GEL FILTRATION ANALYSIS OF EQUINE FERRITIN SUBUNITS

Paolo AROSIO

Istituto di Chimica Biologica, University of Milan, Ospedale San Raffaele, 20090 Segrate, Milano, Italy

Received 24 May 1979

1. Introduction

Conflicting data on ferritin substructure have been produced after the report of ferritin charge heterogeneity [1,2]. In horse spleen ferritin only one subunit type of 18 000–19 000 mol. wt was found by a number of analyses [3]. In the same type of ferritin a consistent amount of 11 000 and 8 000 mol. wt 'subunits' were also found [4]. Moreover 19 000 and 14 000 mol. wt 'subunits' were found in rat ferritins [5]. We showed that human liver ferritin run in reducing conditions on acid–urea gel electrophoresis displayed only one major band, while heart ferritin displayed two: one in common with the liver and another of slower mobility [6]. The ratio between these two bands, in these and other ferritins, was well correlated with their isoelectric points and immunological reactivities [7]. After improving the resolution of sodium dodecylsulphate (SDS)–gel electrophoresis by performing it in a gradient of polyacrylamide, we found that two major bands with similar mobility were present in all the ferritins analyzed, one band was called H subunit and of mol. wt ~21 000, the other L, of 19 000 [8]. Interfacing acid–urea- with SDS-electrophoresis it was found that the two systems separated the same peptides, and a good correlation was evident between H:L ratio and the isoelectric points of isoferritins from various species [8,9]. Other evidence suggested that the smaller peptides may arise from proteolytic digestion of the 19 000 and 21 000 mol. wt subunits [8,10].

Another form of subunit heterogeneity has been suggested from isoelectric focusing analyses in urea gels, in which up to 7 bands have been observed [11]; however it is not clear whether this heterogeneity is artifactually elicited by aggregation during the analyses or represents genuine subtypes of H and L subunits [8,11].

In a reappraisal of the SDS–gel electrophoresis patterns of horse spleen ferritin it was suggested that a 22 000 mol. wt peptide found in this ferritin derives from dimerization of the 11 000 mol. wt peptide [12].

Here subunits obtained by acidic dissociation of equine spleen, liver and heart ferritins are compared, after reduction, by analyses on gel filtration in denaturing conditions, and the effluents from the columns analyzed by SDS electrophoresis. The results support our previous findings on the presence of two separate and independent subunits in the horse ferritins (H, L) with different structures and characteristics.

2. Materials and methods

Ferritins from horse spleen, liver and heart were prepared as in [8]. Ferritins so obtained were electrophoretically pure as judged by coincidence of iron and protein staining [13].

Ferritins were denatured by a modified procedure [14]: after incubation for 18 h in 50% acetic acid at room temperature, they were centrifuged at 50 000 × *g* for 20 min to precipitate the iron cores, 2-mercaptoethanol was then added to 5% final conc. Samples so prepared were loaded on a Sephadex G-200 column (1.6 × 60 cm) equilibrated in 5% acetic acid. The flow rate was 7 ml/h, and 1.3 ml fractions were collected. Protein concentration in the effluents of the columns was detected using a modification of the

method in [15]: to 0.1 ml sample 1.0 ml 0.01% Coomassie brilliant blue G-250 in 8.5% phosphoric acid and 0.5% ethanol was added. After mixing the solution was read at 595 nm.

Ferritins and fractions of the columns were analyzed by polyacrylamide gradient pore SDS–gel electrophoresis following the method in [8].

3. Results and discussion

3.1. *Gel filtration of denatured ferritins*

The conditions used in gel filtration experiments: dissociation in 50% acetic acid and run at pH 2.5 are known to dissociate ferritin into subunits and to prevent their reassociation [11,14,16]. Figure 1 shows the typical elution profiles of denatured equine spleen and heart ferritins from a column of Sephadex G-200 equilibrated in 5% acetic acid. The two profiles had only one peak in common: peak 1, which was coincident with the void volume of the column and had SDS–electrophoresis patterns always very similar to the original ferritins (not shown). Thus it seems that peak 1 represents undissociated ferritin shells. Beside that component, spleen ferritin displayed one major symmetrical peak (peak 4) that probably represented the major subunit. Heart profile was rather different: besides the undissociated ferritin (peak 1) it showed two other peaks (2, 3) which were eluted before the major spleen peak.

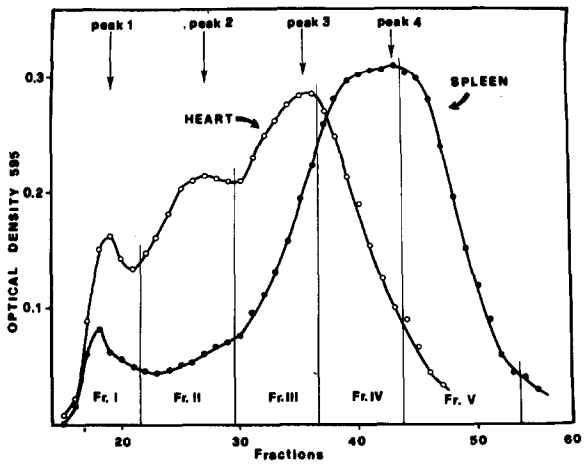

Fig.1. Elution profiles of denatured ferritins from horse spleen and heart from a column of Sephadex G-200 equilibrated in 5% acetic acid. Protein (2 mg) was loaded in 1 ml on each run.

A rough calibration of the column, performed by running bovine serum albumin and cytochrome *c*, indicated that peak 4 was ~20 000–30 000 app. mol. wt, while peaks 2 and 3 were 100 000–200 000.

The effluents of the columns were pooled in fractions I–V as indicated in fig.1, liophylized, resuspended in 6 N urea and further analyzed.

3.2. *Analyses by SDS electrophoresis of gel filtration fractions*

Protein concentration of pooled fractions was measured by the method in [15], using BSA as standard. Figure 2 shows the patterns on SDS gradient pore acrylamide electrophoresis of fractions II–IV from spleen, liver and heart ferritins, compared with the starting unfractionated samples. It appears that for each ferritin there was a clear enrichment in H subunit in fractions II, III. Moreover, in spite of relevant differences in corresponding fractions of the 3 ferritins, due to the very different H:L ratios in the starting ferritins, it was generally found that fraction II contained almost pure H subunit, and fraction III, even containing different amount of L subunit and smaller peptides, was still enriched in H subunit compared with the original ferritins; while fraction IV from liver and spleen was essentially free of H, being composed only of L subunit and the smaller fragments of probable proteolytic origin. Thus it is evident that gel filtration in the conditions described allows the separation of H from L subunits. It is noteworthy that samples which contained essentially pure H subunit, such as fractions II from spleen, liver and heart, and fraction III from heart were free of the smaller molecular weight peptides which were present in variable amounts in the fractions that contained L subunit.

3.3. *Gel filtration analyses of the fractions*

Particular attention was given to fraction II and IV from horse spleen, since they represented fractions containing the 2 subunits essentially not cross-contaminated, and extracted from the most studied ferritin. Figure 3 shows their elution profiles on the same column of fig.1. Both the fractions were eluted as single, wide and symmetrical peaks: fraction IV,

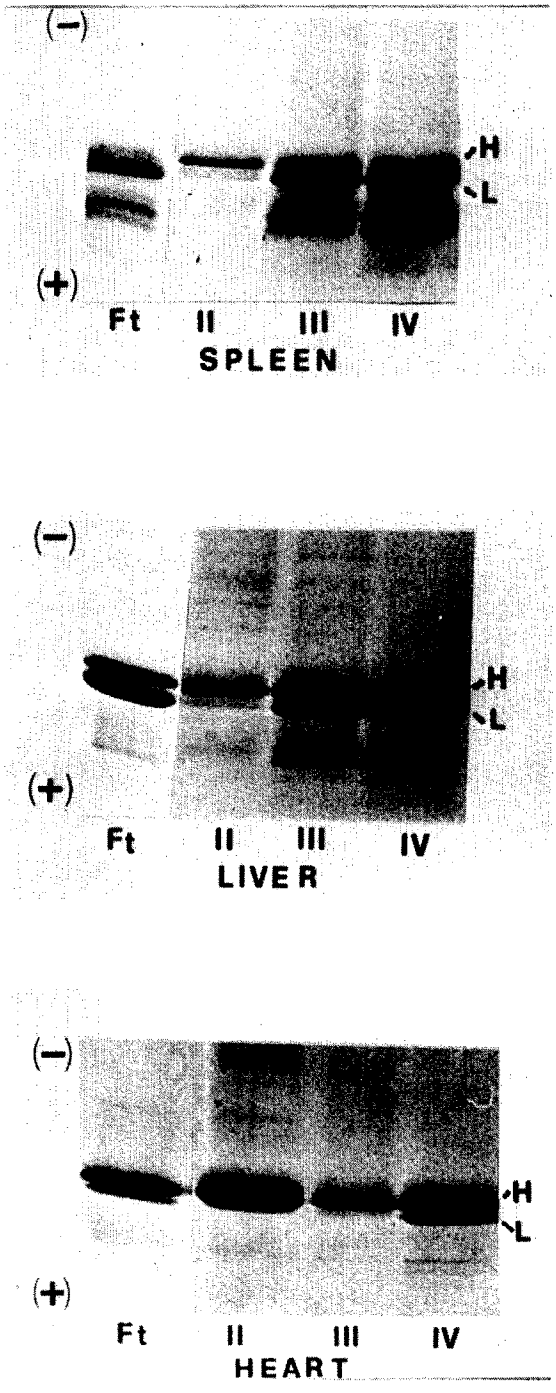

Fig.2. SDS gradient pore electrophoresis: equine spleen, liver and heart ferritins are compared with their fractions from the column of fig.1. Samples of 20–40 μg were applied on each track, except spleen II which contained only 10 μg.

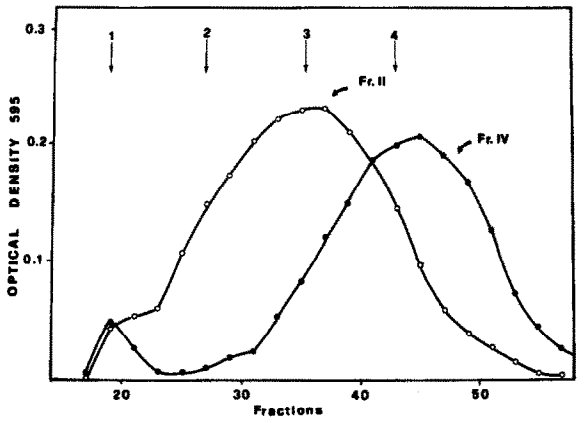

Fig.3. Elution profiles on Sephadex G-200 of fractions II, IV from horse spleen ferritin. Protein (2 mg) was applied in 1 ml on each run.

that basically represented peak 4, eluted in a volume equivalent of the first run; fraction II, that represented peak 2, was instead eluted in the position of peak 3. From this and the finding in fig.2, where fractions II, III of heart ferritin were both essentially composed of pure H subunit, it would appear that peaks 2, 3 represent 2 forms of this subunit, peak 2 probably a higher molecular weight aggregate that may be disrupted after incubation in 6 M urea.

The high apparent molecular weight and its good separation from the main peak of spleen subunit would indicate that peak 3 is an aggregate form of H subunit. Horse ferritin H subunit in acidic conditions was reported to readily form aggregates (fig.3 in [8]).

In conclusion, the findings here presented indicate that:

(i) It is possible to separate H from L subunit also by gel filtration, a method that is well fitted for preparative purposes;

(ii) In the above conditions (presence of 2-mercaptoethanol, acetic acid and urea) H subunit is in the form of aggregates, while L is not. This supports the hypothesis that the two subunits have different structures.

(iii) H subunit is present in two forms with different molecular volumes, and this characteristic allows its separation even from samples, such as spleen ferritin, which contains < 10% H.

(iv) Purified preparations of H subunit do not con-

tain the 11 000 mol. wt peptide (called B in [12]), thus H does not seem to be the product of dimerization of B peptide as suggested [12].

(v) H can now be separated from L subunit by acid–urea, SDS–gel electrophoresis and gel filtration in acetic acid; the 3 methods are known to separate peptides only or mostly on the basis of their molecular weight. The conditions of denaturation and analysis are different in the three procedures, thus it would appear that neither of the two subunits is artifactually produced by either SDS, urea or acetic acid. This strongly supports our hypothesis of ferritin being composed of 2 classes of mol. wt subunit, H and L.

Acknowledgements

This work was partially supported by CNR grant 78. 00453.86 of Progetto Finalizzato Tecnologie Biomediche.

References

[1] Drysdale, J. W. (1970) Biochim. Biophys. Acta 207, 256–258.
[2] Urushizaki, I., Niitsu, Y., Ishitani, K., Matsuda M. and Fukuda, M. (1971) Biochim. Biophys. Acta 243, 187–192.
[3] Bryce, C. F. A. and Crichton, R. R. (1971) J. Biol. Chem. 246, 4198–4205.
[4] Ishitani, K., Niitsu, Y. and Listowky, I. (1975) J. Biol. Chem. 250, 3142–3148.
[5] Linder, M. C., Moor, J. R., Munro, H. N. and Morris, H. P. (1975) Biochim. Biophys. Acta 386, 409–421.
[6] Adelman, T. G., Arosio, P. and Drysdale, J. W. (1975) Biochem. Biophys. Res. Commun. 63, 1056–1062.
[7] Arosio, P., Yokota, M. and Drysdale, J. W. (1976) Cancer Res. 36, 1735–1739.
[8] Arosio, P., Adelman, T. G. and Drysdale, J. W. (1978) J. Biol. Chem. 253, 4451–4458.
[9] Drysdale, J. W. (1977) in: Iron Metabolism, Ciba Found. Symp., vol. 51, pp. 41–67, Elsevier/North-Holland Amsterdam, New York.
[10] Lavoie, D. J., Marcus, D. M., Ishikawa, K. and Listowky, I. (1977) in: Proteins of Iron Metabolism, 71–78 (Brown, E. B., Aisen, P., Fielding, J. and Crichton, R. R. eds) Grune and Stratton, New York.
[11] Lavoie, D. J., Ishikawa, I. and Listowsky, I. (1978) Biochemistry 17, 5448–5454.
[12] Bryce, C. F. A., Magnusson, C. G. M. and Crichton, R. R. (1978) FEBS Lett. 96, 257–262.
[13] Powell, L. W., Alpert, K. L., Isselbacher, K. J. and Drysdale, J. W. (1975) Brit. J. Heamatol. 30, 47–55.
[14] Harrison, P. M. and Gregory, D. W. (1968) Nature 220, 578–580.
[15] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
[16] Crichton, R. R. (1975) in: Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R. R. ed) pp. 253–260, Elsevier/North-Holland, Amsterdam, New York.