ARTIFACTS IN FERRITIN ISOELECTRIC FOCUSSING PROFILES

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1. Introduction

The iron-storage protein, ferritin, consists of a 24 subunit protein shell surrounding a cavity capable of holding up to 4500 Fe(II) atoms as hydrous ferric oxide-phosphate [1]. Electrofocussing patterns of ferritins from several species have been examined [2]. They focus in the pH 4–6 range and often show broad profiles extending over nearly one pH unit. The profiles also exhibit 'microheterogeneity', i.e., they are divided into a number of discrete bands. Ferritins from different tissues of the same species usually focus over a slightly different pI range, and appear to show common 'isoferritins' in regions of overlap. To explain this phenomenon it was proposed [2–4] that all tissue ferritins from one species are heteropolymers of two subunits of different primary structure, designated L and H, giving 25 heteropolymers in all L_{24}, $L_{23}H_1$ - - - L_1H_{23}, H_{24}. L-rich species, which seem to be smaller than H, are said to predominate in liver and H-rich in heart.

After examining the isoelectric focussing patterns of several ferritins and albumins, we have suggested [5,6] that the discrete bands need not be attributed to discrete species of heteropolymer, but may result from steps in the ampholine pH gradients. We present evidence here, which confirms this conclusion.

2. Materials and methods

Ferritin was isolated essentially as described (method I of [6]) and electrofocussing performed in thin slabs [6]. Ampholines used (LKB Produkter, Bromma or Pharmalyte TM, Pharmacia Fine Chemicals AB, Uppsala) were in ranges pH 4.0–6.0 and 3.5–10 in a 2:1 v/v ratio. Staining for protein was carried out with Coomassie blue. Staining for iron was normally carried out as in method I below, but a modification of this procedure (method II) allowed us to see stripes running across the gel, which are important for the interpretation of isoelectric focussing patterns of proteins. In method I the gel was immersed immediately after focussing in the fixing solution (150 ml methanol plus 350 ml distilled water containing 17.25 g sulphosalicylic acid and 57.5 g trichloroacetic acid) for 30–60 min with several changes of fixer and then in a solution of 2% potassium ferrocyanide in 5% HCl for 10–30 min. It was washed for 4 h in 5% HCl in 20% aqueous ethanol. In method II the gel was immersed in fixing solution for 30–60 min without changes. Most of the fixing solution was then removed and the gel covered with 2% potassium ferrocyanide (no HCl) and gently agitated for 3–5 min. The gel was then transferred to a clean glass plate for photography.

3. Results

Figure 1a shows the results of electrofocussing and iron-staining by the normal procedure (method I) for several ferritin samples. A number of iron-staining bands of different intensity and unequal separation are visible in each ferritin. Figure 1b shows another portion of the same gel containing the same ferritin samples as in fig.1a, but stained by method II. Similar iron-stained bands are seen in the samples as in fig.1a, but lighter and darker stripes running right

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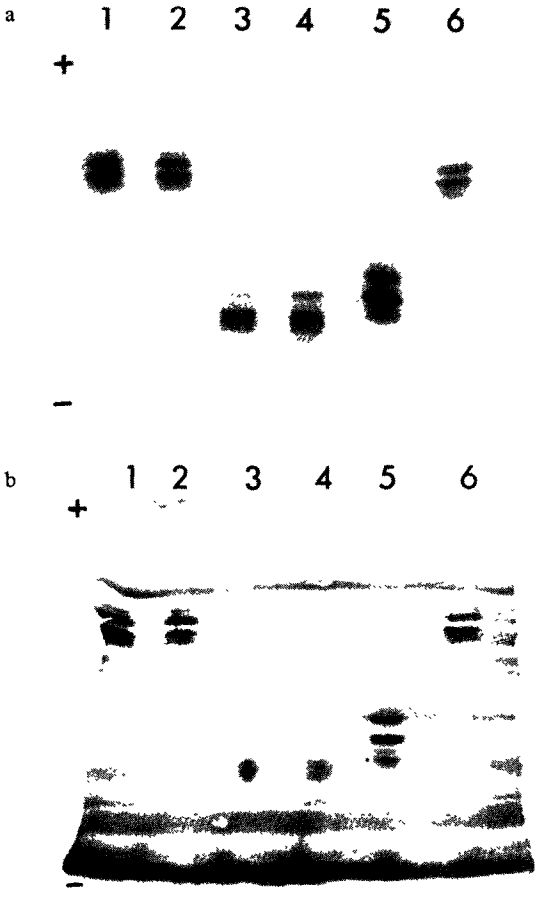

Fig.1. Electrofocussing patterns of gels containing ferritin samples. Gels were treated after focussing: (a) by the normal iron-staining procedure, method I; (b) by method II (see text). Focussing was performed in gels containing 2:1 mixtures of pH 4–6 and pH 3.5–10 LKB ampholines. The ferritin samples in both gels are from: (1,2) horse spleen; (3,4) human liver; (5) rat liver; (6) horse spleen.

across the gel are also clearly visible. In the pH range in which ferritin has focussed (from pH 4.1 on the acid end of horse ferritin to pH 5.9 on the basic end of human ferritin) the darker stripes are continuous with ferritin bands and the lighter regions are continuous with regions of the ferritin sample, which have taken up little or no stain. However, it should be emphasised that although the stripes, like the Prussian blue ferritin bands, appear grey on the photograph, they have not stained for iron. On the gel the dark and light stripes are white (of varying translucency) and transparent, respectively. The stripes remain visible for 1–2 h if method II is followed, but if acid is added, they vanish rapidly and, if 5% HCl is added along with the 2% potassium ferrocyanide, they are not seen (e.g., in the gel shown in fig.1a, stained by method I). Nor are they visible after fixing alone (the gel is clear). If the fixing solution is completely removed before adding ferrocyanide, the stripes may be seen, but the iron-containing protein appears white. Thus, while some acid is required for the development of the Prussian blue colour in ferritin, too much acid prevents the continuous stripes from being seen. The appearance of these stripes is not dependent on the presence of protein in the gel. They can be seen if the ampholines are focussed alone and the gel treated by method II and they are present in regions of the gel, fig.1b, where there is no protein. Although the continuous stripes were only seen when the gels were treated by method II, the phenomenon underlying their formation is not dependent on treatment with potassium ferrocyanide. This is strongly suggested by comparing electrofocussing patterns in fig.2a,b. These were obtained from the same gel, which was cut in two before staining. Ferritin samples in fig.2a were stained for protein with Coomassie blue, those in fig.2b were stained for iron by method I. Similar bands can be seen in all 4 samples

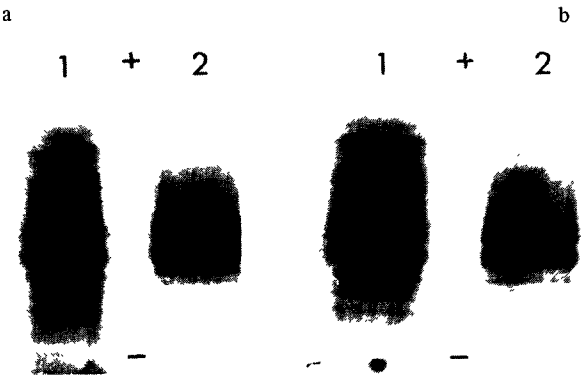

Fig.2. Gel electrofocussing patterns of human liver ferritin. Duplicate samples were applied to 2 halves of the same gel, which was cut in 2 after focussing, and stained with: (a) Coomassie blue for protein; (b) $K_4Fe(CN)_6$ for iron. Only those portions of the gels at which the samples have focussed are shown. Samples are from the same preparation of human liver ferritin, but samples 2 have been crystallised with $CdSO_4$ before focussing. Ampholines used as in fig.1.

(and in others in the same gel, not shown). Taking these results together (and many similar patterns we have obtained) it is clear that the sharp bands in ferritin and the continuous stripes must have the same underlying cause.

The patterns shown in fig.1,2 were obtained with LKB ampholines. We have also examined gels containing Pharmalyte ampholines, with or without ferritin samples, treated by method II after focussing. They too show the same phenomenon of ferritin bands continuous with gel stripes, but their separation is different from that obtained with LKB ampholines, so that the same ferritin samples appear to contain different components or 'isoferritins'. This can also be seen in gels stained with Coomassie blue. Figure 3 shows horse spleen ferritin focussed with: (a) LKB; (b) Pharmalyte ampholines of the same pH range.

4. Conclusion

The results presented here confirm our suggestion [5,6] that the discrete bands seen in isoelectric focussing patterns of ferritin and some other proteins are a methodological artifact. It follows that they should not be taken to represent discrete species, L_nH_{24-n} of heteropolymer ($n = 0–24$). It is also misleading to describe bands common to different tissue ferritins as 'common isoferritins' as some workers do [2]. The continuous stripes across the gel appear to represent discontinuities in the ampholine pH gradients. Although not equally spaced they correspond, on average, in the gels with the LKB ampholine mixtures described, to pH steps of ~0.1 pH unit. It is not entirely clear why they are visible when gels are treated by method II, but presumably the ampholines are more rapidly eluted by other treatments. The fact that iron staining is used infrequently may explain why the stripes have not been noticed previously. It is also possible that with other ampholine mixtures or other pH ranges the pH steps may be more closely spaced and separate bands less clearly distinguished. However, it is clear that isoelectric focussing patterns, whether of ferritin or of other proteins, which do show multiple bands, should be interpreted with caution.

Although the pattern of sharp bands observed by isoelectric focussing seems to result from concentration of the protein during focussing at the pH steps of the ampholines, the method does show that molecules with a range of pI values are present within ferritin preparations. Indeed trends in pI are sometimes associated with trends in iron content [6–8] and these in turn may be associated with charge differences among the 24 subunits [7]. Such differences could arise from post-translational modifications or minor variations in structure affecting sidechain *pK* values and need not have a genetic origin.

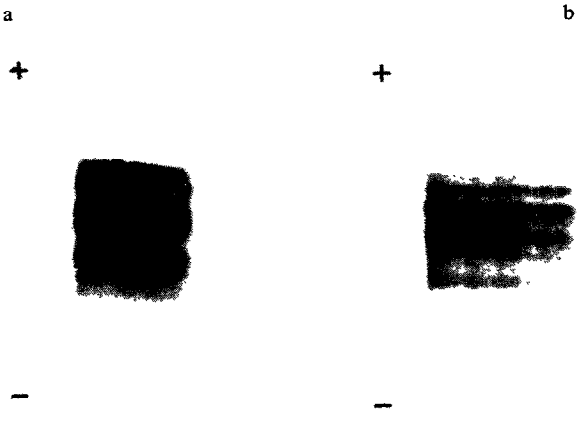

Fig.3. Gel electrofocussing patterns of horse spleen ferritin. Electrofocussing was carried out in gels containing ampholines of the same pH ranges as in fig.1, but the ampholines were: (a) LKB; (b) Pharmalyte TM.

Acknowledgements

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References

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