Characterisation of *Desulfovibrio vulgaris* haem b synthase, a radical SAM family member

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**Abstract**

An alternative route for haem b biosynthesis is operative in sulfate-reducing bacteria of the *Desulfovibrio* genus and in methanogenic Archaea. This pathway diverges from the canonical one at the level of uroporphyrinogen III and progresses via a distinct branch, where sirohaem acts as an intermediate precursor being converted into haem b by a set of novel enzymes, named the alternative haem biosynthetic proteins (Ahb). In this work, we report the biochemical characterisation of the *Desulfovibrio vulgaris* AhbD enzyme that catalyses the last step of the pathway. Mass spectrometry analysis showed that AhbD promotes the cleavage of S-adenosylmethionine (SAM) and converts iron-coproporphyrin III via two oxidative decarboxylations to yield haem b, methionine and the 5'-deoxyadenosyl radical. Electron paramagnetic resonance spectroscopy studies demonstrated that AhbD contains two [4Fe–4S]^{2+/-3+} centres and that binding of the substrates S-adenosylmethionine and iron-coproporphyrin III induces conformational modifications in both centres. Amino acid sequence comparisons indicated that *D. vulgaris* AhbD belongs to the radical SAM protein superfamily, with a GGE-like motif and two cysteine-rich sequences typical for ligation of SAM molecules and iron-sulfur clusters, respectively. A structural model of *D. vulgaris* AhbD with putative binding pockets for the iron-sulfur centres and the substrates SAM and iron-coproporphyrin III is discussed.

**1. Introduction**

All modified tetrapyroles, which include some of the major cofactors of life such as haem, sirohaem, haem d₅, chlorophylls and cobalamin (vitamin B₁₂), are synthesised along a branched biosynthetic pathway. Recently, the discovery of a new branch of the tetrapyrole pathway revealed that haem b can be made by one of two distinct routes. In the classic haem b synthesis pathway, found in eukaryotes and many bacteria, four enzymatic steps are required to transform uroporphyrinogen III, the primogenitor of all modified tetrapyrroles, into haem b. The first step of the classic pathway is catalysed by uroporphyrinogen decarboxylase (HemE), which mediates the sequential decarboxylation of the four acetic acid side chains of the substrate to give coproporphyrinogen III. Next, the two propionic acid side chains attached to C3 and C8 undergo oxidative decarboxylations to generate protoporphyrinogen IX in a reaction catalysed by coproporphyrinogen oxidase (HemF or HemN). Subsequently the macrocycle is oxidised by the removal of six hydrogens to give protoporphyrin IX in a reaction catalysed by protoporphyrinogen oxidase (HemG or HemV) and finally iron is inserted into the core of the substrate template by ferrochelatase (HemH) to give haem b [1,2]. However, in sulfate reducing bacteria of the *Desulfovibrio* genus and in methanogenic Archaea, haem b is produced along a distinct branch of the modified tetrapyrrole pathway where sirohaem acts as an intermediate precursor [3-5]. Along this route uroporphyrinogen III is transformed into sirohaem by methylation at C2 and C7, oxidation of the macrocycle and ferrochelation. Sirohaem is then converted into haem b by the enzymes of the alternative haem biosynthetic pathway (Ahb). Initially, sirohaem is acted upon by the AhbA and AhbB proteins that promote the decarboxylation of the acetic acid chains attached to C12 and C18 yielding 12,18-didecarboxysirohaem (Fig. 1). This decarboxylated form of sirohaem is modified by AhbC, which catalyses the loss of the acetic acid side chains at C2 and C7 in an S-adenosylmethionine (SAM)-dependent reaction, to form iron-coproporphyrin III (Fe-Cp III) (Fig. 1). The final step is also a SAM-dependent reaction promoted by AhbD that converts the two propionate side chains attached to C3 and C8 of Fe-Cp III into vinyl groups forming haem b (Fig. 1) [3]; therefore, the conversion of sirohaem into haem b via the alternative haem pathway involves several reactions that are linked to radical SAM chemistry.

**Abbreviations:** SAM, S-adenosylmethionine; HPLC, high-performance liquid chromatography; MS, mass spectrometry; Ahb, alternative haem biosynthetic proteins; Fe-Cp III, iron-coproporphyrin III

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Radical SAM enzymes are a vast family of proteins with a wide diversity of functions participating, for example, in the biosynthesis of cofactors, vitamins, DNA precursors, and antibiotics. One of the features of these enzymes is the presence of, at least, one $[4\text{Fe}–4\text{S}]^{2+/1+}$ cluster coordinated by only three cysteine residues arranged in a conserved $\text{CxCx}_2\text{C}$ motif located at the N-terminal region of the protein [6–10]. The catalytically active species of the cluster is the reduced $[4\text{Fe}–4\text{S}]^{1+}$ form that promotes the reductive cleavage of SAM to yield methionine and a highly reactive 5′-deoxyadenosyl (dAdo) radical. Due to the oxygen lability of this iron–sulfur cluster these reactions usually occur under anaerobic conditions. This large family of enzymes also includes the oxygen-independent coproporphyrinogen oxidase, HemN, from the canonical haem $b$ pathway [11,12]. Radical SAM enzymes may have additional iron–sulfur centres, dubbed “auxiliary clusters” [13]. For example, the biotin synthase, BioB, contains an extra $[2\text{Fe}–2\text{S}]^{2+/1+}$ cluster [14], whereas the methylthiotransferases RimO and MiaB bind an auxiliary $[4\text{Fe}–4\text{S}]^{2+/1+}$ cluster coordinated by three invariant cysteines, which is proposed to be involved in activation of sulphide or methylsulfide [15,16]. In the molybdenum cofactor biosynthetic enzyme MoaA, the additional $[4\text{Fe}–4\text{S}]^{2+/1+}$ centre is also coordinated by three cysteines [17] while in the anaerobic sulfatase maturing enzyme AnSME from Clostridium perfringens the two auxiliary $[4\text{Fe}–4\text{S}]^{2+/1+}$ centres are coordinated by four cysteine residues [18].

In this work, the AhbD enzyme involved in the last step of the alternative haem $b$ biosynthetic pathway in Desulfovibrio vulgaris Hildenborough was biochemically and spectroscopically characterised and shown to be a member of the radical SAM superfamily of proteins having two $[4\text{Fe}–4\text{S}]^{2+/1+}$ centres. Based on modelling studies, we predicted the tridimensional structure of AhbD and propose the putative pockets for the binding of the iron–sulfur centres and the SAM and iron-coproporphyrin III substrates.

2. Materials and methods

2.1. Cloning and expression of recombinant D. vulgaris AhbD

The gene encoding the D. vulgaris Hildenborough AhbD (gene locus DVU0855) was amplified in a PCR reaction using genomic DNA and two different pairs of oligonucleotides to produce a His-tagged and a non-labelled protein. The DNA fragment amplified with the pair 5′-CAC ATA TGG GAG CGC ATC CCA CTG CCC-3′ and 5′-CAC TCG AGG CGG GAG GGC GTC ATA C-3′ was cloned into NdeI/XhoI pET23b+ vector (Novagen), which allowed production of a C-terminal 6x-HisTag fused protein. The DNA fragment amplified with primers 5′-CGG GCG GCC ATA TGG GAG CG-3′ and 5′-CAT GTC GGT GGA GAA TTC CCC TGC-3′ was cloned into NdeI/EcoRI pET28a+ vector (Novagen), excised with the same restriction enzymes and cloned into pET23b+ to generate a protein with no fusion tags, designated AhbD. All recombinant plasmids were sequenced to confirm absence of errors in the gene sequences and then transformed in E. coli BL21 Star(DE3)pLysS cells (Invitrogen). For protein expression, E. coli cells carrying the recombinant plasmids were grown in 11 flasks fully filled with LB medium, to which the appropriate antibiotics were added. Cells growing at 37 °C that reached an OD$\text{_{600}}$ of approximately 0.5 were supplemented with 400 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 30 μM Fe (in the form of...
FeSO$_4$$\cdot$7H$_2$O), and after an overnight growth, at 20 °C, were harvested by centrifugation.

2.2. Cell free lysates preparation

D. vulgaris cells were grown anaerobically in lactate/sulfate medium [19], at 37 °C, in 1 l closed flasks until an OD$_{600}$ correspondent to the stationary phase (OD$_{600}$ ~ 1.2), at which point cells were collected by centrifugation and kept at ~ 80 °C.

In an anaerobic chamber (Belle Technology, < 2 ppm O$_2$), D. vulgaris and the E. coli BL21 Star(DE3) pLysS cells overexpressing D. vulgaris AhbD were resuspended in degassed 50 mM Tris–HCl pH 8 (buffer A), lysed by sonication and centrifuged for 20 min at ~ 30,000 × g for removal of unbroken cells. The resulting cell free lysates of D. vulgaris and of E. coli overexpressing AhbD were used in the activity assays as described below.

2.3. Protein purification

For protein isolation, E. coli BL21 Star(DE3) pLysS cells overexpressing D. vulgaris AhbD were resuspended in buffer A and disrupted by passing three times through a French Press, at 900 Psi under aerobic conditions, after which the suspensions were centrifuged for 30 min at ~ 30,000 × g. The resulting supernatant was submitted to an argon atmosphere and further manipulated in a Coy model A-2463 anaerobic chamber containing a gas mixture of 95% argon plus 5% hydrogen. The supernatant was applied to a 3 ml Ni$^{2+}$ saturated Chelating chamber containing a gas mixture of 95% argon plus 5% hydrogen. The supernatant was applied to a 3 ml Ni$^{2+}$ saturated Chelating chamber containing a gas mixture of 95% argon plus 5% hydrogen. The supernatant was submitted to an argon atmosphere and further manipulated in a Coy model A-2463 anaerobic chamber containing a gas mixture of 95% argon plus 5% hydrogen. The supernatant was applied to a 3 ml Ni$^{2+}$ saturated Chelating Chamber using a 10 kDa membrane (Millipore), and submitted to a buffer exchange by means of a PD10 column (GE Healthcare) that used buffer A as running buffer. The purity of the protein was evaluated by SDS-PAGE and the protein concentration was determined by the Pierce Bicinchoninic acid Protein Assay Kit (Thermo Scientific) using Sigma protein standards. UV–visible spectra were recorded inside the anaerobic chamber in a Shimadzu UV-1800 spectrophotometer.

The molecular mass and quaternary structure of the protein were determined by passage in a Superdex 200 10/300GL column (GE Healthcare), equilibrated with 50 mM sodium phosphate pH 7 buffer containing 150 mM NaCl. The iron content of the pure protein was determined by the 2,4,6-tripryridyl-1,2,3-triazine (TPTZ) method [20].

2.4. Analysis of the reaction products by high-performance liquid chromatography–mass spectrometry (HPLC–MS)

Independent assays were performed using the purified protein as well as the cell free lysates of D. vulgaris and of E. coli BL21 Star(DE3) pLysS cells overexpressing D. vulgaris AhbD.

Anaerobically purified D. vulgaris AhbD (15 μM) was mixed with 15 μM Fe-Cp III, 1 mM SAM and 0.5 mM sodium dithionite in a final volume of 1 ml and left to react for 3 h and overnight, at room temperature, under anaerobic conditions.

Reaction mixtures of 4 ml in buffer A containing D. vulgaris cell lysate, 20 μM Fe-Cp III (Frontier Scientific), 0.1 mM SAM, 10 mM sodium dithionate and 0.5 mM NADH were prepared and anaerobically incubated overnight, at room temperature. Reaction mixtures of 4 ml in buffer A with E. coli cell lysate, 20 μM Fe-Cp III, 0.5 mM SAM and 10 mM sodium dithionate were also prepared and incubated overnight.

In all cases, after the completion of the reaction, the tetrapyrole intermediates were purified by a haem extraction protocol [21] and analysed by reverse phase chromatography and mass spectrometry. To this end, the final extraction solvent, ethyl acetate, was evaporated and the intermediates were resuspended in a mixture of 90% acetonitrile–10% methanol (HPLC grade) and resolved on an Ace 5 AQ column (2.1 × 150 mm; Advanced Chromatography Technologies Ltd) attached to an Agilent 1100 series HPLC equipped with a diode array detector and coupled to a microTOF-Q II (Bruker) mass spectrometer. The tetrapyrrole derivatives were detected at 390 nm and separation was achieved by applying a binary gradient at a flow rate of 0.2 ml min$^{-1}$ with 0.1% trifluoroacetic acid (TFA) as solvent A and acetonitrile as solvent B. The column was first equilibrated with 20% solvent B and after sample injection the concentration of solvent B was increased to 100%. The total duration of each run was 50 min [3].

For the analysis of SAM derived products, reaction mixtures containing ~40 μM D. vulgaris AhbD, 1 mM sodium dithionite and 15 μM Fe-Cp III in a final 0.5 ml volume of buffer A were anaerobically prepared, and the reaction was initiated by adding 1 mM SAM. After overnight incubation, 100 μl aliquots were taken and immediately quenched by addition of an equal volume of 100 mM H$_2$SO$_4$. The mixtures were then centrifuged for 20 min at 16,000 × g, and 50 μl of the supernatants containing the SAM related products were analysed by HPLC–MS. As a control, a mix of SAM, S′-deoxyadenosine (dAdo), S′-methylthioadenosine (MTA) and S-adenosyl-$\lambda$-homocysteine (SAH) was also prepared and the products were separated and analysed by mass spectrometry as previously described [3], at a flow rate of 0.2 ml/min and with a gradient of acetonitrile in 0.1% TFA as follows: 0–1 min isotropic 0% B (acetonitrile), 1–15 min ramp to 10% B, 15–20 min ramp to 30% B, 20–25 min ramp to 50% B, 25–30 min ramp to 100% B, 30–35 min isotropic 100% B, 35–40 min ramp to 0% B and 40–50 min to 0%. The elution profile was monitored by UV–visible spectroscopy at 260 nm.

2.5. Binding assay

The binding of Fe-Cp III to AhbD was analysed by monitoring the changes of the UV–visible spectrum of the reduced Fe-Cp III (15 μM) upon successive additions of reduced AhbD protein (2 to 36 μM). The reduced form of Fe-Cp III was obtained by previous incubation with 2 molar equivalents of sodium dithionite in buffer A, while reduction of AhbD was achieved by addition of 8 molar equivalents of sodium dithionite. The shift of the Soret band and the variation of the absorbance at 550 nm were monitored. The association constant ($K_a$) and the maximal number of binding sites ($B_{max}$) values were determined by fitting the data to the equation $Y = B_{max} \times X / (X + K_a)$ by nonlinear regression using GraphPad Prism software version 5.0 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com). In this equation Y was calculated from the absorbance at 550 nm or the shift of the Soret band maximum and X represents the concentration of the protein. The titration data were normalised between 0 and 100 in relation to the smallest and the largest values of each data set.

2.6. EPR studies

Four samples prepared under anaerobic conditions were analysed by EPR spectroscopy, all containing the same concentration of AhbD (160 μM): sample 1, constituted by the as-purified AhbD; sample 2, consisting of purified enzyme reduced with sodium dithionite (2 mM); sample 3, a mixture of AhbD with SAM (1 mM) and sodium dithionite (2 mM); and sample 4, a mixture of AhbD, SAM (1 mM), Fe-Cp III (175 μM) and sodium dithionite (2 mM). All samples were prepared in buffer A with a final volume of 300 μl, loaded into EPR tubes, sealed with caps and immediately frozen in liquid nitrogen. EPR spectra were acquired at several temperatures and microwave powers in a Bruker EMX spectrometer equipped with an Oxford Instruments continuous flow helium cryostat. Spectra simulation was done with the SPINCOUNT program (http://www.chem.cmu.edu/groups/hendrich/facilities/index.html). Several independent protein preparations from distinct cell cultures were studied yielding consistently the same results.

The reduction potential of AhbD was determined by means of an anaerobic redox titration (under constant flux of argon) of the purified AhbD, monitored by EPR. To this end, 200 μM AhbD resuspended in
buffer A was titrated by stepwise addition of buffered sodium dithionite (10, 50 and 100 mM), in the presence of a standard redox mediators mixture (100 μM): 1,2 naphtoquinone (E′0 = 180 mV), 1,4-naphtoquinone (E′0 = 60 mV), methylene blue (E′0 = 11 mV), menedione (E′0 = 0 mV), indigo tetrasulfonate (E′0 = −30 mV), plumbagin (E′0 = −40 mV), resorufin (E′0 = −51 mV), indigo disulphonate (E′0 = −70 mV), indigo disulphonate (E′0 = −110 mV), phenazine (E′0 = −125 mV), 2-hydroxy-1,4-naphtoquinone (E′0 = −152 mV), anthraquinone sulfonate (E′0 = −225 mV), phenosafrine (E′0 = −255 mV), safranine (E′0 = −280 mV), neutral red (E′0 = −325 mV), benzyl viologen (E′0 = −345 mV) and methyl viologen (E′0 = −440 mV). A mixture of catalase (130 U/ml), glucose oxidase (4 U/ml) and glucose (3 mM) was also added to the sample mixture to further ensure that anaerobic conditions were maintained. Combined silver/silver chloride and platinum electrodes calibrated against a saturated quinhydrone solution at pH 7 were used. Potentials are reported relative to the standard hydrogen electrode. The experimental data were fitted to a single one-electron Nernst curve (n = 1).

3. Results

3.1. Biochemical and UV–visible spectroscopic analysis of D. vulgaris AhbD

The recombinant D. vulgaris AhbD was purified under anaerobic conditions in order to protect any putative oxygen-sensitive clusters. Nevertheless, the stability of the AhbD clusters was tested by exposure of the protein to oxygen and no degradation was observed. The protein was eluted from an analytical gel filtration chromatography as a mono-mer with an apparent molecular mass of ~40 kDa, in agreement with its gene-derived amino acid sequence. The as-isolated protein exhibits a brown colour with a visible spectrum displaying two absorption bands at 325 and 415 nm (Fig. 2). These features are similar to those of the oxidised form of iron–sulfur cluster containing proteins. Indeed, metal analysis also showed that AhbD contains 6 ± 2 mol of iron per mol of protein, suggesting the presence of more than one iron–sulfur cluster.

3.2. D. vulgaris AhbD binds Fe-Cp III

In the alternative biosynthetic pathway operative in D. vulgaris, AhbD converts iron-coproporphyrin III into haem b. In this work, we have analysed the binding of the substrate by following changes in the absorbance increase at 550 nm (see Materials and methods). The data were monitored by following the alteration of the Soret band and the absorbance increase at 550 nm (see Materials and methods). The data also allowed the determination of an association constant (K_a), consistent with a single (1:1) binding stoichiometry, of 8 ± 1 μM (Fig. 3).

3.3. D. vulgaris AhbD promotes cleavage of SAM and converts Fe-Cp III into haem b

Since the step promoted by AhbD is proposed to be a SAM-dependent reaction, the products of SAM cleavage were analysed by HPLC–MS after incubation of the purified protein with SAM and sodium dithionite. Under these conditions, the formation of S′-deoxyadenosine (dAdo) product was detected (Fig. 4A). The reaction mixture containing the same concentration of all components but done in the presence of the Fe-Cp III substrate was also analysed. We observed that the addition of the second substrate led to an approximately 10-fold increase of the amount of the dAdo formed, indicating that the metalloporphyrin potentiates the SAM cleavage (Fig. 4C). The data show that D. vulgaris AhbD performs a reaction that is typical of the radical SAM enzymes, namely the reductive cleavage of SAM into methionine and the dAdo radical. As with other members of this family of proteins [24] the SAM derived products were observed in the absence of the second substrate.

The products formed during the conversion of Fe-Cp III into haem b catalysed by D. vulgaris AhbD were also determined by HPLC–MS. The reaction was performed separately with D. vulgaris free cell lysates, E. coli cell extracts overproducing an untagged form of D. vulgaris AhbD and purified D. vulgaris AhbD. The reaction mixtures consisting

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Fig. 2. UV–visible spectrum of the as-isolated D. vulgaris AhbD (5 μM) in 50 mM Tris–HCl pH 8, recorded under anaerobic conditions.

Fig. 3. Titration of Fe-Cp III with D. vulgaris AhbD. UV–visible spectra of reduced Fe-Cp III (15 μM) obtained upon successive additions of AhbD (spectral shifts are indicated by arrows) and respective titration curve (inset) considering the shift of the Soret band maximum (black squares) and the absorbance increase at 550 nm (open diamonds); the values were normalised to their final stage. The solid line corresponds to the fitting of the curves with an equation for a 1:1 binding stoichiometry and a binding constant of 8 μM (see Materials and methods).

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D. vulgaris AhbD contains two \([4Fe-4S]^{2+/1+}\) centres

The nature of the iron–sulfur clusters in AhbD was studied in detail by EPR spectroscopy. The EPR spectrum of the as-isolated AhbD exhibits a quasi-isotropic signal with g-values at around 2.02 (2.03, 2015, 2.005) as determined by simulating the spectrum using the program SPINCOUNT (Fig. 6Aa), characteristic of an oxidised \([3Fe-4S]^{1+/0}\) cluster. This resonance accounts 10–15% of the spectral intensity of the dithionite reduced sample, under the same non saturating conditions, and most likely results from a minor oxidative degradation of one or two of the tetranuclear centres, a common feature in low-reduction potential \([4Fe-4S]^{2+/1+}\) centres. Reduction of AhbD with sodium dithionite led to development of a more complex signal with g-values ranging from \(g = -2.06\) to 1.92, which are typical of reduced bi- or tetranuclear iron–sulfur centres (Fig. 6Ab). These resonances were detected without significant line broadening only up to a temperature of ~30 K. Hence, we concluded that they are not originated from binuclear centres but from tetranuclear iron–sulfur clusters which agrees with the cysteine motifs present in the AhbD amino acid sequence (see below). The resonances were deconvoluted by varying the temperature and microwave power. When, at 10 K, the microwave power was increased at 40 mW (Fig. 6Ba) while at lower g-values no further resonances were detected. Also, resonances at low magnetic fields, which can originate from cluster spin states higher than \(\frac{1}{2}\), were not observed even at high microwave powers and low temperatures down to 4.6 K (data not shown). The experimental data at 10 K could be rationalised as resulting from two reduced tetranuclear centres, with distinctly discernible \(g_{\text{max}}\) values and quite different relaxation properties: a fast relaxing species, only observable at low temperatures and high microwave power, with g-values at 2.083, 1.921 and 1.84, and a slower relaxing one with g-values at 2.057, and 1.925 (Fig. 6Ba). Within the limitations resulting from the distinct relaxation properties, these species are present essentially in equimolar amounts.

To further characterise D. vulgaris AhbD, the redox potential of the iron–sulfur centres was determined by performing a redox titration followed by EPR. The results were analysed with a one-electron Nernst curve and allow determining a single reduction potential value of ~390 ± 10 mV (Fig. 7). The data also indicate that the reduction potentials of the two centres are similar as the individual reduction potentials of the two iron–sulfur centres could not be deconvoluted. Moreover, the lack of a plateau observed under the tested conditions shows that the metal centres of AhbD have very low reduction potentials, whose upper limit is ~390 mV.

We have also analysed the EPR spectra of dithionite-reduced AhbD samples upon addition of the substrates. In the presence of SAM, some modifications of the spectra occurred and the data could again be interpreted as resulting from two main components, with g-values only slightly distinct from those of the reduced sample (Fig. 6Ac and Bb). The minor alterations observed upon addition of SAM only are not unexpected as EPR spectroscopy, although sensitive to minute modifications of the paramagnetic centre electronic structure, does not necessarily reflect specific changes of, e.g., the coordination of the iron ions in the clusters.

Major differences were observed when the substrates SAM and Fe-Cp III were both added: two sets of resonances are clearly affected, but to differing extents. By varying the microwave power, two major species were defined, one that was more similar to the previously identified slow relaxing centre, with g-values at 2.064 and 1.92, and a second one from a faster relaxing centre with very broad resonances and g-values of 2.13, and ~1.92, underneath the \(g_{\text{med}}\) of the first species (Fig. 6Ad and Bc).

Above ~40 K, the spectra display solely a signal with g-values of 2.035, 2.023, and 2.01, quite distinct from that of the \([3Fe-4S]^{1+}\) centre detected in the oxidised protein; this species is also observed in the 10 K spectra (Fig. 6Aa and Bb, marked by an asterisk). This species, which has a slow relaxation rate (it is not detected at high microwave powers) and of unknown origin, was observed in all spectra of the reduced protein (including in the presence of the substrates), and in different preparations, albeit accounting always to less than 10% of the total spectral...
Amino acid sequence analysis of D. vulgaris AhbD

A BLAST search against Desulfovibrio spp. and Archaea genomes showed that homologs of D. vulgaris AhbD are widespread in Desulfovibrio spp., sharing 27%–89% identity and 42%–92% similarity when considering the first 100 retrieved amino acid sequences. For example, AhbD from D. vulgaris and D. desulfuricans ATCC 27774 have 70% identity and 77% similarity between them. Orthologs of D. vulgaris AhbD also occur in methanogenic Archaea (e.g., Methanosarcina barkeri AhbD, 50% identity, 68% similarity). In agreement with our data, recent studies showed that the M. barkeri AhbD also catalyses the conversion of Fe-Cp III into haem and the authors proposed that it contains [4Fe–4S]2+/1+ centres [29]. Interestingly, D. vulgaris AhbD shares ~26% identity and 44% similarity with the enzyme involved penultimate step of the alternative haem b pathway, namely the D. vulgaris AhbC that converts 12,18-didecarboxyloisoaem into Fe-Cp III (Figs. 1 and 8).

Fig. 8 compares the amino acid sequence of D. vulgaris AhbD with those of enzymes involved in the synthesis of tetrapyrroles and decarboxylation reactions (Fig. 8). D. vulgaris AhbD shares 25% identity, 39% similarity with Paracoccus denitrificans NirJ, which participates in the synthesis of haem d1. In relation to E. coli HemN, the oxygen-independent coproporphyrinogen III oxidase of the classical haem b pathway that performs the oxidative decarboxylation of the propionate side chains of rings A and B of coproporphyrinogen III to vinyl groups producing protoporphyrinogen IX, a low degree of similarity is seen (10% identity, 20% similarity) [3,26,30]. The low degree of similarity among these proteins suggests that despite the small structural differences of the several tetrapyrrole substrates their conversion requires enzymes with highly specific structures.

A comparative analysis of the amino acid sequence of D. vulgaris AhbD against the NCBI protein database shows that the protein belongs to the radical SAM enzymes superfamily. The protein has a radical SAM motif in the N-terminal region and a SPASM-like motif in the C-terminus (Fig. 8). The SAM motif contains a cysteine rich region (C31xxC35xC38) typical of SAM proteins, namely the Cx3xCxC sequence, where X is an aromatic residue; these three cysteines bind the catalytic [4Fe–4S]2+/1+ centre, leaving the fourth iron free to interact with SAM (e.g., [6,18,31]). The N-terminal region of D. vulgaris AhbD has also a GGD triad in place of the GGE motif, which is present in several radical SAM proteins and is involved in the binding of SAM [31]. The C-terminal region of AhbD houses a second cysteine rich region C326x3C329xC335xC338, characteristic of a SPASM motif, whose name is derived from the following biochemically characterised members of this subfamily: subtilosin A maturase AlbA, pyrroloquinoline quinone E PqqE, anaerobic Sulfatase maturating enzyme, and mycofactocin peptide maturase [32–34]. The SPASM domain is quite...
conserved in members of the radical SAM family and is described to bind auxiliary \[4\text{Fe–}4\text{S}\]2+/1+ centres. The biochemical and spectroscopic studies presented above indicate that in \textit{D. vulgaris} AhbD the SPASM motif is also involved in the binding of one auxiliary \[4\text{Fe–}4\text{S}\]2+/1+ cluster, most possibly coordinated by four cysteine residues. For most of the enzymes, the function of the additional cluster remains, so far, unclear [13].

4. Discussion

In this work, \textit{D. vulgaris} AhbD is shown to be a monomeric cytoplasmic protein and a member of the radical SAM superfamily. The protein harbours two \[4\text{Fe–}4\text{S}\]2+/1+ centres, one located at the N-terminal domain and coordinated by three cysteines and the other at the C-terminus putatively ligated by four cysteines. Under reductive conditions and in the presence of SAM, AhbD promotes the sequential decarboxylation of the two propionate side chains attached to C3 and C8 of Fe-Cp III to form vinyl groups, i.e., generating haem b.

Using two of the more similar structural protein analogues of \textit{D. vulgaris} AhbD, namely the AnSME from \textit{C. perfringens} [18] and MoaA of \textit{S. aureus} [17], we predicted a three dimensional model for the structure of AhbD (Fig. 9). AnSME, which has two auxiliary \[4\text{Fe–}4\text{S}\]2+/1+ clusters each coordinated by four cysteines located at the C-terminus, shares with \textit{D. vulgaris} AhbD 15% identity, 31% similarity, while MoaA that has one additional \[4\text{Fe–}4\text{S}\]2+/1+ coordinated by only three cysteines shares 14% identity and 34% similarity with the \textit{D. vulgaris} enzyme (Fig. 8). The \textit{D. vulgaris} AhbD structure model has \(\beta/\alpha\) motifs arranged in a TIM barrel-like motif, the common feature of most radical SAM enzymes [6], although some of the beta strands appear as loops probably due to the low amino acid sequence similarity. As predicted from the amino acid sequence, the N-terminal region corresponds to the SAM radical protein domain, which includes the three cysteines that bind the catalytic \[4\text{Fe–}4\text{S}\]2+/1+ cluster, the two glycine residues, Gly75 and Gly76, and the aspartate residue Asp77 that interacts with the SAM molecule. The C-terminal domain contains four cysteines (Cys326, Cys329, Cys335 and Cys336) that are predicted to be
Fig. 8. Amino acid sequence alignment of D. vulgaris Hildenborough AhbD (gene locus DVU0855) with D. desulfuricans ATCC 27774 AhbD (Ddes0287), Methanosarcina barkeri AhbD (MbarA1458) and Methanosarcina barkeri AhbC (MbarA1793). The two domains of D. vulgaris AhbD, namely the AdoMet radical domain and the auxiliary iron–sulfur binding SPASM domain are indicated. The amino acid residues of the AdoMet radical motif for the binding of the conserved iron–sulfur centre CX3CX2C are shaded in black and marked with stars. Residues of the GG(E/D)P motif involved in the binding of SAM are shaded in dark grey and indicated with open circles. Cysteine residues of the SPASM motif are shaded in light grey. The alignment was constructed using the amino acid sequence of D. vulgaris AhbD to search for similar sequences against all microbial genomes using NCBI Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/), performed in ClustalX 2.1[37] and edited in GeneDoc[38].
the ligands of the auxiliary [4Fe–4S]$_{1}^{2+/1+}$ cluster equivalent to auxiliary centre II in the C. perfringens AnSME (Fig. 9A). Both iron–sulfur clusters are expected to be close to the protein surface, which may allow exchange of electrons with yet unknown physiological partners. Interestingly, in the place where C. perfringens AnSME binds the auxiliary cluster I, the D. vulgaris enzyme has still two cysteine residues.

**Fig. 9.** 3D model of D. vulgaris AhbD. (A) Cartoon representation of the predicted D. vulgaris AhbD structure. (B) Tridimensional structure of C. perfringens AnSME displayed in the same orientation as in panel A. (C) Surface representation of D. vulgaris AhbD model. (D) Surface representation of D. vulgaris AhbD model rotated by 90° from the view in panel C. The figure depicts a channel (marked with an arrow) and the predicted binding site of the SAM molecule which is located at the end of the channel. The model was generated by the I-TASSER server [39–41] using as template the structure of C. perfringens AnSME (PDB ID: 4K36). The root-mean-square deviation between the two models is 0.987 Å. In panels A and B, the monomer is coloured in gradient, starting from blue (N-terminal) to red (C-terminal) and the region of the conserved cysteines involved on the iron–sulfur clusters is boxed and zoomed. The amino acid residues, the SAM molecule and the iron–sulfur clusters are represented as sticks. Pictures were built with the PyMOL program [42].
Our results show that D. vulgaris AhbD is a two \([4Fe-4S]\) cluster containing haem synthase that adds a new function to the widespread superfamily of radical SAM enzymes.

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**References**


