

A haloarchaeal ferredoxin electron donor that plays an essential role in nitrate assimilation

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Abstract

In the absence of ammonium, many organisms, including the halophilic archaeon *Haloferax volcanii* DS2 (DM3757), may assimilate inorganic nitrogen from nitrate or nitrite, using a ferredoxin-dependent assimilatory $\text{NO}_3^-/\text{NO}_2^-$ reductase pathway. The small acidic ferredoxin *Hv*-Fd plays an essential role in the electron transfer cascade required for assimilatory nitrate and nitrite reduction by the cytoplasmic NarB- and NirA-type reductases respectively. UV-visible absorbance and EPR spectroscopic characterization of purified *Hv*-Fd demonstrate that this protein binds a single [2Fe-2S] cluster, and potentiometric titration reveals that the cluster shares similar redox properties with those present in plant-type ferredoxins.

The role of ferredoxins in nitrate and nitrite assimilation

Nitrogen is essential to all living organisms. This element exists in many reservoirs on earth that are connected by a set of biogeochemical processes collectively termed the nitrogen cycle. Biological access to inorganic nitrogen is predominantly via ammonium (NH_4^+) and nitrate (NO_3^-). Ammonium provides the substrate for biosynthetic cellular pathways that form complex nitrogen-containing compounds, such as nucleic and amino acids required for DNA and proteins.

When the availability of NH_4^+ is limited, many organisms, including plants, fungi and bacteria, may express assimilatory NO_3^- reductase systems and instead assimilate inorganic nitrogen from NO_3^- or NO_2^- . The biochemical basis for this is NAS, a cytoplasmic $\text{NO}_3^-/\text{NO}_2^-$ reductase system that can show a high degree of plasticity between different organisms, but commonly involves a molybdopterin cofactor-dependent nitrate reductase and a sirohaem:ferredoxin-dependent nitrite reductase. In recent years, archaea have been increasingly recognized as important contributors to the global nitrogen cycle. For example, many have been found to contain genes for denitrification and ammonia oxidation [1,2]. Furthermore, *Haloferax* species are halophiles of the Euryarchaeota that inhabit saline environments and assimilate NO_3^- or NO_2^- via an assimilatory $\text{NO}_3^-/\text{NO}_2^-$ reductase pathway [3].

Although NAS systems are dependent on cytoplasmic nitrate and nitrite holoreductases, a high degree of flexibility is observed regarding the nature of the physiological

electron donor to these enzymes. In cyanobacteria, a [2Fe-2S](S^γCys)₄ ferredoxin is reduced by Photosystem I and provides the electron donor to both the nitrate and nitrite reductases NarB and NirA respectively [4]. Similarly, a recent biochemical investigation of the bacterial NAS system from *Paracoccus denitrificans* has shown that a putative [2Fe-2S](S^γCys)₂(N^δHis)₂ ferredoxin, NasG, is essential for coupling of the physiological electron donor, i.e. the reduced NADH pool, to both the nitrate and nitrite reductases NasC and NasB respectively [5]. In haloarchaea such as *Haloferax volcanii*, the physiological electron donor to both the assimilatory nitrate and nitrite reductases is proposed to be a small acidic [2Fe-2S](S^γCys)₄-type ferredoxin, termed *Hv*-Fd. This arrangement is reminiscent of plant and cyanobacterial systems, in which the *Hv*-Fd supplies electrons to both a NarB- and NirA-type nitrate and nitrite reductase respectively.

In the present review, we explore the use of a combination of UV-visible electronic absorbance and continuous wave EPR spectroscopy methodologies to identify the nature of the iron-sulfur site present in the purified *Hv*-Fd and also perform potentiometric experiments to define the redox properties of this metalloprotein.

Purification and spectroscopic properties of *Hv*-Fd

Currently, 87 haloarchaeal genome sequences are available; of these, 13 correspond to *Haloferax* species (<http://www.ncbi.nlm.nih.gov/bioproject?term=Haloferax>). In all species, including *Hfx. volcanii*, it is possible to find more than one open reading frame predicted to encode [2Fe-2S] proteins. The native *Hv*-Fd protein from *Hfx. volcanii* DS2 (DM3757) was purified to homogeneity by anion-exchange (Sephacrose CL-4B followed by Q-Sepharose) and

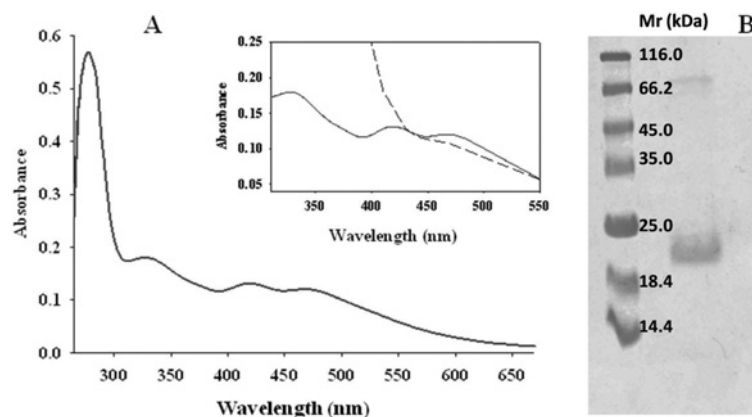
Key words: ferredoxin, haloarchaeon, nitrate assimilation, nitrate and nitrite reduction.

Abbreviations used: *Hm*-Fd, ferredoxin from *Haloferax mediterranei*; *Hv*-Fd, ferredoxin from *Haloferax volcanii*.

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Figure 1 | UV-visible electronic absorbance spectrum (A) and SDS/PAGE analysis (B) of purified *Hv*-Fd

Molecular masses are indicated in kDa. The apparent molecular mass of *Hv*-Fd (~14 kDa, confirmed by analytical gel-filtration chromatography) is overestimated in SDS/PAGE analysis, behaviour that is commonly associated with haloarchaeal proteins.



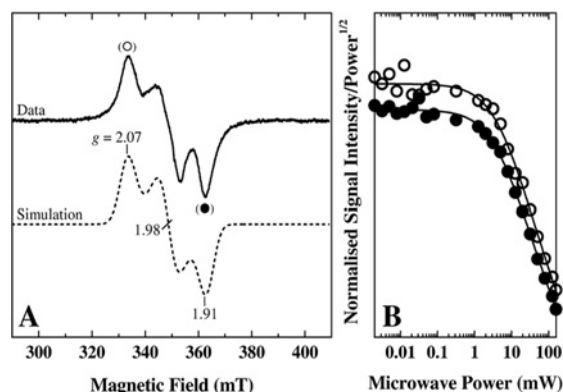
size-exclusion (Sephacryl S-200) chromatography (Figure 1). In order to confirm that the ferredoxin isolated was that involved in assimilatory nitrate and nitrite reductase pathway, the peptide was treated with trypsin and analysed by nano-ESI (electrospray ionization)–LC (liquid chromatography)–MS/MS (tandem MS). The MS results obtained revealed that this protein (129 amino acids) is encoded by the locus tag ‘HVO_2995’ and the predicted molecular mass (14.4 kDa) was consistent with that calculated from gel-filtration chromatography (results not shown). A similar approach was used to identify the previously characterized *Hm*-Fd, i.e. the physiological electron donor to NarB and NirA from *Haloferax mediterranei* [6,7].

The electronic absorbance spectrum of the purified protein contained broad absorbance features between 300 and 600 nm, consistent with the presence of a [2Fe–2S] cluster as described previously for other halophilic ferredoxins [6,7] (Figure 1). Protein concentration was determined by the Bradford assay or spectrophotometrically using an absorption coefficient of $9600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 420 nm [8]. Both methods gave comparable concentrations for the purified protein sample, consistent with near-complete cofactor occupancy.

The X-band EPR spectrum of the oxidized (90 μM , as prepared) protein at 18 K showed no signal, but on addition of 1 equivalent of europium(II) chloride ($E^\circ = -0.4 \text{ V}$ [9]), a rhombic lineshape from a dominant paramagnetic species was observed (Figure 2A), consistent with the presence of a [2Fe–2S]¹⁺ ($S = 1/2$) site. Further addition of europium(II) chloride or the stronger reducing agent europium(II) EGTA ($E^\circ = -0.9 \text{ V}$, prepared as described by Vincent and et al. [9]) failed to alter the magnitude or form of this signal and thus confirmed complete protein reduction. Microwave power saturation curves also measured at 18 K, gave a half-saturation power value of $5.5 \pm 0.3 \text{ mW}$, confirming that all spectra presented were recorded under non-saturating conditions (Figure 2B). Simulation gave

Figure 2 | Continuous wave X-band EPR spectrum (A, continuous line) and power-dependence (B) of the europium(II) chloride-reduced *Hv*-Fd (90 μM) in 2M NaCl, 50 mM Hepes (pH 7.0)

Spectral simulation performed using WINEPR SimFonia (version 1.25, Bruker Analytische Messtechnik) is included and offset for clarity (A, broken line). Experimental conditions for the spectrum presented in (A) were: 9.68 GHz microwave frequency, 0.2 mW power, 1 mT (10 Gs) modulation amplitude, at 18 K.



resonances at $g_{1,2,3} = 2.07, 1.98, 1.91$ and quantification by comparison with a 1 mM copper(II) EDTA standard gave occupancy of approximately 0.9 spin/mol of protein, consistent with *Hv*-Fd binding a single [2Fe–2S]^{2+/1+} cluster co-ordinated by four conserved cysteine residues present in the amino acid sequence. On addition of excess potassium ferricyanide, a featureless spectrum was observed similar to that observed for the oxidized protein (results not shown), indicating reversible redox transformation of the cluster back to the diamagnetic [2Fe–2S]²⁺ ($S = 0$) state. Thus the low-temperature EPR spectrum and relaxation character of this [2Fe–2S]¹⁺ cluster are similar to those determined for other haloarchaeal proteins.

Table 1 | EPR *g*-values and redox properties of selected [2Fe–2S] proteins

Organism	<i>g</i> -values ($g_{1,2,3}$)	Midpoint potential (mV)	Reference
<i>Hfx. mediterranei</i>	2.07, 1.98, 1.91	– 280	[7]
<i>Halobacterium halobium</i>	2.07, 1.97, 1.90	– 345	[12]
<i>Hfx. volcanii</i>	2.07, 1.98, 1.91	– 384	Our study
Spinach	2.04, 1.95, 1.88	– 401	[11]

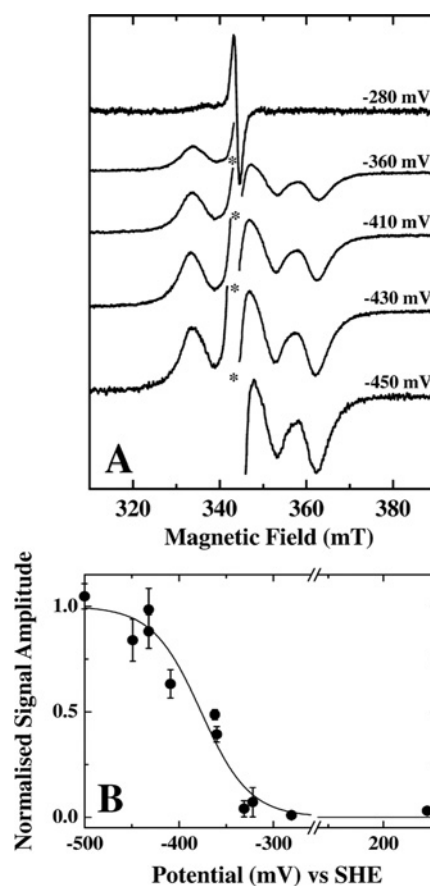
EPR-monitored potentiometric titration experiments were performed to determine the midpoint potential of the iron–sulfur centre present in *Hv*-Fd. Samples were poised in an anaerobic sample vessel and solution potential was monitored using a multimeter that was referenced against a silver/AgCl standard (potentials herein are quoted compared with the standard hydrogen electrode, by addition of + 197 mV [10]). The sample contained the following mediators at 30 μ M each: Methylene Blue, duroquinone, menadione, Indigo Carmine, 9,10-anthraquinone-2,6-disulfonic acid, anthraquinone-2-sulfonic acid, phenosafranin, safranin-O, Benzyl Viologen and Methyl Viologen. Solution potential was decreased by incremental addition of europium(II) chloride and, for desired stable potentials, samples were withdrawn for EPR analysis (Figure 3). Spectra were recorded, and the variation of signal magnitude with respect to solution potential could be fitted to a single $n=1$ Nernstian process to give a midpoint potential (E_m) of -384 ± 10 mV at pH 7. This E_m value is the lowest determined so far for a haloarchaeal ferredoxin and thus the redox properties are similar to other [2Fe–2S](S^γCys)₄ cluster containing ferredoxins present in plants (Table 1). Significantly, the ferredoxins from *Haloarcula marismortui* and plants also share a high degree of structural similarity, notably in the [2Fe–2S] environment and protein core, although the haloarchaeal proteins have a high preponderance of solvent-exposed acidic residues in addition to other surface adaptations that confer halotolerance [13].

Wider roles for ferredoxins in nitrate assimilation

The purification and spectroscopic characterization of the native ferredoxin isolated from *Hfx. volcanii* DS2 revealed that this is a small ferredoxin that binds a single [2Fe–2S] cluster that can undergo a reversible single-electron redox transformation. Taking into account the similarities found between *Hv*-Fd and *Hm*-Fd, this protein is likely to be the physiological electron donor to both the NarB- and NirA-type nitrate and nitrite reductase, as has been described previously for other haloarchaeal species such as *Hfx. mediterranei*, in addition to cyanobacteria and plants [6,7]. As such this protein plays an essential role in the electron-transfer cascade required for assimilatory nitrate reduction in *Hfx. volcanii*.

Figure 3 | EPR-monitored potentiometric titration of *Hv*-Fd

Samples were poised in an anaerobic glove box at the potentials indicated, and EPR spectra were recorded (A). Experimental conditions are as for Figure 2(A). The asterisk denotes the increasing contribution of reduced mediator radicals at $g=2$ observed below approximately – 280 mV. A plot of signal intensity against solution potential was fitted with a single Nernst expression ($n=1$) to give a midpoint potential value of -384 ± 10 mV at pH 7 (B). SHE, standard hydrogen electrode.



Multiple sequence alignment (Figure 4) shows that the ferredoxins in haloarchaea can be divided into two groups, on the basis of sequence. Like that of most Halobacteriaceae, the *Hfx. volcanii* genome encodes both kinds of ferredoxins. We have characterized a member of the first group, i.e. *Hv*-Fd, which is defined by the N- and C-terminal sequences MPTVTYLN and LQNRVI respectively. These proteins

Figure 4 | Sequence alignment of selected haloarchaeal, cyanobacterial and plant [2Fe–2S] ferredoxins

Multiple alignments of family I and II sequences are shown in (A) and (B) respectively. The conserved cysteine residues that serve as ligands to iron atoms are highlighted.

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Haloferax volcanii      -MPTVTYLNVEVLDDNGWDLDDDLGFEQAADAGLDAEDYGEMEYNQGEYI
Halogeometricum borinquense -MPTVEYLNVEVLDDNGWDLDDDLFENAADAGLDGEDYGTLEVNQGEYI
Halobacterium salinarum  -MPTVEYLNVEVLDDNGWDMDDDLFEKAADAGLDGEDYGTMEVVAEGEYI
Halorubrum lacusprofundi MMPTVEYLNVEVLDDHGWSMDDDLFEEAADADLDAEDYGSGLDVNQGEYI
Natronomonas pharaonis  -MPTVEYLNVEVLDDHGWMDDDLFENAADADLDEEDYGSGLDVAEGEYI
Haloarcula japonica     -MPTVEYLNVEVDDNGWDMYDDVFAEASDMDLDGEDYGSLEVNEGEYI
Haloarcula marismortui  -MPTVEYLNVEVDDNGWDMYDDVDFGEASDMDLDEEDYGSLEVNEGEYI
Nostoc sp.              -----MATFKVTLINAEAGTKHEIEVPDDEYI
Anabaena sp.            -----MATFKVTLINAEAGTKHEIEVPDDEYI
Spinacia oleracea       -----ATYKVTLVTPSG--SQVIECGDDEYI
Prochlorococcus marinus -----MASYKVTLISEGEGLNSTIEVPDDQYI

Haloferax volcanii      LEAAEAQGYDWPFS<b>CRAGACAN</b>CAAIVKEGEIDMDMQQILSDEEVNEKNV
Halogeometricum borinquense LEAAEAQGYDWPFS<b>CRAGACAN</b>CAAIVKEGEIQMDMQQILSDEEVSEKNV
Halobacterium salinarum  LEAAEAQGYDWPFS<b>CRAGACAN</b>CAAIVKEGEIDMDMQQILSDEEVVEKDV
Halorubrum lacusprofundi LESAAEAQGYDWPFS<b>CRAGACAN</b>CAIIVMEGDIEMDMQQILSDEEVVEKNV
Natronomonas pharaonis  LEAAEAQGYDWPFS<b>CRAGACAN</b>CAAIVKEGEIDMDMQQILSDEEVVEKDV
Haloarcula japonica     LEAAEAQGYDWPFS<b>CRAGACAN</b>CAAIIVLEGGIDMDMQQILSDEEVDEKNV
Haloarcula marismortui  LEAAEAQGYDWPFS<b>CRAGACAN</b>CAAIIVLEGGIDMDMQQILSDEEVDEKNV
Nostoc sp.              LDAAEQGYDLPPS<b>CRAGACST</b>CAGKLVSGTVDSQSDQSFLLDDQIEAG-Y
Anabaena sp.            LDAAEQGYDLPPS<b>CRAGACST</b>CAGKLVSGTVDSQSDQSFLLDDQIEAG-Y
Spinacia oleracea       LDAAEKGMPLPYS<b>CRAGACSS</b>CAGKVTSGSVDSQSDQSFLEDGQMEEG-W
Prochlorococcus marinus LDAAEQGYDLPPS<b>CRAGACST</b>CAGKVTSGSVDSQSDQSFLLDDQIEAG-F

Haloferax volcanii      RLTCIGSPVEDEVKIVYNAKHLDYLQNRVI
Halogeometricum borinquense RLTCIGSPETDEVKIVYNAKHLDYLQNRVI
Halobacterium salinarum  RLTCIGSPADEVKIVYNAKHLDYLQNRVI
Halorubrum lacusprofundi RLTCIGSPATDEVKIVYNAKHLDYLQNRVI
Natronomonas pharaonis  RLTCIGSPADEVKIVYNAKHLDYLQNRVI
Haloarcula japonica     RLTCIGSPDADEVKIVYNAKHLDYLQNRVI
Haloarcula marismortui  RLTCIGSPDADEVKIVYNAKHLDYLQNRVI
Nostoc sp.              VLT<b>C</b>VAYPTSDVVIQTHKEEDLY-----
Anabaena sp.            VLT<b>C</b>VAYPTSDVVIQTHKEEDLY-----
Spinacia oleracea       VLT<b>C</b>IAYPTGDVTIETHKEEELTA-----
Prochlorococcus marinus VLT<b>C</b>VAYPTSDVTITTHAEEELY-----

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B

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Haloferax mediterranei  -----MTEYTVVEFVGTGETITVSDKQITLKACIEEGIAQEYS<b>CRVGM</b>CLA
Haloferax volcanii      -----MTEYTVVEFVGTGETITVSDKQITLKACIEEGIAQEYS<b>CRVGM</b>CLA
Halogeometricum borinquense -----MTEYTVVEFVGTGESIQVSDKQITLKACIEEGIAQEYS<b>CRVGM</b>CLA
Halorubrum lacusprofundi -----MTEYTVVEFVGTGETIEVADTETILQPCIEEGIAQEYS<b>CRVGM</b>CLA
Natronomonas pharaonis  -----MTDYTVVEFVGTGETITVSDKDTILSRCEEGIAQEFSS<b>CRVGM</b>CLA
Haloarcula marismortui  -----MTEHTVTFVGTGEEITVSEKETILSRCEEGIAQEYS<b>CRVGM</b>CLA
Halobacterium salinarum -----MTEYTVVEFAGAGETIEVSDTETILKACLREGIAQEYS<b>CRVGM</b>CLA

Haloferax mediterranei  <b>CS</b>AEIIEGDVTPAARGLTEEESEK-YALTC<b>M</b>ARPQSDLKLKRGYPPSI
Haloferax volcanii      <b>CS</b>AEIIEGDVTPAARGLTEEEAER-FALTC<b>M</b>ARPQSDLKLKRGYPPSI
Halogeometricum borinquense <b>CS</b>AEIIEGDVVQPAARGLTEEEEEE-YALTC<b>M</b>ARPQSDLKLKRGYPPSI
Halorubrum lacusprofundi <b>CS</b>AEIVEGEVTPAARGLTDEEAEE-YALTC<b>M</b>ARPQSDLKLKRGYPPSI
Natronomonas pharaonis  <b>CS</b>AEIIEGEVTPAARGLTDAERED-YALTC<b>M</b>ARPQSDLKLKRGYPPSI
Haloarcula marismortui  <b>CS</b>AEIVEGSVTPAARGLTDREREN-YALTC<b>M</b>ARPQSDLKLKRGYPPSI
Halobacterium salinarum <b>CS</b>AKIESGDVTPAARGLTDAEADE-YALTC<b>M</b>ARPQTDLVLDKRGYPPSI

Haloferax mediterranei  -----EDDAVTAG-----AAADDD-----
Haloferax volcanii      -----EDDAAAAG-----TAADDD-----
Halogeometricum borinquense -----EEDAASAA-----AAADDD-----
Halorubrum lacusprofundi -----EDDAATDASDVDAADDD-----
Natronomonas pharaonis  -----EDEVVDVADGTDAAAADD-----
Haloarcula marismortui  -----EGDVSPEDG--DPTPADDD-----
Halobacterium salinarum -----EEEAADVAD--QAAADDD-----

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contain approximately 129 amino acids, whereas the second group of ferredoxins are slightly smaller (~108 amino acids) and distinguished by the N- and C-terminal sequences MTEYTVVEF and AGTAADDD respectively. The reason for the presence of two forms of [2Fe–2S](S^γCys)₄ ferredoxin in these micro-organisms is unclear at present. Whereas the role

of family I proteins as electron donor to both assimilatory nitrate and nitrite reductases has been established [6,7], a clear role for the family II ferredoxins is yet to be demonstrated. It also remains to be determined whether these proteins are interchangeable or whether one family shows bias to a specific acceptor; however, one or both may act as electron donor

to glutamate synthase, which performs the final key step in inorganic nitrogen assimilation.

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