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 archaean Haloferax volcanii DS2 (DM3757), may assimilate inorganic nitrogen from nitrate or nitrite, using a ferredoxin-dependent assimilatory NO3\(^-\)/NO2\(^-\) 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 (NH4\(^+\)) and nitrate (NO3\(^-\)). 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 NH4\(^+\) is limited, many organisms, including plants, fungi and bacteria, may express assimilatory NO3\(^-\) reductases to assimilate inorganic nitrogen from NO3\(^-\) or NO2\(^-\). The biochemical basis for this is NAS, a cytoplasmic NO3\(^-\)/NO2\(^-\) reductase system that can show a high degree of plasticity between different organisms, but is predominantly a molybdoenzyme 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 NO3\(^-\) or NO2\(^-\) via an assimilatory NO3\(^-\)/NO2\(^-\) 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\(^\gamma\)Cys)\(^4\)-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\(^\gamma\)Cys)\(^2\)(N\(^\delta\)His)\(^2\)-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 haloharcea 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\(^\gamma\)Cys)\(^2\)-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 (Sepharose CL–4B followed by Q-Sepharose) and

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Abbreviations used: Nar-Fd, ferredoxin from Halobacterium mediterranei; Hv-Fd, ferredoxin from Haloferax volcanii.
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 M$^{-1}$·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 ($E^0' = -0.4$ V [9]), a rhombic lineshape from a dominant paramagnetic species was observed (Figure 2A), consistent with the presence of a [2Fe–2S]$^{1+} (S = \frac{1}{2})$ site. Further addition of europium(II) chloride or the stronger reducing agent europium(II) EGTA ($E^0' = -0.9$ V, prepared as described by Vincent and 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 ± 0.3 mW, confirming that all spectra presented were recorded under non-saturating conditions (Figure 2B). Simulation gave 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.

**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.

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

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

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 \( \mu \)M each: Methylene Blue, duroquinone, menadione, Indigo Carmine, 9,10-anthraquinone-2,6-disulfonic acid, anthraquinone-2-sulfonic acid, phenosafranine, safranine-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 \( \left( E_m \right) \) of \(-384 \pm 10\) 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]\( (S^\gamma Cys)_4 \) 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.

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...
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 MTEYTVEF and AGTAADDD respectively. The reason for the presence of two forms of [2Fe–2S](SγCys)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

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**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.

**A**

Haloferax volcanii
Halogemetricum borengense
Haloarcula salinarum
Halobacterium lacusprofundi
Metronomonas pharosinis
Haloarcula japonica
Haloarcula marismortui
Nostoc sp.
Anabaena sp.
Spinacia oleracea
Prochlorococcus marinus

Haloferax volcanii
Halogemetricum borengense
Haloarcula salinarum
Halobacterium lacusprofundi
Metronomonas pharosinis
Haloarcula japonica
Haloarcula marismortui
Nostoc sp.
Anabaena sp.
Spinacia oleracea
Prochlorococcus marinus

**B**

Haloferax mediterranei
Haloferax volcanii
Halogemetricum borengense
Metronomonas pharosinis
Haloarcula marismortui
Haloarcula salinarum
Haloferax mediterranei
Haloferax volcanii
Halogemetricum borengense
Metronomonas pharosinis
Haloarcula marismortui
Haloarcula salinarum
Haloferax mediterranei
Haloferax volcanii
Halogemetricum borengense
Metronomonas pharosinis
Haloarcula marismortui
Haloarcula salinarum
Haloferax mediterranei
Haloferax volcanii
Halogemetricum borengense
Metronomonas pharosinis
Haloarcula marismortui
Haloarcula salinarum

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

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**References**


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