A REAPPRAISAL OF THE ELECTROPHORETIC PATTERNS OBTAINED FROM FERRITIN AND APOFERRITIN IN THE PRESENCE OF DENATURANTS

Charles F. A. BRYCE, Carl G. M. MAGNUSSON and Robert R. CRICHTON

Department of Molecular and Life Sciences, Dundee College of Technology, Dundee, Scotland and *Unité de Biochimie, Université de Louvain, Place Louis Pasteur, 1, 1348 Louvain-la-Neuve, Belgium*

Received 4 October 1978

1. Introduction

Some years ago we demonstrated by polyacrylamide gel electrophoresis that the detergent sodium dodecyl sulphate (SDS) was capable of denaturing ferritin and apoferritin into subunits and that the molecular weight of these subunits was of the order of 18 000–19 000 [1]. This finding was later confirmed using a variety of techniques [2,3] and is now the generally accepted value for the molecular weight of the protein subunit.

Since that time a number of groups, using SDS–polyacrylamide gel electrophoresis, have also demonstrated the presence of two smaller polypeptide species, B and C, of mol. wt 11 000 and 7000–8000, respectively [3–6]. Although at present there is no definitive evidence as to the nature of the origin of peptides B and C, it is clear that they are derived from the subunit of mol. wt 18 000–19 000 [4–6]. Peptides B and C have been purified and it has been shown that the sum of their amino acid compositions agrees well with the amino acid composition of the subunit [5,6]. It has also been shown that a tryptic fingerprint of peptide C yields a ninhydrin-negative arginine-positive peptide characteristic of the *N*-acetyl blocked serine of the N-terminus of apoferritin.

Whilst the origin of the two lower molecular weight peptides from the subunit is of considerable interest to us, we also felt that these peptides could be used to good advantage in the protein primary sequence studies provided they could be isolated in high yields in a purified form. In the course of such peptide isolation and purification we observed, on deliberate overloading of SDS gels, a number of additional molecular weight species.

We describe here a novel interpretation of these electrophoretic patterns as discussed in relation to a number of independent studies by other groups of workers both in terms of fundamental subunit structure and of the alleged protein microheterogeneity.

2. Materials and methods

Ferritin was purchased from Miles-Pentex Corp., Mann Research Labs and Boehringer-Mannheim. Ferritin from horse spleen, horse liver and human liver were also prepared by a modified method [7] of that in [8].

Apoferritin was prepared from ferritin by reduction with thioglycollic acid/acetate buffer, pH 5.5, followed by extensive dialysis against several changes of 0.01 M phosphate buffer, pH 7.0.

Gel electrophoresis in the presence of sodium dodecyl sulphate was carried out by the method in [9] as in [2] whilst electrophoresis in acetic acid–urea was performed as in [10].

3. Results and discussion

3.1. *Effect of denaturation time on the electrophoretic pattern*

Ferritin was treated with 1% SDS, 1% 2-mercaptoethanol in 0.01 M phosphate buffer (pH 7.0) for 4 h at room temperature. When 20 μg protein

were applied to a gel and electrophoresed (10 mA/gel) for 8 h, three bands were visible on staining the gels (see fig.1a). These were shown to represent undissociated protein, subunit dimer (mol. wt 37 000) and subunit.

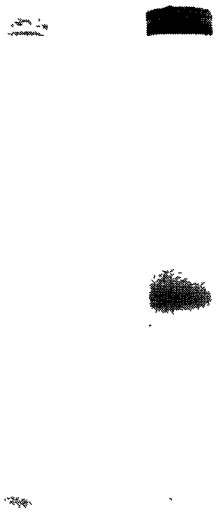


Fig.1. SDS–polyacrylamide gel electrophoresis of horse spleen ferritin. Effect of denaturation time on the electrophoretic pattern. Gel (a), 4 h incubation at room temperature. Gel (b), 18 h incubation at room temperature. In all figures shown, the anode is at the bottom and all gels are stained with Amido Black.

If the sample was allowed to incubate at room temperature overnight prior to electrophoresis then the subunit dimer band disappeared (see fig.1b).

This observation also holds true for samples prepared by heating at 100°C for 3 min in the sample buffer. If the sample is electrophoresed immediately, the band corresponding to subunit dimer exists whereas no such band is present in material which has been left to incubate overnight following this treatment.

Two findings here that are worth stressing are that under these conditions of sample preparation (1% SDS, 1% 2-mercaptoethanol), undissociated protein still exists. This observation differs from [11] where it is argued that complete degradation of apoferritin into subunits can be attained at >0.5% SDS. It should be pointed out, however, that these workers also include 6 M urea in their sample buffer. The subunit dimer band is also visible in the gels published elsewhere and it would appear that it is necessary to include a fairly lengthy incubation and/or dialysis step in order to eliminate this molecular species from the electrophoretic band pattern.

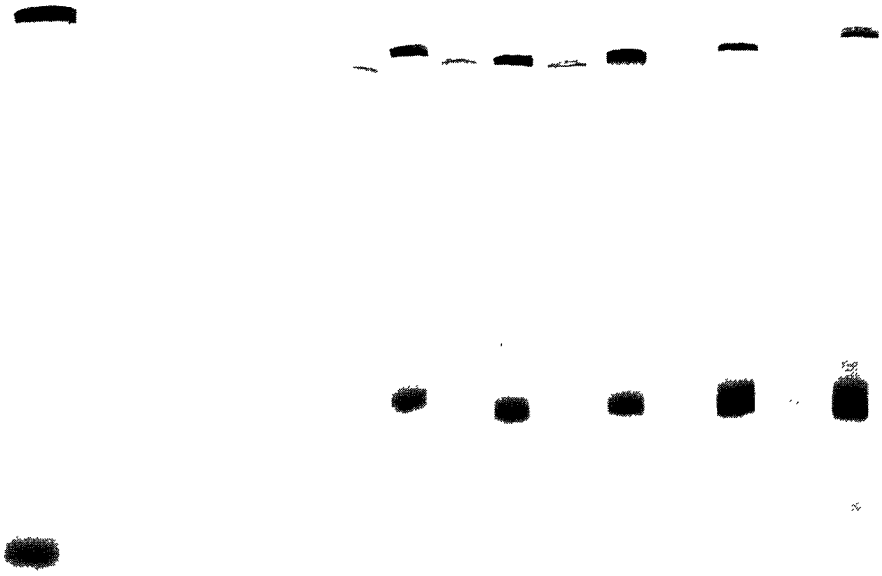
3.2. *Effect of protein concentration on the electrophoretic pattern*

In our earlier work on the subunit structure of apoferritin by SDS–polyacrylamide gel electrophoresis [2] by the method in [9] we routinely saw one band only, that representing the 18 000–19 000 mol. wt species (see fig.2a). In order to detect peptides B and C and the additional bands it was necessary to deliberately overload the gels with ~100–200 μg total protein (see fig.2b). Clearly then it is important to know the quantity of protein being electrophoresed in order to interpret the band pattern accordingly.

3.3. *Effect of incubation temperature on the electrophoretic pattern*

Figure 3 shows the effect that increasing the temperature of the sample buffer has on the distribution of molecular species.

With gels which are normally loaded, little change appears in the electrophoretic pattern (i.e., a single band) until ~90°C. At >90°C trace amounts of the



a b

Fig.2. SDS–polyacrylamide gel electrophoresis of horse spleen ferritin. Effect of protein concentration on the electrophoretic pattern. Gel (a), 5 μl sample. Gel (b), 50 μl sample.

Fig.3. SDS–polyacrylamide gel electrophoresis of horse spleen ferritin. Effect of incubation temperature on the electrophoretic pattern. Gels (from left to right): 5 μl sample at 62°C, 50 μl sample at 62°C; 5 μl sample at 82°C, 50 μl sample at 82°C; 5 μl sample at 87°C, 50 μl sample at 87°C; 5 μl sample at 92°C, 50 μl sample at 92°C; 5 μl sample at 100°C, 50 μl sample at 100°C; (sample maintained at each temperature for 30 min).

other components (7000–8000, 11 000, 15 000 and 22 000–23 000) are just visible.

With gels which are deliberately overloaded it appears that as the temperature increases the amounts of B and C increase. This observation previously led us to believe that B and C were the results of a specific heat-generated scission of the apoferritin polypeptide chain (C.F.A.B. and W. Kiddie, unpublished observation). However, the amount of subunit also increases while the amount of undissociated protein decreases. If we have an equilibrium between undissociated protein, dissociated subunit and B and C peptides then an increase in temperature merely moves the equilibrium over in favour of dissociated material. Since the proportion of B and C to subunit does not vary significantly over this temperature range, this would argue against the view that B and C peptides are the results of thermal degradation of the intact subunit polypeptide.

In the course of this study we also tested the apoferritin sample by treating it at 100°C for 3 min since this appears to be the method most often used

nowadays in sample preparation. The results are shown in fig.4. In a sample so treated we can clearly identify 7 specific bands representing undissociated protein, subunit dimer, 22 000–23 000 mol. wt polypeptide, subunit, 15 000 mol. wt polypeptide, peptide B and peptide C.

Fig.4. SDS–polyacrylamide gel electrophoresis of horse spleen ferritin. Sample (50 μl) prepared by incubating at 100°C for 3 min.

We can account for each unique electrophoretic band in the following way:

(i) 1% SDS, 1% 2-mercaptoethanol is not sufficient to dissociate all the protein, especially in the case of short incubation times.

(ii) The subunit dimer is an intermediate in the dissociation of apoferritin/ferritin monomer [12]. We have already shown that this molecular weight species can be eliminated by increasing the time of incubation in detergent.

(iii) The subunit is the basic structural component of the apoferritin/ferritin monomer.

(iv) Peptides B and C are derived from the subunit by some form of specific proteolysis.

(v) The 22 000–23 000 mol. wt species is the result of dimerization of peptide B.

(vi) The 15 000 mol. wt species is the result of dimerization of peptide C.

In terms of the dissociated material, then the subunit, peptide B and peptide C are, to all intents and purposes, basic to the structure whilst the other bands are secondary and result from polymerization either via disulphide interchange or hydrophobic interaction of peptides or SDS–peptide complexes.

3.4. *Effect of the concentration of SDS and 2-mercaptoethanol on the electrophoretic pattern*

Preliminary work in this study indicated that an increase in SDS concentration resulted in a decrease in the concentration of B. This is at present being studied more extensively in a quantitative manner. Certainly what appears to be the case is that at $\leq$0.05% SDS the gel patterns become uninterpretable smears. At $\gtrsim$ 7.5% SDS, the resolution between subunit, peptide B and peptide C is greatly improved (see fig.5).

At low concentrations of 2-mercaptoethanol it proved difficult to discern discrete bands. This again improved by raising the concentration.

Interestingly, we also showed in preliminary studies that BB was not apparent when denaturation times were short but was visible in the same sample following more extensive incubation. It can be argued that in a short denaturation time, the concentration of B is not very high and hence the concentration of BB will be correspondingly low whereas with longer incubation times the concentration of B is higher and so BB is also present at a higher concentration and

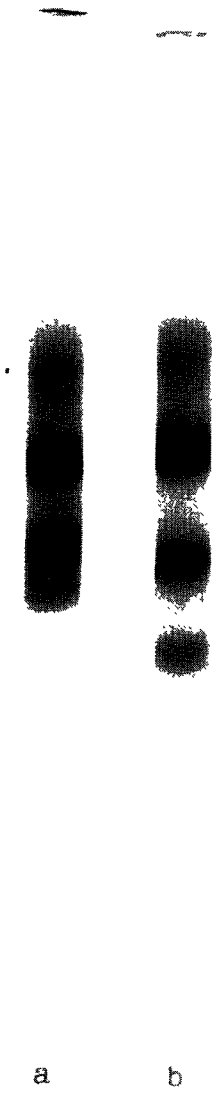


Fig.5. SDS–polyacrylamide gel electrophoresis of horse spleen apoferritin. Effect of (SDS) on electrophoretic pattern. Gel (a), 1% SDS. Gel (b), 10% SDS.

therefore more likely to be detected on the gels.

In all our studies on the effect of various parameters on the gel electrophoretic patterns we were able to interpret the experimental data in terms of our proposed model.

Similar studies have been carried out [4,5] although only the 23 000 mol. wt species was found using SDS–Tris–glycine buffer system. They found that they could eliminate this band by prior treatment with 2-mercaptoethanol. These workers interpretation of the 15 000 mol. wt component is different from the one discussed here. They found that the 15 000 mol. wt component was not present following treatment with 2-mercaptoethanol or dithiothreitol and that it was possible on isolation and purification to generate from this material components of mol. wt 19 000, 11 000 and 7000–8000. They argue that the '15 000' species has in fact mol. wt 19 000 but because of a tight intact disulphide bridge the molecule has a higher mobility than expected. This we have been unable to demonstrate. An alternative explanation is that on isolation of CC (the 15 000 mol. wt species) a small amount of band B was included. This mixture could then give rise to BC (19 000 mol. wt), B and C.

During these studies and independent ones with ferritins and apoferritins isolated from a variety of sources, we observed a remarkable parallel between the gel patterns obtained on electrophoresis in the presence of SDS and those obtained on electrophoresis in acetic acid–urea, both in terms of the number of bands present and the intensity of staining.

For this reason we would like to present an alternative interpretation of the electrophoretic patterns obtained in acetic acid–urea gels to that proposed [10]. In our interpretation we assume that the L-subunit (as proposed [13] is in fact the basic 18 000–19 000 mol. wt subunit whereas the H subunit represents the dimer of B rather than an isoferritin subunit. The two bands which are apparent below the subunit are peptides B and C as in the case of SDS–polyacrylamide gel electrophoresis. In some acetic acid–urea gels a band corresponding to subunit dimer is also present.

The significance of this interpretation cannot be overestimated. In an earlier study [14] it was reported that horse liver ferritin appeared to give rise to two bands corresponding to mol. wt 18 000 and 21 000 although any attempts to isolate the heavier molecular weight species were unsuccessful. The reason for this in part could be explained by BB being converted back to B some of which may be lost on extensive dialysis.

We are at present isolating each molecular weight species described in this communication by a variety

of techniques and once we have these in high yields and purified it should be possible to characterize these extensively and so provide an unequivocable interpretation of the gel electrophoretic patterns.

Acknowledgements

We would like to thank Professor A. Goffeau and Dr J.-M. Van Dijk for their interest in this study. One of us (C.F.A.B.) would like to thank the European Molecular Biology Organisation for the award of a short-term fellowship.

References

[1] Crichton, R. R. and Bryce, C. F. A. (1970) FEBS Lett. 6, 121–124.
[2] Bryce, C. F. A. and Crichton, R. R. (1971) J. Biol. Chem. 246, 4198–4205.
[3] Bjork, I. and Fish, W. W. (1971) Biochemistry 10, 2844–2848.
[4] Niitsu, Y., Ishitani, K. and Listowsky, I. (1973) Biochem. Biophys. Res Commun. 55, 1134–1140.
[5] Ishitani, K., Niitsu, Y. and Listowsky, I. (1975) J. Biol. Chem. 250, 3142–3148.
[6] Collet-Cassart, D. and Crichton, R. R. (1975) in: Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R. R ed.) pp. 185–192, North-Holland, Amsterdam.
[7] Crichton, R. R., Miller, J. A., Cumming, R. L. C. and Bryce, C. F. A. (1973) Biochem J. 131, 51–59.
[8] Granick, S and Michaelis, L. (1943) J. Biol. Chem. 147, 91–97.
[9] Weber, K and Osborn, M. (1969) J Biol. Chem. 244, 4406–4412.
[10] Adelman, T. G , Arosio, P. and Drysdale, J. W. (1975) Biochem. Biophys. Res. Commun. 63, 1056–1062
[11] Niitsu, Y., Ohtsuka, S., Watanabe, N., Koseki, J., Kohgo, V. and Urushizaki, I (1977) in: Proteins of Iron Metabolism (Brown, E B. et al. eds), pp. 65–70, Grune and Stratton, New York.
[12] Crichton, R. R. (1975) in· Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R. R. ed), pp. 253–260, North-Holland, Amsterdam.
[13] Drysdale, J. W (1977) in CIBA Iron Metabolism Found. Symp., no. 51, pp. 41–57, Excerpta Medica, Amsterdam.
[14] Crichton, R. R., Collet-Cassart, D., Ponce-Ortiz, Y., Wauters, M., Roman, F. and Paques, E. (1977) in: Proteins of Iron Metabolism (Brown, E. B. et al. eds), pp. 13–22, Grune and Stratton, New York.