Biochimica et Biophysica Acta, 634 (1981) 41—51

BBA 47982

CHARACTERIZATION OF A NEW TYPE OF FERREDOXIN FROM *DESULFOVIBRIO AFRICANUS*

E.C. HATCHIKIAN and M. BRUSCHI

Laboratoire de Chimie Bactérienne, C.N.R.S., 13274 Marseille Cedex 2 (France)

(Received July 15th, 1980)

Key words: Ferredoxin; Iron-sulfur protein; Absorption spectrum; Amino acid composition; (Desulfovibrio africanus)

Summary

A new ferredoxin designated ferredoxin III has been isolated from *Desulfovibrio africanus* grown on media high in iron. Native ferredoxin III is a dimer constituted by two identical subunits of approx. 7500. It is distinguished from the two other ferredoxins (I and II) isolated from this microorganism by its amino acid composition, N-terminal sequence, spectral properties and iron-sulfur content. The amino acid composition of *D. africanus* ferredoxin III is typical of ferredoxins with an excess of acidic over basic residues and the absence of histidine and arginine residues.

The absorption spectrum of ferredoxin III exhibits two maxima, at 408 nm ($\epsilon = 58.5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) and 285 nm ($\epsilon = 82 \cdot 10^3\ M^{-1} \cdot cm^{-1}$), with a shoulder at 305 nm ($\epsilon = 75 \cdot 10^3\ M^{-1} \cdot cm^{-1}$). Its A_{408}/A_{285} absorbance ratio is 0.78. Ferredoxin III contains approx. 12—13 atoms each of iron and labile sulfur. This is in agreement with the high value of the extinction coefficient at 408 nm, which is slightly higher than 3-fold that of one [4Fe-4S] cluster. However, the number of cysteine residues of the protein (six residues), which is about the half that of iron atoms, is indicative of the presence of a new type of iron-sulfur cluster in ferredoxin III. The protein is unstable in a low ionic strength environment; the addition of neutral salts stabilizes the protein conformation.

The data on the biological activity of ferredoxin III as compared to the two other ferredoxins from *D. africanus* show that the three iron-sulfur proteins function with equal effectiveness as electron carrier in the phosphoroclastic reaction and the H_2-sulfite reductase system.

Introduction

Evidence has been accumulating that ferredoxins, like cytochromes, may occur in the cell in several forms. In the last few years, the occurrence of two

distinct ferredoxins has been reported in several microorganisms [1—8] including *Desulfovibrio* [9—11]. Two different ferredoxins were detected in *D. desulfuricans* strain Norway 4 [10], whereas two distinct oligomeric forms of the same ferredoxin exhibiting different biological and physico-chemical properties have been isolated and characterized from *D. gigas* [9,12,13].

Desulfovibrio africanus strain Benghazi has been reported by Campbell et al. [14] to be in most respects typical of a *Desulfovibrio*. Recently, different electron carrier proteins have been characterized in this microorganism, including a rubredoxin [15], two distinct ferredoxins [15], a cytochrome c_3 [16] and a molybdenum-containing iron-sulfur protein [17]. The two ferredoxins (ferredoxin I and ferredoxin II) are dimers constituted by two identical subunits with a molecular weight of approx. 6000. The absorption spectra, amino acid compositions and N-terminal sequencing data of the two ferredoxins clearly indicate that ferredoxin I and ferredoxin II are different protein species. The proteins appear to contain one four-iron, four-sulfur cluster, however ferredoxin I exhibits a high molar extinction coefficient at 390 nm which is in agreement with the presence of nearly six non-heme iron atoms per molecule [15].

In the present paper, we report the purification and some properties of a new ferredoxin which is synthesized by *D. africanus* grown on media high in iron. This iron-sulfur protein, which is a new type ferredoxin containing approx. 12—13 atoms each of iron and labile sulfur per molecule, is quite different from ferredoxin I and II, although it exhibits similar biological activities.

Materials and Methods

Desulfovibrio africanus strain Benghazi (NCIB 8401) was grown at 37°C on the lactate-sulfate medium of Starkey [18] using an iron concentration of 3.6 mg/l instead of 0.7 mg/l in the previous study [15]. Cells were stored at −20°C for several weeks until being used.

Purification procedure. The purification was performed at 4°C and Tris-HCl buffers (pH 7.6) of appropriate molarity were used unless otherwise stated.

The frozen cells (1700 × *g*, wet weight) were thawed and suspended in 1200 ml of 25 mM Tris-HCl. The crude extract (2500 ml) and the soluble protein fraction (1700 ml) were subsequently prepared as already described [17]. A settled volume of DEAE-cellulose equal to 400 ml was added to the soluble protein fraction and the acidic protein extract was then prepared as reported previously [15].

Step I. The dialyzed acidic protein extract was applied to a DEAE-cellulose column (5.2 × 21 cm) which had been equilibrated with 0.05 M NaCl/0.05 M Tris-HCl buffer. The proteins were eluted with a discontinuous NaCl gradient (1600 ml) from 100 mM NaCl in 50 mM Tris-HCl to 1 M NaCl in 50 mM Tris-HCl. At this stage two bands of ferredoxin, very close to each other, were eluted together between 350 and 450 mM NaCl in 50 mM Tris-HCl buffer in a volume of 380 ml.

Step II. The ferredoxin-containing fraction was dialyzed against 10 mM Tris-HCl and further purified by chromatography on a DEAE-cellulose column (5 × 25 cm) previously equilibated with 50 mM Tris-HCl. Proteins were eluted with a discontinuous gradient from 100 mM to 500 mM NaCl in 50 mM Tris-HCl.

This gradient enables the separation of a band comprising mainly ferredoxin I and ferredoxin II (eluted between 400 and 500 mM NaCl in 50 mM Tris-HCl) from other components of the acidic extract still present at this stage (desulfoviridin, rubredoxin and an acidic cytochrome). Fractions containing ferredoxin, recognized by their characteristic dark brown colour, were localized on the top of the column and were eluted with 400 mM NaCl/50 mM Tris-HCl buffer.

Step III. The fraction containing the ferredoxins (470 ml) was dialyzed against 10 mM Tris-HCl and readsorbed on a third DEAE-cellulose column (4.5 × 21 cm). Proteins were eluted with a discontinuous gradient from 100 mM to 300 mM NaCl in 50 mM Tris-HCl. The acidic cytochrome still present in the ferredoxin fraction was finally separated from the band containing the ferredoxins and eluted at approx. 230—240 mM NaCl in 50 mM Tris-HCl. The less acidic band of ferredoxin (ferredoxin II) was then eluted at a higher molarity (270 mM NaCl/50 mM Tris-HCl) whereas the band containing ferredoxin I spread on the column and appeared to be subdivided into two bands which exhibit different colours. At this stage, ferredoxin I cannot be separated from a new reddish brown ferredoxin which is slightly more acidic. The two ferredoxins were eluted together between 270 and 300 mM NaCl in 50 mM Tris-HCl in a volume of about 350 ml. The ferredoxin fraction was then dialyzed against 5 l of 10 mM Tris-HCl buffer, concentrated on a small DEAE-cellulose column (3 × 12 cm) and eluted with 0.4 M Tris-HCl, giving a volume of 85 ml.

Step IV. The more acidic ferredoxin-containing fraction was then placed on a calcinated alumina column (3 × 14 cm) equilibrated with 400 mM Tris-HCl, in order to remove a contaminant with a strong absorption at 260 nm. After this step, the ferredoxin fraction presented an A_{380}/A_{283} absorbance ratio close to 0.65 which is higher than that of pure ferredoxin I which exhibits a ratio of about 0.56 [15].

Step V. The separation of ferredoxin I from the new ferredoxin was achieved using a DEAE-Sephadex A-50 column (4.2 × 25 cm) equilibrated with 0.34 M Tris-HCl buffer. The ferredoxin fraction from step IV was brought to approximately the same Tris-HCl molarity and applied to the column. Elution was performed with a discontinuous gradient (2500 ml) from 340 mM to 450 mM Tris-HCl buffer. The ferredoxin fraction adsorbed at the top of the column separated into two bands at about 0.4 M Tris-HCl. The first, and the strongest, which was dark brown coloured, contained ferredoxin I and was collected in a volume of 400 ml. The protein was judged to be pure both from its spectrum (A_{380}/A_{283} = 0.56—0.57) and its amino acid composition. The yield of ferredoxin I was about 70 mg.

The second band, exhibiting a reddish brown colour, is constituted by a new ferredoxin which appears to be more acidic than ferredoxin I. It was collected in a volume of 300 ml. This new ferredoxin, which exhibits a high absorbance ratio (A_{408}/A_{285} = 0.78), was judged to be pure both from polyacrylamide gel electrophoresis and from its amino acid composition. The yield of this ferredoxin was approx. 25 mg. It will subsequently be referred to as *D. africanus* ferredoxin III.

Analytical procedures. The molecular weight of ferredoxin III was estimated by gel filtration on a Sephadex G-50 column according to the method of

Whitaker [19] and by SDS-polyacrylamide gel electrophoresis, using the procedure of Weber and Osborn [20]. Analytical gel electrophoresis was performed according to the method of Davis [21] on 7% polyacrylamide gels at pH 8.8.

Absorption spectra were measured on a Cary 14 spectrophotometer. Molar extinction coefficients of ferredoxin III 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 Varian A175 spectrometer. Inorganic sulfide was estimated by the method of Lovenberg et al. [22], as modified by Suhara et al. [23]. In the extracts, protein was determined according to the procedure of Lowry et al. [24].

Amino acid analysis. Protein samples were hydrolyzed in 200 μl of 6 M HCl at 110°C for 18, 20, 24 and 48 h in evacuated sealed tubes [25]. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs [26]. The values for serine, threonine and tyrosine were corrected for decomposition during hydrolysis. Amino acid analyses were performed on a LKB 3201 amino acid analyzer.

Sequence determination. Sequence determination was performed in the Socosi Protein Sequencer (P.S. 100). *N,N*-Dimethylbenzylamine buffer was used. To minimize the losses during solvent washing of the protein, apocytochrome *c* was added in the cup as described by Bonicel et al. [27]. The quantitative determination of the phenyl thiohydantoin derivatives was done relative to known amounts of the appropriate standards on gas chromatography (Beckman gas chromatography GC45 using SP400 as stationary phase according to the technique of Pisano et al. [28] with and without silylation. Phenyl thiohydantoin derivatives were also analyzed by thin-layer chromatography on silica gel containing an ultraviolet fluorescent indicator (Silica Gel GF 254, Merck) as described by Edman and Sjoquist [29] and Edman and Begg [30] particularly for the identification of Glu/Gln and Asp/Asn residues [31]. If necessary, these methods were completed by amino acid analysis after conversion to the parent amino acid by hydrolysis with hydrochloric acid [32] or hydroiodic acid [33].

Measurement of pyruvate dehydrogenase activity. Pyruvate dehydrogenase activity was determined by measuring the hydrogen produced from pyruvate by the manometric method reported previously [15].

Measurement of sulfite reductase activity. Sulfite reductase activity was determined by measuring the hydrogen absorption in the presence of sulfite using the manometric method as previously described [34]. The main compartment of the Warburg flasks contained: potassium phosphate buffer (pH 7.0), 150 μmol; ferredoxin-free extract and ferredoxin as indicated. Flasks were flushed with H_2 for 15 min and allowed to equilibrate for an additional 15 min. Reactions were started by tipping in sulfite (4 μmol) from the sidearm. H_2S was absorbed by an NaOH wick in the center well.

Preparation of ferredoxin-free extract from D. africanus. The non-adsorbed protein fraction obtained after treatment of the soluble extract from *D. africanus* on DEAE-cellulose during the purification procedure was used as ferredoxin-free enzymatic extract.

Results

Molecular weight. The molecular weight of native-ferredoxin III estimated by gel filtration on a Sephadex G-50 column was found to be approx. 15 000. The results of SDS-polyacrylamide gel electrophoresis indicates that native ferredoxin III is a dimer of molecular weight 14 500 constituted by two identical monomeric units with a molecular weight of approx. 7500. The minimal molecular weight calculated from the amino acid composition was 6800, excluding the iron-sulfur content of the protein.

Amino acid composition. The amino acid composition of *D. africanus* ferredoxin III is given in Table I. It is typical of ferredoxins with an excess of acidic over the basic residues, the absence of histidine and arginine residues and low aromatic amino acid content. Four amino acids are absent in the amino acid composition (histidine, arginine, proline and phenylalanine). Ferredoxin III contains six cysteine residues which should imply the presence of only one [4Fe-4S] cluster in the protein.

Table I allows a comparison with the two others ferredoxins isolated from *D. africanus*. As the ferredoxins from other species, the three ferredoxins have a large content of acidic amino acids but ferredoxins I and II have an unusually high content of the aromatic residues tyrosine and phenylalanine. Ferredoxins

TABLE I

AMINO ACID COMPOSITION OF *D. AFRICANUS* FERREDOXIN III IN COMPARISON WITH THE TWO OTHER *D. AFRICANUS* FERREDOXINS

Amino acid analysis were carried out using general methods with 18 h hydrolysis (6 M HCl) at 110°C under vacuum. Other analysis after 20, 24 and 48 h are in accordance with the average value. N.D., not determined.

Amino acids	Ferredoxin III		Ferredoxin I **	Ferredoxin II **
	from analysis	nearest integer		
Lysine	3.0	3	3	1
Histidine	0	0	1	1
Arginine	0	0	1	1
Tryptophan	n.d.	n.d.	n.d.	n.d.
Aspartic acid	7.94	8	5	6
Threonine	3.36	4	1	2
Serine	1.4	2	2	4
Glutamic acid	13.9	14	13—14	8
Proline	0	0	3	3
Glycine	3.85	4	2	3
Alanine	3.1	3	6	5
Cystine (half) *	5.1	6	5—6	4
Valine	8.5	9	7	5
Methionine *	0.9	1	2	1
Isoleucine	2.7	3	3	4
Leucine	2.8	3	0	0
Tyrosine	1.85	2	2	1
Phenylalanine	0	0	2	2
Total residues		62	58	51

* Calculated after performic acid oxidation.

** From Hatchikian et al. [15].

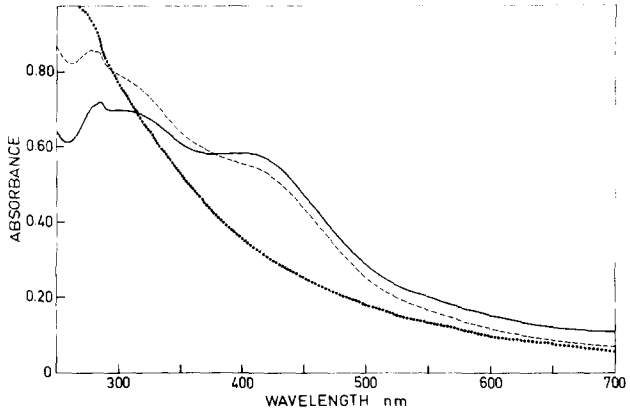

Fig. 1. Absorption spectra of *D. africanus* ferredoxin III; ———, oxidized protein freshly purified, in 100 mM Tris-HCl buffer (pH 7.6); - - - - - -, freshly dialyzed protein; ●●●●, denatured ferredoxin after 8 h incubation of the dialyzed sample at room temperature. Both spectra were recorded at similar protein concentration (9.8 μM).

I and II contain one histidine, one arginine and three proline residues whereas ferredoxin III is devoid of these residues. Ferredoxin II differs from the two other ferredoxins by its low content of cysteine residues.

Absorption spectrum and extinction coefficients. The ultraviolet-visible absorption spectrum of ferredoxin III in the oxidized form (Fig. 1) appears to be typical of four-iron, four-sulfur proteins. Ferredoxin III exhibits two broad absorption bands centered around 408 and 305 nm. In contrast to ferredoxins I and II [15], it shows a small peak in the ultraviolet region at 285 nm with a shoulder at 279 nm. Addition of dithionite resulted in a partial bleaching of the chromophore.

The freshly purified preparation exhibited an absorbance ratio, A_{408}/A_{285}, of 0.78. The molar extinction coefficients of ferredoxin III at 408, 305 and 285 nm are 58 500, 75 000 and 82 000, respectively. These data indicate that optical properties of this ferredoxin are quite different from those reported for *D. africanus* ferredoxins I and II [15]. The value of the molar extinction coefficient of ferredoxin III at 408 nm is much higher than those found for the one [4Fe-4S] cluster ferredoxin from *Desulfovibrio* [9,10,15] and the two [4Fe-4S] cluster ferredoxins from *Clostridia* [22]. It corresponds approximately to the absorption of a three [4Fe-4S] cluster containing protein.

Ferredoxin III proved to be extremely unstable at low ionic strength. As indicated in Fig. 1, the absorbance ratio decreased by approx. 17% after dialysis of the protein for 10 h at 4°C. In addition, the spectrum of the dialyzed sample shows a variation of the chromophore absorbance in the ultraviolet region with a shift of the peak at 285 nm to 280 nm.

After a few hours of incubation at room temperature under an air atmosphere (Fig. 1) there is a complete disappearance of the absorption bands centered around 400 and 305 nm (see Fig. 1) with a concomitant bleaching of the protein which corresponds to the destruction of the iron-sulfur cluster. Tris-HCl buffer or NaCl at a concentration of approx. 0.4—0.5 M stabilizes the protein. With protein samples in such a high ionic strength environment no detectable change occurs in the absorption spectrum for many hours under air at

about 25°C. Tris-HCl buffer is more efficient than NaCl in preventing denaturation of the protein.

These results suggest that the modification of the protein conformation occurring at low ionic strength leads to an increase of the oxygen sensitivity of the iron-sulfur protein. This hypothesis was supported by the increase of stability of dialyzed samples of ferredoxin III when they were stored under argon.

Iron and sulfur content. Iron and acid-labile sulfide were determined as reported in Materials and Methods, whereas the concentration of ferredoxin was calculated from amino acid analysis.

The average value of iron content of ferredoxin III estimated from several analyses has been found to be approx. 12—13 atoms per mol of protein.

The complete release of labile sulfide from ferredoxin III requires an incubation of the protein sample of about 2 h with the alkaline zinc reagent as reported by Suhara et al. [23]. In these conditions, the analyses revealed the presence of 12—13 mol of acid-labile sulfide per mol.

This iron-sulfur content of ferredoxin III is in good agreement with the value of the extinction coefficient at 408 nm which is indicative of the presence of at least three [4Fe-4S] clusters per mol. However as compared with other ferredoxins, the binding of these clusters to the protein appears to be different since only six cysteine residues have been found in ferredoxin III.

N-terminal sequence analysis. The N-terminal sequence of ferredoxin III established by automatic Edman degradation on apoferredoxin was determined up to 20 residues with some residues not identified (Fig. 2). Apoferredoxin was obtained by precipitating native ferredoxin III with 3% HCl at 80°C for 10 min.

The N-terminal sequence of *D. africanus* ferredoxin III has been compared to those of ferredoxins I and II from the same organism as well as to those of ferredoxins from *D. gigas*, *D. desulfuricans* Norway strain and *Clostridium pasteurianum* (Fig. 3). Ferredoxin I and ferredoxin II show a high degree of homology. When the two sequences are aligned for maximum homology with the other known sequences of ferredoxins from *D. gigas* [35], *D. desulfuricans* [11] and *C. pasteurianum* [36] there is an additional basic sequence of three residues: Ala-Arg-Lys for ferredoxin I and Ala-Arg-Val for ferredoxin II. When compared to ferredoxin III, this additional sequence is not observed and the sequence is aligned for homology with tyrosine residue (No. 2) which is observed in other ferredoxins. The amino acid sequence of ferredoxin III differs from the two other ferredoxins isolated from *D. africanus*. However, homology is apparent when compared to the N-terminal amino acid sequences from *C. pasteurianum* ferredoxin [36] and from *D. desulfuricans* Norway ferredoxin II [11].

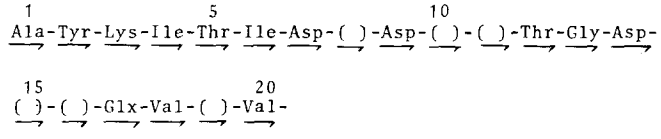

Fig. 2. N-terminal sequence of ferredoxin III. Parentheses indicate that the residues in these positions could not be identified.

D. africanus Fd I	Ala-Arg-Lys-Phe-Tyr-Val-Asp-Glx-Asp-Gln-( )-Ile-Ala-( )-Glu-Ser-Cys-Val-Glu-Ile-( )-Pro-Gly
D. africanus Fd II	Ala-Arg-Val-Val-Tyr-Val-Asp-( )-Asp-( )-( )-Ile-( )-( )-Ala-Ala-( )-Val-Glu-Ile-( )-Pro-Asp
D. africanus Fd III	Ala-Tyr-Lys-Ile-Thr-Ile-Asp-( )-Asp-( )-( )-Thr-Gly-Asp-( )-( )-Glx-Val-( )-Val
D. gigas	Pro-Ile-Glu-Val-Asn-Asp-Asp-Cys-Met-Ala-Cys-Glu-Ala-Cys-Val-Glu-Ile-Cys-Pro-Asp
D.d. Norway Fd II	Gly-Tyr-Ser-Val-Ile-Val-Asp-( )-Asp-Lys-( )-Ile-Gly-Ser-( )-Glu-Ala-Val-( )-( )
C. pasteurianum	Ala-Tyr-Lys-Ile-Ala-Asp-Ser-Cys-Val-Ser-Cys-Gly-Ala-Cys-Ala-Ser-Glu-Cys-Pro-Val

Fig. 3. N-terminal sequencing data of *D. africanus* ferredoxins in comparison with *D. gigas*, *D. desulfuricans* (Norway strain) and *C. pasteurianum* ferredoxins. Brackets indicate that the residues in these positions could not be identified.

Biological activity. The results on the coupling activity of ferredoxin III between pyruvate dehydrogenase and hydrogenase on the one hand and hydrogenase and sulfite reductase on the other are reported in Table II. Ferredoxin III appears to be an efficient electron carrier in these two redox reactions. From the data obtained, no significant difference of activity could be detected between ferredoxin III and ferredoxins I and II. The stimulation of both H_2 evolution from pyruvate and H_2 consumption in the presence of sulfite was quite similar with the three ferredoxins (Table II).

The effect of ferredoxin III concentration on pyruvate dehydrogenase activity of the ferredoxin-free extract has been investigated. The results reported on Fig. 4 indicate that under our experimental conditions the saturation level of the electron carrier was obtained with a concentration of approx. 3 nmol of ferredoxin.

TABLE II

EFFECT OF FERREDOXIN (Fd) I, II AND III ON PYRUVATE DEHYDROGENASE AND SULFITE REDUCTASE ACTIVITIES OF DEAE-TREATED EXTRACTS FROM *D. AFRICANUS*

Enzymatic activities were determined as reported in Materials and Methods. Pyruvate dehydrogenase activity: The crude extract and the ferredoxin free extract contained: 16.2 mg protein. Each ferredoxin was assayed using a concentration of 4 nmol. Sulfite reductase activity: The following enzymatic extracts were utilized in these assays: crude extract, 9.4 mg protein; DEAE-cellulose-treated extract (free of ferredoxin) 9.4 mg protein; ferredoxins I, II and III, 20 nmol.

Enzymatic extract	Pyruvate dehydrogenase activity *	Sulfite reductase activity **
Crude extract	3.4	3.9
DEAE-cellulose-treated extract	1.1	0.7
DEAE-cellulose-treated extract + Fd I	3.3	2.9
DEAE-cellulose-treated extract + Fd II	3.5	2.9
DEAE-cellulose-treated extract + Fd III	3.6	2.7

* H_2 (μmol) evolved in 12 min under the assay conditions.
** H_2 (μmol) consumed in 30 min under the assay conditions.

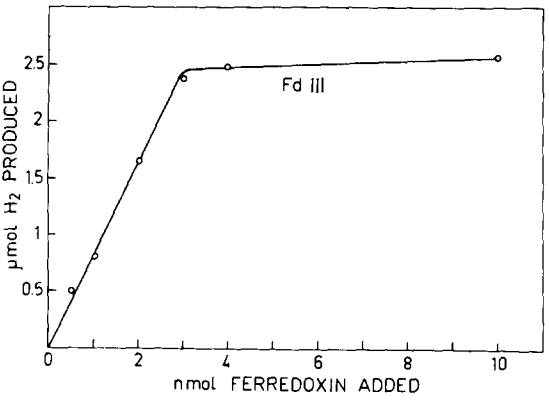

Fig. 4. Effectiveness of *D. africanus* ferredoxin III in the pyruvate phosphoroclastic reaction. Pyruvate oxidoreductase activity was determined as reported in Methods. The ferredoxin-free extract was used as enzymatic extract. The values of hydrogen production indicated in the figure for each concentration of ferredoxin represent the activity after 12 min of reaction and after subtraction of the endogenous activity.

Discussion

A new ferredoxin (ferredoxin III) has been characterized, in addition to the previously reported ferredoxins I and II, from *D. africanus* grown on media high in iron. In *Desulfovibrio*, the regulatory mechanism of the synthesis of iron-sulfur proteins linked to iron concentration is unknown [37]. Under our growth conditions with *D. africanus* (3.6 mg iron/l), in addition to the biosynthesis of ferredoxin III, we observed a marked decrease in the amount of the blue green molybdenum containing iron-sulfur protein previously characterized [17]. This suggests that the Mo-Fe-S protein could play a role in the synthesis of iron sulfur-proteins.

Ferredoxins III is slightly more acidic than ferredoxin I and as with the two other ferredoxins from *D. africanus* [15] the native protein appears to exist as a dimer comprising two identical monomeric units with a molecular weight of approx. 7500.

The amino acid composition of ferredoxin III clearly indicate that it is a different protein species. It is characterized by the absence of histidine, arginine, proline and phenylalanine residues, a low aromatic amino acid content and the presence of six cysteine residues. The N-terminal sequencing data of the protein and the comparison with the N-terminal sequences of known *Desulfovibrio* ferredoxins show more homology with ferredoxin II from *D. desulfuricans* Norway strain than with ferredoxins I and II from *D. africanus*. Homology is also observed with *C. pasteurianum* ferredoxin.

The optical properties of ferredoxin III differ clearly from those of ferredoxins I and II [15]. The spectrum of the protein is characterized by its high absorbance ratio ($A_{408}/A_{285} = 0.78$), its absorption band in the visible region centered around 408 nm, its weak absorption in the ultraviolet aromatic region and its high molar extinction coefficients at 408 and 305 nm. It should be noted that in ferredoxin III, the absorption band in the visible region is shifted

to higher wavelengths when compared to that of ferredoxins I and II [15]. This spectral pattern has already been described in *D. gigas* ferredoxin II and I' [9].

D. africanus ferredoxin III is distinguished from all the bacterial ferredoxins previously characterized by its high molar extinction coefficients at 408 and 305 nm reflecting the presence of a high content of non heme iron in the protein. Considering the molar extinction coefficient at 400 nm per iron in ferredoxin to be close to $4 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [38], the molar extinction coefficient of ferredoxin III ($\epsilon_{408} = 58.5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) is indicative of the presence of at least 12 iron atoms per molecule. This is in agreement with the iron content of the protein, since a value slightly higher than 12 atoms per mol has been found.

Our results do not allow a description of the nature of the iron-sulfur cluster present in the protein. Indeed, no significant spectral characteristics appear to exist between a [4Fe-4S] cluster containing protein and the recently reported [3Fe-3S] cluster containing ferredoxins [39,40]. Moreover, ferredoxin III contains at most only six cysteine residues and not the 12 or more required for the binding of three or four clusters. It is therefore a new type of ferredoxin containing a number of cysteine residues lower than the number of iron atoms. This implies a binding of the iron-sulfur clusters of the protein which is different from that occurring in the previously reported ferredoxins. The elucidation of the structure of the cluster and its binding to the protein must await further physico-chemical investigations on the iron-sulfur centers and more information on the sequence of the protein, including its cysteine distribution.

Ferredoxin III proved to be unstable at low ionic strength during incubation at room temperature, as was shown for other iron-sulfur proteins [10,11,41, 42]. This was evidenced by the bleaching of the visible absorption which is usually taken as an index of denaturation of the protein. The results suggest that the sensitivity of the protein to oxygen is enhanced in low ionic strength environment. The addition of neutral salts markedly protects ferredoxin III from denaturation. As postulated by Hasumi et al. [42], the stabilization of ferredoxin by a high ionic strength environment could be due to the modulation of the interaction between the chromophore and the protein moiety.

The biological activity of ferredoxin III deserves some comment. Ferredoxin III serves as electron carrier in the phosphoro-clastic reaction, which implies that it has a low redox potential. It also mediates electron transfer between cytochrome c_3 and the sulfite reductase system. The comparative study of the biological activity of the three ferredoxins from *D. africanus* has shown that both proteins function with equal effectiveness in the two redox reactions mentioned. Thus, distinction between these ferredoxins on the basis of biological activity has not been established.

However, one has to point out that our enzymatic system is purely artificial. It is quite possible that a compartmentalization exists in the intact cell with a different subcellular localization of the dehydrogenases and the reductases, each having its own electron chain: disruption of the cells would completely change the distribution of the carriers leading thus to misleading activities.

Acknowledgements

We are indebted to G. Bovier-Lapierre for skilful technical help. Thanks are due to P. Couchoud and J. Bonicel for the analysis with liquid phase sequencer. We are grateful to N. Forget, Dr. M. Scandellari and R. Bourrelli for growing the bacteria.

References

1 Yoch, D.C. and Arnon, D.I. (1972) J. Biol. Chem. 247, 4514—4520
2 Shanmugam, K.T., Buchanan, B.B. and Arnon, D.I. (1972) Biochim. Biophys. Acta 256, 477—486
3 Yoch, D.C., Arnon, D.I. and Sweeney, W.V. (1975) J. Biol. Chem. 250, 8330—8336
4 Stombaugh, N.A., Burris, R.H. and Orme-Johnson, W.H. (1973) J. Biol. Chem. 248, 7951—7956
5 Yoch, D.C. (1973) Arch. Biochem. Biophys. 158, 633—640
6 Bothe, H. and Yates, M.G. (1976) Arch. Microbiol. 107, 25—31
7 Yates, M.G., O'Donnel, M.J., Lowe, D.J. and Bothe, H. (1978) Eur. J. Biochem. 85, 291—299
8 Berndt, H., Lowe, D.J. and Yates, G.M. (1978) Eur. J. Biochem. 86, 133—142
9 Bruschi, M., Hatchikian, E.C., Le Gall, J., Moura, J.J.G. and Xavier, A.V. (1976) Biochim. Biophys. Acta 449, 275—284
10 Bruschi, M., Hatchikian, E.C., Golovleva, L.A. and Le Gall, J. (1977) J. Bacteriol. 129, 30—38
11 Guerlesquin, F., Bruschi, M., Bovier-Lapierre and Fauque, G. (1980) Biochim. Biophys. Acta 626, 127—135
12 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
13 Moura, J.J.G., Xavier, A.V., Hatchikian, E.C. and Le Gall, J. (1978) FEBS Lett. 89, 177—179
14 Campbell, L.L., Kasprzycki, M.A. and Postgate, J.R. (1966) J. Bacteriol. 92, 1122—1127
15 Hatchikian, E.C., Jones, H.E. and Bruschi, M. (1979) Biochim. Biophys. Acta 548, 471—483
16 Singleton, R., Jr., Campbell, L.L. and Hawkridge, F.M. (1979) J. Bacteriol. 140, 893—901
17 Hatchikian, E.C. and Bruschi, M. (1979) Biochim. Biophys. Res. Commun. 86, 725—734
18 Starkey, R.L. (1938) Arch. Micrbiol. 9, 268—304
19 Whitaker, J.R. (1963) Anal. Chem. 35, 1950—1953
20 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
21 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
22 Lovenberg, W., Buchanan, B.B. and Rabinowitz, J.C. (1963) J. Biol. Chem. 238, 3899—3913
23 Suhara, K., Kanayama, K., Takemori, S. and Katagiri, M. (1974) Biochim. Biophys. Acta 336, 309—317
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
25 Moore, S. and Stein, W.H. (1963) Methods Enzymol. 6, 819—831
26 Hirs, C.H.W. (1967) Methods Enzymol. 11, 59—62
27 Bonicel, J., Bruschi, M., Couchoud, P. and Bovier-Lapierre, G. (1977) Biochimie 59, 111—113
28 Pisano, J.J., Bronzert, T.J. and Brewer, H.B., Jr. (1972) Anal. Biochem. 45, 43—59
29 Edman, P. and Sjoquist, J. (1956) Acta Chem. Scand. 10, 1507—1509
30 Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80—91
31 Edman, P. (1970) in Protein Sequence Determination (Needleman, S.B., ed.), pp. 211—255, Springer-Verlag, Berlin
32 Van Orden, H.O. and Carpenter, F.H. (1964) Biochem. Biophys. Res. Commun. 14, 399—403
33 Smithies, O., Gibson, D., Fanning, E.M., Goodfliesh, R.M., Gilman, J.G. and Ballantyne, D.L. (1971) Biochemistry 10, 4912—4921
34 Hatchikian, E.C., Le Gall, J., Bruschi, M. and Dubourdieu, M. (1972) Biochim. Biophys. Acta 258, 701—708
35 Bruschi, M. (1979) Biochem. Biophys. Res. Commun. 91, 623—628
36 Tanaka, M., Nakashima, T., Benson, A., Mower, H.F. and Yasunobu, K.T. (1966) Biochemistry 5, 1666—1681
37 Fauque, G.D., Barton, L.L. and Le Gall, J. (1980) in Sulphur in Biology, Ciba Foundation Symposium, Vol. 72, pp. 71—86, Elsevier/North-Holland, Amsterdam
38 Palmer, G. (1975) in The Enzymes (Boyer, P.D., ed.), Vol. 12, pp. 1—56, Academic Press, New York
39 Emptage, M.H., Kent, T.A., Huynh, B.H., Rawlings, J., Orme-Johnson, H. and Münck, E. (1980) J. Biol. Chem. 255, 1793—1796
40 Huynh, B.H., Moura, J.J.G., Moura, I., Kent, T.A., Le Gall, J., Xavier, A.V. and Münck, E. (1980) J. Biol. Chem. 255, 3242—3244
41 Probst, I., Moura, J.J.G., Moura, I., Bruschi, M. and Le Gall, J. (1978) Biochim. Biophys. Acta 502, 38—44
42 Hasumi, H., Nakamura, S., Koga, K. and Yoshizumi, H. (1979) Biochem. Biophys. Res. Commun. 87, 1095—1101