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ISOLATION AND CHARACTERIZATION OF A RUBREDOXIN AND AN (8Fe-8S) FERREDOXIN FROM *DESULFUROMONAS ACETOXIDANS*

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Summary

A two cluster (4Fe-4S) ferredoxin and a rubredoxin have been isolated from the sulfur-reducing bacterium *Desulfuromonas acetoxidans*. Their amino acid compositions are reported and compared to those of other iron-sulfur proteins.

The ferredoxin contains 8 cysteine residues, 8 atoms of iron and 8 atoms of labile sulfur per molecule; its minimum molecular weight is 6163. The protein exhibits an absorbance ratio of A_{385}/A_{283} = 0.74. Storage results in a bleaching of the chromophore; the denatured ferredoxin is reconstitutable with iron and sulfide. The instability temperature is 52°C.

The rubredoxin does not differ markedly from rubredoxins from other anaerobic bacteria.

Introduction

Recently, several organisms capable of reduction of elemental sulfur in anaerobic environments have been described: *Desulfuromonas acetoxidans* [1], *Spirillum* 5175 [2] and members of the genus *Desulfovibrio* [3]. Reduction of elemental sulfur as electron acceptor is linked to the oxidation of either organic substrates or hydrogen, as in the case of *Spirillum* 5175. Whereas the oxidation of lactate by *Desulfovibrio* species is incomplete and yields acetate as end product, *D. acetoxidans* carries out the complete oxidation of organic acids to CO_2. Despite this fundamental difference both organisms contain electron transfer proteins including low-potential *c*-type cytochromes. The occurrence of the four-haem cytochrome c_3 in *Desulfovibriones* [4] and of the three-

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haem cytochrome c_7 [5] in *Desulfuromonas*, both having histidines as iron ligands, is unique for this type of sulfur- and sulfate metabolism and may point to a phylogenetic relationship between the two species. Therefore, purification and characterization of additional electron transfer proteins seemed necessary.

Ferredoxin and rubredoxin from *Desulfovibrio* species have been utilized for the establishment of phylogenetic relationships [6]. Ferredoxins from *Desulfovibrio* species are different from clostridial type ferredoxins in that they contain one (4Fe-4S) cluster instead of two clusters [7—11]. It was thus of taxonomic and biochemical interest to use these two iron-sulfur proteins for determining the possible relationship between *D. acetoxidans* and other ferredoxin- and rubredoxin-containing bacteria, namely *Desulfovibrio* and *Clostridium* species. In the present work we report the purification of an (8Fe-8S) ferredoxin and of a rubredoxin, which by amino acid composition is closely related to that of *Desulfovibrio gigas*.

Methods

D. acetoxidans strain 5071 was kindly provided by Professor N. Pfennig, Göttingen, G.F.R. Cell culture and the preparation of a cell-free extract from 400 g cells (wet weight) have recently been described [1,5].

Purification procedure

All purification steps were performed at 4°C under an argon atmosphere, using argon-saturated buffers, to minimize the aerobic degradation of native *Desulfuromonas* ferredoxin. The cell-free extract (800 ml) was applied to an Al_2O_3 column (4 × 20 cm) equilibrated with 0.01 M Tris · HCl (pH 7.6) to remove cytochrome *c*-551.5 (c_7). The unadsorbed proteins were eluted with equilibration buffer and applied to a DEAE-cellulose column (5 × 19 cm) equilibrated in the same buffer. The column was initially washed with 200 ml of 0.01 M Tris · HCl (pH 7.6) and then eluted with a discontinuous gradient from 0.1 to 0.5 M Tris · HCl (pH 7.6) in 0.05 M increments with 150-ml volumes. Ferredoxin, rubredoxin and various *c*-type cytochromes eluted as one fraction (450 ml) between 0.3 and 0.4 M Tris · HCl. This fraction was dialyzed against 0.01 M Tris · HCl (pH 7.6) for 12 h and adsorbed on a DEAE-cellulose column (5 × 19 cm) equilibrated with the dialysis buffer. The column was washed with 200 ml of 0.15 M Tris · HCl (pH 7.6). The black-coloured top of the resin was then transferred to the top of another DEAE-cellulose column (3 × 10 cm) equilibrated with 0.15 M Tris · HCl (pH 7.6). Separation of ferredoxin from rubredoxin was achieved by elution with a discontinuous gradient as described above using 60-ml volumes. Rubredoxin was eluted between 0.25 and 0.3 M Tris · HCl and ferredoxin at approximately 0.4 M Tris · HCl.

Rubredoxin. Rubredoxin (50 ml) was freed from remaining cytochromes by chromatography on a silica gel column (3 × 11 cm) equilibrated with 0.3 M Tris · HCl (pH 7.6). The protein was eluted with 140 ml of the same buffer, diluted with an equal volume of distilled water and adsorbed on a DEAE-cellulose column (4 × 10 cm) equilibrated with 0.1 M Tris · HCl (pH 7.6). Rubredoxin was eluted free from ferredoxin by a discontinuous gradient as described above. The rubredoxin fraction was purified by a final chromatography

on a DEAE-cellulose column (2.5 × 15 cm) equilibrated and developed with 0.3 M sodium citrate (pH 5.0).

Ferredoxin. The ferredoxin-containing fraction (50 ml) was dialyzed against 0.01 M Tris · HCl (pH 7.6) and further purified by chromatography on a DEAE-cellulose column (3 × 10 cm) using a discontinuous gradient from 0.2 to 0.5 M Tris · HCl (pH 7.6) as described above with 50 ml volumes. The ferredoxin fraction (0.4 Tris · HCl) was concentrated on a hydroxyapatite column (2 × 12 cm) equilibrated with 0.4 M Tris · HCl (pH 7.6). The protein was eluted with 0.1 M sodium phosphate buffer (pH 7.0) and then dialyzed against 0.01 M Tris · HCl (pH 7.6). The concentrated ferredoxin (20 ml) was finally absorbed on to an Al_2O_3 column (2 × 10 cm) equilibrated with 0.01 M Tris · HCl (pH 7.6). The column was washed with 50 ml 0.4 M Tris · HCl (pH 6.7) and the ferredoxin eluted with 0.01 M sodium phosphate (pH 7.0).

Analytical procedures

The molecular weight was estimated by gel filtration on a Sephadex G-50 column according to the method of Whitaker [12], using the following standards [9,11,13]: *Desulfovibrio gigas* rubredoxin (6000), *Des. vulgaris* cytochrome c_{553} (9100), *Des. gigas* cytochrome c_3 (13 000) and *Des. gigas* ferredoxin II (24 000). Analytical gel electrophoresis was performed according to the method of Davis [14] on 7% (w/v) gels at pH 8.8. Absorption spectra were measured on a Cary 14 spectrophotometer. Amino acid analyses were performed on a Beckman Multichrom amino acid analyser. Protein samples were hydrolyzed in 6 M HCl at 110°C for 24 h, according to the method of Moore and Stein [15]. The values of threonine, serine and tyrosine were corrected for decomposition during hydrolysis. Iron was determined by atomic absorption spectrophotometry using a Unicam model SP 1900 spectrometer. Inorganic sulfide was determined by the method of Lovenberg et al. [16], as modified by Fogo and Popowsky [17].

Results

Ferredoxin

Ferredoxin was sensitive toward oxygen especially during the last steps of purification. The purification, therefore, had to be carried out under argon and

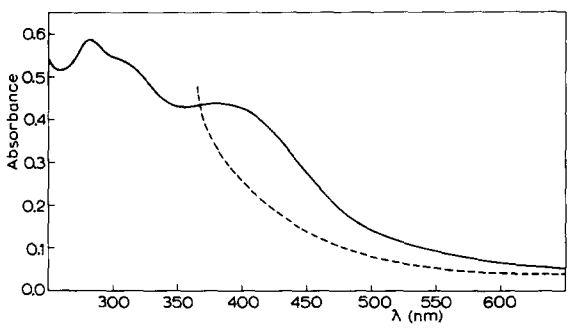

Fig. 1. Absorption spectra of the *D. acetoxidans* ferredoxin (14.6 μM, in 50 mM Tris · HCl, pH 7.6). ———, oxidized protein; - - - - -, dithionite-reduced protein.

TABLE I

MOLAR EXTINCTION COEFFICIENTS OF FERREDOXIN AND RUBREDOXIN FROM *D. ACETOXIDANS*

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

Component	λ(nm)	$\epsilon(M^{-1} \cdot cm^{-1})$	Ratio
Ferredoxin	283	40 100	
	300 *	37 300	$A_{385}/A_{283} = 0.74$
	385	29 800	
Rubredoxin	280	16 700	
	370	7 100	
	490	6 020	$A_{280}/A_{490} = 2.77$
	570 *	2 600	

* Shoulder.

chemical properties were analyzed without delay after the last purification step. The absorption spectra of oxidized and reduced ferredoxin are shown in Fig. 1. The oxidized form exhibited absorption peaks at 385 and 283 nm with a distinct shoulder at 300 nm closely resembling the spectrum of *Clo. acidiurici* ferredoxin [18]. The weak absorption in the ultraviolet aromatic region is

TABLE I

AMINO ACID COMPOSITION OF *D. ACETOXIDANS* FERREDOXIN

Amino acid	*D. acetoxidans*	*C. butyricum* [16,20]	*Des. gigas* [21]
Lysine	1	0	1
Histidine	0	0	0
Arginine	0	0	1
Tryptophan	n.d. ††	0	0
Aspartic acid	7	9	11
Threonine	4	3	0
Serine	4	3	3
Glutamic acid	6	5	9
Proline	2	3	4
Glycine	5	5	1
Alanine	6	7	6
Cysine (half) *	8	8	6
Valine	3	6	5
Methionine *	0	0	2
Isoleucine	3	4	5
Leucine	0	0	1
Tyrosine	2	0	0
Phenylalanine	0	2	1
Total residues	51	55	56
Molecular weight	6163 **	6096 **	6545 **
	8500 ***		
Labile sulfur †	8	8	4
Non-haem iron †	7—8	8	4

* Calculated after performic acid oxidation.
** Minimum molecular weight calculated from amino acid composition.
*** Estimated by gel filtration.
† Atoms per molecule.
†† n.d., not determined.

reflected by the low content of aromatic residues (Table II) and serves as purity control index. The freshly purified preparation exhibited an absorbance ratio of $A_{385}/A_{283} = 0.74$ (Table I). Addition of dithionite resulted in a partial bleaching of the chromophore. The molar extinction coefficients, based on the minimum molecular weight of 6163, as determined by amino acid analysis, agree well with those reported for (8Fe-8S) ferredoxins [16,18,19]. The analytical results show the presence of 7—8 iron atoms, 8 labile sulfur atoms and 8 cysteines per minimum molecular weight of 6163, clearly identifying the *Desulfuromonas* ferredoxin as an (8Fe-8S) ferredoxin. The molar extinction coefficient at 385 nm per g atom iron is 3.725; this value is close to that (3.85) found in clostridial [18,19] ferredoxins. The amino acid composition of the *Desulfuromonas* ferredoxin is typical of ferredoxins with an excess of acidic over the basic residues, the absence of histidine residues and low aromatic amino acid content. Like the anaerobic clostridial type of ferredoxin it contains neither methionine nor leucine and shows a high degree of similarity with the ferredoxin of *Clo. butyricum* [20]. The *Des. gigas* ferredoxin is quite different from the clostridial type in that it contains no threonine, but both arginine and methionine; the presence of only 6 cysteine residues and one (4Fe-4S) cluster is noteworthy.

As previously mentioned, the ferredoxin proved to be extremely unstable. Even during storage under argon, the absorbance ratio decreased by approx. 20% within one week. The spectrum of the denatured ferredoxin (Fig. 2, spectrum A) shows a shift of the chromophore absorbance to longer wavelengths (from 385 nm to 405 nm) and the appearance of a shoulder at approx. 450 nm. A sample of denatured ferredoxin was reconstituted with iron and sulfide in the presence of mercaptoethanol by the method of Hong and Rabinowitz [22] except the conversion to apoprotein by treatment with trichloroacetic acid was omitted. After purification of the reconstituted protein on a G-25

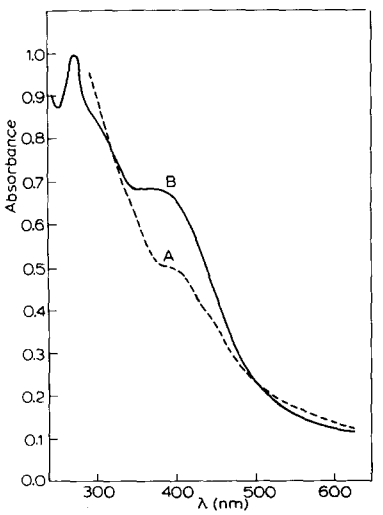

Fig. 2. Absorption spectra of the *D. acetoxidans* ferredoxin, A (- - - - -) denatured; B (———) after reconstitution. Both spectra were recorded at similar protein concentration (24 μM), the 283-nm peak of the denatured ferredoxin is masked by the absorption of mercaptoethanol added to stabilize the ferredoxin.

Sephadex column equilibrated with 0.01 M Tris · HCl (pH 7.6), the main band presented spectrum B shown in Fig. 2. A large increase of the chromophore contribution to the visible spectrum was observed. Eight iron atoms per molecule were determined and the ratio A_{385}/A_{283} was 0.68.

The thermal stability of the ferredoxin was determined in temperature-controlled spectrophotometer cells. An instability temperature of 52°C was obtained. No appreciable spectrophotometric changes were observed until the instability temperature was reached. The bleaching of the chromophore was irreversible, but the protein was stable for at least one hour at intermediate temperatures.

Rubredoxin

The anaerobic rubredoxin contains four cysteine residues responsible for the maintenance of the rubredoxin type cluster and is characterized by the absence of labile sulfur and the presence of one iron atom linked in a tetrahedral arrangement to the sulfur of cysteine residues. The oxidized *D. acetoxidans* rubredoxin exhibited the characteristic absorption spectrum with maxima at 280, 370 and 490 nm (Table 1). The amino acid composition obtained was: Lys_2, Asp_8, $Thre_2$, Ser_2, Glu_4, Pro_5, Gly_6, Ala_4, Cys_4, Val_4, Met_{2-3}, $Ileu_2$, Leu_1, Tyr_3, Phe_2. Tryptophane was not measured. The *D. acetoxidans* rubredoxin closely resembles the rubredoxin of *Des. gigas* in amino acid composition [13].

Discussion

The *D. acetoxidans* ferredoxin contains 8 cysteine residues, 8 iron and 8 labile sulfur atoms per molecule, thus representing a two cluster (4Fe-4S) ferredoxin of the anaerobic clostridial type. Its lability in the presence of oxygen is partially abolished under anaerobic conditions. Rapid destruction of the ferredoxin chromophore by oxygen has also been reported for the ferredoxins from *Clo. cylindrosporum* [16], *Bacillus polymyxa* ferredoxin II [23] and alfalfa [24]. *Clo. pasteurianum* ferredoxin dimerizes under aerobic conditions with loss of iron and labile sulfur giving a product with half as much iron and sulfide content per 6000 g of protein as the native monomeric molecule [19]. During deterioration of alfalfa ferredoxin only labile sulfur is released with a concomitant polymerization of the protein.

Different rates of removal of iron and sulfide by chemical methods are reflected by differences in heat stability [25]; during thermal denaturation both components are released from the ferredoxins. The very low instability temperature of 52°C for the *D. acetoxidans* ferredoxin agrees well with the lability of its chromophore and coincides with the above theory.

Contrary to strictly anaerobic bacteria and desulfovibriones, *D. acetoxidans* carries out the complete oxidation of acetate to CO_2 [1]; ferredoxin is involved in both the degradation and synthesis of pyruvate [26].

Ferredoxins from various microorganisms and plants have been utilized for the establishment of phylogenetic relationships. An evolutionary development of ferredoxins from obligate anaerobic bacteria to anerobic microorganisms and plants has been proposed, with the (4Fe-4S) ferredoxins as a link between the

(8Fe-8S) ferredoxins of *Clostridia* and photosynthetic bacteria and the (2Fe-2S) ferredoxins of blue-green algae and plants [27]. Bacteria with (4Fe-4S) ferredoxins are considered to exist in a transition between anaerobic and aerobic environments. The occurrence of an (8Fe-8S) ferredoxin of the anaerobic clostridial type in *D. acetoxidans* and of an (4Fe-4S) ferrodoxin in *Clo. thermoaceticum* [28] complicates this evolution model.

Acknowledgments

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