HORSE SPLEEN FERRITIN: A CHANGE OF CRYSTAL FORM DURING CRYSTALLISATION OF MONOMER IS ASSOCIATED WITH THE APPEARANCE OF OLIGOMERS

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1. Introduction

The conventional preparation of ferritin from horse spleen includes crystallisation in the presence of cadmium sulphate. The product is heterogeneous to electrophoresis on starch or polyacrylamide gel because some of the spherical units exist as dimers, trimers and higher oligomers [1–3].

The nature and origin of ferritin oligomers is not known. We have now separated the ferritin monomer and the individual polymeric forms by preparative polyacrylamide gel electrophoresis and have found that formation of oligomers occurs during the crystallisation of ferritin monomer.

2. Materials and methods

Ferritin, twice-crystallised from cadmium sulphate, was purchased from Koch-Light Lab. or prepared by the method in [4]. As is well known, this material is heterogeneous to electrophoresis on starch gel or polyacrylamide gel because of the presence of oligomers [2,3]. We prepared monomer essentially free of oligomers by means of preparative polyacrylamide gel electrophoresis in an apparatus which provides for intermittent, automatic collection of fractions as they emerge from the end of the gel [5]. The concentration of gels is described in terms of % *T* (g total acrylamide monomer/100 ml solution). All were 5% cross-linked (5% C); i.e., 5 g *N,N'*-methylene–bis-acrylamide/100 g total monomers. The conditions used were: gel 5% T, 5% C of cross-section 5.9 × 1.0 cm [5], buffer 25 mmol/l borate (sodium) (pH 8.4), voltage gradient 4 V/cm, path-length 4 cm, 5°C. Sample was applied in a warm solution of agarose, to prevent electro-decantation. The product was freed of buffer by dialysis against water and concentrated by ultrafiltration.

Crystallisation was carried out by adding 250 μl cadmium sulphate solution ($CdSO_4 \cdot SH_2O$, 20%, w/v) to 1.0 ml of ferritin solution (30 mg/ml, pure monomer or preparations containing oligomers, as appropriate). After 5–10 min, 20 min or 24 h a sample of this solution was withdrawn for microscopic study and the remainder was centrifuged rapidly to recover the crystals which were then dissolved in 1% (w/v) ammonium sulphate solution, dialysed against a large excess of distilled water for 24 h and finally centrifuged at 100 000 × *g* to clarify the solution before electrophoresis.

Analytical electrophoresis was carried out in horizontal polyacrylamide gel slabs of cross-section 4.0 × 0.6 cm, and conditions were otherwise as for preparative electrophoresis. Gels were stained for iron with Perls' reagent [6] or for protein with amido black 10B.

Apoferritin was prepared from ferritin (whether pure monomer or preparations containing oligomers) as in [7], with slight modifications. The procedure involves dithionite reduction, precipitation by ammonium sulphate (50% saturation) and cadmium crystallisation under conditions resembling those above.

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To explore the occurrence of ferritin oligomers in vivo, extracts were tested which had not been exposed to high salt concentrations. Spleens were obtained from freshly killed horses and homogenised as in [4]. Aliquots of such homogenates were mixed with twice their volume of distilled water. One extract was heated to 80°C for 2–3 min and another was kept at 0°C throughout. Both extracts were strained through cheese-cloth, dialysed against running tap water for 4 days and centrifuged at 10 000 × *g* for 30 min before analytical electrophoresis.

3. Results

3.1. *Preparation of monomer*

Separation of ferritin monomer and oligomers is shown in fig.1. The peaks are distinct and the compositions as revealed by electron microscopy may be regarded as showing minimum estimates of purity, because conditions during specimen preparation for microscopy are such that either association and dissociation could occur, or both, and because accidental juxtaposition of monomers may give a slight overreading of oligomer numbers. No cross-contamination could be detected by analytical electrophoresis. In this experiment, which was typical, the proportions of monomer, dimer and trimer were as 90.2:7.5:2.2; based on absorbance at 280 nm and neglecting tetramer and higher oligomers.

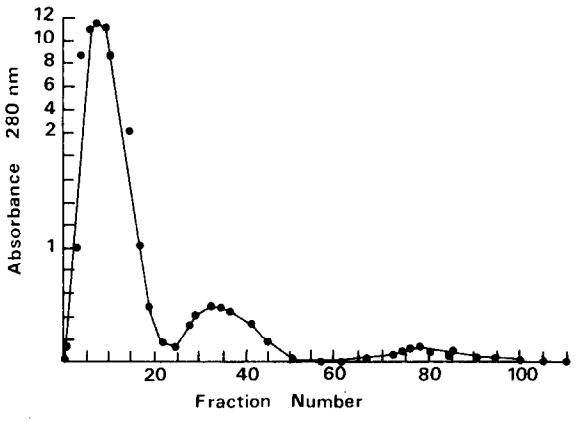

Fig.1. Preparative electrophoresis was conducted as in the text and [5]. Sample, 45 mg ferritin [4]. Fractions from each peak (and from the position of the tetramer peak as predicted by extrapolation) were pooled, concentrated and examined by electron microscopy:

Peak	Distribution of iron 'cores' (%)			
	Monomer	Dimer	Trimer	Tetramer
1	96	4	–	–
2	15	82	3	–
3	22	20	58	–
–	–	–	–	100

3.2. *Crystal form and appearance of oligomers*

When pure ferritin monomer was subjected to the crystallisation procedure, the first crystals appeared to be tetrahedral and later showed 3 triangulate lobes. Crystals of the accepted normal ferritin form were seen only after 24 h. But if whole ferritin were subjected to the same conditions, crystals of the normal form appeared at once. Fig.2 shows these results, also that solutions made from the early crops of monomer crystals were free of oligomers, which appear, however, in the older preparations, after the change to normal crystal shape.

3.3. *Production of oligomers under other conditions*

In view of these results, it was natural to try the effect of treating ferritin monomer in various ways, to determine whether oligomers were produced. The results quoted are by visual assessment of the results of analytical electrophoresis:

- 25 mmol/l borate (sodium) pH 8.4 at 4°C for 3 months; no dimer
- Freezing and thawing, buffers as above; no dimer
- Drying in ultrafiltration sac for 48 h; ~1% dimer
- Dialysis against 9 g/l NaCl solution, 4°C, 2 days; ~4% dimer
- Reduction to apoferritin; all oligomeric forms of apoferritin in the usual proportions
- Reduction to apoferritin but omitting precipitation and crystallisation steps; ~3% apoferritin dimer plus a trace only of trimer

3.4. *Occurrence of oligomers in vivo?*

Fig.3 suggests that extracts prepared from fresh horse (avoiding exposure to ammonium or cadmium sulphate) spleen contain less oligomers than the usual ferritin preparations. Heating to 80°C has little effect except for increasing the proportion of material which fails to penetrate the gel.

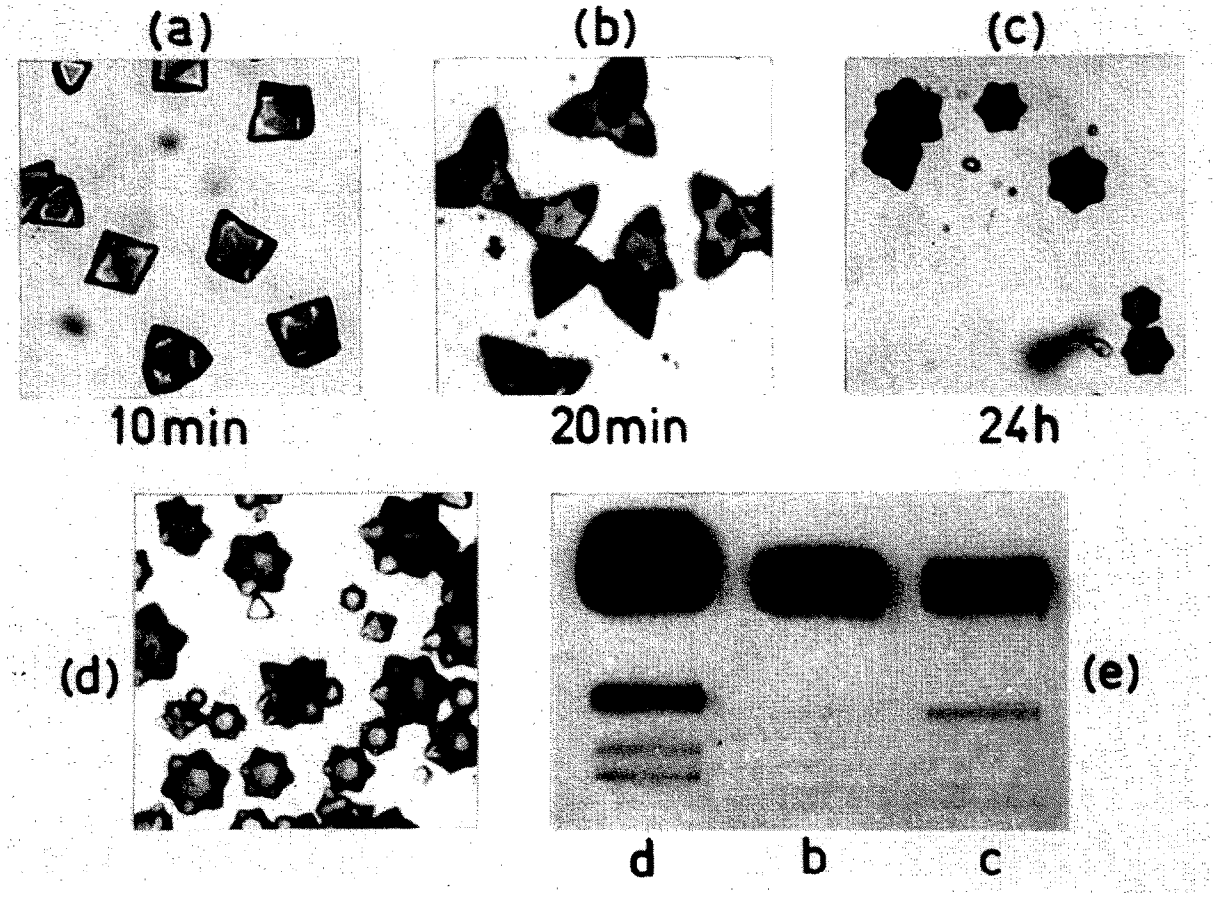

Fig.2. (a–c) Photographs through microscope of crystals obtained from ferritin monomer after contact with cadmium sulphate for the time stated. (d) Crystals from standard preparation of ferritin (containing oligomers), after 10 min. (e) Analytical electrophoresis of ferritin solution derived from crystals (b–d).

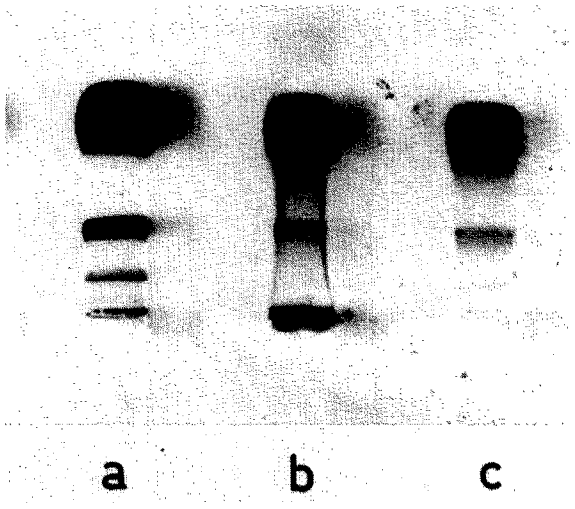

Fig.3. Analytical electrophoresis of: (a) horse spleen ferritin prepared according to [4]; (b,c) horse spleen extracts prepared as described in the text, without exposure to ammonium sulphate or cadmium sulphate: (b) heated to 80°C for 3 min; (c) kept at 0°C.

4. Discussion

Ferritin monomer is usually made by recycling gel filtration chromatography. Using Sepharose 6B [8], preparations were obtained comparable with ours according to electron microscopic appearances, but the qualitative electrophoresis experiments in [8] suggested slight, persistent cross-contamination between adjacent 'peaks', which we cannot detect in

our material, obtained in a single operation. This emphasises the extraordinary power of preparative gel electrophoresis for separating proteins which differ chiefly in molecular size, and may also have a bearing on the lack of previous reports of a peculiar crystal form of freshly crystallised monomer ferritin.

Probable explanations of our findings are that of oligomer formation is favoured by moderate to high salt concentrations (perhaps also by close approximation of spherical units in the crystal) and that the lattice of the usually observed type of crystal selects a certain amount of each species of oligomer. This would explain the consistency of the proportions of each oligomer type in samples of ferritin prepared by the conventional method. There seems little doubt that oligomer formation is powerfully induced by the process of crystallisation.

Anomalous patterns obtained on X-ray diffraction of ferritin or apoferritin crystals led to great difficulty in establishing the structure of the monomer. Although the simple 24-subunit structure was suggested at a very early date, it was not finally confirmed [9] until after chemical analytical data proved that the alternative 20-subunit structure, of lower symmetry, was impossible. It seems likely that the observed pattern from ferritin crystals was due to the presence of oligomers and associated with this an unexpectedly complex lattice structure. This suggestion is reinforced by our finding of a different crystal habit for the pure monomer. In apoferritin, and perhaps also in ferritin, cadmium ions occupy positions in the crystal which allow them to act as bridges between adjacent spherical units [9]. They might also act as agents in the process of oligomerisation.

Dimer is formed by other treatments besides crystallisation. There may be more than one route to oligomer formation, and the presence of any oligomer, however formed, might promote crystallisation in the usually observed habit.

The final question is whether oligomers are wholly artefactual. A conclusive answer cannot yet be given. Fresh horse spleen homogenate appeared to contain some dimer, but the amount was less than in conventional ferritin preparations, and it is conceivable that dimer formation occurred during the homogenisation process. Ferritin molecules in vivo may well be exposed to conditions under which we would, in view of the above results, now expect dimer formation. Probably, however, if any is actually present in vivo, it is in lower proportion than seen in conventional preparations of purified ferritin.

All the above results throw new light on the nature and origin of ferritin oligomers and on anomalies in the X-ray diffraction pattern of ferritin.

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