FEBS Letters 356 (1994) 342–344

FEBS 14926

Characterization and determination of the redox properties of the 2[4Fe-4S] ferredoxin from *Methanosarcina barkeri* strain MS

Piet J.H. Daas[a,*], Wilfred R. Hagen[b], Jan T. Keltjens[a], Godfried D. Vogels[a]

[a]*Department of Microbiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, NL-6525 ED, Nijmegen, The Netherlands*
[b]*Department of Biochemistry, Agricultural University, Wageningen, The Netherlands*

Received 13 October 1994; revised version received 16 November 1994

Abstract Ferredoxin was purified from methanol-grown *Methanosarcina barkeri* strain MS. It was isolated as a dimer with a subunit molecular weight of 6,200. The protein contained 7.4 mol iron and 7.2 mol acid-labile sulfur per monomer. In the reduced state the ferredoxin exhibited an EPR spectrum characteristic of two spin-coupled $[4Fe\text{-}4S]^{1+}$ clusters. The E_m of the $[4Fe\text{-}4S]^{2+,1+}$ couple was −322 mV ± 3 mV vs. NHE at 21°C and pH 7.0. The midpoint potential was temperature but not pH dependent. At the physiological temperature of 37°C the E_m was −340 mV.

Key words: Ferredoxin; Iron-sulfur cluster; Cyclic voltammetry; Redox potential; *Methanosarcina barkeri*

1. Introduction

Ferredoxins are small, acidic proteins involved in the electron transport in a wide variety of redox reactions. The proteins typically contain non-heme iron and acid-labile sulfur coordinated by cysteines. Ferredoxins have been isolated from many organisms including methanogenic bacteria [1,2].

Ferredoxins have been purified from various *Methanosarcina* species, including *M. barkeri* strain MS [3,4], *M. barkeri* strain Fusaro [5], and *M. thermophila* [6]. The ferredoxin of *M. barkeri* strain MS is composed of 59 amino acids with a molecular mass of 6,000. It has a 41% sequence homology with the 2[4Fe-4S] ferredoxin from *Clostridium pasteurianum* [7]. The eight cysteine residues involved in cluster formation in the *C. pasteurianum* ferredoxin are completely conserved in the ferredoxin from M. barkeri [1,7]. However, as isolated the latter protein was found to contain a 3Fe cluster [3]. The ferredoxin from M. barkeri strain Fusaro has a molecular mass of 6,100. The protein contains 7 iron and 7–8 acid-labile sulfur atoms per molecule indicative of the presence of 2[4Fe-4S] clusters [5]. The ferredoxin gene from M. thermophila has been sequenced and encodes a 6,200 molecular weight protein with an eight cysteine-spacing typical for a 2[4Fe-4S] protein [8]. Resonance Raman and electronic paramagnetic resonance (EPR) spectroscopy studies demonstrated that the protein indeed contained two [4Fe-4S] clusters per monomer [9].

From the above it is clear that there is a notable difference between the amount of iron and sulfur expected on the basis of the amino acid sequence, and the iron-sulfur content present in the as-isolated ferredoxin from *M. barkeri* strain MS [3]. In this communication we demonstrate that the ferredoxin from *M. barkeri* strain MS can be purified as a 2[4Fe-4S] protein. Its redox properties were determined.

2. Materials and methods

2.1. Organism

Methanosarcina barkeri strain MS (DSM 800) was grown in a mineral medium with methanol as a substrate. Cell-free extract was prepared in 50 mM TES/K^+ buffer (pH 7.0) containing 15 mM $MgCl_2$ and 1 mM dithiothreitol and stored at −70°C under H_2 [10].

2.2. Purification of ferredoxin

Because of the oxygen sensitivity of ferredoxin, all handlings were performed under anaerobic conditions [10]. Cell-free extract (10 ml) was applied to a DEAE-Sepharose-Cl-6B column (12 by 2.8 cm) equilibrated in 50 mM TES/K^+ buffer (pH 7.0) containing 15 mM $MgCl_2$ and 1 mM dithiothreitol. Bound proteins were eluted with a 400-ml linear gradient of 0 to 0.6 M NH_4Cl as described before [10]. The ferredoxin-containing fractions, eluting between 0.50 and 0.56 M NH_4Cl, were concentrated by Amicon YM-3 ultrafiltration to a final volume of 1 ml. Ethylene glycol was added as a stabilizing agent to a final concentration of 10%. Ferredoxin was further purified by gel filtration on Superose 6 (30 by 1 cm) equilibrated in 50 mM TES/K^+ buffer (pH 7.0) containing 15 mM $MgCl_2$, 1 mM dithiothreitol, 150 mM NH_4Cl, and 10% ethylene glycol. Ferredoxin fractions that were judged to be pure according to native PAGE were concentrated by Amicon UM-05 ultrafiltration. Ferredoxin was stored at −20°C under H_2 in 10 mM TES/K^+ (pH 7.0) buffer containing 65 mM NH_4Cl and 10% ethylene glycol.

2.3. Spectroscopy

EPR spectroscopy was carried out on a Bruker 200 D spectrometer equipped with cryogenics, peripheral equipment and data-acquisition/manipulation facilities as described previously [11]. Ultraviolet-visible absorption spectra were measured at room temperature using a Hitachi U-3200 spectrophotometer.

2.4. Electrochemistry

Direct electrochemistry (cyclic voltammetry) of 12 μl amounts of ferredoxin (0.87 mg/ml) was performed under argon atmosphere with the nitric acid treated glassy carbon electrode and the micro-apparatus and methodology described before [12]. The response of the reduction potential as a function of the temperature was determined by immersing the electrochemical cell in a thermostatted water bath. For the determination of the pH dependence, the protein stock solution was diluted 1 : 3 into 100 mM buffer mixtures (pH 6–10) of the desired pH.

2.5. Other procedures

Ferredoxin activity was assayed as described by Van der Meijden et al. [4]. Protein concentrations were determined by means of amino acid analysis. Ferredoxin was hydrolyzed in vacuo with 5.7 N HCl at 105°C for 24 h. Amino acids were analyzed after derivatization with 9-fluorenylmethyl chloroformate on a Lichrosorb RP-8 column with L-norleucine as an internal standard [13]. Total iron and acid-labile sulfur were determined as described by Fish [14] and Rabinowitz [15], respectively. Native and denaturing SDS-PAGE and isoelectric focusing were performed with pre-fabricated minigels using the Pharmacia PhastSystem equipment. The gels were fixed with 20% trichloroacetic acid and stained with Coomassie brilliant blue R-350. The native molecular

*Corresponding author. Fax: (31) (80) 553450.

SSDI 0014-5793(94)01313-6

weight was determined by gel filtration on Sephadex-G50 (120 by 2.5 cm) equilibrated in 10 mM TES/K^+ (pH 7.0) containing 100 mM NH_4Cl and 1 mM dithiothreitol.

3. Results and discussion

3.1. Physical and chemical properties

Ferredoxin was anaerobically purified from *M. barkeri* strain MS by a factor of 116 with a recovery of 16%. The native molecular weight, estimated by gel filtration chromatography, was 12,000. From SDS-PAGE a subunit molecular weight of 6,200 was measured. This suggested that ferredoxin was isolated as a dimer. Determination of the total iron and acid-labile sulfur content showed the presence of 7.4 Fe/mol and 7.2 S/mol of monomer, respectively. From isoelectric focusing a pI of 4.6 was obtained.

3.2. Spectral properties

The ultraviolet-visible absorption spectrum of the as-isolated oxidized ferredoxin showed absorption maxima at 276 and 384 nm, and A_{384}/A_{276} of 0.84. After addition of dithionite the absorption at 384 nm decreased as the result of reduction of the iron-sulfur clusters. From the absorption spectrum of the as-isolated oxidized ferredoxin a molar extinction coefficient of ε_{384} = 28,700 $M^{-1} \cdot cm^{-1}$ per monomer (ε_{384} = 3,900 $M^{-1} \cdot cm^{-1}$ per iron atom) could be calculated. This type of spectrum and extinction coefficient per iron atom is typical of iron-sulfur proteins containing $[4Fe\text{-}4S]^{2+}$ centers [16].

The results described above suggested that the ferredoxin was isolated with two [4Fe-4S] clusters per monomer. Therefore, the protein was studied by EPR in two oxidation states. In the oxidized state no signal was observed. After reduction with dithionite a signal with g values at 2.03, 1.99, 1.96, 1.92, and 1.88 appeared (Fig. 1). The complex spectrum had the characteristics of a signal resulting from the spin-spin interaction of two paramagnetic $[4Fe\text{-}4S]^{1+}$ clusters [17,18], and it was nearly identical to the one reported for the *M. thermophila* ferredoxin [9]. Except for the absence of a broad lateral line at g = 2.12, the signal was similar to that reported for the fully reduced *C. pasteurianum* ferredoxin [18,19]. The lateral line was also absent in the spectrum of the *M. thermophila* ferredoxin [9], which has a 92% sequence identity to the protein described here [8]. From these results we concluded that the ferredoxin of *M. barkeri* was isolated with 2[4Fe-4S] clusters per subunit.

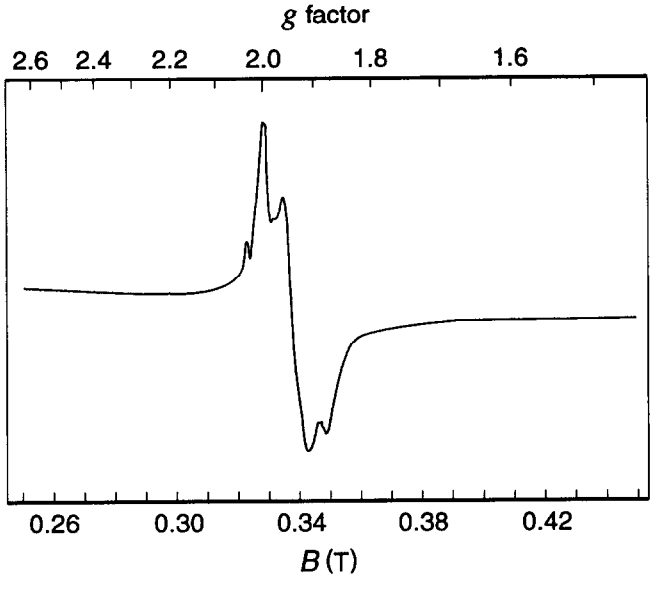

Fig. 1. EPR-spectrum of the reduced *M. barkeri* ferredoxin. The protein (0.66 mg/ml) was reduced with 2 mM dithionite and frozen in liquid nitrogen. EPR conditions: microwave frequency, 9.1799 GHz; modulation frequency, 100 kHz; modulation amplitude, 2 mT; microwave power, 3.2 mW; temperature, 13 K.

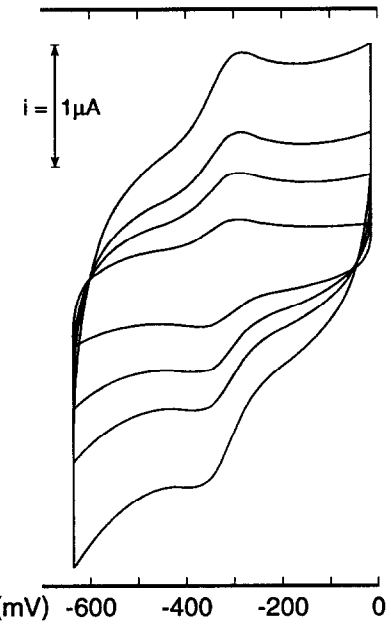

Fig. 2. Cyclic voltammograms of ferredoxin from *M. barkeri*. Conditions: ferredoxin (12 μl, 0.87 mg/ml) in 10 mM TES/K^+ (pH 7.0) containing 65 mM NH_4Cl, 10% ethylene glycol, and 3.3 mM neomycin; potential scan rates, from the inside outward, 10, 25, 40, and 75 mV/s, respectively; temperature, 21°C. The potential axis is defined versus the normal hydrogen electrode.

3.3. Reversible electrochemistry

Ferredoxin showed a direct response at the glassy carbon electrode in the absence of promotors or mediators. However, in the presence of neomycin (3.3 mM), the signal became more stable and fully reversible. Representative voltammograms are shown in Fig. 2. At low scan rates (typically 10 mV/s) the n = 1 electron transfer is reversible, as judged from the reproducible cathodic-to-anodic peak potential separation of 57 mV ± 5 mV. At ambient temperatures and at neutral pH, no significant deterioration of this response was observed over a period of one hour. The reduction potential (E_m) was −322 mV ± 3 mV at 21°C and pH 7.0 versus the normal hydrogen electrode (NHE). From Fig. 2 it is also clear that the midpoint redox potentials of the individual [4Fe-4S] clusters do not differ significantly, since only one symmetrical wave is observed.

The response of the reduction potential was also measured as a function of the temperature. At all temperatures measured between 2°C and 50°C, the peak-to-peak distance was 58 mV ± 6 mV for a scan rate of 10 mV/s. For temperatures above 50°C reproducibility was not warranted due to desiccation of the sample under the argon flow. At temperatures up to 18°C the midpoint reduction potential decreased linearly with a slope

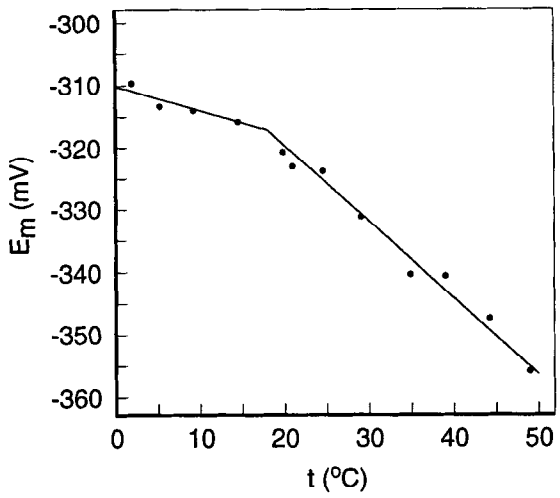

Fig. 3. Temperature dependence of the midpoint redox potential of the ferredoxin from *M. barkeri*. The midpoint redox potentials were obtained from voltammograms at a scan rate of 10 mV/s. Except for the temperature all other conditions were as described in Fig. 2.

of −0.39 mV/°C (Fig. 3). Hereabove, a linear decrease of −1.22 mV/°C was observed. This effect is probably the result of a change in protein conformation at 18°C which influences the immediate vicinity of the iron-sulfur clusters [16]. Ferredoxin showed a midpoint potential of −340 mV versus NHE at the physiological growth temperature of 37°C [20]. The E_m was pH independent between pH 6–10 which indicates that the electron transfer mechanism does not involve proton exchange. Grahame [21], who isolated a ferredoxin from acetate-grown *M. barkeri*, measured a significantly lower E_m of −420 mV at pH 8.0. This ferredoxin may be different from the one discussed here that was obtained from methanol-grown cells.

In conclusion, we have demonstrated that the ferredoxin from methanol-grown *M. barkeri* strain MS contains 2[4Fe-4S] clusters per monomer, which accords the eight cysteine-spacing in the amino acid sequence [1,7]. The previously reported presence of a 3Fe cluster [3] could be an isolation artifact. Degradation of [4Fe-4S] clusters was also found for the ferredoxin of *M. thermophila* [8,9].

Acknowledgements: We would like to thank Rien van de Gaag for performing the amino acid analysis. Johan Baars and Johnny Visscher are acknowledged for their valuable suggestions and assistance in the protein hydrolyzation.

References

[1] Bruschi, M. and Guerlesquin, F. (1988) FEMS Microbiol. Rev. 54, 155–176.
[2] Bruschi, M., Bonicel, J., Hatchikian, E.C., Fardeau, M.L., Belaich, J.P. and Frey, M. (1991) Biochim. Biophys. Acta 1076, 79–85.
[3] Moura, I., Moura, J.J.G., Huynh, B.H., Santos, H., LeGall, J. and Xavier, A.V. (1982) Eur. J. Biochem. 126, 95–98.
[4] Van der Meijden, P., van der Lest, C., van der Drift, C. and Vogels, G.D. (1984) Biochem. Biophys. Res. Commun. 118, 760–766.
[5] Hatchikian, E.C., Brischi, M., Forget, N. and Scandellari, M. (1982) Biochem. Biophys. Res. Commun. 109, 1316–1323.
[6] Terlesky, K.C. and Ferry, J.G. (1988) J. Biol. Chem. 263, 4080–4082.
[7] Hausinger, R.P., Moura, I., Moura, J.J.G., Xavier, A.V., Santos, M.H., LeGall, J. and Howard, J.B. (1982) J. Biol. Chem. 257, 14192–14197.
[8] Clements, A.P. and Ferry, J.G. (1992) J. Bacteriol. 174, 5244–5250.
[9] Clements, A.P., Killpatrick, L., Lu, W.-P., Ragsdale, S.W. and Ferry, J.G. (1994) J. Bacteriol. 176, 2689–2693.
[10] Daas, P.J.H., Gerrits, K.A.A., Keltjens, J.T., van der Drift, C. and Vogels, G.D. (1993) J. Bacteriol. 175, 1278–1283.
[11] Pierik, A.J. and Hagen, W.R. (1991) Eur. J. Biochem. 195, 505–516.
[12] Hagen, W.R. (1989) Eur. J. Biochem. 182, 523–530.
[13] Einarsson, S., Josefsson, B. and Lagerkvist, S. (1983) J. Chromatogr. 282, 609–618.
[14] Fish, W.W. (1988) in: Methods Enzymol. 158 (Riordan, J.F. and Vallee, B.L. Eds.) pp. 357–364, Academic Press, San Diego, London.
[15] Rabinowitz, J.C. (1978) in: Methods Enzymol. 53 (Fleischer, S. and Packer, L. Eds.) pp. 275–277, Academic Press, San Diego, London.
[16] Sweeney, W.V. and Rabinowitz, J.C. (1980) Annu. Rev. Biochem. 49, 139–161.
[17] Cammack, R. and Cooper, C.E. (1993) in: Methods Enzymol. 227 (Riordan, J.F. and Vallee, B.L. Eds.) pp. 353–384, Academic Press, San Diego, London.
[18] Mathews, R., Charlton, S., Sands, R.H. and Palmer, G. (1974) J. Biol. Chem. 249, 4326–4328.
[19] Prince, R.C. and Adams, M.W.W. (1987) J. Biol. Chem. 262, 5125–5128.
[20] Keltjens, J.T. and Vogels, G.D. (1993) in: Methanogenesis, Ecology, Physiology, Biochemistry and Genetics (Ferry, J.G. Ed.) pp. 253–303, Chapman and Hall, New York, London.
[21] Grahame, D.A. (1991) J. Biol. Chem. 266, 22227–22233.