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NMR studies of a ferredoxin from *Haloferax mediterranei* and its physiological role in nitrate assimilatory pathway

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Abstract

Haloferax mediterranei is a halophilic archaeon that can grow in aerobic conditions with nitrate as sole nitrogen source. The electron donor in the aerobic nitrate reduction to ammonium was a ferredoxin. This ferredoxin has been purified and characterised. Air-oxidized *H. mediterranei* ferredoxin has a UV–visible absorption spectra typical of 2Fe-type ferredoxins with an A_{420}/A_{280} of 0.21. The nuclear magnetic resonance (NMR) spectra of the ferredoxin showed similarity to those of ferredoxins from plant and bacteria, containing a [2Fe–2S] cluster. The physiological function of ferredoxin might be to serve as an electron donor for nitrate reduction to ammonium by assimilatory nitrate (EC 1.6.6.2) and nitrite reductases (EC 1.7.7.1). The apparent molecular weight (M_r) of the ferredoxin was estimated to be 21 kDa on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Keywords: Halophile; Archaea; Ferredoxin; Iron–sulfur cluster; Assimilatory nitrate pathway; Nuclear magnetic resonance

1. Introduction

The nitrate assimilatory pathway represents a fundamental biological process in most bacteria [1], yeast [2], cyanobacteria [3], fungi [4], algae [5] and higher plants [6]. This pathway is mediated by nitrate reductase (Nas, EC 1.6.6.2) and nitrite reductase (NiR, EC 1.7.7.1), which catalyse the stepwise reduction of nitrate to nitrite and nitrite to ammonia, respectively. Two classes of assimilatory nitrate and nitrite reductases are found in bacteria: NADH-dependent enzymes [7,8], and the ferredoxin- or flavodoxin-dependent enzymes [9]. In non-photosynthetic organisms electron transfer is mediated by NAD(P)H [10]. On the contrary, a ferredoxin (Fd, hereafter) is typically found as the physiological electron donor in photosynthetic organisms [10]. Moreover, Fds are also present in anaerobic nitrogen-fixing bacteria [8], anaerobic parasitic and free-living protozoa, and even in vertebrates [11].

Ferredoxins are iron–sulfur electron-transfer proteins of low molecular weight (around 12 kDa), which are versatile from both structural and functional points of view [12]. These proteins are involved in a large number of physiological events, such as in the regulation of gene expression [13], oxygen and iron sensing, generation and stabilization of radical intermediates [14], or in peptide metabolism [15]. However, the most important function of ferredoxins is electron transfer. In fact, they play the important role of carrying one electron from the photosynthetic electron transport chain to several metabolic pathways in cyanobacteria and plant chloroplasts [16]. In the reduced state, ferredoxin transfer electrons to a number of different enzymes such as nitrate reductase, nitrite reductase, thioredoxin reductase, sulfate reductase, and glutamate synthase [17].

Fds have been typically classified in bacterial- and plant-type. Bacterial-type Fds contains clusters with four or three iron ions, while plant-type ferredoxins only possess two iron ions (bridged by two sulfide atoms, Fe_2S_2) per molecule [11]. This kind of Fds are highly acidic, being the acidic

Abbreviations: Fd, ferredoxin; Fe–S, iron–sulfur cluster; MV, methyl viologen; Nas, assimilatory nitrate reductase; NiR, assimilatory nitrite reductase; NMR, nuclear magnetic resonance

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residues involved in the interaction of Fds with Fd-dependent enzymes [18]. Some ferredoxins isolated from non-plant sources, such as bacteria [19] and mitochondrial Fds also contain a 2Fe cluster. Halophilic archaea ferredoxins belong to the plant type [20,21], although differences between these two groups have been emphasised [22]. Ferredoxins from halophilic *Archaea* such as *Halobacterium halobium* [20], *Haloarcula marismortui* [23], and *Haloarcula japonica* [24] have been purified and characterised. All these Fds contain a [Fe_2S_2] cluster.

We report here the purification and characterisation of a ferredoxin from *Haloferax mediterranei* (*H. mediterranei*). We had previously suggested that this Fd participates in the assimilatory reduction of nitrate and nitrite by nitrate reductase (Nas) [25] and nitrite reductase (NiR) [26], respectively. Here, we present the kinetic analysis of the interaction between these proteins. This study has allowed us to determine the specific role of this Fd in the assimilatory nitrate reduction pathway.

2. Materials and methods

2.1. Protein purification

H. mediterranei culture growth and the purification of its Fd were performed as previously reported [20,25,26]. Assimilatory nitrate and nitrite reductase purification was carried out as described by the Martínez-Espinosa et al. [25,26].

2.2. Protein determination and enzymatic assays

The protein content was determined by the Bradford method. Nas and NiR activities were measured according to Martínez-Espinosa et al. [25,26], using the diazo coupling method. The salt concentration was 1 M NaCl for the Nas assay [25] and 3.2 M NaCl for the Nir assay [26], and the temperature was 60 °C for both. Nas and Nir activities were measured by using the methyl viologen (MV) or the ferredoxin from *H. mediterranei* as electron donors. The final concentration of Fd in assay mixtures was 200 nM. All the assays were carried out by triplicate and against a control assay without enzyme.

The apparent K_m for ferredoxin (assimilatory nitrate and nitrite reductases assays) was determined by a linear regression analysis of Lineweaver–Burk plots with the SigmaPlot program (Jandel Scientific, v.1.02). The concentration range of ferredoxin was from 13 up to 270 nM ($\varepsilon = 9600\ M^{-1}\ cm^{-1}$ at 420 nm, [22]).

2.3. Gel electrophoresis

The M_r of ferredoxin was estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% w/v) by using molecular mass markers from Promega.

2.4. Spectroscopic methods

Absorption UV–visible spectra were obtained on a Pharmacia Biotech ultrospec 2000 UV/Visible. The oxidized ferredoxin (in phosphate buffer 50 mM, containing 4.3 M NaCl at pH 7.5) was reduced with a few crystals of sodium dithionite.

Reduced samples for nuclear magnetic resonance (NMR) experiments were measured in D_2O solutions with phosphate buffer 20 mM and KCl 2 M at pH 7.2, 293 K. ^1H NMR spectra were performed on a Bruker Avance 400 spectrometer operating at 400.13 MHz. The superWEFT pulse sequence [27] was applied in order to observe the hyperfine shifted signals. The recycle rates typically were ca. 20 s^{-1}, with 32 000 Hz of spectral window.

3. Results and discussion

3.1. Isolation

H. mediterranei Fd was purified under aerobic conditions as described by Kerscher et al. [20]. The protein was easily recognised along the purification process by its dark brown colour. The ratio A_{420}/A_{275} of the purified final sample was 0.21. A similar ratio has been found in other halophilic ferredoxins [20,22,24]. The apparent molecular weight (M_r) of the ferredoxin, determined by SDS-PAGE, was 21 kDa. This method is known to overestimate the molecular weight of halophilic proteins, owing to the presence of an excess of acidic amino acids [28,29]. The archaeon ferredoxin from *H. japonica* [24] with a larger molecular weight has been purified. In this case, the authors propose that this Fd is a dimer in aqueous solution.

3.2. Spectroscopic studies

The optical absorption spectrum (Fig. 1) of the oxidized ferredoxin from *H. mediterranei* shows the absorption maxima at 275, 325, 420 and 465 nm. Upon reduction with sodium dithionite, the absorption decreased by 44% at 420 nm and 57% at 465 nm. Plant, adrenodoxin and other halobacteria-type ferredoxins present similar spectra [20,23,24,30,31]. Thus, the structure of the polypeptide chains surrounding the chromophore in the ferredoxins from all these organisms is quite conserved. On the contrary, bacterial ferredoxins typically show a broad absorption band centered at ca. 400 nm [32]. This broad absorption band also appear in the UV–visible spectrum of the [Fe_4S_4]-ferredoxin from different hyperthermophilic archaeon organisms [33]. The present results clearly suggests that *H. mediterranei* Fd is a 2Fe Fd.

Fig. 2 shows the superWEFT ^1H NMR spectrum of the reduced ferredoxin from *H. mediterranei* in D_2O. Thirteen hyperfine shifted signals can be clearly identify. Nine of them (called *a*–*i*) are downfield shifted, while the other four

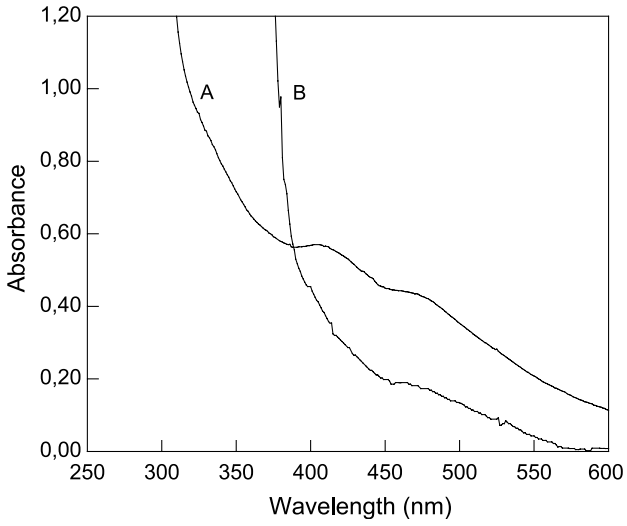

Fig. 1. (A) Absolute spectra of purified ferredoxin (30 μg of protein/ml) in 50 mM phosphate pH 7.5, containing 4.3 M NaCl. The oxidized spectrum was obtained first and the reduced by re-running the same sample after the addition of a few crystals of dithionite (B).

(signals *w*–*z*) display upfield contribution to their hyperfine shifts. It is well established that hyperfine shifts in iron–sulfur proteins are almost completely due to a contact or Fermi (through bonds) contribution. Hence, these signals necessarily belong either to the α or β protons of the coordinated cysteines or to protons belonging to residues forming hydrogen bonds with the sulfur atoms of the cluster (inorganic or cysteinil sulfur atoms). The ^{1}H NMR spectrum presented here is similar to those previously reported for plant-like ferredoxins, clearly indicating that this ferredoxin contains a two-iron [Fe_2S_2] cluster [34].

The temperature dependence of these signals is described in Table 1. Four of them (signals *a*, *b*, *c*, and *g*) display the so-called Curie pattern, i.e., the chemical shift decreases when increasing the temperature. The other four signals (*d*, *e*, *f*, and *h*) have the opposite behaviour (anti-Curie pattern). Signals *w*–*z* are classified in the pseudo-Curie group, i.e., the absolute values of their chemical shifts decreases with the inverse of the temperature, but they are upfield shifted. Finally, signal *i* displays a non-Curie behaviour, i.e., its chemical shift is not altered by temperature.

Table 1
Temperature dependence of the NMR spectra signals

Signal	Pattern	Temperature (24 °C)	Temperature (30 °C)
a	Curie	148.5	147.9
b	Curie	101.5	100.3
c	Curie	95.7	94.9
d	anti-Curie	59.9	61.6
e	anti-Curie	53.4	54.9
f	anti-Curie	49.9	51.1
g	Curie	45.5	45.2
h	anti-Curie	36.7	37.9
i	non-Curie	15.1	15.1
w	pseudo-Curie	− 1.65	− 1.48
x	pseudo-Curie	− 6.1	− 5.9
y	pseudo-Curie	− 18.5	− 18.3
z	pseudo-Curie	− 22.6	− 21.9

The causes of the temperature dependence of the hyperfine shifted signals in iron–sulfur proteins are very well established [34,35]. Concretely, in the reduced two-iron ferredoxins, the highest electronic spin (the ferric ion, S = 5/2) is aligned with the magnetic field. Thus, signals belonging to the cysteines coordinated to the iron(III) show the typical Curie-like behaviour. On the contrary, the smaller spin (S = 2) of the ferrous ion, antiferromagnetically coupled to the iron(III) ion, is aligned against the magnetic field. Proton signals belonging to residues attached through bonds to the iron(II) ion will experience either upfield shifts or anti-Curie behaviour with the temperature.

For the above discussion, it follows that signals *a*, *b*, *c* and *g* belong to cysteines coordinated to the iron(III) ion. The

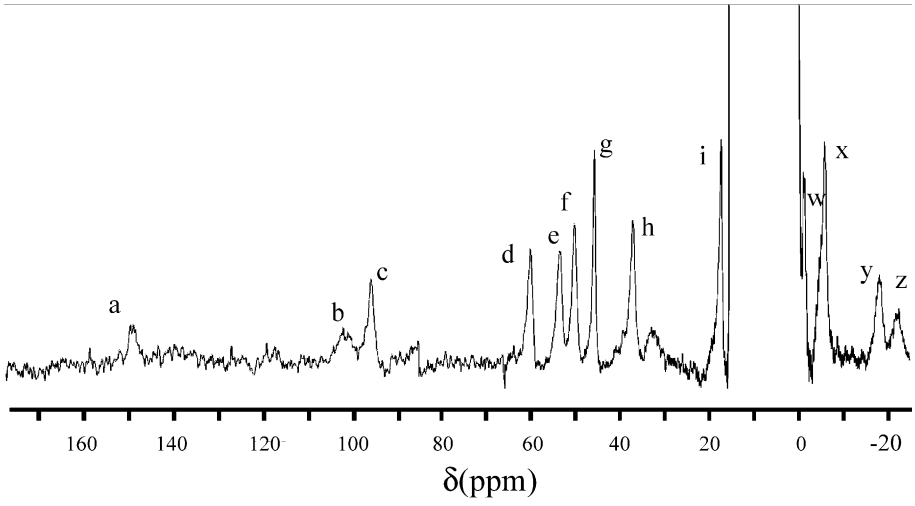

Fig. 2. SuperWEFT ^{1}H NMR (400 MHz) spectrum at 298 K of the reduced ferredoxin Fe_2S_2 (500 μM) from *H. mediterranei* in D_2O (phosphate buffer 20 mM, KCl 2M, pH 7.2).

signal *g* is sharper than the other three, and thus, corresponds to a proton found relatively far from the metal ion, i.e., to an α proton. Signals *a*, *b*, and *c*, more shifted and broader than signal *g*, should correspond to three of the four β protons of the two cysteines coordinated to the ferric ion. Signals *d*, *e*, *f*, and *h* (with anti-Curie behaviour, Table 1) and signal *i* (non-Curie), correspond to protons belonging to the cysteines coordinated to the iron(II) ion. According to their chemical shifts and line widths, it is easily deducible that signal *i* corresponds to an α proton, while the others are cysteine β protons. Signals *w*–*z* are due to protons belonging to residues hydrogen bonded to sulfur atoms, in turn, bound to the iron(II) ion, i.e., they are amide protons. Since this spectrum has been recorded in D_2O, the fact that these protons are observed with intensity close to one strongly suggests that iron(II) is completely buried to the solvent, even more than in other 2Fe Fds. It has been suggested that halophilic proteins at high salt concentrations are stabilized by the formation of a hydrated salt ion network coordinated by the acidic groups of the protein surface [36]. This network would make water molecules less labile and hence, water exchange would be more difficult to take place. Our findings are consistent with this hypothesis. In fact, water exchange in this Fd (and maybe in halophilic proteins in general) is much slower than in Fd from other mesophilic organisms.

3.3. Physiological role in the assimilatory nitrate pathway

We had previously demonstrated that reduced ferredoxin from *H. mediterranei* was almost as efficient as MV as an electron donor for the reduction of nitrate and nitrite by Nas [25] and NiR [26], respectively. In order to check that this pattern is physiologically relevant, we have carried out an enzymatic analysis of these reactions with *H. mediterranei* Fd. The apparent K_m values for *H. mediterranei* Fd were 44 ± 14 and 32 ± 11 nM for the Nas and Nir assays, respectively. When an artificial electron donor as MV was used, the K_m values obtained were much higher: 1.0 ± 0.2 and 1.9 ± 0.2 mM in the Nas and NiR assays [25,26], respectively.

Nas and NiR from different organisms are NAD(P)H- or ferredoxin-dependent [8,37]. We have already shown that these enzymes in *H. mediterranei* are not able of use the reduced nucleotidic coenzymes in the assimilatory pathway of nitrate. The very low values of K_m ferredoxin in *H. mediterranei* indicate that this protein is the natural electron donor in this metabolic pathway.

When the dithionite was the electron donor in the ferredoxin reduction, the efficacy in the Nas and NiR assays were 100% in comparison with the assay with MV [25,26]. However, if MV and ferredoxin from *H. mediterranei* were present in the assay medium, the efficacy in the reduction of nitrate and nitrite was around 280%, which may suggest that MV could act as an electron transfer bridge between dithionite and Fd, as previously reported for the spinach GOGAT [38] and *Monoraphidium braunii* GOGAT [39].

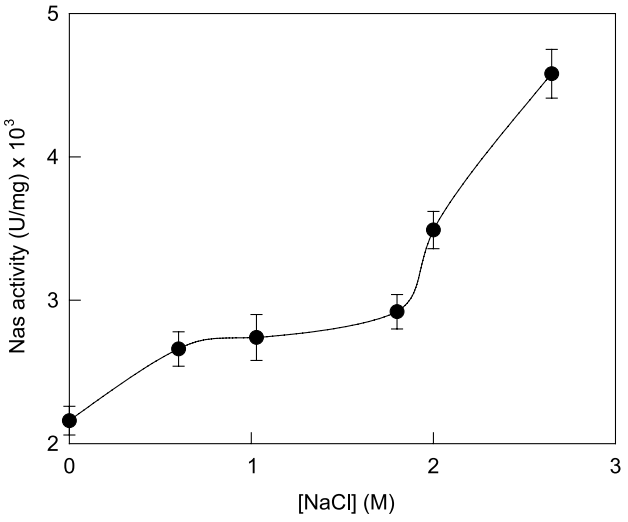

Fig. 3. Effect of salt concentration on Nas activity from *H. mediterranei* using ferredoxin as electron donor at different NaCl concentrations. The temperature was 60 °C.

The activities of extreme halophilic enzymes show a strong dependence on ionic strength: when the ionic strength increased, the enzymatic activities also increased [36]. Nas and Nir from *H. mediterranei* displayed this dependence when MV was used as an electron donor [25,26]. As it is displayed in Fig. 3, the Nas from *H. mediterranei* showed this typical halophilic behaviour. The formation of electron transfer complex ferredoxin–Nas was more effective at high ionic strength, increasing the enzymatic nitrate reduction (Fig. 3). In the same way, this reaction was carried out at 60 °C, indicating that this ferredoxin–Nas complex was stable in the assay conditions.

In conclusion, the present results suggest that ferredoxin from *H. mediterranei* is a 2Fe–2S protein. We propose that this ferredoxin is the electron carrier in the assimilatory nitrate pathway and the formation of the ferredoxin–enzyme complex would be driven by the high ionic strength of the medium. This study sheds light on the physiological roles played by ferredoxin in *Archaea*.

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