Bacterial ferritin contains 24 haem groups

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Received 30 July 1990

Pseudomonas aeruginosa bacterioferritin, also known as cytochrome b_1 or cytochrome b_{557}, has been isolated with 9 haems per 24 subunits. Various forms of the protein have been prepared including the completely haem-free protein and the fully haem-loaded protein with 24 haems per 24 subunits. The presence of the core does not significantly affect haem addition or removal. The absorbance ratio of the non-haem-iron-loaded protein, 278 nm:417 nm (oxidised), can be used to estimate the haem loading.

Bacterial ferritin; Cytochrome b_1; Haem content

1. INTRODUCTION

The bacterial equivalent of animal ferritin consists of 24 subunits of molecular mass 18–22 kDa that pack together in a similar way to their animal counterparts; namely, they form a protein shell of ~20 Å thickness surrounding a cavity of ~80 Å diameter [1–5]. The non-haem iron storage mineral is contained in the central cavity. A major difference between the animal and bacterial ferritins is that, as isolated, the latter contain intrinsic haem groups whilst the former do not. It is these that give bacterial ferritin its alternative names of cytochrome b_1 and cytochrome b_{557} [3,6,7]. We are engaged on a study of the role of the haem in the function of bacterial ferritin and during the course of this work have found that the haem content of the *Pseudomonas aeruginosa* protein is variable; samples ranging from 5 haems per 24 subunits to 9 haems per 24 subunits have been isolated [6,8]. In contrast to this, the ferritins from *Escherichia coli* and *Azotobacter vinelandii* have been reported to contain 12 haems per 24 subunits [2,3,9]. These observations have prompted us to investigate the removal, and replacement, of the haems of *Ps. aeruginosa* bacterial ferritin. Our results, reported in the present paper, show that bacterial ferritin can accommodate 24 haems in 24 subunits.

2. MATERIALS AND METHODS

Ps. aeruginosa bacterioferritin was isolated and purified as previously described [8]. As isolated, the bacfer was iron-loaded and contained 9.2% (w/w) iron with a haem content of 9 per 24 subunits.

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Haemin chloride and sodium dithionite were obtained from the Sigma Chemical Co. (Poole, Dorset, UK), and acetone and methyl ethyl ketone from BDH Ltd. (Poole, Dorset, UK). The organic solvents were used as supplied. All spectra were obtained with a Hitachi 557 double-beam spectrophotometer or a Hitachi U-2000 spectrophotometer. Protein concentrations were determined by the method of Lowry et al. [10].

Four forms of bacfer were studied in the present work: the protein as isolated, holo-holobacfer; holo-apobacfer, which had the haems present in the isolated protein attached but the non-haem-iron core removed; apo-holobacfer with the haems removed and the non-haem-iron in place; and apo-apobacfer with both the haem and non-haem-iron components removed. The holo-apobacfer was prepared by dialysis of the isolated protein at 4°C against 0.12 M thioglycollic acid at pH 5, followed by dialysis against 0.025 M sodium phosphate buffer containing 0.15 M sodium chloride at pH 7.4, to remove excess thioglycollic acid and the Fe^{2+}-containing species.

Apo-apobacfer was prepared using both the methyl ethyl ketone method described by Rossi-Fanelli et al. [11] and the acid-acetone method described by Teale [12]. The latter procedure gave the best results. In a typical preparation 5 ml of the stock holo-apobacfer (12 mg protein per ml, 0.025 M sodium phosphate buffer at pH 7.4) was added to a mixture of 197 ml of acetone and 3 ml 0.2 M HCl at −20°C. The precipitate was collected by centrifugation and dissolved in 2 ml of distilled H_2O at 4°C. The UV-visible spectrum of the resulting solution was measured and if bands due to haem were still present the treatment was repeated. 3–5 cycles were required to remove >97% of the haem. The solution resulting from the final cycle was left for 5 h at 4°C and then dialysed against 0.0016 M sodium bicarbonate at 4°C for 20 h. It was then dialysed against 0.025 M phosphate buffer at pH 7.4 for 24 h. Finally, the sample was spun at 4°C in a centrifuge at 6000 rpm for 30 min to remove the small quantity of insoluble protein.

Apo-holobacfer was prepared by the acid-acetone method as described above for apo-apobacfer.

Incorporation of haem into bacfer was carried out by the following procedure. A solution of haemin was freshly prepared each day by dissolving 5 mg of haemin chloride in a minimal volume of 0.1 M NaOH, followed by the addition of distilled H_2O to a final volume of 5 ml. This gave a haemin concentration of 1.5 mM. This was confirmed by measurement of its optical absorbance; ϵ_{390} = 50 $mM \cdot cm^{-1}$ [13]. The pH was then adjusted to 7.9. 10^{-2} ml aliquots of the haemin solution were added to the protein solutions and the haem incorporation monitored spectrophotometrically. This was

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00145793/90/$3.50

achieved using a difference-spectrum method. The sample cuvette contained a solution of 6.4×10^{-6} M protein dissolved in 0.025 M phosphate buffer (pH 7.4) and the reference cuvette contained only buffer. Aliquots of haemin were added to both cuvettes and either the absorbance measured over the wavelength range 350–650 nm 5 min after mixing, or the absorbance was measured at 417 nm as a function of time, starting immediately after mixing. The final solutions from each titration were kept for periods of 1–7 days and their absorbance spectra, before and after addition of sodium dithionite to reduce the ferrihaem, recorded with buffer only in the reference cuvette.

3. RESULTS AND DISCUSSION

Visible absorption spectra for the addition of aliquots of a haemin chloride solution to apo-apobacfer are shown in Fig. 1. Positive peaks arising from haem binding to bacfer occur at 417 nm and approx. 530 nm, and negative peaks due to the loss of free haemin chloride occur at <400 nm and approx. 620 nm. The spectral change with increasing concentrations of haemin chloride occurs with minimal dilution of the bacfer and thus a graph of absorbance at 417 nm vs the haem/protein molar ratio indicates the haem content of the protein at a given point during the titration. Such a graph is given in Fig. 2.

The shape of the graph indicates that the maximum number of haem groups per 24 subunit bacfer is 24, i.e. one haem per subunit. A Hill-plot analysis of the data shows that the haem binding is non-cooperative with a dissociation constant of 7×10^{-5} M.

Analogous spectra to those of Fig. 1 for the addition of haemin chloride to solutions of holo-holobacfer, holo-apobacfer and apo-holobacfer show that the maximum haem content of bacfer is one haem per subunit

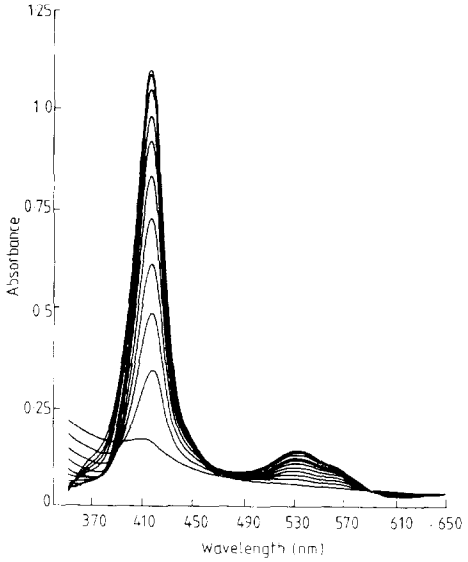

Fig. 1. UV-visible difference spectra for the interaction of 1.5×10^{-3} M haemin chloride with 6.7×10^{-6} M apo-apobacfer at pH 7.4 (0.1 M phosphate). The spectra correspond to successive 10×10^{-3} ml additions of the haemin chloride solution.

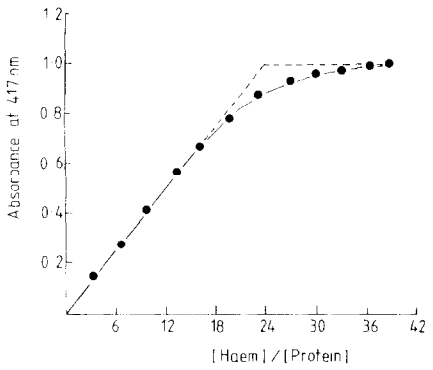

Fig. 2. Plot of absorbance at 417 nm vs the [haem]/[protein] ratio for the data of Fig. 1. The haem/protein stoichiometry is indicated by the intersection of the broken lines. The dissociation constant for the binding, 7×10^{-5} M, was taken from a Hill plot.

irrespective of whether the core is present or not. Addition of haemin chloride beyond this ratio leads to the appearance of bands in the spectrum at approx. 400 and 530 nm indicating that the excess haem remains in the high-spin form but with somewhat different spectral characteristics to those of haemin chloride in the reference cuvette. This may be a result of aggregation of the reference haemin.

The absolute UV-visible spectra of the reconstituted bacfer, obtained with buffer in the reference cuvette, are indistinguishable from those of the isolated bacfer over the region 250–750 nm, as far as absorbance wavelength maxima are concerned (Fig. 3). The absorbance ratios are different however; for the fully reconstituted protein the spectral characteristics are given in the caption to Fig. 3.

The marked similarity between the spectra, particularly the appearance of the band at 690–700 nm, indicates that the haem has the same axial ligands in the

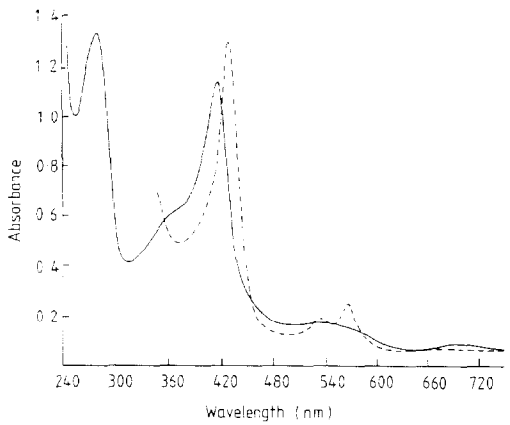

Fig. 3. UV-visible difference spectra of fully haem-loaded holo-holobacfer, 6.7×10^{-6} M, at pH 7.4 (0.1 M phosphate). The solid line is the spectrum of the oxidised form and the broken line the spectrum of the dithionite reduced form. The absorbance ratios 278/417 (oxidised), 417 (oxidised)/424 (reduced) and 424 (reduced)/557 (reduced) are 1.17, 0.88 and 5.16, respectively.

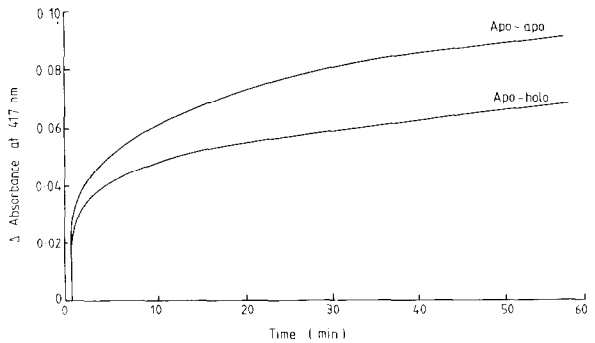

Fig. 4. Time course of the change in absorbance at 417 nm for the addition of 30×10^{-3} ml of 1.5×10^{-3} M haemin chloride to 6.75×10^{-6} M apo-apobacfer and apo-holobacfer at pH 7.4 (0.1 M phosphate). The ratio of haem/24 protein subunits was 9:24. The haemin chloride was added at $t = 0$ min.

reconstituted and native proteins. These have recently been shown to be two methionine residues [14].

Haem binding to bacfer with a non-haem-iron core proceeds with different thermodynamic and kinetic parameters to those for haem binding to apo-apobacfer. The dissociation constant for apo-holobacfer is 1.5×10^{-4} M, showing that the haem binding is weaker. Similarly the rate of haem binding is less. Fig. 4 shows the time-course of the absorbance change for addition of haem to apo-apobacfer and apo-holobacfer at a molar ratio of added haem to protein of 6:24. The time-course is biphasic with a rapid absorbance change followed by a slower change. Since the total absorbance change should be the same for the two proteins, binding to apo-holobacfer is slower than binding to apo-apobacfer.

The differences between haem binding to the two forms of bacfer are unlikely to reflect a major conformational difference between them, especially in view of the fact that the haem visible, EPR and NIR-MCD spectral characteristics of the haem are not significantly different in the presence and absence of the core (Cheesman, M.R., Kadir, F.H.A., Moore, G.R. and Thomson, A.J., unpublished data). Thus more likely origins for the differences in haem binding are the electrostatic field of the core and/or the different dynamic properties of the protein shell. For example, it seems highly probable that the subunit interfaces and channels of the protein shell have some flexibility and this would be reduced by the non-haem-iron core acting as an anchor within the shell.

A haem content of one haem per subunit has important implications for the structure of bacfer. Smith et al. [8,9] have shown from X-ray studies that *E. coli* bacfer has 432 symmetry, implying the 24 subunits have the same structure, even though the haem content of the crystalline bacfer is less than one haem per subunit. They suggest possible structures of bacfer to accommodate these results including the subunits being structurally equivalent because the haems are shared between pairs of subunits. An alternative explanation, also considered by Smith et al., that each of the subunits has a haem binding site but in only some of the subunits of the crystalline bacfer is this occupied, is supported strongly by the work reported herein.

Acknowledgements: We thank the Wellcome Trust for supporting our work on ferritin and bacterioferritin, and the Science and Engineering Research Council for their support of the UEA Metallobiology Unit via the SERC Molecular Recognition Initiative. We also thank Professor C. Greenwood and Mr A. Thompson for their help in obtaining pure bacterioferritin.

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