Iron-coproporphyrin III is a natural cofactor in bacterioferritin from the anaerobic bacterium *Desulfovibrio desulfuricans*

Célia V. Romão[a], Ricardo Louro[a], Russel Timkovich[b], Mathias Lübben[c], Ming-Yih Liu[d], Jean LeGall[a,d], António V. Xavier[a], Miguel Teixeira[a,*]

[a] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apt 127, 2780-156 Oeiras, Portugal*
[b] *Department of Chemistry, University of Alabama, Tuscaloosa, AL 35487-0336, USA*
[c] *Lehrstuhl für Biophysik, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44780 Bochum, Germany*
[d] *Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA*

Received 5 July 2000; accepted 26 July 2000

Edited by Thomas L. James

Abstract A bacterioferritin was recently isolated from the anaerobic sulphate-reducing bacterium *Desulfovibrio desulfuricans* ATCC 27774 [Romão et al. (2000) Biochemistry 39, 6841–6849]. Although its properties are in general similar to those of the other bacterioferritins, it contains a haem quite distinct from the haem B, found in bacterioferritins from aerobic organisms. Using visible and NMR spectroscopies, as well as mass spectrometry analysis, the haem is now unambiguously identified as iron-coproporphyrin III, the first example of such a prosthetic group in a biological system. This unexpected finding is discussed in the framework of haem biosynthetic pathways in anaerobes and particularly in sulphate-reducing bacteria.

Key words: Bacterioferritin; Coproporphyrin; Heme biosynthesis; *Desulfovibrio*

1. Introduction

Iron homeostasis in living organisms involves a series of specialised proteins, which assure efficient uptake, transport, storage and utilisation of this metal ion. Ferritin, the iron storage protein, plays a key role in iron metabolism; its ability to sequester this element gives ferritin the dual function of providing a storage of this metal ion and of precluding its undesirable reactivity towards oxygen, leading to the production of highly hazardous reactive oxygen species. This iron storage protein has been found in different aerobic organisms, in the three life domains, eukarya, bacteria and archaea [1]. The holoprotein is constituted in general by 24 subunits which form the protein shell harbouring the Fe(III) mineral core [1]. Ferritins are part of a large superfamily of proteins, which includes another type of iron-storage proteins first identified in bacteria and thus known as bacterioferritins (BFRs) [2–6]. However, bacterioferritins are not specific of bacteria, as it was recently identified in a eukaryote [7]. BFRs have as their major distinctive characteristic the presence of iron-protoporphyrin IX as a cofactor, located between each two subunits and having a methionine from each monomer as the iron axial ligands [8,9]. The role of the haem remains unknown.

In contrast to aerobes, the function of (bacterio)ferritins in anaerobes is still poorly understood. Since during the iron storage process oxygen is concomitantly consumed, (bacterio)ferritins may act also as oxygen scavengers. Thus, it may be speculated that it plays a role as part of the antioxidant protective systems, capturing iron and consuming oxygen once anaerobes become exposed to oxygen. The only known example of a bacterioferritin from an anaerobe was found when we started investigating the iron metabolism of the strictly anaerobic, sulphate-reducing bacterium *Desulfovibrio desulfuricans* (ATCC 27774) [10]. Like other members of the superfamily, this protein is constituted by 24 identical subunits, which although having a low amino acid sequence similarity towards other BFRs, still contain the residues involved in the binding of the iron-binuclear centre, necessary for the oxidation of iron in the uptake process. It was isolated containing a stable di-iron centre and, quite unexpectedly, did not contain a b-type haem in contrast with all other known BFRs. Instead, it has a haem with chromatographic properties that resemble those of iron-uroporphyrin [10,11] and with a pyridine haemochrome spectrum quite distinct from those of haems present in biological systems. Here, we report the identification of this haem as iron-coproporphyrin III, which, to our knowledge, is the first example for the presence of such a haem in living organisms.

2. Materials and methods

Bacterioferritin was purified as previously described [10]. For haem extraction and NMR characterisation 25 mg of protein was used. Coproporphyrin methyl ester was purchased from Aldrich and used without further purification.

2.1. Haem extraction

Acidic acetone extraction was performed as in [12]. The ethylacetate was removed by a rotary evaporator at room temperature. Demetalisation and esterification reactions were done as in [13]. After the esterification step, a solution of 2 M Na_2CO_3 was added to the haem solution, until the pH became slightly basic; four volumes of chloroform were then added, followed by washing with several portions of water. The organic layer was dried in a rotary evaporator at room temperature. The purification was performed by chromatography on a silica gel (70–230 mesh) column (15×1.5 cm), equilibrated in solvent A (hexane:chloroform 1:1). The methyl ester porphyrin was dissolved in a minimal volume of solvent A and then applied to the column, which was eluted with chloroform. The red band obtained was dried in a rotary evaporator and stored until further use.

2.2. Preparation of Fe-coproporphyrin

Iron-coproporphyrin was prepared by hydrolysis of commercial

*Corresponding author. Fax: (351)-21-4428766.
E-mail: miguel@itqb.unl.pt

PII: S0014-5793(00)01939-6

coproporphyrin methyl ester and iron was inserted as described in [14]. Haem extraction and purification was done as in [11].

2.3. Spectroscopic methods

Visible spectra were recorded on a Shimadzu UV 3100 spectrophotometer. The alkaline pyridine haemochromogen method was performed as in [15]. NMR spectra were obtained in a Bruker AMX300 spectrometer, at 293 K. 1D ^{1}H-NMR experiments were performed using either d_1-chloroform or d_2-dichloromethane as solvent. 64k data points were acquired in the time domain and the spectrum processed using exponential multiplication. 2D ^{1}H-double quantum filtered COSY was performed with 2k points in F2 and 1k experiments in the F1 and processed with cosine on both dimensions without zero filling. 2D ^{1}H-NOESY was performed with a mixing time of 500 ms (4k×512 data size). The 2D map was processed with 4k by 4k data points in F1 and applying cosine in F1 and exponential multiplication in F2. On all experiments the spectral sweep width was 6 kHz. Chemical shifts were referenced using the residual signal of chloroform as internal reference at 7.24 ppm.

2.4. Mass spectra

For mass spectrometry analysis, small portions of the haem extracts were mixed with the same volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile and were placed for 10 min on the target for matrix crystallisation. Matrix-assisted laser desorption ionisation (MALDI) spectroscopy was performed with a Voyager DE-RP instrument (Perseptive Biosystems, Framingham), operated at an accelerating voltage of 25 000 V in the reflector mode.

3. Results and discussion

The haem from *D. desulfuricans* ATCC 27774 bacterioferritin was previously shown to have quite unique properties [10] and in fact the extracted haem and porphyrin are now identified as iron-coproporphyrin III and coproporphyrin III (Fig. 1A), respectively.

The pyridine haemochrome of *D. desulfuricans* bacterioferritin gives an α-band at 545 nm [10], different from that of cytochrome *c* (550 nm) or iron-uroporphyrin (548 nm), that matches Fe-coproporphyrin prepared from commercial coproporphyrin methyl ester (Fig. 2A,B). The visible spectrum of the purified porphyrin methyl ester in chloroform has maxima at 399.5 nm (Soret band) and four Q-bands at 497.5, 531, 566.5 and 621 nm, identical to those of commercially available coproporphyrin ester (Fig. 2C,D). The haem is not covalently bound, and is easily extractable to ethyl acetate; its elution behaviour on a reverse-phase column indicates a quite hydrophilic haem, similar to iron-uroporphyrin [10], as expected for iron-coproporphyrin.

Table 1
^{1}H-NMR chemical shifts, multiplicity and coupling constants (*J*) of coproporphyrin III methyl ester isolated from *D. desulfuricans* bacterioferritin

Proton type	Chemical shift (ppm)	Multiplicity
Meso	10.09–10.06	Singlet
Methyl	3.60–3.54	Singlet
Propionate α CH_2	3.22	Triplet; $J=7.5$ Hz
Propionate β CH_2	4.35	Triplet; $J=7.5$ Hz
NH	−3.93	Singlet

The extracted haem gave a single peak in a MALDI mass spectrum, corresponding to a molecular ion with a nominal mass of 710 Da (Fig. 3, calculated 710.57 for $C_{36}H_{38}N_4O_8Fe$ with a prominent $^{12}C_{35}$ $^{13}C_1$ satellite).

The NMR data unequivocally identified the porphyrin as the type III isomer (Fig. 4). The resonances were assigned to the substituents in the porphyrin depicted in Fig. 1 according to Table 1. The chemical shift multiplicity, and coupling constants of the resonances (Table 1) are in agreement with previous assignments for haem substituents reported in the literature [11,16]. Confirmation of the isomer of the porphyrin

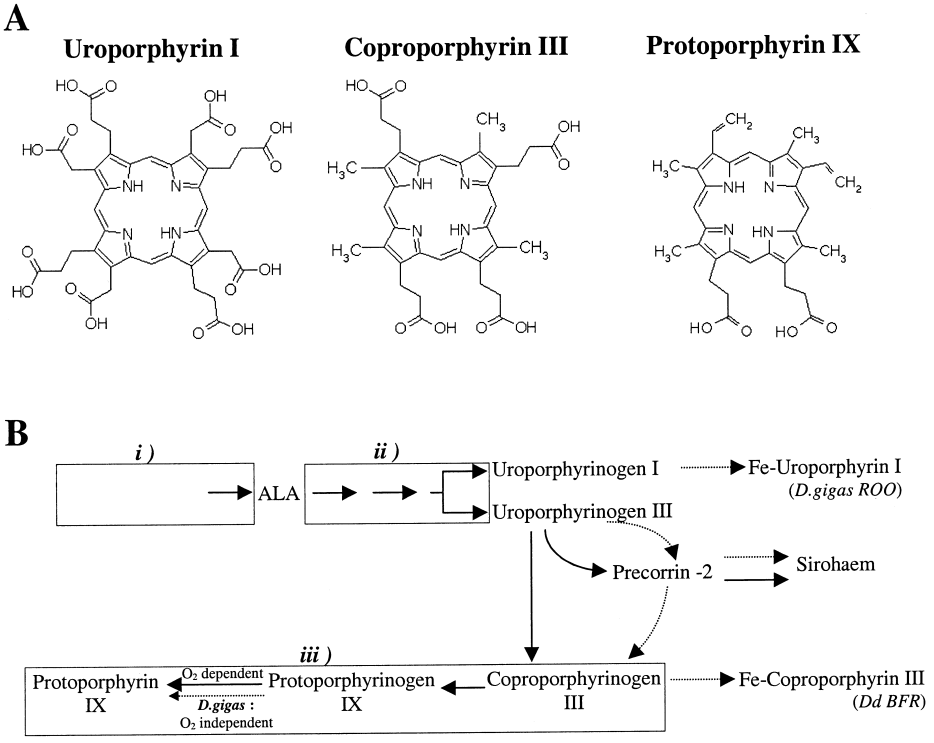

Fig. 1. (A) Chemical structures of uroporphyrin III, coproporphyrin III and protoporphyrin IX. (B) Schematic representation of haem biosynthesis pathway in aerobic organisms (full arrows) and of the alternative routes already established in *Desulfovibrio* species (dotted arrows).

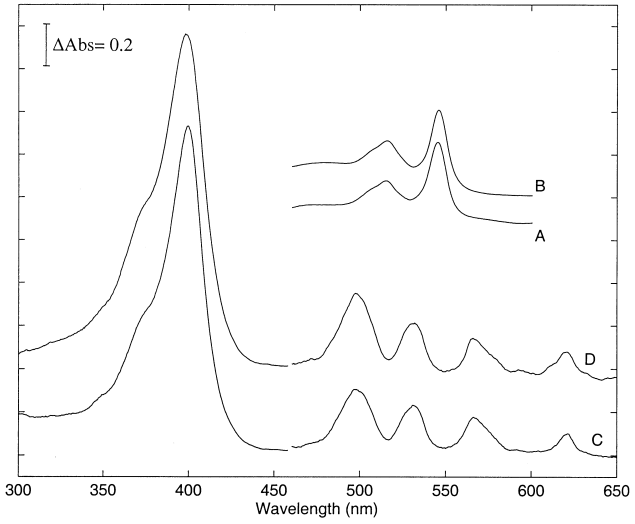

Fig. 2. (A,B) Pyridine haemochrome of *D. desulfuricans* bacterioferritin (A) and iron-coproporphyrin (B). (C,D) Visible spectra of the methyl ester of the porphyrin extracted from *D. desulfuricans* bacterioferritin (C) and commercial coproporphyrin methyl ester (D), in dichloromethane.

was obtained via 2D ^{1}H-NMR by observation of the relevant cross-peaks in the NOESY and COSY spectra (not shown), and by comparison with the 1D ^{1}H-NMR of the commercial coproporphyrin III methyl ester.

Dd BFR is the second example of a protein isolated from a sulphate-reducing bacterium that contains as a cofactor a so far unprecedented haem. The unusual properties of *Dd* BFR haem, i.e. its high reduction potential of +140 mV [10] when compared to that of ca. −220 mV in haem-B containing BFRs [17,18], and the split of its Soret band in the reduced protein [10] do not appear to be related to the porphyrin type. In fact, the porphyrin ring of coproporphyrin has the same degree of aromaticity as protoporphyrin and the presence of the two extra propionates, substituting for the vinyl groups of protoporhyrin (Fig. 1A), cannot account for the rise of the reduction potential; its high value has to be due either to the presence of the binuclear centre [10] or the influence of the protein environment. Also, the symmetry of the coproporphyrin substituents cannot account for the split of the Soret band, which again may result from the presence of the diiron centre. It should be noted that the visible spectrum of the haem centre in *Escherichia coli* bacterioferritin is sensitive to modifications at the di-iron centre [19].

Haem biosynthesis in aerobic organisms has been extensively investigated and the pathway is well established. The haem biosynthesis pathway can be divided into three parts: (i) the synthesis of 5-aminolevulinic acid (ALA); (ii) the assembly of eight ALA molecules to yield uroporphyrinogen III; and (iii) the modification of tetrapyrrole side chains, ring oxidation and iron (Fe^{2+}) insertion to yield haem (Fig. 1B) [20]. In aerobic organisms two steps, both in the third part, are oxygen dependent: the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX, catalysed by coproporphyrinogen oxidase (Hem F), and the six-electron oxidation of protoporphyrinogen IX to protoporphyrin by the protoporphyrinogen IX oxidase (Hem G) [20]. Anaerobic bacteria utilise a different coproporphyrinogen oxidase, an oxygen-independent enzyme, Hem N, which requires Mg^{2+}, methionine, ATP, and $NAD(P)^+$ for its activity [21–24]. *Bacillus subtilis* has at least two genes (*hemN* and *hemZ*), coding for oxygen-independent coproporphyrinogen oxidases [25,26].

In the anaerobic sulphate-reducing bacteria, haem biosynthesis is far from being understood, but a distinct pathway has already been shown to be operative in these bacteria [27]. In *D. vulgaris*, it deviates from the known pathway at the level of uroporphyrinogen III, which is converted into precorrin-2 (precursor of sirohaem) and then, through several intermediates, is converted into coproporphyrinogen III, a common intermediate to all known pathways [28,29] (Fig. 1B). This by-pass, through precorrin-2, was proposed to be particularly important in sulphate-reducing bacteria, due to the presence of large amounts of sulfite reductases in these organisms, which contain sirohaem and/or siroporphyrin as prosthetic groups [28,29]. However, the next steps have not yet been clarified.

A protoporphyrinogen oxidase was isolated from *Desulfovibrio gigas*, which is oxygen independent and does not donate electrons either to $NAD(P)^+$ or flavin nucleotides [30]. The oxidation of protoporphyrinogen, by *D. gigas* membrane extracts, with nitrite as electron acceptor is coupled with ATP synthesis [31]. The finding of iron-uroporphyrin and iron-co-

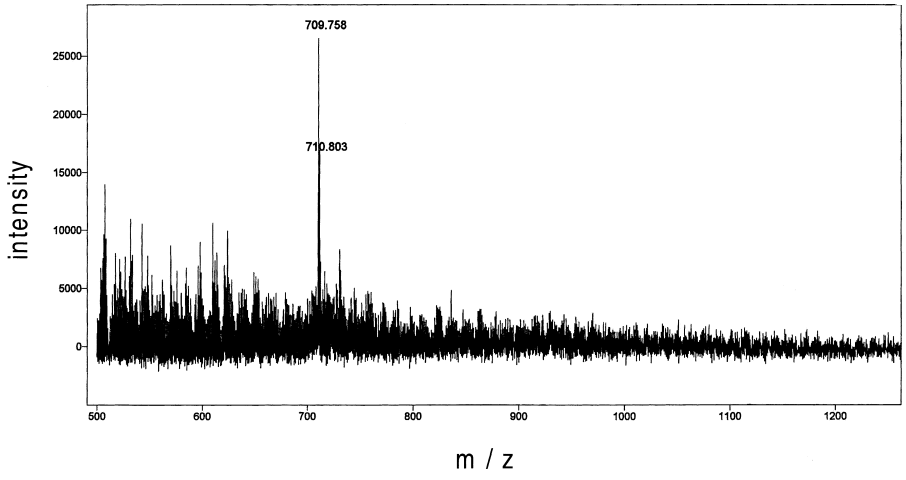

Fig. 3. MALDI nominal mass spectrum of the haem extracted from *D. desulfuricans* bacterioferritin, averaged from 100 scans.

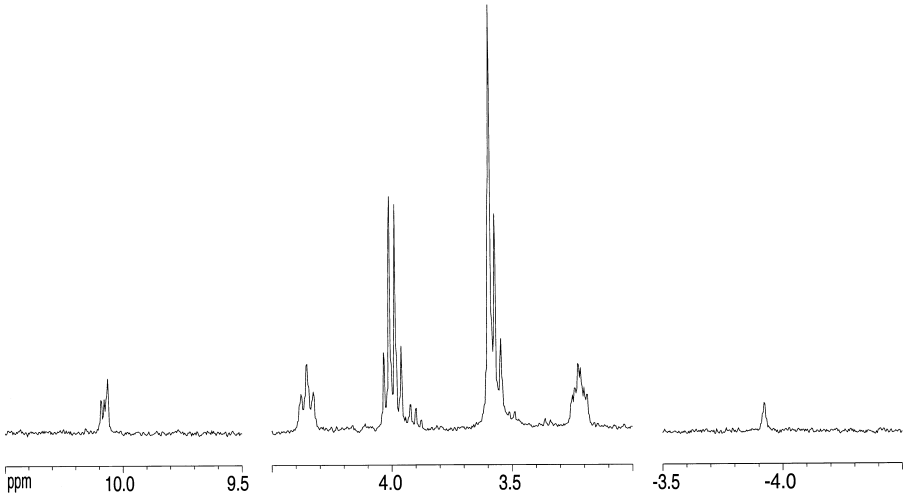

Fig. 4. ^{1}H-NMR spectrum of coproporphyrin III methyl ester, in deuterated dichloromethane, extracted from *D. desulfuricans* bacterioferritin. The intense signals close to 4 ppm arise from residual ethyl acetate present in the sample.

proporhyrin III in proteins from *D. gigas* [11] and *D. desulfuricans,* respectively, suggests the presence in these bacteria of uroporphyrinogen and coproporphyrinogen oxidases, to generate the respective porphyrins. Whether the last step in haem synthesis, the insertion of iron into the porphyrin core, is achieved by one, unspecific, ferrochelatase or several, specific such enzymes, is still an open question.

In conclusion, the present work proved that iron-coproporphyrin is the cofactor of *Desulfovibrio desulfuricans* bacterioferritin. The identification of this new type of haem in a natural biological system shows that coproporphyrin is not just a non-functional intermediate of haem biosynthesis, but that it is also used as part of a protein cofactor.

Acknowledgements: This work was supported by PRAXIS XXI Grant BIO/37/96 to M.T. and by NIH Grants GM56000-03 to J.L.G. and GM59035 to R.T. M.L. was supported by DFG Grant SFB394-C6. C.V.R. acknowledges a grant from Praxis XXI (BD/19879/99).

References

[1] Harrison, P.M. and Arosio, P. (1996) Biochim. Biophys. Acta 1275, 161–203.
[2] Stiefel, E.I. and Watt, G.D. (1979) Nature 279, 81–83.
[3] Yariv, J., Kalb, A.J., Sperling, R., Bauminger, E.R., Cohen, S.G. and Ofer, S. (1981) Biochem. J. 197, 171–175.
[4] Moore, G.R., Mann, S. and Bannister, J.V. (1986) J. Inorg. Biochem. 28, 329–336.
[5] Kurokawa, T., Fukumori, Y. and Yamanaka, T. (1989) Biochim. Biophys. Acta 976, 135–139.
[6] Andrews, S. (1998) Adv. Microb. Physiol. 40, 281–351.
[7] Carrano, C.J., Bohnke, R. and Matzanke, B.F. (1996) FEBS Lett. 390, 261–264.
[8] Frolow, F., Kalb, A.J. and Yariv, J. (1994) Nat. Struct. Biol. 1, 453–460.
[9] Andrews, S.C., Le Brun, N.E., Barynin, V., Thomson, A.J., Moore, G.R., Guest, J.R. and Harrison, P.M. (1995) J. Biol. Chem. 270, 23268–23274.
[10] Romão, C.V., Regalla, M., Liu, M.-Y., LeGall, J., Xavier, A.V. and Teixeira, M. (2000) Biochemistry 39, 6841–6849.
[11] Timkovich, R., Burkhalter, R.S., Xavier, A.V., Chen, L. and LeGall, J. (1994) Bioorg. Chem. 22, 284–293.
[12] Lübben, M. and Morand, K. (1994) J. Biol. Chem. 269, 21473–21479.
[13] Timkovich, R., Cork, M.S. and Taylor, P.V. (1984) J. Biol. Chem. 259, 1577–1585.
[14] Fuhrhop, J.H. and Smith, K.M. (1975) in: Porphyrins and Metalloporphyrins (Smith, K.M., Ed.), pp. 757–869, Elsevier Science, Amsterdam.
[15] Berry, E.A. and Trumpower, B.L. (1987) Anal. Biochem. 161, 1–15.
[16] Jason, T.A. and Katz, J.J. (1979) in: The Porphyrins, Vol. IV (Dolphin, D., Ed.), pp. 1–59, Academic Press, New York.
[17] Watt, G.D., Frankel, R.B., Papaefthymiou, G.C., Spartalian, K. and Stiefel, E.I. (1986) Biochemistry 25, 4330–4336.
[18] Meyer, T.E. and Cusanovich, M.A. (1985) Biochim. Biophys. Acta 807, 308–319.
[19] Le Brun, N.E., Wilson, M.T., Andrews, S.C., Guest, J.R., Harrison, P.M., Thomson, A.J. and Moore, G.M. (1993) FEBS Lett. 333, 197–202.
[20] Dailey, H.A. (1997) J. Biol. Inorg. Chem. 2, 411–417.
[21] Tait, G.H. (1969) Biochem. Biophys. Res. Commun. 37, 116–122.
[22] Tait, G.H. (1972) Biochem. J. 128, 1159–1169.
[23] Seehra, J.S., Jordan, P.M. and Akhtar, M. (1983) Biochem. J. 209, 709–718.
[24] Coomber, S.A., Jones, R.M., Jordan, P.M. and Hunter, C.N. (1992) Mol. Microbiol. 6, 3159–3169.
[25] Hippler, B., Homuth, G., Hoffmann, T., Hungerer, C., Schumann, W. and Jahn, D. (1997) J. Bacteriol. 179, 7181–7185.
[26] Homuth, G., Rompf, A., Schumann, W. and Jahn, D. (1999) J. Bacteriol. 181, 5922–5929.
[27] Akutsu, H., Park, J.-S. and Sano, S. (1993) J. Am. Chem. Soc. 115, 12185–12186.
[28] Ishida, T., Yu, L., Akutsu, H., Ozawa, K., Kawanishi, S., Seto, A., Inubushi, T. and Sano, S. (1998) Proc. Natl. Acad. Sci. USA 95, 4853–4858.
[29] Matthews, J.C., Burkhalter, R.S. and Timkovich, R. (1998) Bioorg. Chem. 26, 221–231.
[30] Klemm, D.J. and Barton, L.L. (1989) FEMS Microbiol. Lett. 61, 61–64.
[31] Klemm, D.J. and Barton, L.L. (1987) J. Bacteriol. 169, 5209–5215.