed rather than preceded reduction with sodium dithionite or thioglycollate (Fig. 3f).

Reconstitution of horse spleen and liver ferritin

Further evidence that the reduced fractions differed in their ability to take up iron came from an analysis of reconstituted ferritins obtained by the addition of iron to dithionite- and thioglycollate-reduced horse liver and spleen apoferritins. Ferrous ammonium sulphate was added to give on average 2500 Fe atoms/molecule and, after reconstitution, the resulting ferritin was fractionated on a density gradient according to its iron content, and Fe and protein were measured in each of the fractions. Fig. 5 shows that molecules did not take up equal amounts of iron. The distribution of molecules and their Fe contents are quite similar to those of native ferritin.

Fractions from the density gradient were examined by isoelectric focusing and patterns very similar to those in Fig. 1, gels (e)–(n), were obtained. Fractions near the top of the gradient with low Fe content were relatively acidic, whereas those that had taken up a greater proportion of the iron had a more basic isoelectric-focusing profile. Since it had been established that isoelectric-focusing patterns were unaltered if the fractions were reduced and then reconstituted, these experiments show that fractions of different isoelectric point differed in their ability to take up iron and that fractions which originally contained more iron competed more effectively for iron than did apoferritins from iron-poor molecules.

Fig 6. Kinetics of iron uptake of apoferritins from different organs

(a) Initial rates of iron uptake by apoferritins obtained from unfractionated horse heart (■), kidney (▼) and spleen (●); ferritins were reduced to apoferritin by thioglycollate. Conditions were as for Figs. 3(a)–3(d). Original Fe atoms/molecule: heart, 690; kidney, 790; spleen, 2300. (b) Double-reciprocal plot of initial velocities of iron uptake against Fe concentration for horse heart (●) and kidney (■) apoferritins. Conditions and Fe contents were as for (a).

Kinetics of iron uptake of horse kidney and heart ferritins

The yield of ferritins from kidney (0.13 mg/g of tissue) and heart (0.22 mg/g of tissue) was small compared with that from liver (0.44 mg/g of tissue) or spleen (0.62 mg/g of tissue), and insufficient protein was available for fractionation. In any case

Fig. 7. Initial velocities of iron uptake by horse spleen apoferritins

Conditions were as for Figs. 3(a)–3(d). ●, Horse spleen ferritin from which native apoferritin had been removed, ferritin reduced to apoferritin by thioglycollate. ▲, Native apoferritin obtained from same ferritin sample as (●) by differential centrifugation (Fe atoms/molecule ≤ 26). ■, Native apoferritin, as ▲, but treated with thioglycollate to remove residual Fe.

the measured iron contents of these ferritins were quite low. We therefore compared the kinetics of iron uptake for the unfractionated ferritins from these tissues after reduction to apoferritin with thioglycollate. Initial velocities of iron uptake at concentrations corresponding to 10–100 Fe atoms/molecule of apoferritin are plotted against Fe(II) concentration in Fig. 6(a) for apoferritins from horse spleen, kidney and heart. The average original iron contents of the preparations were 2300, 790 and 690 Fe atoms/molecule respectively (molecules containing 2300 ± 600 Fe atoms accounted for 70% of the spleen preparation). The V_0 values were in order spleen > kidney > heart throughout the range of iron concentrations examined, and this is the same order as the iron contents of the ferritins. With fractions of horse spleen or liver ferritin, rates of iron uptake also increased with increasing (previous) iron content, but this was also in order of increasing basicity or surface charge. Since kidney and heart ferritins are more basic than those of spleen (see Fig. 1), but had lower rates of iron uptake, it seems that consistent correlations can be made only with Fe content and not with surface charge. A double-reciprocal plot (Fig. 6b) shows apparent K_m values similar to those of liver and spleen apoferritins.

Kinetics of iron addition to native apoferritin

Native apoferritin obtained by repeated differential centrifugation of horse spleen ferritin had an average Fe content of up to 26 Fe atoms/molecule,

measured either as the bipyridyl-Fe(II) complex or as the ferric micelle absorption at 420 nm. The remaining iron-containing molecules left after native apoferritin had been removed were reduced with thioglycollate. Rates of iron uptake by this apoferritin were greater than that of native apoferritin, but the latter oxidized iron considerably faster than did the same protein that had been treated with thioglycollate or FMNH₂ to remove its remaining iron, as shown in Fig. 7.

Addition of iron to low-iron fractions

Low-iron fractions (containing less than 500 Fe atoms/molecule) were reconstituted to an iron content of 2500 atoms (if more iron was added to these fractions precipitation occurred). After dialysis the reconstituted and original low-iron ferritins were reduced to apoferritin with thioglycollate. The kinetics of iron addition to these two apoferritins were identical.

Discussion

The photographs reproduced in Fig. 1, gels (a)–(d), show that in horse, as in other species, ferritins from different organs have different average isoelectric points, with the liver protein resembling spleen ferritin more closely than that from kidney or heart. In horse, spleen ferritin is slightly more acidic and those of heart and kidney are slightly more basic than is liver ferritin. In humans the order of increasing basicity is heart < kidney < spleen < liver (Powell *et al.*, 1975a). Moreover the iron-poor species of horse spleen and liver ferritins [Fig. 1, gels (e)–(n), Fig. 2, gels (a)–(c); see also Ishitani *et al.*, 1975] lie at the acidic ends of their electrofocusing profiles, whereas in human ferritins from these organs the iron-poor molecules are the most basic species present (Drysdale *et al.*, 1977). Human kidney ferritin, like horse spleen ferritin, seems to have its iron-poor species at the acidic end of its spectrum (Powell *et al.*, 1975a). Drysdale (1977) reports a trend for human organs of increasing iron content with increasing basicity (which he attributes to an increasing content of L subunit), but notes that the progression ends abruptly with natural liver apoferritin at the end of the spectrum. We find, however (S. Shinjo, S. M. Russell & P. M. Harrison, unpublished work), that when both human liver and spleen ferritins are fractionated by sucrose-density-gradient centrifugation the iron content of individual fractions increases as they become more acidic. A similar trend has been noted by A. Bomford, M. Berger, Y. Lis & R. Williams (personal communication). In horse, we find that the average iron contents of heart and kidney ferritins are less than those of liver or spleen in the preparations that we have examined, but that within the last two organs the correlation with

increasing acidity lies in the opposite direction to that of the human ferritins (the more basic species have the higher iron contents). Our results differ from those of Ishitani *et al.* (1975) in that we find a smooth relationship between iron content and average isoelectric point, whereas they found that iron content did not vary systematically from band to band except that iron-poor ferritin banded at the acidic end of the spectrum. This difference may have resulted from differences in techniques used to separate the components.

Our observation that serial (NH₄)₂SO₄ precipitation (or solubilization) fractionates horse spleen and liver apoferritin [Fig. 2, gels (f) and (g), as well as ferritin (Fig. 2, gels (a)–(c))] into species of different average isoelectric point, which do not redistribute on electrofocusing, confirms the conclusion that such preparations contain a spectrum of molecules with small differences in surface charge. Serial (NH₄)₂SO₄ precipitation had previously been shown to fractionate ferritin with respect to iron content (Mazur *et al.*, 1950). That fractionation can be achieved also with apoferritin indicates that separation by this method is governed by surface-charge differences and not by the differences in iron content that happen to be associated with them [Table 1 and Fig. 1, gels (e)–(n)]. The fact that a very low iron content (less than 200 Fe atoms/molecule) species was not obtained by serial precipitation of ferritin may have been due to some natural apoferritin remaining in solution even at 55% (w/v) (NH₄)₂SO₄ or to incomplete separation of the species.

Before discussing the implications of our finding that ferritins from a single organ are also heterogeneous in their intrinsic ability to take up iron, it is necessary to review the evidence relating to structural inhomogeneity, within and between tissue-specific ferritins. Essentially there are two possibilities: that both arise from some factor, or factors, continuously varying within and between organs, or that the heterogeneity is superimposed on an organ-specific variation of different origin.

In the most recent version of the hybrid-molecule scheme of Drysdale (1977), which falls into the first category, it is proposed that all horse and human ferritins are hybrids of two subunits of differing size and primary structure, called L (mol.wt. 19000) and H (mol.wt. 21000), predominating respectively in liver and heart. The 25 hybrids expected for the 24-subunit molecule would account for the complete spectrum of bands seen on isoelectric focusing in the various tissues, including malignant tissue. Other evidence cited in favour of the hybrid model are differences in amino acid composition of ferritins from different tissues and the finding of more than one subunit band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (e.g. Drysdale, 1977). However, although the amino acid analyses suggest

there may be genetically different subunits none of these observations in themselves proves the existence of hybrids.

Several reports of differences in subunit molecular weight have appeared. To quote the most recent, Crichton *et al.* (1977) and Drysdale (1977) found two subunits of mol.wts. 18000–19000 and 21000 in horse liver ferritin, but only one band in horse heart ferritin, either 18000 or 21000. Niitsu *et al.* (1977) report subunits of 19000 and 21000 mol.wt. in horse spleen ferritin and also the formation of hybrids from spleen and heart subunits by co-assembly *in vitro* (they do not quote a molecular weight for the heart subunit). In an earlier study Crichton *et al.* (1973) found a single 19000-mol.wt. component in horse liver and spleen ferritins. Rat liver ferritin apparently has a subunit weight between those of horse spleen and heart (about 20000; Drysdale, 1977). The very close similarity in X-ray-diffraction patterns of the octahedral crystals of the horse liver and spleen ferritins, human spleen and rat liver ferritins and also of the apoferritins derived from iron-rich and iron-poor spleen ferritin (Hoare *et al.*, 1975a; S. H. Banyard & P. M. Harrison, unpublished work) suggests close similarity in the subunit size, primary structure and arrangement in the 24-subunit molecules present in these crystals. Octahedral crystals of horse kidney ferritin have also been grown, although not large enough yet for X-ray photography. In the present study we tested the proposition that crystallization selected a homopolymer from a hybrid mixture of horse spleen ferritins. With one sample there was a loss of a few basic bands on electrofocusing, but this was to some extent compensated for by the appearance of an additional band on the acid side of the original pattern. With another sample there appeared to be little or no alteration in the electrofocusing pattern (containing seven or eight bands) after several cycles of crystallization. It seems to us unlikely that hybrid molecules containing subunits differing by as much as 2000 mol.wt. would satisfactorily crystallize together if the additional 2000 mol.wt. were on the external surface of the molecule. Conceivably the extra peptide might pack inside the hollow molecule in place of part of the iron core (the protein shell itself is rather tightly packed; Hoare *et al.*, 1975a,b). The tentative interpretation of our current electron-density map (Banyard *et al.*, 1978) has the *N*-terminus of each subunit at the outside surface and the *C*-terminus in the central cavity of the molecule. It is possible, however, that the estimated difference of 2000 in subunit molecular weight is exaggerated and the differences in gel migration rates result largely from folding to alternative more-or-less compact conformations.

Tissue-specific differences in amino acid composition of ferritins suggest the presence of more than one apoferritin gene, and the expression of these

genes must be controlled, whether different cells produce homopolymers of single specific subunit types or heteropolymers of two subunits in different amounts. If different tissues produce characteristic homopolymers then an explanation must be found for the surface-charge heterogeneity observed by isoelectric focusing, serial $(\text{NH}_4)_2\text{SO}_4$ precipitation or ion-exchange chromatography. It is possible that the sharp bands seen on electrofocusing are more a consequence of discontinuities in the Ampholine pH gradient (Russell *et al.*, 1978) than of discrete hybrids and hence that the number of bands should not be equated with a specific number of hybrid molecules. However, it is clear that there is a real distribution of isoelectric points. This could arise independently in each cell type as a result of post-transcriptional modification, which could be related both to the age of the molecule and to its iron accumulation. Thus low-iron acidic molecules may be converted into high-iron more-basic ferritin molecules in horse liver or spleen. On the face of it the experiments of Drysdale & Munro (1966), who showed in rat liver that [^{14}C]leucine initially incorporated into iron-poor ferritin gradually passed to iron-rich species over a 72h period, taken together with our findings relating iron content and surface charge (for horse liver and spleen ferritins), suggest such a conversion. Possibly, however, the same result could be achieved starting from a range of 'isoapoferritins' by differential iron uptake followed by differential loss of iron-poor species.

Several types of modification have been found in erythrocyte enzymes to produce charge differences, e.g. loss of amide groups (Funakoshi & Deutsch, 1969) or attachment of glutathione to surface thiol groups (Hopkinson & Harris, 1969), which are related to age or length of storage of the erythrocyte preparation. The reversible effect of thioglycollate on the isoelectric-focusing patterns suggests that reduction/oxidation may influence ferritin isoelectric points. However, such modifications must be related in an opposite sense to iron content in horse and human ferritin. On the assumption that older molecules have higher iron contents, then deamidation could not explain the variation in pI in horse spleen and liver ferritins, since iron-rich molecules are the more basic. Attachment of different amounts of carbohydrate to different subunits (Cynkin & Knowlton, 1977) is probably not the cause of heterogeneity, since Shinjo *et al.* (1975) found the same amounts of sugars and amino sugars in horse spleen ferritin fractions of different iron content. Moreover, when bovine serum albumin, which is devoid of carbohydrate (Sharon, 1975) and which contains a single polypeptide chain, is subjected to electrofocusing alongside ferritins, it shows a comparable distribution of isoelectric points as well as sharp bands (Russell *et al.*, 1978). Other proteins are also

known to exhibit charge heterogeneity, e.g. ion-exchange chromatography of sperm-whale myoglobin gave five components, at least four of which had identical amino acid compositions (Edmundson & Hirs, 1962). Perhaps the variations in ferritin surface charge may simply be accounted for by variations in side-chain pK values resulting from small conformational differences among the 24 subunits.

We now turn to the question of the differential rates of iron uptake by apoferritins of different surface charge. We first thought that, if the Drysdale (1977) hybrid scheme were correct, then some functional correlation might be expected between surface charge (a putative indicator of the proportions of H and L subunits) and ability to sequester iron. It has been reported that in rats and humans iron overload leads to a replacement of heart-type by liver-type ferritins in heart tissue itself (Powell *et al.*, 1975*b*, 1977), which might suggest a difference in ability of different ferritins to sequester iron. However, Bomford *et al.* (1977) observed no consistent correlation between iron-loading and the proportion of L-type ferritin. Adelman & Drysdale (1977) have found an increased [3H]leucine labelling of L subunit compared with the H subunit in response to iron injection. They suggest that H subunit may be a precursor of L subunit with iron mediating the conversion. There are some difficulties in accommodating all the observations with the Drysdale (1977) heteropolymer hypothesis. Our results show that within a single tissue ferritin (liver or spleen) there is both an increasing iron content and, once the iron has been removed, an increasing ability to sequester iron (measured by rates of iron uptake *in vitro*) which parallels the trend in isoelectric point towards heart ferritin. However, in horse ferritins the trend in iron content and in velocity of iron uptake cannot be extrapolated from liver and spleen to results for the kidney and heart. Rates of iron uptake correlate better with original iron content than with surface charge. This could be consistent with tissue differences in ability to sequester iron if tissue ferritins were distinct, but it does not correlate with the proposed progressive increase in H subunits from liver to heart. The suggestion of an H-to-L-subunit conversion enhanced by iron is an interesting one with respect to structure-function relationships. The *N*-terminal amino acid (serine) in horse spleen ferritin is acetylated (Suran & Tarver, 1965; Harrison *et al.*, 1962). Huberman & Franco (1975) report the isolation of *N*-acetylseryl-tRNA from rat liver and find its concentration to be increased in iron-stimulated rats. On the assumption that this *N*-acetylserine is incorporated at the *N*-terminus early in synthesis, then the additional amino acids of the supposed precursor must be attached at the C-terminal end of the chain. If this is tucked inside the molecule

(Banyard *et al.*, 1978), then molecules containing large amounts of H subunit would have a lower capacity for iron, and perhaps also a lower avidity for iron (if iron-binding sites inside the molecule were altered). This might explain differences in function between heart and liver ferritins, but it would not explain differences in surface properties (such as in immunological differences in acidic and basic ferritins; e.g. Worwood *et al.*, 1977) if this were the only difference between the proteins, nor would it explain the trends in iron contents within the liver and spleen, which are in the opposite direction. We also found on adding successive amounts of iron to ferritin fractions that, whereas iron-rich molecules could accommodate 4000 Fe atoms, iron-poor ferritins were precipitated after 2500 Fe atoms/molecule had been added.

It seemed to us possible that the latter observation and the low rates of iron uptake by originally iron-poor apoferritins could be due to selective partial degradation *in vivo* or denaturation on isolation (which involves heat treatment) or storage, or on removal of iron. However, a preparation made without a heating step, fractions treated with reducing agents for various lengths of time and iron-poor fractions to which extra iron had been added before reduction all gave the original differential patterns of iron uptake. Several enzymes have been shown to lose activity with cell age (e.g. purine nucleoside phosphorylase; Turner *et al.*, 1971). Within liver cells iron-rich molecules may be longer-lived than iron-poor ones (Munro & Drysdale, 1970), but it is the iron-rich molecules whose protein has the greatest intrinsic ability to take up iron. If ferritins of low iron content are more susceptible to turnover *in vivo* then there may be some means of distinguishing iron-rich and iron-poor molecules within cells, such as differences in the protein surface, since iron is stored within the molecules. Electron micrographs of lysosomal ferritins in iron-loaded liver cells (Iancu *et al.*, 1977) suggest that iron-rich ferritin molecules may be preferentially taken up by lysosomes. Iron-poor ferritin may also be 'compartmentalized', in respect to its accessibility to iron-pools (Hoy & Harrison, 1976). It could be that some of this apoferritin never acquires an ability to take up iron rapidly, whereas those molecules that do, have in some way been activated.

A final word must be said about the implications of the present study for models of iron uptake based on work with unfractionated apoferritins (Macara *et al.*, 1972, 1973; Crichton *et al.*, 1977). Examination of Table 1 shows that most of the protein present in horse spleen ferritin can be accounted for by molecules containing between 1600 and 3000 Fe atoms, so results obtained with unfractionated apoferritins would be weighted towards molecules within this range. In previous studies with both unfractionated

(Macara *et al.*, 1972) and fractionated ferritins (Harrison *et al.*, 1974b, 1977) we noted that rates of iron uptake of ferritin molecules depend on the amounts of iron added or already present. This is also indicated in the results in Fig. 5, where lower rates were obtained after iron had been removed from iron-poor molecules. Initial rates of iron uptake by apoferritin plotted against original iron content do not follow the same curve as rates for similar fractions which still contain iron (Harrison *et al.*, 1974b; S. M. Russell & P. M. Harrison, unpublished work). Thus the presence of iron within a ferritin molecule, and its amount, are important factors in determining rates of iron incorporation, and the effects that we observe here do not in themselves explain our earlier results with unreduced ferritin fractions.

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