Animal ferritin and bacterioferritin contain quinones

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The origin of the 440 nm fluorescence of horse spleen ferritin and of *Pseudomonas aeruginosa* and *Azotobacter vinelandii* bacterioferritin has been investigated using a Nitro Blue Tetrazolium/glycinate colorimetric test specific for quinones [Paz, Flückiger, Boak, Kagan & Gallop (1991) J. Biol. Chem. 266, 689–692]. The results of the analysis indicate that ferritin and bacterioferritins contain quinones. A possible functional role of these quinones in iron uptake and release is described, as is the possibility that the presence of quinones in these proteins results from oxidative damage.

INTRODUCTION

The iron-storage protein of animals, ferritin, and its bacterial equivalent, bacterioferritin (BFR), consists of a protein shell surrounding a non-haem iron core (Stiefel & Watt, 1979; Ford *et al.*, 1984; Harrison *et al.*, 1989). The protein shell is constructed from 24 subunits, each of molecular mass 18–22 kDa. Iron uptake into the core, and iron release from it, involves the passage of iron through the intact protein shell (Clegg *et al.*, 1980; Ford *et al.*, 1984). This raises a number of mechanistic questions, including ‘does the movement of iron require the action of a cofactor bound to the ferritin?’ Such a cofactor could be involved in electron transfer, so that the iron could move as Fe²⁺, but be bound in the core as Fe³⁺, or make direct contact with the iron as part of an iron-transfer channel.

We have explored the possibility that ferritins contain cofactors by a series of spectroscopic examinations of a variety of proteins (Moore *et al.*, 1986; Cheesman *et al.*, 1990; Kadir & Moore, 1990). During the course of this survey we have confirmed the original observation of Maruyama & Listowsky (1982) that animal ferritins contain a fluorescent group with an emission maximum at ~ 440 nm, and showed that *Pseudomonas aeruginosa* BFR also contains such a group (Moore *et al.*, 1986). In the present paper we extend this work by a chemical and fluorimetric study of horse spleen ferritin and *P. aeruginosa* and *Azotobacter vinelandii* BFRs. The results indicate that the fluorescent group is a quinone that is common to all ferritins and BFRs for which fluorescence data are available.

EXPERIMENTAL

P. aeruginosa and *A. vinelandii* BFRs were isolated and purified by a combination of the procedures described by Moore *et al.* (1986) and Smith *et al.* (1988). This involved heating the cell extract at 65 °C for 15 min to precipitate contaminating proteins, followed by precipitating BFR with 40–60 % (w/v) (NH₄)₂SO₄, redissolving the BFR and, after chromatography on cellulose DEAE-52 and Sephacryl S-300 columns, spinning at 150 000 g for 16 h. Horse spleen ferritin, sodium glycinate, Nitro Blue Tetrazolium (NBT), human serum albumin and pyrroloquinoline quinone (PQQ) were obtained from Sigma (Poole, Dorset, U.K.). NaBH₄ and phenanthrenequinone were obtained from Aldrich Chemicals (Poole, Dorset, U.K.). Apoferritin was prepared by dialysing holoferritin with 0.12 M-thioglycolic acid, pH 4.25, at 4 °C for 4 days, followed by dialysis against 0.02 M-phosphate, pH 7.4.

Colorimetric test for quinones

The NBT/glycinate test (Paz *et al.*, 1991) was used to detect quinoproteins in animal ferritin and BFR. A 0.5 ml portion of ferritin at a concentration of 2 mg·ml⁻¹ was mixed with the following reagents, at 4 °C: 1 ml of sodium glycinate (2 M, pH 10.0), 0.5 ml of albumin (10 mg·ml⁻¹; dialysed overnight against 2 M-sodium glycinate at pH 10.0 and 4 °C) and 3 ml of NBT (0.24 mM in 2 M-sodium glycinate at pH 10.0). The reaction was started by incubation of tubes in a water bath at 30 °C in the dark. After 1 h the absorbance at 530 nm was determined using a Hitachi 577 u.v.–visible spectrophotometer. The test was run with and without NaBH₄-reduced albumin. A number of quinones, including PQQ and phenanthrenequinone, were used in a control test.

SDS/15 %-(w/v)-PAGE was also used to identify the protein staining for quinones. The gels were run in 0.025 M-Tris/HCl buffer, pH 8.6, containing 0.192 M-glycine and 0.1 % SDS. Electrophoretic transfer of proteins from polyacrylamide gel on to nitrocellulose paper (0.45 µm-pore-size membrane) was obtained at 100 V for 2.5 h and at 20 °C using a pH 8.3 blotting buffer containing 25 mM-Tris, 192 mM-glycine and 20 % (v/v) methanol. Quinone detection was done by staining the nitrocellulose paper with NBT (0.24 mM in 2 M-sodium glycinate at pH 10.0) for 1 h in the dark. The result was a blue–purple stain of the quinoprotein bands and no staining of the bands of other proteins. The nitrocellulose paper was washed and stored in 0.1 M-sodium borate at pH 10.0 and 4 °C. The other proteins stained red when Ponceau S (0.5 % in 5 % acetic acid) was applied, whereas the colour of the blue–purple-stained quinoproteins remained the same.

Fluorescence spectra of ferritin and BFR were recorded with a Perkin–Elmer luminescence spectrophotometer (model LS 50).

RESULTS AND DISCUSSION

Fluorescence spectra

Fig. 1 shows fluorescence emission spectra of *P. aeruginosa* apo-BFR. Excitation at 290 nm produces a relatively strong emission at 340 nm with a broad tail out to ~ 500 nm. Excitation at 340 nm causes emission at 445 nm and ~ 500 nm. Corresponding spectral features were observed for *A. vinelandii* apo-BFR (results not shown), with the main emission at 360 nm, and *Escherichia coli* BFR (F. H. A. Kadir, G. R. Moore, J. Guest & P. M. Harrison, unpublished work). In emission spectra for some preparations of BFR resulting from excitation at 280–290 nm, a relatively weak peak was observed at 440–450 nm, as

Abbreviations used: NBT, Nitro Blue Tetrazolium; PQQ, pyrroloquinoline quinone; BFR, bacterioferritin.

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Fig. 1. Fluorescence emission spectra of *P. aeruginosa* apo-BFR

The protein concentration was $1.2 \text{ mg} \cdot \text{ml}^{-1}$ at pH 7.0 in 0.025 M-phosphate buffer/0.15 M-NaCl. Fluorescence intensities are reported relative to the intensity of the 280 nm peak in the excitation spectrum (Fig. 2a). (a) Excitation at 290 nm; the wavelength maximum is 340 nm; (b) excitation at 340 nm; the wavelength maxima are 445 and 520 nm.

Fig. 2. Fluorescence excitation spectra of *P. aeruginosa* apo-BFR

The protein concentration was $1.2 \text{ mg} \cdot \text{ml}^{-1}$ at pH 7.0 in 0.025 M-phosphate buffer/0.15 M-NaCl. Fluorescence intensities are reported relative to the intensity of the 280 nm peak in spectrum (a), where emission was at 360 nm and the wavelength maximum 280 nm. (b) Emission at 450 nm; wavelength maxima 280 and 350 nm.

well as the intense peak at 340–360 nm. The 440–450 nm emission was $\leq 25\%$ of the intensity of the 360 nm peak. For the majority of BFR samples the spectra resembled those in Fig. 1. These spectra are similar to those reported previously for a variety of

Fig. 3. Electronic absorption spectra of horse spleen apoferritin after reaction with NBT/glycinate

The final ferritin concentration was $0.2 \text{ mg} \cdot \text{ml}^{-1}$ at pH 10.

animal ferritins (Maruyama & Listowsky, 1982) and *P. aeruginosa* BFR (Moore *et al.*, 1986).

Fig. 2 shows the corresponding fluorescence excitation spectra to the emission spectra of Fig. 1. Monitoring emission at 360 nm gives an excitation maximum at 280 nm, and monitoring emission at 450 nm gives excitation maxima at 280 nm and 350 nm.

In simple cases fluorescence emission spectra are mirror images of absorption spectra, and fluorescence excitation spectra closely resemble absorption spectra. However, this is not always so, and there are many reasons why these parallels do not always occur (Ingle & Crouch, 1988). In the present case of BFR the situation is complicated because the fluorophore is bound tightly to the protein, as shown by the observation that dialysis, chromatography and ultracentrifugation does not remove it, and absorption spectra of intact apo-BFR are dominated by the strong absorbance of the aromatic amino acids. Nevertheless, we can make some progress in interpreting the fluorescence spectra. These are consistent with the presence of two fluorophores (Cantor & Schimmel, 1980). The emission at 340–360 nm resulting from excitation at 280–290 nm is typical of tryptophan fluorescence; the free amino acid absorbs at 280 nm and fluoresces at 348 nm (Cantor & Schimmel, 1980). The longer-wavelength fluorescence at 440–450 nm resembles the properties of a variety of types of group. For example, phenanthrenequinone has a broad absorption band at 325 nm and fluoresces at 420 nm (results not shown). We suggest that the second fluorophore in ferritin and BFR has an absorption band overlapping the fluorescence band of tryptophan, so that energy transfer can take place between the two fluorophores.

The widespread distribution of the long-wavelength fluorescence indicates that it is intrinsic to the protein, though whether from a functional cofactor or from degraded amino acid side chains remains to be determined. The strong resemblance between the fluorescence spectra of Figs. 1 and 2 and those of amino acid derivatives, such as modified tryptophans (Finazzi-Agrò & Avigliano, 1984), pyridinoline (Tshuchikura *et al.*, 1981) and functional cofactors, such as quinones (Anthony & Zatman, 1967; Duine *et al.*, 1987) and folates (Uyeda & Rabinowitz, 1963), does not resolve the assignment. Thus it is necessary to employ chemical methods to identify the fluorophore.

Test for quinones

The NBT/glycinate test for quinones (Paz *et al.*, 1991) gave a positive result for horse spleen ferritin and *P. aeruginosa* and *A. vinelandii* BFRs. The reaction of apoferritin with NBT/glycinate generated a deep blue colour with an optical absorption maximum of 530 nm (Fig. 3). Such an absorption could be generated

Fig. 4. SDS/PAGE of ferritin and BFR

The gel contained 15% polyacrylamide and SDS (0.1%). The running buffer was 0.025 M-Tris/HCl, pH 8.6, containing 0.192 M-glycine and 0.1% SDS. Samples were denatured with 10% (v/v) glycerol/5% (v/v) mercaptoethanol and boiled for 10 min. Electrophoresis was run for 16 h at 100 V. (a) The gel was stained with Brilliant Blue R stain. Lane 1, *P. aeruginosa* holo-BFR; lane 2, *P. aeruginosa* apo-BFR; lane 3, horse spleen apoferritin. (b) NBT/glycinate staining of a SDS/PAGE electroblot. Lanes 1–3 are as for (a); lanes M in (a) and (b) contained molecular-mass (*M*) markers.

by a range of quinones (Paz *et al.*, 1991), including, as we confirmed, PQQ and phenanthrenequinone. The next step in the analysis was to demonstrate that the quinone forms part of the protein shell of ferritin and BFR, and this was achieved by using the SDS/PAGE procedure described by Paz *et al.* (1991). Fig. 4(a) shows an SDS/PAGE gel of horse spleen apoferritin and *P. aeruginosa* BFR stained with Brilliant Blue R stain. Fig. 4(b) illustrates a similar gel after electroblotting on to nitrocellulose paper and staining with NBT/glycinate.

Fig. 4 clearly shows that both horse spleen ferritin and BFR contain quinones. It also indicates that the protein standards do not contain quinones. Thus our analysis supports the claim of Paz *et al.* (1991) that the NBT/glycinate test is specific for quinones, since the only proteins giving a positive result for this test in our study were those exhibiting fluorescence properties similar to quinones. The fact that the quinone remained with the proteins on the SDS/PAGE gels indicate that they are tightly bound together, probably by covalent bonds.

General discussion

The discovery that ferritins and BFRs contain quinones suggests that these are the origin of their long-wavelength fluorescence. However, it is possible that there is a further component of ferritin and BFR producing the fluorescence, perhaps a flavin, with the fluorescence of the quinone being quenched. This seems to us to be unlikely, and therefore we assign the long-wavelength fluorescence to the presence of quinones. This raises a number of questions, the chief among these being 'does the quinone have a functional role?' This cannot be answered until the identity and quantity of the quinone have been determined.

A number of enzymes, called quinoproteins, have been discovered to contain PQQ (Anthony, 1988; Duine & Jongejan, 1989). The list is extensive and includes many metalloproteins, including copper-containing enzymes such as lipoxygenase (van der Meer & Duine, 1988) and nitrile hydratase (Nagasawa & Yamada, 1987). PQQ can bind metal ions and take part in a range of proton-coupled redox reactions, as can other quinones. Thus it is tempting to propose that, in ferritins and BFR, quinones are involved in iron uptake and release by channelling protons and electrons, and perhaps iron, to and from the core. This can only really be resolved when the nature of the quinone is established, and this is not a straightforward task. For example, many of the copper-containing enzymes previously proposed to contain PQQ have now been shown not to (Klinman *et al.*, 1991). In at least one case, serum amine oxidase, the quinone is protein-derived and arises from a modified tyrosine or phenylalanine residue (Janes *et al.*, 1990), and in another case, galactose oxidase (Ito *et al.*, 1991), a novel tyrosine–cysteine linkage is present at the active site and is probably responsible for the previous indication of PQQ.

Partly because of previous errors in identifying quinone cofactors, McIntire *et al.* (1991), in their report of the identification of tryptophan tryptophylquinone in methylamine dehydrogenase, stress that cofactor identification must involve the conversion of the putative quinone into a stable intermediate, its removal from the protein, and its structural analysis. This has not yet been achieved for ferritin or BFR.

The detected presence of quinones in ferritins may not imply a direct functional role. Their presence may result from damage to the protein. This could arise by, for example, oxidation of tyrosine residues by free radicals, such as the hydroxyl radical, generated by reactions of the non-haem iron. Such a proposal is consistent with the finding by O'Connell *et al.* (1986) that ferritin incubated with the free-radical-generating system of liposomes and ascorbate exhibited enhanced fluorescence of the type shown in Fig. 1. Similar oxidative reactions may be responsible for the presence of quinones in other metalloproteins. Alternatively, the presence of quinones in ferritins may result from damage to the protein incurred during its isolation. Crichton *et al.* (1980) report that horse spleen ferritin prepared by a method involving heat treatment contains a fluorescent material that is probably a degradation product of a flavin, whereas ferritin prepared by a method in which the heat-treatment step is replaced by an acid treatment does not contain a fluorescent group. If the quinones are formed on heating, then it is probable that the non-haem iron catalyses the formation.

In summary, then, ferritins and BFRs contain quinones, and their presence in these proteins may be a consequence of oxidative damage resulting from iron storage and not a functional requirement for their normal activity.

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