Heterogeneity in Tissue Ferritins Displayed by Gel Electrofocusing

By JAMES W. DRYSDALE

*Department of Biochemistry and Pharmacology, Tufts University School of Medicine,
136 Harrison Avenue, Boston, Mass. 02111, U.S.A.*

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1. Horse spleen ferritin and human liver ferritin were examined by gel electrofocusing under conditions that demonstrated equilibrium focusing. Both ferritins were resolved into multiple isoferritins. Both families of isoferritins were separable from one another.
2. Horse spleen ferritin was also resolved into five components by ion-exchange chromatography on DEAE-Sephadex A-50. Each of the major chromatographic fractions contained only a few of the isoferritins seen on gel electrofocusing. Each chromatographic fraction corresponded to different portions of the isoferritin profile.
3. These results indicate that the heterogeneity seen in many ferritins by gel electrofocusing represents structural heterogeneity in the ferritin population as isolated from the tissues.

Ferritin is a red-brown protein that stores iron in many plant and animal tissues. In mammals it is found, or may be induced by iron administration, in many organs such as liver, spleen, heart, intestine and brain (Granick, 1951; Munro & Drysdale, 1970). Structurally, the molecule resembles a small icosahedral virus, being fashioned from a multimeric protein shell, apoferritin, within which variable amounts of iron may be sequestered. Studies with the readily available horse spleen apoferritin indicate that this ferritin has a molecular weight of about 440 000 and contains about 20 or 24 subunits, each of approx. 18 500 daltons (Harrison, 1964; Crichton, 1971).

For some time it has been thought that all subunits in all apoferritin molecules in a single organism were chemically identical. This view is changing, however, in the light of the finding of tissue-specific ferritins of different structure and metabolism. At least six tissue ferritins (liver, spleen, heart, kidney, bone marrow and pancreas) have been distinguished electrophoretically. Most migrate as single, although rather broad, bands, often with considerable overlap. Some, such as those from heart and bone marrow, appear to separate into two components (Alfrey *et al.*, 1967; Gabuzda & Gardner, 1967; Gabuzda & Pearson, 1968; Linder-Horowitz *et al.*, 1970; Righetti *et al.*, 1971; Lee & Richter, 1971a; Linder *et al.*, 1972; Powell *et al.*, 1974). This heterogeneity is not due to differences in iron content or aggregate size, but appears to represent structural differences in the apoferritin shells. Recent evidence indicates that some isoferritins differ in primary structure and that more than one gene codes for apoferritin subunits in several mammals (Linder *et al.*, 1972; Crichton *et al.*, 1973; Linder & Munro, 1973; Niitsu & Listowsky, 1973; Powell *et al.*, 1974). At present it is not known whether all ferritins

are homogeneous with respect to subunit composition or whether some exist as hybrid molecules.

In investigating the phenomenon of tissue-specific ferritins at the higher resolution offered by isoelectric focusing, we and others have found a more extensive heterogeneity in many ferritins than is apparent from electrophoretic analysis (Drysdale, 1970; Urushizaki *et al.*, 1971). The isoferritin profiles are characteristic of the tissue of origin, but vary in normal and diseased states (Makino & Konno, 1969; Alpert *et al.*, 1973; Urushizaki *et al.*, 1973a; Powell *et al.*, 1974). The validity of these results has, however, been repeatedly questioned (Lee & Richter, 1971b; Crichton, 1972). Bryce & Crichton (1973) purport to show that the heterogeneity displayed in horse spleen and human liver ferritins by electrofocusing is an artifact. The present paper refutes this contention and establishes that both of these ferritins are resolved into multiple components on gel electrofocusing, provided that equilibrium focusing conditions are achieved. Evidence is also presented that a related heterogeneity may be shown in horse spleen ferritin by the more conventional technique of ion-exchange chromatography.

Materials and Methods

Materials

Horse spleen ferritin (twice crystallized) was obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A. Human liver ferritin was obtained from fresh tissue *post mortem* from the Department of Pathology, Massachusetts General Hospital, Boston, Mass., U.S.A. This ferritin was isolated essentially by the method of Drysdale & Munro (1965), except that a different chromatographic procedure on CM-cellulose was used to take account of differences

in properties of rat and human liver ferritins (Drysdale & Ramsay, 1965). Both ferritins were further purified by chromatography on Sephadex G-200 or Sepharose 6B and analysed by electrophoresis in 5% (w/v) polyacrylamide-gel slabs in 30 mM-Tris-HCl, pH 8.0. The only bands found in both ferritin preparations stained for both iron and protein (Smith-Johannsen & Drysdale, 1969). Virtually all of both ferritins were in the monomeric form, i.e. single shells, as judged by gel electrophoresis (Williams & Harrison, 1968).

Analytical methods

Isoelectric focusing. Isoelectric focusing in a sucrose density gradient was performed in a 110 ml-capacity column by using ampholytes (Ampholines, pH 3–5, from LKB Produkter, Bromma, Sweden) at a concentration of 2% (w/v). The ferritin (about 7 mg) was introduced into the middle of the sucrose gradient before electrolysis was started. The column was maintained at 4°C and electrolysis was continued over 4 days. The initial voltage was 250 V and this was gradually increased to 600 V as the conductivity in the column decreased. At no time was the power delivered to the column allowed to exceed 2.5 W.

The column contents were drained by downward displacement and collected in 1 ml fractions. The E_{340} of the eluate was monitored to distinguish the yellow-brown ferritin from ampholytes, which interfere with protein absorbances at 280 nm (Righetti & Drysdale, 1971). It should be noted that this procedure monitors the distribution of ferritin iron rather than ferritin protein, and therefore discriminates against ferritin fractions of low iron content (Drysdale & Munro, 1965). The pH of the fractions was measured at 20°C.

Analytical gel electrofocusing in slabs or cylinders of polyacrylamide gel was performed essentially by the method of Righetti & Drysdale (1971, 1973) in apparatus from Medical Research Apparatus, Boston, Mass., U.S.A. High-porosity gels (4%) were used to minimize sieving effects on the large ferritin molecule and to allow equilibrium focusing in the allotted period. Gels were cooled by an antifreeze solution circulated with a Haake pump (Berlin, Germany) and maintained at 0°C by a cold finger (Neslab, Portsmouth, N.H., U.S.A.). Generally 10–50 µg of protein was used for each analysis. Protein samples were usually applied to the top of the gels in dilute alkaline buffer e.g. 20 mM-Tris-HCl, pH 8.0, in 10% (v/v) glycerol. Electrolysis conditions were controlled with a high-voltage pulse power supply capable of maintaining a constant average power delivered to the cell by regulating the pulse frequency of a predetermined potential difference. This system allows much better control of the amount of joule heating generated in the electrofocusing

cell than power supplies that regulate only voltage or current. A voltage of 400 V was applied for 6–20 h with an Automatic Regulated Pulse Power Supply (Medical Research Apparatus). The initial power delivered to each tube was limited to 0.15 W. Equilibrium was reached after 6 h, as shown by a fall in power to a low but constant value and the voltage regulation changing to d.c. mode. The pH gradient and the protein-banding pattern remained essentially the same for a further 18 h. Protein was stained with Coomassie Blue (Fairbanks *et al.*, 1968) or with Ponceau S after removing ampholytes by washing the gels for 24 h in repeated changes of 5% (w/v) trichloroacetic acid. Ferritin iron was detected by the Prussian Blue stain by immersing the gels for 10 min in a solution of 5% trichloroacetic acid, 5% HCl and 2% potassium ferrocyanide. Gels were then washed for 4 h in a mixture of 5% trichloroacetic acid and 5% HCl and finally in 5% trichloroacetic acid.

Ion-exchange chromatography

Ferritin (10 mg) was adsorbed on a column (20 cm × 3 cm) of DEAE-Sephadex A-50 equilibrated at pH 7.4 with 10 mM-sodium phosphate buffer at 4°C. The column was developed with increasing concentrations of the buffer up to a limiting buffer of 2 M-sodium phosphate. The entire fractionation was accomplished in about 1 h and recoveries of 60–80% were obtained. Longer elution periods gave substantially poorer recoveries.

Results

Fig. 1 shows a fractionation of horse spleen ferritin by isoelectric focusing in a sucrose density gradient. In agreement with Urushizaki *et al.* (1971)

Fig. 1. Isoelectric fractionation of horse spleen ferritin in sucrose-density-gradient system, pH range 3–5

Experimental details are given in the Materials and Methods section and in the LKB manual. ○, E_{340} ; ●, pH.

EXPLANATION OF PLATE I

Isoelectric fractionation of horse spleen and human liver ferritins in 4% polyacrylamide gels, pH range 3-6

(1) Mixture of horse spleen ferritin and human liver ferritin. Samples were mixed and loaded on top of gels after 30min electrolysis to remove residual persulphate. Gels were stained for non-haem iron with potassium ferrocyanide. (2) Mixture of horse spleen ferritin and human liver ferritin. Samples were mixed throughout gel solution before polymerization. Gels were stained for protein with Coomassie Blue. (3) Horse spleen ferritin. (4) Human liver ferritin. (5) Mixture of horse spleen ferritin and human liver ferritin. Samples 3, 4 and 5 were applied to the top of the gels after 30min electrolysis. After focusing, gels were stained for protein with Coomassie Blue. Gel compositions and electrolysis conditions are given in the Materials and Methods section.

EXPLANATION OF PLATE 2

Analysis of ferritin fractions obtained from DEAE-Sephadex chromatography by isoelectric focusing in a slab of 4% polyacrylamide gel

C, Unfractionated ferritin; (1) fraction from tubes 27–32, eluted near 0.075 M-phosphate; (2) fraction from tubes 45–50 eluted near 0.1 M-phosphate; (3) fraction from tubes 62–67, eluted near 0.2 M-phosphate. The samples were analysed in a horizontal slab (12 cm × 9 cm × 0.2 cm) of 4% polyacrylamide gel with parallel tracks according to the protocol given by the Medical Research Apparatus manual. Ampholyte pH range was 3–5. Total electrolysis period was 18 h at 400 V, regulated power 1 W. After focusing, the gel was washed for 6 h in 5% trichloroacetic acid to precipitate proteins and remove ampholytes, then in 1% trichloroacetic acid overnight. The gel was stained for protein with Ponceau S and overstained for iron with potassium ferrocyanide (Smith-Johannsen & Drysdale, 1969).

we could distinguish about three general areas which together comprised about seven discrete peaks with apparent isoelectric points between pH 4.1 and 4.7. Much of the resolution evident in the column was, however, lost during the rather lengthy elution period. Because of these practical difficulties we have preferred a small-scale analytical system for isoelectric focusing in polyacrylamide gels. This system gives highly reproducible pH gradients and protein patterns and offers faster and more convenient analyses at a higher resolution than obtainable in the sucrose density gradients (Righetti & Drysdale, 1973).

Plate 1 shows banding patterns given by subjecting human liver and horse spleen ferritin to gel electrofocusing in the pH range 3–6. Both ferritins were resolved into multiple components. The banding pattern and the pI range of the horse spleen iso-ferritins (Plate 1, gel 3) are in good agreement with results obtained above and with other analyses in sucrose density gradients (Urushizaki *et al.*, 1971), polyacrylamide gels (Drysdale, 1970) or Sephadex beds (Radola, 1973). The iso-ferritins from human liver banded in the pH range 5.1–5.6. Again, both the banding pattern and pH range are similar to previous results given by subjecting human liver ferritin to electrofocusing in polyacrylamide gels or sucrose density gradients (Righetti & Drysdale, 1973; Alpert *et al.*, 1973).

The banding patterns of horse spleen and human liver iso-ferritins given by iron staining (Plate 1, gel 1) show some quantitative differences from the iso-ferritin profiles given by protein staining (Plate 1, gel 5). These differences, which are most evident in the more acidic iso-ferritins in horse spleen, reflect a lower iron content of these iso-ferritins (Powell *et al.*, 1974; P. G. Righetti, J. T. Hazard & J. W. Drysdale, unpublished work).

Both sets of iso-ferritins from horse spleen and human liver were readily separable from a mixture of the two ferritins (Plate 1, gels 2 and 5). Moreover, essentially the same pattern resulted when samples were loaded on top of the gel or distributed throughout the gel solution before polymerization (Plate 1, gels 5 and 2 respectively). These results indicate that equilibrium focusing was achieved.

In attempts to demonstrate multiple forms by procedures other than electrofocusing, we investigated the behaviour of horse spleen ferritin on various ion-exchange resins. Fig. 2 shows a fractionation achieved by DEAE-Sephadex chromatography in which five peaks were eluted at different ionic strengths of a phosphate buffer, pH 7.4. Although a linear gradient was not used, transitions in ionic strength were made gradually. Each fraction was eluted cleanly from the ferritin bound to the top of the column. No 'roll up' of trailing peaks was observed.

Fig. 2. Fractionation of horse spleen ferritin on DEAE-Sephadex A-50

Details are given in the Materials and Methods section.

The three peaks eluted between 0.075M- and 0.2M-phosphate were dialysed against starting buffer and rechromatographed after storage at 4°C for 3 days. Each fraction rechromatographed essentially as a homogeneous peak, although each appeared to contain about 5–15% of fractions eluted at higher ionic strengths. Further experiments with more refined conditions are required to determine whether this represents cross-contamination or redistribution. These three chromatographic fractions were then analysed by electrofocusing in a flat slab of polyacrylamide gel. Although some of the separation in the gel is lost in the double-staining procedure, the results (Plate 2) clearly indicate a correlation between chromatographic behaviour and electrofocusing behaviour. It was noteworthy that fractions eluted at low ionic strengths contained iso-ferritins of lower pI than fractions eluted at higher ionic strengths.

Discussion

The results presented here confirm that both horse spleen and human liver ferritin are resolved into multiple components by isoelectric focusing in polyacrylamide gels and demonstrate that both sets of iso-ferritins are readily separable from one another. Our experiments also show that a similar fractionation of horse spleen ferritin may be obtained by ion-exchange chromatography. The electrofocusing analyses contrast with those of Bryce & Crichton (1973), who failed to distinguish between these ferritins and who could not resolve either into multiple iso-ferritins.

The resolution of this conflict seems entirely explicable by procedural differences. It is generally recognized that gel electrofocusing gives superior resolution to that obtained in the conventional sucrose density gradient (Awdeh *et al.*, 1968; Dale & Latner, 1969; Hayes & Wellner, 1969; Catsimpooulas, 1969; Righetti & Drysdale, 1971). However, in contrast with the latter method, where stable pH gradients

and protein patterns are readily obtained, many systems for gel electrofocusing suffer from a marked instability (usually a cathodic shift) in the pH gradient. This gradient instability may prevent proteins from reaching their isoelectric positions in the experimental period (Righetti & Drysdale, 1971, 1973). This problem is magnified when one attempts to focus large molecules such as ferritin (mol.wt. 440000), whose mobility is severely restricted in polyacrylamide gels.

We have largely overcome these problems by using a method that gives highly reproducible pH gradients that remain stable for at least 18 h (Righetti & Drysdale, 1971, 1973). Molecular sieving is minimized by use of a gel of high porosity. Most proteins, including ferritin, will generally reach their equilibrium positions in about 8 h in 4% gels containing 2% (w/v) ampholytes. Because we do not have to compromise with electrolysis periods, we conduct our experiments for 18 h to ensure equilibrium focusing. This system has been successfully applied to a number of well-characterized proteins (Righetti & Drysdale, 1973). Patterns given from mixtures of closely related haemoglobins showed that all of the bands detected by gel electrofocusing either had unknown counterparts in electrophoretic or chromatographic separations or were identifiable as different molecular species (Drysdale *et al.*, 1971; Bunn, 1973).

That equilibrium focusing was indeed achieved in the present experiments with ferritin is evident from the demonstration that essentially the same pattern was obtained when samples were introduced by loading on top of preformed gels or by mixing throughout the gel solution before polymerization. We prefer the former technique, since it prevents interaction with potentially harmful persulphate (Mitchell, 1967; Fantes & Furminger, 1967) which is removed from the gel by an initial period of electrolysis before sample application.

In our studies both horse spleen and human liver ferritins were resolved into multiple components with no overlap. Horse spleen isoferritins banded between pH 4.1 and 4.6, whereas human liver isoferritins banded between pH 5.1 and 5.6. Bryce & Crichton (1973) found by another method of gel electrofocusing that these ferritins appeared homogeneous and banded in similar positions. The latter result deserves comment. It has been known for some years that these ferritins differ in pI by about 1 pH unit (Mazur *et al.*, 1950) and are consequently readily separable by electrophoresis (Mazur *et al.*, 1950; Richter, 1965). They should therefore be readily distinguishable on isoelectric focusing, even in the widest pH ranges. Although Bryce & Crichton (1973) did not publish measurements of pH gradients developed in their gels, it is significant that they found human liver ferritin to band in gels

whose nominal pH range (3–5) was below the isoelectric range of this ferritin. These discrepancies suggest that Bryce & Crichton (1973) did not attain equilibrium focusing, possibly because they used a 7.5% gel which severely restricts the electrophoretic mobility of the large ferritin molecule (Drysdale, 1968).

The controversy about the heterogeneity in ferritins on isoelectric focusing has not been confined to results given in gels. Makino & Konno (1969) and Urushizaki *et al.* (1973a) found multiple forms in ferritins from rat liver and hepatoma by isoelectric focusing in sucrose density gradients. Lee & Richter (1971b) also examined ferritins from rat liver and hepatoma by the same procedure, but concluded that each ferritin was homogeneous. However, this seems unlikely, since their results show that both ferritins were eluted over a range of about 1 pH unit, a spread much greater than might be expected for a single molecular species (Vesterberg & Svensson, 1966).

Given that these ferritins are resolved into multiple components on isoelectric focusing, the question now arises as to whether the banding patterns represent a heterogeneity in the protein population before electrofocusing, or, as suggested by Lee & Richter (1971b), some artifact caused by interaction with the ampholytes. The latter possibility may now be ruled out by the findings that a related heterogeneity may be obtained by ion-exchange chromatography. To date we have been unable to separate all of the isoferritins in horse spleen by ion-exchange chromatography. We do not know whether this is due to incomplete separation on chromatography or to a partial interconversion of separated fractions. The former seems more likely, since isolated isoferritins are not redistributed when re-run (Alpert *et al.*, 1973) or stored at 4°C for 1 month (J. T. Hazard & J. W. Drysdale, unpublished work). Several other groups have also observed a heterogeneity in ferritins by ion-exchange chromatography. Theron *et al.* (1963) and Suran & Tarver (1965) obtained three fractions from horse spleen ferritin by DEAE-cellulose chromatography. Yu & Fineberg (1955) obtained three chromatographic fractions from rat liver that could be distinguished electrophoretically. A similar result has been obtained by Urushizaki *et al.* (1973b) who showed a correlation between rat liver isoferritins separated by ion-exchange chromatography and by isoelectric focusing.

The structural basis for such heterogeneity has not yet been established. Much of our present evidence suggests that the heterogeneity resides in the apoferritin moiety. Although one might expect the large and variable iron content of ferritin molecules to be reflected in differences in pI, it does not seem to be. Chemically reduced apoferritin shows the same heterogeneity (Drysdale, 1970; Urushizaki *et al.*, 1971). Moreover, although the iron content

of individual isoferritins in a tissue may differ substantially, there is no systematic variation in iron content through the isoferritin profile. Finally, the iron content of specific isoferritins may be markedly altered in pathological conditions such as secondary iron overload, with little effect on the characteristic distribution of isoferritins in different tissues (Powell *et al.*, 1974). Although we cannot exclude post-synthetic modifications, e.g. deamidation or conformational differences, much evidence points to small differences in the primary structure of the subunits. This is consistent with evidence suggesting that tissue-specific isoferritins differ in primary structure (Crichton *et al.*, 1973; Linder & Munro, 1973; Powell *et al.*, 1974). Although it is generally accepted that each ferritin is homogeneous with respect to subunit composition, others have found evidence to the contrary (Shafritz *et al.*, 1973; Niitsu & Listowsky, 1973).

Our results indicate that most tissues contain multiple isoferritins that migrate together on electrophoresis but are separable by gel electrofocusing. Each tissue ferritin population appears to consist of at least six isoferritins, many of which are common to several tissues. Those tissue ferritins that are most easily distinguished electrophoretically, e.g. those of liver and heart, contain few isoferritins in common. Those with similar electrophoretic mobilities, e.g. those of liver and spleen, have many isoferritins in common (Righetti & Drysdale, 1973; Alpert *et al.*, 1973; Powell *et al.*, 1974). It seems unlikely that these various isoferritins will each prove homogeneous with respect to subunit composition and unlikely that each represents a different gene product. A simpler explanation is that many tissue isoferritins represent families of hybrid molecules, each containing differing proportions of two or more dissimilar subunits.

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