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ISOLATION AND CHARACTERIZATION OF A RUBREDOXIN AND TWO FERREDOXINS FROM *DESULFOVIBRIO AFRICANUS*

CLAUDE E. HATCHIKIAN [a], HOWARD E. JONES [b,*] and MIREILLE BRUSCHI [a]

With the technical assistance of GISELE BOVIER-LAPIERRE, JACQUES BONICEL, PAULE COUCHOUD and NICOLE FORGET

[a] *Laboratoire de Chimie Bactérienne, C.N.R.S., 13274 Marseille Cedex 2 (France) and*
[b] *Baas Becking Laboratory, C.S.I.R.O., Canberra, A.C.T. (Australia)*

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Summary

Rubredoxin and two distinct ferredoxins have been purified from *Desulfovibrio africanus*. The rubredoxin has a molecular weight of 6000 while the ferredoxins appear to be dimers of identical subunits of approximately 6000 to 7000 molecular weight. Rubredoxin contains one iron atom, no acid-labile sulfide and four cysteine residues per molecule. Its absorbance ratio A_{278}/A_{490} is 2.23 and its amino acid composition is characterized by the absence of leucine and a preponderance of acidic amino acids.

The two ferredoxins, designated I and II, are readily separated on DEAE-cellulose. The amino acid compositions of ferredoxins I and II show them to be different protein species; the greater number of acidic amino acid residues in ferredoxin I than in ferredoxin II appears to account for separation based on electronic charge. Both ferredoxins contain four iron atoms, four acid-labile sulfur groups and either four (ferredoxin II) or six (ferredoxin I) cysteine residues per molecule. Spectra of the two ferredoxins differ from those of ferredoxins of other *Desulfovibrio* species by exhibiting a pronounced absorption peak at 283 nm consistent with an unusual high content of aromatic residues. The A_{385}/A_{283} absorbance ratio of ferredoxins I and II are 0.56 and 0.62, respectively.

The N-terminal sequencing data of the two ferredoxins clearly indicate that ferredoxins I and II are different protein species. However, the two proteins exhibit a high degree of homology.

* Present address: W.A. Marine Research Laboratories, Waterman, W. Australia, 6020.

The physiological activity of ferredoxins I and II appears to be similar as far as the electron transfer in the phosphoroclastic reaction is concerned.

Introduction

The dissimilatory sulfate-reducing bacterium *Desulfovibrio africanus* was first described by Campbell et al. [1] and later by Jones [2] who showed that, in addition to a soluble *c*-type cytochrome and desulfoviridin characteristic of *Desulfovibrio* species [1,3], it contained particulate *b*-*c* and *d*-type cytochromes. No further studies on the electron carriers of *D. africanus* have been reported.

Ferredoxin and rubredoxin from *Desulfovibrio* species have been utilized for the establishment of phylogenetic relationships [4]. Ferredoxins from *Desulfovibrio* species differ from Clostridial type ferredoxins in that they contain one 4 iron-4 sulfur cluster instead of two clusters [5—9]. The occurrence of two distinct ferredoxins has been reported in some organisms: *Azotobacter vinelandii* [10], *Rhodospirillum rubrum* [11,12], *Bacillus polymyxa* [13,14], *Mycobacterium flavum* [15,16] and *Corynebacterium autotrophicum* [17]. These ferredoxins are in all cases two proteins with different amino acid compositions. Recently, two ferredoxins were detected in the sulfate reducing bacterium *D. desulfuricans* strain Norway 4, however only the more stable ferredoxin was well characterized [9]. On the other hand, two distinct oligomeric forms of the same (4Fe-4S) ferredoxin exhibiting different biological and physico-chemical properties have been isolated and characterized from *D. gigas* [18,19].

It was thus of taxonomic and biochemical interest to compare the electron carriers of *D. africanus* with those of other bacteria including *Desulfovibrio* species. In the present work, we report the purification and characterization of two distinct ferredoxins and a rubredoxin from *D. africanus*.

Methods

D. africanus strain Benghazi, NCIB (8401) was grown at 32°C in the medium of Starkey [20]. The preparation of the cell-free extract from 1500 g cells (wet weight) has recently been described [21].

Initial purification procedure. All purification steps were performed at 4°C and Tris-HCl and potassium phosphate buffers, pH 7.6, of appropriate molarity were used. The cell free extract (1180 ml) was applied to a calcinated alumina column to remove the Mo-Fe-S protein and the cytochromes as previously reported [21]. A settled volume of DEAE-cellulose, equal to 350 ml of DEAE-cellulose was added to the unadsorbed proteins obtained after chromatography on the calcinated alumina column and the mixture was stirred overnight. The DEAE-cellulose was then decanted, washed several times with 10 mM Tris-HCl buffer and the adsorbed acidic proteins eluted with 1 M Tris-HCl buffer, giving a volume of 360 ml. This extract was dialyzed against 10 litres of distilled water, placed on a DEAE-cellulose column (4.5 × 21 cm) and the proteins eluted with a discontinuous gradient (1500 ml) from 100 mM

to 1 M Tris-HCl. Rubredoxin was eluted between 200 and 250 mM Tris-HCl buffer followed by a band of cytochrome *c* which was eluted with 350 mM Tris-HCl. Two other bands of more acidic proteins, very close to each other, and exhibiting the typical absorption spectrum of ferredoxin, were eluted together at approximately 450 mM Tris-HCl.

Purification of rubredoxin. The rubredoxin-containing fraction from the DEAE-cellulose column was dialyzed against 10 mM Tris-HCl and applied to a DEAE-cellulose column (3 × 16 cm). Proteins were eluted with a discontinuous gradient from 150 to 400 mM Tris-HCl, and the rubredoxin (110 ml) was applied to a silica gel column (2.5 × 25 cm) equilibrated with 300 mM Tris-HCl. The rubredoxin migrated slowly in the column and was eluted in a volume of 90 ml. It was subsequently applied to a calcinated alumina column (3 cm × 8 cm) equilibrated with 300 mM Tris-HCl and eluted by the same buffer in a volume of 80 ml. The volume of the extract was brought to 180 ml with distilled water and the rubredoxin was adsorbed on a DEAE-cellulose column (3.2 × 20 cm). It was eluted with a discontinuous gradient from 150 to 500 mM Tris-HCl and collected in a volume of 18 ml. The rubredoxin was finally passed through a Sephadex G-50 column (2.5 × 100 cm) equilibrated with 10 mM Tris-HCl and collected in a volume of 72 ml. The protein was judged to be pure both from its spectrum (A_{278}/A_{490} = 2.23) and from polyacrylamide gel analysis. The yield was 10 mg.

Purification of ferredoxins. The ferredoxins-containing fraction from the DEAE-cellulose column was dialyzed against 10 mM Tris-HCl and further purified by chromatography on a DEAE-cellulose column (4.5 × 20 cm) using a discontinuous gradient from 200 to 500 mM Tris-HCl with 200 ml volumes. A small band of ferredoxin was eluted at approximately 300—350 mM whereas the main, more acidic ferredoxin band was eluted at a higher molarity (400—450 mM Tris-HCl). Subsequently, each band of ferredoxin was purified separately.

The more acidic ferredoxin-containing fraction (220 ml) was placed on a calcinated alumina column (3 × 12 cm) equilibrated with 400 mM Tris-HCl, in order to remove a contaminant with a strong absorption at 260 nm. The ferredoxin was collected in a volume of 185 ml which was brought to 360 ml with distilled water. It was then adsorbed on a DEAE-cellulose column (3 × 20 cm), washed with a discontinuous gradient from 250 to 360 mM Tris-HCl and eluted with 550 mM Tris-HCl buffer in a volume of 50 ml. The ferredoxin fraction was finally passed through a Sephadex G-50 column (5 × 100 cm) and collected in a volume of 190 ml. At this stage, the protein was judged to be pure both from polyacrylamide gel analysis and from its amino-acid composition. The yield of the more acidic ferredoxin was 32 mg.

The less acidic ferredoxin-containing fraction (150 ml) was placed on a calcinated alumina column (2.5 × 10 cm) equilibrated with 300 mM Tris-HCl. The protein was collected in a volume of 130 ml and the volume was brought to 260 ml with distilled water. It was then further purified by chromatography on a DEAE-cellulose column (3 × 20 cm) using a discontinuous gradient from 200 to 350 mM Tris-HCl with 80 ml volumes and eluted with 500 mM Tris-HCl buffer giving a volume of 40 ml. The ferredoxin fraction was subsequently passed through a Sephadex G-50 column (5 × 100 cm) equilibrated

with 10 mM Tris-HCl buffer and collected in a volume of 155 ml. The protein was judged to be pure both from polyacrylamide gel analysis and from its amino acid composition. The yield of the less acidic ferredoxin was 10 mg.

The two ferredoxins will subsequently be referred to as *D. africanus* ferredoxins I and II. Under the growth conditions used ferredoxin I which corresponds to the more acidic ferredoxin is the main ferredoxin component of *D. africanus* cells, being about three times more abundant than ferredoxin II.

Analytical procedures. Molecular weights were estimated by gel filtration on a Sephadex G-50 column according to the method of Whitaker [22], using the following molecular weight standards: *D. desulfuricans* Norway rubredoxin (6700), *D. vulgaris* cytochrome *c*-553 (9100), horse heart cytochrome *c* (12 500), soybean trypsin inhibitor (20 100), chymotrypsin (25 500), ovalbumin (43 000). The molecular weights were also estimated in the presence of sodium dodecyl sulfate (SDS), using the procedure of Weber and Osborn [23]. Analytical gel electrophoresis was performed according to the method of Davis [24] on 7% polyacrylamide gels 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 optical densities 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 SP 1900 spectrometer. Inorganic sulfide was estimated by the method of Fogo and Popowsky [25] as modified by Lovenberg et al. [26]. In the extracts, protein was determined according to the procedure of Lowry et al. [27].

Amino acid analysis were performed on an LKB 3201 amino acid analyzer. Protein samples were hydrolyzed in 6 N HCl at 110°C for 24 h and 48 h according to the method of Moore and Stein [28]. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs [29]. The tryptophan content of rubredoxin was estimated by the method of Edelhoch [30].

Sequence determination. Sequence determination was performed on an LKB 4020 solid phase peptide sequencer. In the solid phase procedure, the protein is attached to the support either through the C-terminal carboxyl group using carbodiimide or through a side-chain function using *p*-phenylene diisothiocyanate to cross link the NH_2 groups. The protein has been coupled by both methods. Best results were obtained after performic acid oxidation of the protein and attachment to an amino ethyl propyl glass support using the carbodiimide procedure [31]. The yield of attachment was 50% and the sequence was determined with 150 nmol of protein.

Phenylthiohydantoin derivatives were analyzed by thin layer chromatography on silica gel containing an ultraviolet fluorescent indicator (Silica gel GF 254, Merck) as described by Edman [32,33], especially for the identification of Glu/Gln and Asp/Asn residues [34]. All the phenylthiohydantoin derivatives have been characterized by amino acid analysis after conversion to the parent amino acid by hydrolysis with hydriodic acid [35].

Measurement of pyruvate dehydrogenase activity. Pyruvate dehydrogenase activity was determined by measuring the hydrogen produced from pyruvate

by 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 and ferredoxin-free extract and ferredoxin as indicated. Flasks were flushed with O_2-free argon for 15 min and allowed to equilibrate for an additional 15 min. Reactions were started by tipping in pyruvate (30 μmol) from the sidearm. CO_2 was absorbed by an NaOH wick in the center well. All assays were performed at 37° C and the final volume was 3 ml.

Preparation of ferredoxin-free extract from D. africanus. To prepare the pyruvate dehydrogenase-containing fraction free of ferredoxin, the crude soluble extract from *D. africanus* (10 ml containing 45 mg/ml protein) obtained from 10 g of cells was passed through a small DEAE-cellulose column (10 × 10 mm) previously equilibrated with 10 mM Tris-HCl pH 7.6. The non-adsorbed protein fraction (36 mg/ml protein) was used as pyruvate dehydrogenase free of ferredoxin. The acidic proteins were obtained after elution from the column with 1 M Tris-HCl.

Results

Molecular weight

Rubredoxin. The molecular weight of rubredoxin from *D. africanus* estimated by molecular filtration on a Sephadex G-50 column was 5800. This value is in good agreement with the minimum molecular weight of 6144 determined by amino acid analysis.

Ferredoxins. The molecular weights of native ferredoxin I and II estimated by gel filtration were approximately 11 000 and 9600, respectively. After incubation with both 1% SDS and 2-mercaptoethanol followed by SDS gel electrophoresis, each ferredoxin gave two bands of protein after staining: a main protein band exhibiting a mobility identical to that of *D. desulfuricans* (Norway 4) ferredoxin which is isolated as a monomeric unit (M_r = 6728) and a small protein band with a mobility similar to that of cytochrome *c* (M_r = 12 500). The minimal molecular weights calculated from the amino acid compositions were 6838 and 5828 for ferredoxin I and ferredoxin II, respectively, including one (4Fe-4S) cluster per molecule. This suggests that in the cell free extracts, the two ferredoxins from *D. africanus* exist as dimers constituted by two identical monomeric units of molecular weight approximately 6000 to 7000. The molecular weight values obtained for ferredoxins I and II by gel filtration are significantly lower than those obtained by doubling the molecular weights of the monomeric units calculated from the amino acid compositions. However, such discrepancies have been previously reported with some homologous proteins [14,36].

Absorption spectra and extinction coefficients

Rubredoxin. The absorption spectrum of *D. africanus* rubredoxin is shown in Fig. 1. It is similar to the spectra of rubredoxins from other desulfovibrio species [5,9,37] and exhibits absorption maxima at 490, 378 and 278 nm with molar extinction coefficients of 11 450, 13 380 and 25 530 $M^{-1} \cdot cm^{-1}$ respec-

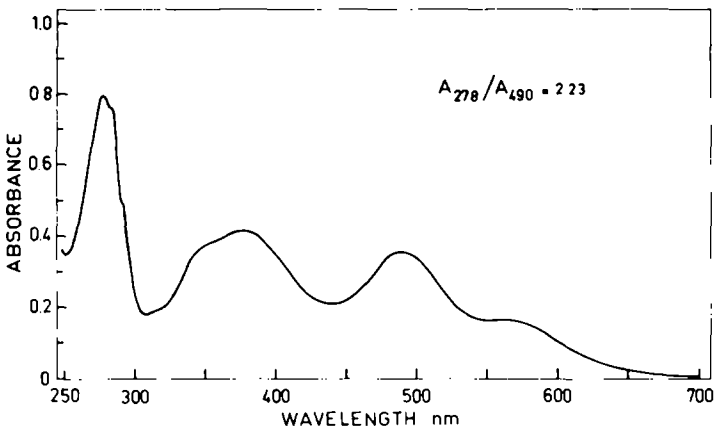

Fig. 1. Absorption spectrum of *D. africanus* rubredoxin. The spectrum of rubredoxin, 31 μM in 25 mM Tris-HCl (pH 7.6) was measured with a Cary 219 spectrophotometer, using 1 cm light path cuvettes.

tively (Table I). Shoulders occur at 355, 291 and 283 nm and the absorbance ratio A_{278}/A_{490} is 2.23. The pronounced shoulder in the ultraviolet region at 291 nm is consistent with the presence of tryptophan residues in the protein. Indeed, the spectrophotometric estimation of tryptophan [30] indicates the presence of 3 tryptophan residues per molecule.

Ferredoxins. The absorption spectra of *D. africanus* ferredoxin I and ferredoxin II are presented in Fig. 2. Ferredoxin I shows a broad absorption band centered at 380 nm in the visible region of the spectrum and a peak at 283 nm in the ultraviolet region with shoulders at 280, 290 and 308 nm. Its absorbance ratio A_{380}/A_{283} is 0.56. Ferredoxin II exhibits a maximum absorption at 385 nm, a minimum at 360 nm and absorption peaks at 283.5 and 290.5 nm

TABLE I

MOLAR EXTINCTION COEFFICIENTS OF RUBREDOXIN AND FERREDOXINS FROM *D. AFRICANUS*

The molar extinction coefficients were obtained by measuring the values of the optical densities of the absorption maxima of a solution of kwown protein concentration. The protein content was calculated from the total amino acid content of the solution determined from amino acid analysis.

Component	λ (nm)	ε ($M^{-1} \cdot cm^{-1}$)	Ratio
Rubredoxin	278	25 530	
	355 *	12 280	
	378	13 380	A_{278}/A_{490} = 2.23
	490	11 450	
Ferredoxin I	283	37 870	
	308 *	28 560	A_{380}/A_{283} = 0.56
	380	21 280	
Ferredoxin II	283.5	30 150	
	290.5	28 950	
	308 *	23 510	$A_{385}/A_{283.5}$ = 0.62
	385	18 650	

* Shoulder.

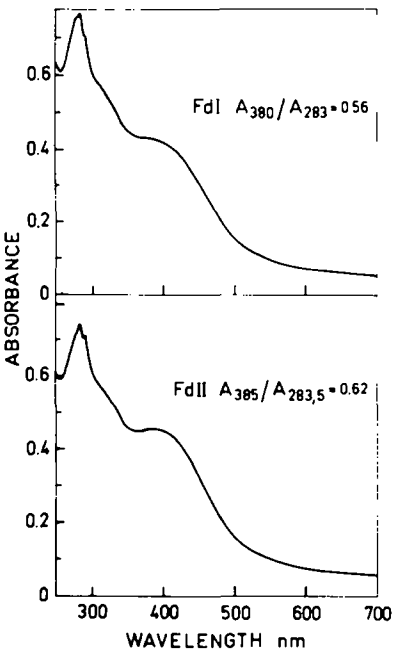

Fig. 2. Absorption spectra of *D. africanus* ferredoxin I (FdI) and ferredoxin II (FdII). The cuvettes (1 cm light path) contained FdI, 20.2 μM in 25 mM Tris-HCl (pH 7.6) or FdII, 24.5 μM in the same buffer. The spectra were measured with a Cary 219 spectrophotometer.

in the ultraviolet region; shoulders occur at 279 and 308 nm. Its absorbance ratio $A_{385}/A_{283.5}$ is 0.62. Although the spectra of the two proteins are similar, ferredoxin I has no minimum at 360 nm and lacks the peak at 290.5 nm. The presence of a shoulder at 290 nm in the spectrum of ferredoxin I and a peak at 290.5 nm in that of ferredoxin II is consistent with the presence of tryptophan in the two proteins.

The molar extinction coefficients at 380 nm for ferredoxin I and 385 nm for ferredoxin II are 21 280 and 18 650 $M^{-1} \cdot cm^{-1}$, respectively (Table I).

Amino acid composition

Rubredoxin. The amino acid composition of rubredoxin from *D. africanus* is listed in Table II. It is compared with those of the other four *Desulfovibrio* species rubredoxins so far reported. Acidic amino acids are preponderant in these proteins and all the rubredoxins lack histidine and arginine. *D. africanus* rubredoxin has 54 residues and, like the other rubredoxins, it contains four cysteine residues responsible for the maintenance of the rubredoxin type cluster. Furthermore it is characterized by the absence of leucine and a remarkably high content of tryptophan.

Ferredoxins. Table III gives the amino acid composition of the two *D. africanus* ferredoxins and allows a comparison with those from *D. gigas*, *D. desulfuricans* (Norway 4) and *D. desulfuricans* (*azotovorans*). Like the other ferredoxins, ferredoxins I and II have a large content of acidic amino acids but differ through the absence of leucine and the presence of tyrosine and tryptophan (see earlier). In addition to the presence of one histidine residue, which is

TABLE II

AMINO ACID COMPOSITION OF *D. AFRICANUS* RUBREDOXINS IN COMPARISON WITH OTHER DESULFOVIBRIO RUBREDOXINS

	D. africanus	*D. gigas* [a]	*D. vulgaris* [b]	*D. desulfuricans* [c] (Norway)	*D. desulfuricans* [d] *azotovorans*
Lysine	4	5	4	5	4
Histidine	0	0	0	0	0
Arginine	0	0	0	0	0
Tryptophan [e]	3	1	1	ND	1
Aspartic acid	9	8	6	13	7
Threonine	1	2	3	4	2
Serine	2	2	2	0	2
Glutamic acid	5	4	3	5	8
Proline	6	5	6	5	6—7
Glycine	5	5	5	7	6
Alanine	2	4	5	5	6
Cystine (half)	4	4	4	4	4
Valine	6	3	5	6	5
Methionine	1	1	1	1	1
Isoleucine	1	2	0	0	2
Leucine	0	1	1	1	0
Tyrosine	3	3	3	4	3
Phenylalanine	2	2	2	2	3
Total residues	54	52	51	62	60
Non-heme iron [f]	1	1	1	1	1
Acid-labile sulfide [f]	0	0	0	0	0

[a] From Bruschi [38]; [b] from Bruschi [39]; [c] from Bruschi et al. [9]; [d] from Newman and Postgate [37]; [e] based on ultraviolet absorbance [30]; [f] atoms per molecule.

also present in *D. desulfuricans* (*azotovorans*) protein, ferredoxin I and ferredoxin II exhibit an unusually high content of the aromatic residues tyrosine and phenylalanine compared with the other ferredoxins.

Despite similarities to each other, the two *D. africanus* proteins differ in their amounts of certain amino acids, especially in the greater number of lysine, glutamic acid and cysteine residues in ferredoxin I which contains seven more residues than ferredoxin II. The more acidic properties of ferredoxin I, compared with ferredoxin II, could be due to its higher content of glutamic acid.

The presence of six and four cysteine residues for ferredoxin I and ferredoxin II, respectively, implies that, like the other *Desulfovibrio* ferredoxins so far reported [7—9], the two ferredoxins have only one (4Fe-4S) cluster.

Iron and sulfur content. Iron and acid-labile sulfide were determined as reported in materials and methods whereas the concentrations of rubredoxin and ferredoxins were calculated from amino acid analysis.

As with the other homologous proteins *D. africanus* rubredoxin contains one iron atom per molecule. No acid-labile sulfide was detected.

Results with ferredoxin I and ferredoxin II show the presence of four iron atoms and four moles of acid-labile sulfide per mol of protein which is consistent with a single (4Fe-4S) cluster (see earlier).

TABLE III

AMINO ACID COMPOSITION OF *D. AFRICANUS* FERREDOXINS IN COMPARISON WITH OTHER *DESULFOVIBRIO* FERREDOXINS

	D. africanus		*D. gigas*	*D. desulfuricans* [b] (Norway)	*D. desulfuricans* [c] *azotovorans*
	Fd I	Fd II			
Lysine	3	1	1	2	2
Histidine	1	1	0	0	1
Arginine	1	1	1	0	0
Tryptophan	ND	ND	0	ND	0
Aspartic acid	5	6	11	5	10
Threonine	1	2	0	3	3
Serine	2	4	3	3	2
Glutamic acid	13—14	8	9	11	11
Proline	3	3	4	3	3
Glycine	2	3	1	2	6
Alanine	6	5	6	7	2
Cystine (half)	5—6	4	6	6	6
Valine	7	5	5	3	5
Methionine	2	1	2	2	0
Isoleucine	3	4	5	5	4
Leucine	0	0	1	1	2
Tyrosine	2	1	0	0	0
Phenylalanine	2	2	1	1	0
Total residues	58	51	56	54	57
Non-heme iron [d]	4	4	4	4	4
Acid-labile sulfide	4	4	4	4	4

[a] From Bruschi [7]; [b] from Bruschi et al. [9]; [c] from Zubieta et al. [8]; [d] atoms per molecule.

N-terminal sequencing data of ferredoxin I and ferredoxin II

26 residues of ferredoxin I and 23 residues of ferredoxin II were identified using automatic protein Sequencer. The cysteine residues in the sequence of the oxidized ferredoxin are identified as cysteic acid by back hydrolysis. For some experiments the yield was low and only the cysteic acid in position 17 was identified, but we suggest that the positions 11, 14 and 21 are also occupied by cysteic acid.

Comparison of the N-terminal sequence of the two ferredoxins (Fig. 3) shows that there is a great degree of homology. However the sequence Lys-Phe (residues 3 and 4) in ferredoxin I is replaced by Val-Val in ferredoxin II. Other

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          1                   5                  10                  15
Fd I      Ala-Arg-Lys-Phe-Tyr-Val-Asp-Glx-Asp-Gln-( )-Ile-Ala-( )-Glu-

Fd II     Ala-Arg-Val-Val-Tyr-Val-Asp-( )-Asp-( )-( )-Ile-( )-( )-Ala-

                              20                  25
Fd I      Ser-Cys-Val-Glu-Ile-( )-Pro-Gly-( )-Phe-Lys

Fd II     Ala-( )-Val-Glu-Ile-( )-Pro-Asp
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Fig. 3. N-terminal sequencing data of ferredoxin I and II (FdI and FdII). Brackets indicate that the residues in these positions could not be identified.

TABLE IV

STIMULATION OF H_2 EVOLUTION FROM PYRUVATE BY *D. AFRICANUS* FERREDOXINS I AND II

The reactions mixtures contained the components as described in Methods. The following enzymatic extracts were utilized in these assays as indicated on the Table: crude extract, 11.5 mg of protein; DEAE-cellulose treated extract (free of ferredoxin) 11.5 mg; acidic proteins, 0.8 mg; ferredoxins I and II, a, 4.2 nmol; b, 17 nmol.

Enzymatic extract	H_2 evolved * (μmol)
Crude extract	4
DEAE-cellulose treated extract	1.5
DEAE-cellulose treated extract + acidic proteins	3.3
DEAE-cellulose treated extract + ferredoxin I	4.1 a
	7.8 b
DEAE-cellulose treated extract + ferredoxin II	3.2 a
	7.8 b

* H_2 evolved in 20 min in the assay condition.

differences appear in positions 15, 16 and 23. When we aligned the two sequences for maximum homology with the other known sequences of ferredoxins from *D. gigas* (Bruschi, M., personal communication) and *Clostridium pasteurianum* [40], there is an additional sequence of three residues in the N-terminal sequence: Ala-Arg-Lys for ferredoxin I and Ala-Arg-Val for ferredoxin II. Homologies are observed on the positions of cysteine residues and several amino acids are conserved in the same position in these homologous sequences.

Coupling activity of ferredoxins I and II in the phosphoroclastic reaction

Results on the coupling activity of ferredoxin I and ferredoxin II between pyruvate dehydrogenase and hydrogenase are reported in Table IV. Compared with the endogenous activity of the control containing ferredoxin-free extract, the systems containing ferredoxin I or ferredoxin II exhibited significant stimulation of H_2 evolution from pyruvate. This implies that both ferredoxins function as electron carriers in the pyruvate phosphoroclastic reaction; however, ferredoxin I appears to be slightly more effective than ferredoxin II at a protein concentration lower than the saturation level. It should be noted that the systems containing ferredoxin at a saturation concentration exhibited a higher activity than that shown by the crude extract.

Discussion

In its molecular weight, iron content and number of cysteine residues *D. africanus* rubredoxin is similar to the other rubredoxins characterized from *Desulfovibrio* species. However, as well as an unusually large content of tryptophan it exhibits remarkably high molar extinction coefficients at the maximum absorptions compared with those of homologous proteins [9,37,41]. These higher values, especially for the maximum absorptions in the visible region of the spectrum (490 and 378 nm), could be due to possible interaction of an aromatic residue, such as tryptophan, with the (1 Fe-4 Cys) cluster resulting in an increased chromophore contribution to the visible region absorption.

Perhaps these spectral characteristics indicate that significant structural differences exist between *D. africanus* rubredoxin and other rubredoxins.

Two distinct ferredoxins, differing by their chromatographic behaviour, absorption spectrum and amino acid composition have been isolated from *D. africanus*. This is the first time that two different protein species ferredoxins are clearly identified from a *Desulfovibrio*.

Flavodoxin was not detected in the cell extract and, in this respect, *D. africanus* is similar to *D. desulfuricans* Norway strain which also contains two ferredoxins [9].

The spectra of the two ferredoxins differ from those of the homologous proteins reported from other *Desulfovibrio* species [5,7,9] by exhibiting a pronounced absorption peak at 283 nm. In both ferredoxins, the presence of a shoulder (ferredoxin I) or a peak (ferredoxin II) at about 290 nm is consistent with the presence of tryptophan which is responsible with tyrosine of the unusually (for a ferredoxin) high absorbance in the 280 nm region. It is noteworthy that the ferredoxins of most anaerobes so far reported [7, 42—44] lacks tryptophan except *Clostridium thermoaceticum* [45]. The spectral properties of ferredoxins I and II are similar to those of ferredoxins rich in tyrosine and/or containing tryptophan which have been isolated from *Bacillus polymyxa* [13,14], *Rhodospirillum rubrum* [11,12] and *Azotobacter vinelandii* [10].

The value of the molar extinction coefficient of ferredoxin II at 385 nm (18 650) is close to the maximum value (16 800—17 500) reported for one (4Fe-4S) cluster ferredoxins [9,45]. That of ferredoxin I at 380 nm (21 280) is clearly higher but still below those found for ferredoxins with two (4Fe-4S) clusters [46]. As with *D. africanus* rubredoxin it can be postulated that the increased chromophore contribution to the visible region absorption which occurs in spectra of ferredoxin I is a result of interaction of an aromatic residue with the iron-sulfur cluster.

The determination that only one (4Fe-4S) cluster occurs per molecule of each ferredoxin is based on analyses of iron and inorganic sulfur content, the number of cysteine residues and the molar extinction coefficient at about 385 nm. Ferredoxin II differs from all *Desulfovibrio* ferredoxins so far characterized by containing four rather than six cysteine residues. Since the iron-sulfur centers of *Desulfovibrio* ferredoxins have not been well characterized so far, one cannot conclude that only a (4Fe-4S) cluster will account for all these characteristics. One has to point out that the possibility of the existence of (2Fe-2S) centers in these proteins should be considered. Additional information on the nature of these centers could come from extrusion experiments and X-ray analysis.

The N-terminal sequencing data of the two ferredoxins clearly indicate that ferredoxins I and II are different protein species. The comparison of the two sequences shows that differences occur on positions 3, 4, 15, 16 and 23. However, it should be noted that the two proteins exhibit a high degree of homology.

Ferredoxins participate in a variety of electron transfer reactions such as photosynthesis, nitrogen fixation and fermentative metabolism [42]. Like *Clostridium pasteurianum* ferredoxin [47] ferredoxins I and II can function as

electron carriers in the phophoroclastic reaction, which implies that they have a low redox potential. Since ferredoxin I is slightly more effective than ferredoxin II at a low protein concentration and is the main ferredoxin component in *D. africanus* cells, it probably plays the major role in coupling pyruvate dehydrogenase with hydrogenase via cytochrome c_3 [48,49].

Evidence has been accumulating that ferredoxins, like cytochromes, may occur in the cell in several forms [8,9,10—16]. However, distinction between ferredoxins from the same organism on the basis of biological activity has not always been established [10,14]. The significance of two ferredoxins from *D. africanus* functioning in the same electron transfer reaction is not yet understood. Further information on the physico-chemical properties of ferredoxins I and II as well as on the electron transfer systems of *D. africanus* are required before the precise role of each ferredoxin may be specified.

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References

1 Campbell, L.L., Kasprzycki, M.A. and Postgate, J.R. (1966) J. Bacteriol. 92, 1122—1127
2 Jones, H.E. (1971) Arch. Mikrobiol. 80, 78—86
3 Postgate, J.R. and Campbell, L.L. (1966) Bacteriol. Rev. 732—738
4 Vogel, H., Bruschi, M. and Le Gall, J. (1977) J. Mol. Evol. 9, 111—119
5 Le Gall, J. and Dragoni, N. (1966) Biochem. Biophys. Res. Commun. 23, 145—149
6 Laishley, E.C., Travis, J. and Peck, H.D., Jr. (1969) J. Bacteriol. 98, 302—303
7 Zubieta, J.A., Mason, R. and Postgate, J.R. (1973) Biochem. J. 133, 851—854
8 Bruschi, M., Hatchikian, E.C., Le Gall, J., Moura, J.J.G. and Xavier, A.V. (1976) Biochim. Biophys. Acta 449, 275—284
9 Bruschi, M., Hatchikian, E.C., Golovleva, L.A. and Le Gall, J. (1977) J. Bacteriol. 129, 30—38
10 Yoch, D.C. and Arnon, D.I. (1972) J. Biol. Chem. 247, 4514—4520
11 Shanmugam, K.T., Buchanan, B.B. and Arnon, D.I. (1972) Biochim. Biophys. Acta 256, 477—486
12 Yoch, D.C., Arnon, D.I. and Sweeney, W.V. (1975) J. Biol. Chem. 250, 8330—8336
13 Stombaugh, N.A., Burris, R.H. and Orme-Johnson, W.H. (1973) J. Biol. Chem. 248, 7951—7956
14 Yoch, D.C. (1973) Arch. Biochem. Biophys. 158, 633—640
15 Bothe, H. and Yates, M.G. (1976) Arch. Microbiol. 107, 25—31
16 Yates, M.G., O'Donnel, M.J., Lowe, D.J. and Bothe, H. (1978) Eur. J. Biochem. 85, 291—299
17 Berndt, H., Lowe, D.J. and Yates, G.M. (1978) Eur. J. Biochem. 86, 133—142
18 Cammack, R., Rao, K.K., Hall, D.O., Moura, J.J.G., Xavier, A.V., Bruschi, M., Le Gall, J., Deville, A. and Gayda, J.P. (1977) Biochim. Biophys. Acta 490, 311—321
19 Moura, J.J.G., Xavier, A.V., Hatchikian, E.C. and Le Gall, J. (1978) FEBS Lett. 89, 177—179
20 Starkey, R.L. (1938) Arch. Microbiol. 9, 268—304
21 Hatchikian, E.C. and Bruschi, M. (1979) Biochem. Biophys. Res. Commun. 86, 725—734
22 Whitaker, J.R. (1963) Anal. Chem. 35, 1950—1953
23 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
24 Davis, B.J. (1964) Ann. N.Y. Acad. Sci., 121, 404—427
25 Fogo, J.J. and Popowski, M. (1949) Anal. Chem. 21, 732—734
26 Lovenberg, W., Buchanan, B.B. and Rabinowitz, J.C. (1963) J. Biol. Chem. 238, 3899—3913
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275

28 Moore, S. and Stein, W.H. (1963) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 6, pp. 819—831, Academic Press, New York, NY
29 Hirs, C.H.W. (1967) in Methods in Enzymology (Hirs, C.H.W., ed.), Vol. 11, Academic Press, New York, NY
30 Edelhoch, H. (1967) Biochemistry 6, 1948—1954
31 Previero, A., Derancourt, J., Coletti-Previero, M.A. and Laursen, R.A. (1973) FEBS Lett. 33, 135
32 Edman, P. and Sjoquist, J. (1956) Acta Chem. Scand. 10, 1507—1509
33 Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80—91
34 Edman, P. (1970) in Protein Sequence Determination, (Needleman, S.B., ed.), pp. 211—255, Springer-Verlag, Berlin
35 Smithies, O., Gibson, D., Fanning, E.M., Goodfliesh, R.M., Gilman, J.G. and Ballantyne, D.L. (1971) Biochemistry, 10, 4912—4921
36 Probst, I., Moura, J.J.G., Bruschi, M. and Le Gall, J. (1978) Biochim. Biophys. Acta 502, 38—44
37 Newman, D.J. and Postgate, J.R. (1968) Eur. J. Biochem. 7, 45—50
38 Bruschi, M. (1976) Biochem. Biophys. Res. Commun. 70, 615—621
39 Bruschi, M. (1976) Biochim. Biophys. Acta 434, 4—17
40 Tanala, M., Nakashima, T., Benson, A., Mower, H.F. and Yasunobu, K.T. (1966) Biochemistry 5, 1666—1681
41 Lovenberg, W. and Sobel, B.E. (1965) Proc. Natl. Ac. Sci. U.S. 54, 193—199
42 Buchanan, B.B. and Arnon, D.I. (1970) Adv. Enzymol. 33, 119—176
43 Johnson, P.W. and Canale-Parola (1973) Arch. Microbiol. 89, 341—353
44 Travis, J., Newman, J.D., Le Gall, J. and Peck, H.D. Jr. (1971) Biochem. Biophys. Res. Commun. 45, 452—458
45 Yang, S.S., Ljungdahl, L.G. and Le Gall, J. (1977) J. Bacteriol. 130, 1084—1090
46 Hong, J.S. and Rabinowitz, J.C. (1970) J. Biol. Chem. 245, 4982—4987
47 Mortenson, L.E., Valentine, R.C. and Carnahan, J.E. (1962) Biochem. Biophys. Res. Commun. 7, 448—452
48 Akagi, J.M. (1967) J. Biol. Chem. 242, 2478—2483
49 Bell, G.R., Lee, J.P., Peck, H.D. Jr. and Le Gall, J. (1978) Biochimie 60, 315—320