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Isolation and characterization of a rubredoxin and a two-(4Fe-4S) ferredoxin from *Thermodesulfobacterium commune*

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Two non-heme iron proteins, ferredoxin and rubredoxin, have been isolated from the thermophilic non-sporeforming sulfate-reducer *Thermodesulfobacterium commune*. In most respects, the rubredoxin is similar to the corresponding mesophile proteins from other anaerobic bacteria but it differs by its higher content of proline residues. Native ferredoxin from *T. commune* is a dimer comprising two identical subunits of approx. 7000 molecular weight. Its absorption spectrum exhibits two maxima at 385 nm (29500 $M^{-1} \cdot cm^{-1}$) and 280 nm (36100 $M^{-1} \cdot cm^{-1}$) and its A_{385}/A_{280} absorbance ratio is 0.82. The protein contains 8 atoms each of iron and labile sulfur per molecule. The absorption at 385 nm and the content of iron of the protein are in agreement with the presence of two (4Fe-4S) clusters in *T. commune* ferredoxin. Its amino acid composition shows the presence of six cysteine residues and is characterized by the absence of histidine and a high content of aromatic residues. The N-terminal amino acid sequence of *T. commune* ferredoxin has been established. The comparison of amino acid sequences shows that it presents more homology with the one-(4Fe-4S) ferredoxin from *Clostridium thermoaceticum* than with the two-(4Fe-4S) ferredoxin from *Desulfovibrio desulfuricans* Norway. *T. commune* ferredoxin shows thermal stability and retains its full activity in the phosphoroclastic reaction after treatment at 70°C. The protein exhibits sensitivity towards oxygen at 25°C.

Introduction

A new thermophilic sulfate-reducing bacterium, designed *Thermodesulfobacterium commune,* was recently isolated from volcanic hot springs environments [1]. This rod-shaped, non-sporulating extreme thermophilic organism (optimum 70°C) differs from all bacteria described so far because it contains non-isopranoid branched diethers and monoethers [2]. Although several cellular and biochemical properties suggest that *T. commune* is a unique sulfate-reducing species [1,3], it contains a typical cytochrome c_3 [4] which is characteristic of the genus *Desulfovibrio* [5,6].

Ferredoxin and rubredoxin are low-molecular-weight electron-carrier proteins which have been utilized for the establishment of bacterial phylogenetic relationships [7]. Ferredoxins from *Desulfovibrio* species generally differ from clostridial type ferredoxins in that they contain one (4Fe-4S) cluster instead of two clusters [8–11]. However, some species have been found recently to contain in addition a two-(4Fe-4S) ferredoxin [11,12]. Although these iron-sulfur proteins are homologous proteins they show significant differences in their primary structure [11,13]. The various ferredoxins present in sulfate-reducing bacteria are electron carriers for low-potential oxidation-reduction reac-

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tions, including pyruvate: ferredoxin oxidoreductase and hydrogen sulfite reductase activity [9,12,14], whereas the physiological function of rubredoxin, which exhibits a redox potential approx. 400 mV higher than that of ferredoxin, remains unknown [15].

Our knowledge of the biochemistry of the electron-carrier proteins of thermophilic sulfate-reducing bacteria is very limited. It was thus of taxonomic and biochemical interest to compare the electron carriers of *T. commune* with those of various species of *desulfovibrio* and thermophilic *Clostridia*. In this paper, the isolation, characterization and some molecular properties of ferredoxin and rubredoxin of the thermophilic sulfate-reducer *T. commune* are described.

Materials and Methods

T. commune type strain YSRA-1 (DSM 2178, ATCC 33708) was grown at 65°C on a lactate-sulfate medium as previously described [4].

Initial purification procedure

Unless otherwise noted, all buffers were at pH 7.6 and all operations were performed at 4°C. Precautions were taken against oxygen by flushing the buffers and the columns with purified argon and maintaining all fractions under the same atmosphere.

Freshly thawed cells of *T. commune* (350 g wet weight) were suspended in 200 ml of 20 mM Tris-HCl buffer containing 100 μM DNAase and passed through a French pressure cell at 100 MPa. The extract was centrifuged at 100 000 $\times$ *g* for 1.5 h in a Beckman model L3 ultracentrifuge and the pellet was discarded. A settled volume of DEAE-cellulose, equal to 60 ml, was added to the supernatant corresponding to the soluble extract (300 ml) and the mixture was stirred for 4 h. The suspension of DEAE-cellulose was then poured into a column (4 $\times$ 12 cm) and the adsorbed acidic proteins were eluted as follows: a protein fraction containing APS reductase and the major part of bisulfite reductase was eluted with 210 mM Tris-HCl buffer; the more acidic fraction containing ferredoxin and rubredoxin was subsequently eluted with 520 mM Tris-HCl buffer.

This acidic fraction (300 ml) was loaded onto a DEAE-cellulose column (3 $\times$ 20 cm) equilibrated with 10 mM Tris-HCl buffer. The adsorbed proteins were eluted stepwise using a Tris-HCl buffer gradient (750 ml) from 100 to 450 mM. The rubredoxin and the ferredoxin fractions were eluted with 300 and 450 mM Tris-HCl buffer, respectively.

Purification of ferredoxin

The ferredoxin-containing fraction (90 ml) eluted from the second DEAE-cellulose column was diluted to 150 ml with distilled water previously flushed with argon, and applied to a DEAE-Sephadex A-50 column (4 $\times$ 13 cm) previously equilibrated with 300 mM Tris-HCl buffer. Ferredoxin was then eluted from the DEAE-Sephadex column and subsequently purified on a calcinated alumina column as previously reported for homologous proteins [11,12]. At this stage the protein was judged to be pure both by polyacrylamide gel electrophoresis and from its amino acid composition. The A_{385}/A_{280} absorbance ratio was 0.82 and the yield was 27 mg.

When *T. commune* ferredoxin is isolated in the absence of strictly anaerobic conditions, specially when dialysis is utilized through the purification procedure, three major bands of ferredoxin are separated on the DEAE-cellulose column instead of a single ferredoxin band. These ferredoxin bands have been proved to be different oligomeric forms of the same subunit which are apparently induced by oxidative modification of the native protein.

Purification of rubredoxin

The rubredoxin-containing fraction eluted from the second DEAE-cellulose column was dialyzed overnight against 10 mM tris-HCl and applied to a DEAE-cellulose column (3 $\times$ 10 cm). Proteins were eluted with a discontinuous gradient from 100 to 340 mM Tris-HCl buffer, and the rubredoxin was eluted with 225 mM of the same buffer. The protein was subsequently purified as reported previously [14] using successively chromatography on silica gel, calcinated alumina and DEAE-cellulose. The protein was judged to be pure from its spectrum ($A_{280}/A_{493} = 2.23$), amino acid composition and polyacrylamide gel electrophoresis. The yield was 6 mg.

Analytical procedures

The molecular weights of the isolated proteins were estimated by gel filtration on a Sephadex G-50 column according to the method of Whitaker [16], using the following molecular weight standards: horse heart cytochrome *c* (12500), soybean trypsin inhibitor (20100), α-chymotrypsinogen (25000) and ovalbumin (43000). The molecular weight of the ferredoxin was also estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using the procedure of Weber and Osborn [17]. Analytical gel electrophoresis was performed according to the method of Davis [18] on 7% polyacrylamide gel at pH 8.8.

Absorption spectra were measured on a Cary 219 spectrophotometer. Molar extinction coefficients of the proteins were obtained by measuring the values of the absorbances of their absorption maxima using a solution of known protein concentration calculated from amino acid analysis.

Iron was determined by atomic absorption spectrometry using a Unicam model SP1900 spectrometer. Inorganic sulfide was estimated by the method of Fogo and Popowski [19] as modified by Lovenberg et al. [20]. In the extracts, protein was determined according to the procedure of Lowry et al. [21].

Amino acid analysis were performed on an LKB 4150 amino acid analyzer. Ferredoxin and rubredoxin samples were hydrolyzed for 24 and 48 h in 6 M HCl at 110°C in evacuated, sealed tubes by the method of Moore and Stein [22]. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs [23].

Sequence determination

Sequence determination was performed on a Socosi Protein Sequencer (P.S. 100) using 0.3 M Quadrol buffer. The degradation was carried out on 200 nmol of the apoferredoxin obtained by precipitating native ferredoxin with 3% HCl at 80°C for 10 min. The quantitative determination of the phenylthiohydantoin derivatives was carried out relative to known amounts of the appropriate standards by HPLC (high-pressure liquid chromatography, Waters) as described by Bonicel et al. [24].

Measurement of pyruvate dehydrogenase activity

Pyruvate dehydrogenase activity was determined by measuring the hydrogen produced from pyruvate using the manometric method. The main compartment of the Warburg flasks contained (in micromoles) the following components: potassium phosphate buffer (pH 7.0) (150), thiamine pyrophosphate (5), coenzyme A (4), $MgCl_2$ (20), 2-mercaptoethanol (25), ferredoxin-free extract (10.5 mg protein) and ferredoxin as indicated. Flasks were flushed with O_2-free argon for 10 min and allowed to equilibrate for 20 min. Reactions were started by tipping in pyruvate (30 μmol) from the side-arm. CO_2 was absorbed by an NaOH wick in the center well. All assays were performed at 45°C and the final volume was 3 ml. Additional assays were performed using samples of ferredoxin after heating at 60, 70, 75 and 80°C for 1 h.

Measurement of sulfite reductase activity

Sulfite reductase activity of the extracts was determined by measuring the hydrogen absorption in the presence of sulfite using the manometric method as previously described [25]. The main compartment of the Warburg flasks contained 150 μmol potassium phosphate buffer (pH 7.0), ferredoxin-free extract (17.5 mg protein) and ferredoxin as indicated. Flasks were flushed with H_2 for 10 min and allowed to equilibrate for an additional 10 min. Reactions were started by tipping in sulfite (4 μmol) from the side-arm. H_2S was absorbed by an NaOH wick in the center well. All assays were performed at 45°C and the final volume was 3 ml.

Measurement of NAD(P)H : rubredoxin oxidoreductase activity

NAD(P)H : rubredoxin oxidoreductase activity of the crude and soluble extracts was measured at 55°C according to the method of Petitdemange et al. [26].

Preparation of ferredoxin-free extract

The pyruvate dehydrogenase- and sulfite reductase-containing fractions free of ferredoxin were prepared according to the following procedure: the crude soluble extract from *T. commune* (15 ml, 39 mg/ml) obtained from 15 g (wet weight)

of cells was passed through a small DEAE-cellulose column (10 × 15 mm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.6). The non-adsorbed protein fraction (35 mg/ml) free of ferredoxin was used as the enzymatic extract.

Results

Molecular weight

Rubredoxin. The molecular weight of rubredoxin from *T. commune* estimated by gel filtration on a Sephadex G-50 column was 5800. This value is in good agreement with the minimum molecular weight of 5774 calculated from the amino acid composition, including one iron atom per molecule.

Ferredoxin. The molecular weight of native ferredoxin estimated by gel filtration was 12500. After incubation with 1% SDS followed by SDS-gel electrophoresis, the ferredoxin gave one band of protein of molecular weight approx. 6000. The minimal molecular weight calculated from the amino acid composition is 6970, including two (4Fe-4S) clusters per molecule. This suggests that *T. commune* native ferredoxin exists as a dimer comprising two identical monomeric units of molecular weight approx. 7000.

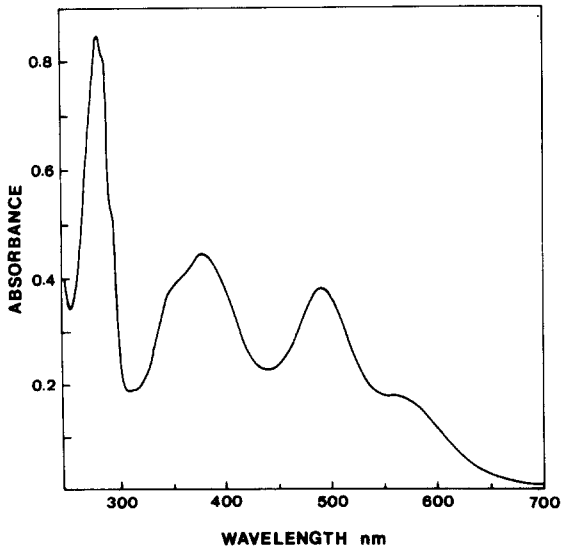

Fig. 1. Absorption spectrum of *T. commune* rubredoxin. The spectrum of rubredoxin, 51 μM in 10 mM Tris-HCl (pH 7.6), was measured with a Cary 219 spectrophotometer, using 1-cm light-path cuvettes.

Absorption spectra and extinction coefficients

Rubredoxin. The absorption spectrum of *T. commune* rubredoxin is shown in Fig. 1. It is similar to the spectra of rubredoxins from *Desulfovibrio* species [10,27,28] and exhibits absorption maxima at 493, 380 and 280 nm with molar extinction coefficients of 7480, 8720 and 16730 $M^{-1} \cdot cm^{-1}$, respectively (Table I). Shoulders occur at 284, 291, 353 and 560 nm. The absorbance ratio A_{280}/A_{493} is 2.23.

Ferredoxin. The absorption spectrum of *T. commune* ferredoxin is presented in Fig. 2. It shows a broad absorption band centered at 385 nm in the visible region of the spectrum and a peak at 280 nm in the ultraviolet region with a shoulder at 305 nm. Its absorbance ratio A_{385}/A_{280} is 0.82, and the molar extinction coefficient at 385 nm is 29500 $M^{-1} \cdot cm^{-1}$ (Table I).

Amino acid composition

Rubredoxin. The amino acid composition of rubredoxin from *T. commune* is listed in Table II and compared with those of five other homologous proteins from *Desulfovibrio* and *Clostridium* species. Acidic amino acids are preponderant and all the rubredoxins lack histidine and arginine residues. *T. commune* rubredoxin has 54 residues and, like the other rubredoxins, it contains four cysteine residues responsible for the maintenance of the rubredoxin-type cluster characterized by the absence of labile sulfur and the presence of one iron atom ligated in a presumed tetrahedral

TABLE I

MOLAR EXTINCTION COEFFICIENTS OF RUBREDOXIN AND FERREDOXIN FROM *T. COMMUNE*

Component	nm	$M^{-1} \cdot cm^{-1}$	Ratio
Rubredoxin	280	16730	$A_{280}/A_{493} = 2.23$
	353	7680	
	380	8720	
	493	7480	
Ferredoxin	280	36100	$A_{385}/A_{280} = 0.82$
	305	34550	
	385	29500	

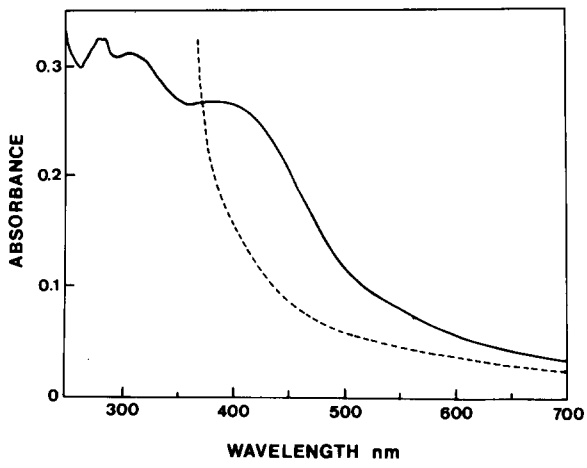

Fig. 2. Absorption spectrum of *T. commune* ferredoxin in oxidized and dithionite-reduced state. ———, oxidized protein spectrum; - - - - - -, dithionite-reduced protein spectrum. The spectrum was measured with a Cary 219 spectrophotometer. The cuvettes (1-cm light-path) contained 9 μM ferredoxin in 20 mM Tris-HCl (pH 7.6).

arrangement to the sulfur of cysteine residues. It differs from the other rubredoxins by its higher content of proline, a property shared only by rubredoxin 1 from *Clostridium thermoaceticum.*

Ferredoxin. Table III gives the amino acid composition of the *T. commune* ferredoxin and allows a comparison with those from some *Desulfovibrio* and *Clostridium* species. The protein has 56 residues and is characterized by the lack of histidine, as are the other ferredoxins except that of *C. thermoaceticum. T. commune* ferredoxin shows a low content of acidic amino acids and a high level of threonine. More importantly, our analyses show the presence of only six cysteine residues instead of eight required for the binding of the two (4Fe-4S) clusters. This could be due to an underestimation of the total number of cysteine residues using the performic acid oxidation method.

Iron and sulfur content

T. commune rubredoxin contains 1.1 ± 0.1 iron

TABLE II

AMINO ACID COMPOSITION OF *T. COMMUNE* RUBREDOXIN IN COMPARISON WITH THOSE FROM SOME *DESULFOVIBRIO* AND *CLOSTRIDIUM* SPECIES
N.D., not determined

Amino acids	*T. commune*	*D. africanus* [a]	*D. gigas* [b]	*D. vulgaris* [c]	*C. thermoaceticum* 1 [d]	*C. thermoaceticum* 2 [d]
Lys	4	4	5	4	2	4
His	0	0	0	0	0	0
Arg	0	0	0	0	0	0
Trp	N.D.	3	1	1	1	2
Asp	8	9	8	6	7	7
Thr	2	1	2	3	3	2
Ser	3	2	2	2	2	1
Glu	3	5	4	3	7	5
Pro	9	6	5	6	9	6
Gly	4	5	5	5	6	9
Ala	5	2	4	5	6	5
Cys	4	4	4	4	6	4
Val	3	6	3	5	3	2
Met	1	1	1	1	1	1
Ile	2	1	2	0	1	1
Leu	1	0	1	1	2	3
Tyr	3	3	3	3	3	2
Phe	2	2	2	2	2	2
Total residues	54	54	52	51	61	56

[a] From Hatchikian et al. [14].
[b] From Bruschi [51].
[c] From Bruschi [52].
[d] From Shiow-Shong Yang et al. [38].

TABLE III

AMINO ACID COMPOSITION OF *I. COMMUNE* FERREDOXIN IN COMPARISON WITH THOSE FROM SOME *DESULFOVIBRIO* AND *CLOSTRIDIUM* SPECIES
N.D., not determined

Amino acids	*T. commune*	*D. africanus* [a] Fd I	*D. desulfuricans* [b] Norway Fd II	*D. gigas* [c]	*C. thermoaceticum* [d]	*C. thermocellum* [e]
Lys	3	3	2	1	2	1
His	0	0	0	0	1	0
Arg	1	1	0	1	1	0
Trp	N.D.	N.D.	N.D.	N.D.	1	0
Asp	6	5	6	11	9	6
Thr	4	1	0	0	3	1
Ser	3	3	3	3	3	3
Glu	6	15	11	9	9	6
Pro	3	3	3	4	4	5
Gly	4	2	5	1	3	3
Ala	3	8	2	6	4	7
Cys	6	4	8	6	6	8
Val	6	6	10	6	8	6–7
Met	1	2	1	2	1	0
Ile	5	4	4	5	4	4
Leu	1	0	2	1	3	0
Tyr	2	2	2	0	0	2
Phe	2	2	0	1	1	2
Total residues	56	61	59	57	63	54–55

[a] From Bruschi et al. [45].
[b] From Guerlesquin et al. [13].
[c] From Bruschi [29].
[d] From Shiow-Shong Yang et al. [42].
[e] From Forget [53].

atom per molecule (average of three estimations). No acid-labile sulfide was detected.

Ferredoxin from *T. commune* exhibits a content of 8.1 ± 0.1 iron atoms and 7.2 ± 0.1 mol of acid-labile sulfide per monomer (average of two estimations), which is consistent with the presence of two (4Fe-4S) clusters.

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                          1                5                10               15
T. commune                Met-Phe-Glu-Ile-Thr-Val-Asn-Tyr-Asp-Thr-( )-Asp-Ala-( )-Gly-
D.d. Norway Fd II   Met-Gly-Tyr-Ser-Val-Ile-Val-Asp-Ser-Asp-Lys-Cys-Ile-Gly-Cys-Gly-
D. gigas                  Pro-Ile-Glu-Val-Asn-Asp-  -   -Asp-Cys-Met-Ala-Cys-Glu-
C. pasteurianum           Gly-Tyr-  -Lys-Ile-Ala-Asp-Ser-  -  -Cys-Val-Ser-Cys-Gly-
C. thermosaccharolyticum  Ala-His-  -Ile-Ile-Thr-Asp-  -Glu-  -Cys-Ile-Ser-Cys-Gly-
C. thermoaceticum         Met-Lys-  -Val-Thr-Val-Asp-Gln-Asp-Leu-Cys-Ile-Ala-Cys-Gly-

                                20               25               30
T. commune                Thr-( )-Ile-Glu-Ser-( )-Pro-Ala-Glu-Val-Tyr-Asp-Lys-Gly-Asp---
D.d. Norway Fd II         Glu-Cys-Val-Asp-Val-Cys-Pro-Val-Glu-Val-Tyr-Glu-Leu-Gln-Asn---
D. gigas                  Ala-Cys-Val-Glu-Ile-Cys-Pro-Asp-  -Val-Phe-Glu-Met-Asn-Glu---
C. pasteurianum           Ala-Cys-Ala-Ser-Glu-Cys-Pro-Val-Asn-Ala-Ile-Ser-  -  -  ---
C. thermosaccharolyticum  Ala-Cys-Ala-Ala-Glu-Cys-Pro-Val-Glu-Ala-Ile-His-Glu-Gly-  ---
C. thermoaceticum         Thr-Cys-Ile-Asp-Leu-Cys-Pro-Ser-  -Val-Phe-Asp-Trp-Asp-Asp---
```

Fig. 3. N-terminal amino acid sequence of *T. commune* ferredoxin in comparison with those from some *Desulfovibrio* and *Clostridium* species. Brackets indicate that the residues in these positions could not be identified. Deletions are juggled to improve the apparent match. Fd, ferredoxin.

N-terminal sequence of T. commune ferredoxin

The N-terminal sequence established by automatic Edman degradation on apoferredoxin was determined up to 30 residues, with some residues not identified (Fig. 3). We suggest that the positions 11, 14, 17 and 21 are occupied by cysteine residues from the comparison with the other known sequences of ferredoxins.

The N-terminal sequence of *T. commune* ferredoxin has been compared to those of the sulfate-reducing bacteria *D. desulfuricans* Norway [13] and *D. gigas* [29] as well as to those of the *Clostridium* species including *C. pasteurianum* [30], *C. thermosaccharolyticum* [31] and *C. thermoaceticum* [32]. When these sequences are aligned for maximum homology on the positions of cysteines (residues 11, 14, 17, 21) and proline (Pro-22) which are conserved in all ferredoxins (Fig. 3), 12 amino acids out of 30 are in identical positions in *T. commune* ferredoxin and *D. desulfuricans* Norway ferredoxin II, whereas only 8 out of 30 are conserved in *D. gigas*. The presence of two (4Fe-4S) clusters makes *T. commune* ferredoxin more similar to *D. desulfuricans* Norway ferredoxin II than to the one-(4Fe-4S)-cluster ferredoxins from various *Desulfovibrio* species [29,14,33]. In contrast to these data, when compared to the clostridial ferredoxins (Fig. 3), the primary structure of *T. commune* ferredoxin exhibits striking homology with the four-iron ferredoxin from *C. thermoaceticum*, whereas only poor homology is observed with the two clusters containing ferredoxins from *C. pasteurianum* and *C. thermosaccharolyticum*. 17 amino acids out of 30 are in identical positions in *T. commune* and *C. thermoaceticum* ferredoxins, instead of 8 and 6 out of 30 in *C. thermosaccharolyticum* and *C. pasteurianum*, respectively.

Coupling activity of ferredoxin in the phosphoroclastic reaction

Results on the coupling activity of ferredoxin between pyruvate dehydrogenase and hydrogenase are shown in Table IV. Compared with the endogenous activity of the control containing ferredoxin-free extract, the system containing ferredoxin exhibited significant stimulation of H_2 evolution from pyruvate. This implies that ferredoxin functions as electron carrier in the pyruvate phosphoroclastic reaction.

TABLE IV

EFFECT OF FERREDOXIN ON PYRUVATE DEHYDROGENASE AND SULFITE REDUCTASE ACTIVITIES OF FERREDOXIN-FREE EXTRACTS FROM *T. COMMUNE*

Enzymatic activities were determined as reported in Materials and Methods. The reaction mixture used in the pyruvate dehydrogenase assay contained crude extract (11.6 mg protein), ferredoxin-free extract (10.5 mg) and ferredoxin (9 nmol). The reaction mixture used in the sulfite reductase assay contained crude extract (18.5 mg protein), ferredoxin-free extract (17.5 mg) and ferredoxin (15 nmol).

Enzymatic extract	Pyruvate dehydrogenase activity [a]	Sulfite reductase activity [b]
Crude extract	1.8	2.9
Ferredoxin-free extract	0.2	0.5
Ferredoxin-free extract + Fd	1.6	1.9

[a] H_2 (μmol) evolved in 20 min under the assay conditions.
[b] H_2 (μmol) consumed in 24 min under the assay conditions.

The effect of ferredoxin concentration on pyruvate dehydrogenase activity of the ferredoxin-free extract has been investigated. The results reported in Fig. 4 indicate that under our experimental conditions the saturation level of the

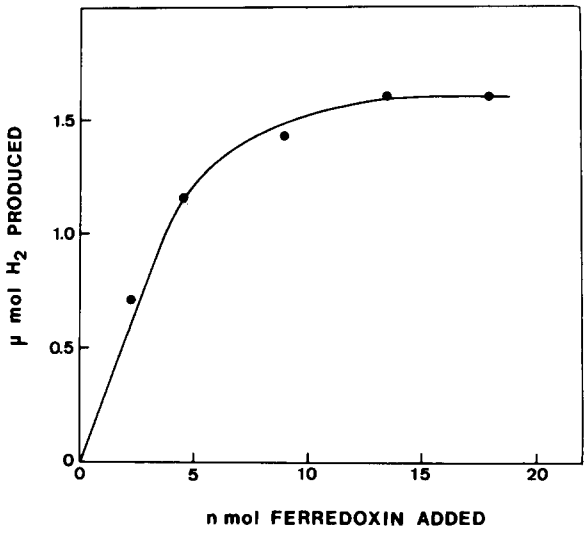

Fig. 4. Effectiveness of *T. commune* ferredoxin in the pyruvate phosphoroclastic reaction. Pyruvate oxidoreductase activity was determined as reported in Materials and Methods. The ferredoxin-free extract was used as enzymatic extract. The values of hydrogen production indicated in the figure for each concentration of ferredoxin represent the activity after 20 min of reaction and after subtraction of the endogenous activity.

electron carrier was obtained with a concentration of approx. 15 nmol of ferredoxin. It should be noted that the systems containing ferredoxin at a saturation concentration exhibit a higher activity than that shown by the crude extract.

Coupling activity of ferredoxin between hydrogenase and sulfite reduction

The results on the hydrogen sulfite reductase activity in the presence of ferredoxin are reported in Table IV. As compared with the endogenous activity of the control containing ferredoxin-free extract, the system containing ferredoxin exhibited a significant stimulation of sulfite reduction.

Thermal stability

Ferredoxin. The thermostability of *T. commune* ferredoxin has been studied with reference to its physiological activity using its coupling activity in the phosphoroclastic reaction. This activity was stable up to 1 h heating at 70°C, a temperature corresponding to the optimum of growth of *T. commune.* However, after 1 h at 80°C the activity decreased rapidly to 20% of its initial rate.

Rubredoxin. After 1 h at 80°C, the absorbance of *T. commune* rubredoxin at 493 nm had decreased to 89% of the original, whereas for the *D. gigas* rubredoxin this figure was 74%.

Stability of T. commune ferredoxin towards oxygen

As previously mentioned in the purification procedure, *T. commune* ferredoxin is unstable in the presence of oxygen. In the absence of strictly anaerobic conditions it gives three major bands on DEAE-cellulose column chromatography. These different oligomeric forms of the protein which are due to oxidative damage could be separated on the basis of their electronic charge. These different bands of ferredoxin have been isolated and characterized by their spectral properties and their molecular weights. The UV-visible spectrum of the more acidic damaged ferredoxin shows a shift of the chromophore absorbance to longer wavelengths (maximum at 405 nm) and exhibits a molecular weight of approx. 21 000, corresponding to a trimer. The two other altered ferredoxins appear to be in the dimeric form (M_r 12 000); however, they differ from each other and from the more acidic protein by their absorbance ratio and their UV-visible spectrum. These three damaged ferredoxins show the same amino acid composition and comprise the same subunit. Electron spin resonance spectrometry has allowed detection of a high level of 3-Fe clusters in these molecules in addition to (4Fe-4S) clusters as compared to a control sample (Guigliarelli, B., Bertrand, P., Papavassiliou, P., Hatchikian, E.C. and Gayda, J.P., unpublished data). The total integrated intensity of the $g = 2.01$ signal detected from the oxidized samples gave average values of 1 ± 0.1 3-Fe cluster per monomer of the more acidic ferredoxin and 0.3 3-Fe cluster per monomer of the less acidic protein. These data indicate most probably that interconversion of (4Fe-4S) clusters into (3Fe-XS) cluster occurred easily in *T. commune* ferredoxin exposed to oxygen, leading to different forms of altered ferredoxins.

The measurement of the decrease of absorbance at 385 nm was utilized to estimate the sensitivity of *T. commune* ferredoxin to oxygen. The protein shows 25% and 50% loss of absorbance after 7 and 26 h under air at 25°C, respectively. This was apparently related to the destructive effect of oxygen on Fe-S clusters. The period of 'optical' half-inactivation ($T_{1/2}$) of *T. commune* ferredoxin under air was about 26 h. However, the bleaching of the chromophore slows down considerably as the degradation process continues.

NAD(P)H : rubredoxin oxidoreductase activity

The NAD(P)H : rubredoxin oxidoreductase activity was investigated in *T. commune* extract in order to determine the physiological activity of rubredoxin. Under our experimental conditions (see Materials and Methods), no activity has been detected.

Discussion

In contrast to thermophilic clostridial species, our knowledge of thermophilic sulfate-reducing bacteria is very limited [34]. Only two species, *Desulfotomaculum nigrificans* [35] and *Desulfovibrio thermophilus* [36], have been studied in pure culture and practically nothing is known about their electron-carrier proteins. *T. commune* differs from both of these thermophilic microorganisms by its cellular and biochemical properties [1–3]

although it contains a cytochrome c_3 [4] which is characteristic of the genus *Desulfovibrio* [5]. In the present work we have shown that *T. commune* contains a rubredoxin and a two-(4Fe-4S) ferredoxin. Flavodoxin has not been detected in these bacteria grown in high-iron media (90 μM) [37].

Rubredoxin from *T. commune* does not differ essentially from *Desulfovibrio* and *Clostridium* species rubredoxins in molecular weight, optical properties, number of cysteine residues and amino acid composition. However, it exhibits a higher content of proline as compared to the other homologous proteins, a property shared only by rubredoxin 1 from *C. thermoaceticum* [38]. The proline residues known to be involved in the reverse turns of proteins [39] could induce an increase of hydrophobicity in the cluster environment, leading to a higher stability. No data have been reported so far on the thermostability of rubredoxins, which are considered to be very stable proteins [40]. However, it is noteworthy that *T. commune* rubredoxin showed a higher thermal stability than rubredoxin of the mesophilic sulfate-reducing bacterium *D. gigas,* based on spectrophotometric changes at the maxima absorption in the visible region. The various species of the genus *Desulfovibrio* studied so far contain at least one ferredoxin with a single (4Fe-4S) cluster [8,9,11,14], which has been distinguished from the clostridial ferredoxins containing two (4Fe-4S) clusters [31,41]. However, a two-(4Fe-4S)-cluster ferredoxin has been recently characterized in some *Desulfovibrio* species [12,13] in addition to a four-iron ferredoxin, whereas a ferredoxin containing only one (4Fe-4S) cluster has been isolated from *C. thermoaceticum* [42] and *C. formicoaceticum* [43]. In contrast to the mesophilic sulfate-reducing bacteria of the genus *Desulfovibrio, T. commune* contains a single ferredoxin exhibiting two (4Fe-4S) clusters per monomeric subunit of molecular weight 7000.

The presence of two four-iron–four-sulfur clusters in *T. commune* ferredoxin is based on analysis of iron and inorganic sulfur content which reveals eight atoms of each per subunit. Moreover, the molar extinction coefficient of the protein at 385 nm ($29.5 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$) is indicative of two (4Fe-4S) clusters, since the molar extinction coefficient per iron in ferredoxins has been reported to be $4 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ [44]. However, the six cysteine residues found per subunit of the molecule is lower than the eight cysteines required to bind two (4Fe-4S) clusters. The definitive answer about the number of cysteine residues in *T. commune* ferredoxin must await the establishment of the complete sequence of the protein, since underestimation of this amino acid after performic acid oxidation [23] has been previously observed with ferredoxin II of *D. desulfuricans* Norway [11,13].

The amino acid composition of *T. commune* ferredoxin shows a low content of acidic amino acids and a high content of threonine as compared to the homologous proteins. It is to be noted that the protein lacks a histidine residue in contrast to the thermophilic clostridial ferredoxins [32] except *C. thermocellum,* and exhibits, as does the latter ferredoxin, a higher level of aromatic residues (see Table III).

The comparison of the N-terminal sequence of *T. commune* ferredoxin with the other known sequences of ferredoxins from sulfate-reducing bacteria indicates that the presence of two (4Fe-4S) clusters makes *T. commune* ferredoxin more similar to *D. desulfuricans* Norway ferredoxin II [13] than to the one-(4Fe-4S)-cluster ferredoxins [29,33,45] (Fig. 3). On the other hand, it shows striking similarity to the primary structure of the one-(4Fe-4S) ferredoxin from the thermophilic *Clostridium* species *C. thermoaceticum* [32], whereas poor homology is observed with the typical two-(4Fe-4S)-cluster clostridial ferredoxins originating from both mesophilic and thermophilic species [32].

Although *T. commune* ferredoxin shows instability towards O_2, the process of Fe-S destruction under air (25°C) is slow ($T_{1/2}$ = 26 h) as compared to highly sensitive ferredoxins [20,46–48]. As reported in the purification procedures, different oligomeric forms of damaged ferredoxins exhibiting various contents of (3Fe-XS) clusters are observed under limited oxidative conditions [49].

T. commune ferredoxin appears to be a thermostable iron-sulfur protein, since it retains its full activity in the phosphoroclastic reaction after 1 h heating at 70°C. Thermal stability has been proposed to be related to a number of different factors. A positively charged group located at residue 2 (histidine or lysine) appears to be critical for

thermal stability of ferredoxins [32,50]; however, this is not sufficient itself to impart thermostability [31,32]. It is to be noted that *T. commune* ferredoxin, which exhibits somewhat lower thermostability than ferredoxins from *C. thermoaceticum* [32] and *C. thermosaccharolyticum* [50], has an aromatic residue at position 2, as observed in mesophilic ferredoxins from sulfate-reducing bacteria and clostridia [50]. The establishment of more detailed relationships between *T. commune* ferredoxin and the homologous thermostable proteins requires the determination of its complete amino acid sequence.

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