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FLAVODOXIN AND RUBREDOXIN FROM *DESULPHOVIBRIO SALEXIGENS*

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Summary

A flavodoxin and a rubredoxin have been isolated from the sulfate-reducing bacterium *Desulphovibrio salexigens* (strain British Guiana, NICB 8403). Their amino acid composition and spectral characteristics did not differ markedly from the homologous proteins presented in other *Desulphovibrio* spp. Flavodoxin was shown to be active in the electron transport of the sulfite reductase system.

Introduction

The function of electron transfer proteins from *Desulphovibrio* spp. is far from being understood. The heterogeneity of the *Desulphovibrio* spp. allows a large variation in the enzymatic equipment of the different strains [1]. There is always a number of homologous electron transfer proteins but important differences have been noted in their amino acid sequences and physiological role and it has been suggested that these proteins have not maintained the same physiological role through evolution [1,2].

A better understanding of the structure-function relationship may be achieved by a comparative study of the largest number of homologous electron transfer proteins from different *Desulphovibrio* spp. Table I shows the state of purification of homologous electron transfer proteins from these bacteria.

Physico-chemical characterization of these proteins allowed a better understanding of their redox properties [3—10]. Structural studies by X-ray

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TABLE I

ELECTRON CARRIER HOMOLOGOUS PROTEINS IN *DESULPHOVIBRIO* spp.

S, sequenced; NF, not found; PNP, present not purified; P, purified; NR, not reported.

Desulphovibrio species	Ferredoxin	Flavodoxin	Rubredoxin	Cytochrome c_3 (M_r 13 000)	Cytochrome c_3 (M_r 26 000)	Cytochrome *c*-553
gigas	S	P	S	S	P	NF
vulgaris Hildenborough	PNP	S	S	S	P	S
vulgaris Miyazaki	NF	PNP	NR	P	NR	NR
desulphuricans Norway 4	P	NF	P	S	NF	P
desulphuricans El Agheila Z	NR	NR	NR	S	NR	NR
salexigens	PNP *	P *	P *	S	PNP *	PNP *

* This work.

crystallography have been reported for flavodoxin [11], rubredoxin [12] and cyochrome c_3 (M_r 13 000) [13]. A comparative study between cytochrome c_3 (M_r 13 000) isolated so far from *Desulphovibrio* spp. is under way by the analysis of the different kinetics of reoxidation made by NMR techniques and comparison of EPR and NMR spectral data [14].

In this paper we report the purification and preliminary characterization of two electron transfer proteins from *Desulphovibrio salexigens*: a flavodoxin and a rubredoxin. Comparison with other homologous proteins is presented.

Materials and Methods

Organism and growth conditions. *D. salexigens* (strain British Guiana NCIB 8403) was grown in a lactate/sulfate medium as previously described [15].

Enzyme assays. A manometric assay was utilized to determine the biological activity of the flavodoxin and rubredoxin isolated from *D. salexigens*, following the reduction of sulfite by hydrogen. 4 μmol of freshly prepared sodium sulfite were added from the side arm to the main compartment of each manometric vessel containing 150 μmol of phosphate buffer (pH 7.0), the electron carrier, pure hydrogenase and the reductase preparation in a final volume of 2.8 ml. The center well containing 50 μmol NaOH (10 N) and 50 μmol of $CdCl_2$ (10%). Before addition of sodium sulfite the flask was incubated for 30 min under hydrogen at 37°C. The *D. salexigens* sulfite reductase containing extract devoid of acidic electron carriers was prepared by passing the crude extract (10 ml containing 36 mg/ml protein) on a small diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman) column (1 × 1.5 cm) equilibrated with 0.01 M Tris-HCl, pH 7.6. Of this extract, 22 mg protein were used in each assay performed. Pure hydrogenase, prepared from *D. gigas* by the method of Bell [16] was added in all cases to the system to insure an excess of this activity.

Amino acid analysis. Amino acid analyses were carried out using a LKB

amino acid analyser. Protein samples were hydrolyzed in 6 M HCl at 110°C for 20 h in evacuated, sealed tubes according to Moore and Stein [17]. Cysteine and methionine were analysed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs [18].

Acrylamide gel electrophoresis and electrofocussing. Analytical gel electrophoresis was performed according to Davis [19] on 7% (v/v) gels at pH 8.8. Analytical thin-layer gel electrofocussing in polyacrylamide gel was used to estimate the isoelectric points and purity of the proteins with a LKB Multiphor apparatus [20]. A pH gradient between 2.5 and 6.0 was achieved using ampholine.

Molecular weight determinations. The molecular weight of the purified proteins was determined by gel filtration on a Sephadex G-50 column, according to Whitaker [21] using the following standards: chymotrypsin (M_r 25 000), soybean trypsin inhibitor (M_r 21 000), horse heart cytochrome *c* (M_r 12 500) and *D. vulgaris* rubredoxin (M_r 6000).

Optical spectra and molar extinction coefficients. Ultraviolet and visible spectra were recorded with a Cary 14 spectrophotometer (Varian Associates, Palo Alto, CA, U.S.A.). The molar extinction coefficients of the proteins were obtained by measuring the values of absorbances at the absorption maxima of a solution of known protein concentration. The molarity of the protein solutions was calculated from amino acid analysis.

Results

Protein purification scheme

Unless otherwise stated, all buffers were at pH 7.6 and all operations were performed at 4°C. 800 g of cells (wet weight) were suspended in 1 l of 10 mM Tris-HCl, DNAase was added and the mixture was passed twice through a French pressure cell. The disrupted cells suspension was centrifuged at 13 200 × *g* for 2 h. The supernatant (1900 ml) was applied to an alumina oxide (Merck) column (4.5 × 25 cm) equilibrated with 10 mM Tris-HCl to remove cytochrome c_3 (M_r 13 000) [15] and a blue protein containing molybdenum and iron-sulfur centers (Le Gall, J., unpublished results). A cytochrome reducible by ascorbic acid is fixed in this step and was not further purified. The unadsorbed proteins were eluted with the equilibration buffer. A settled volume of DEAE-52 cellulose equal to 300 ml was added to the solution of unadsorbed proteins on the alumina and the mixture was stirred for 4 h. The supernatant was then decanted and the DEAE-cellulose washed several times with 10 mM Tris-HCl buffer. The adsorbed acidic protein extract was dialysed overnight against 20 l of destilled water (final volume after dialysis 600 ml) and adsorbed onto a DEAE-52 cellulose column (5 × 36 cm) previously treated according to the manufacturer instructions and equilibrated with 10 mM Tris-HCl. After elution with a discontinuous gradient of 200 ml each of 0.10, 0.15, 0.20 ... up to 0.50 M Tris-HCl, four main fractions were collected: one containing mainly desulfoviridin (sulfite reductase analogous to the enzyme present in *D. gigas* [22]) (0.10—0.15 M); one containing traces of desulfoviridin and an acidic cytochrome (0.10—0.25 M); one containing rubredoxin and flavodoxin (0.30—0.35 M) and the last one containing mainly ferredoxin (0.40—0.45 M).

The fraction containing rubredoxin and flavodoxin was dialyzed overnight against distilled water, adsorbed onto a second DEAE-52 column (4.5 × 10 cm) and eluted with a discontinuous gradient of Tris-HCl buffer.

A subdivision of the main band into close bands was obtained at 0.40 M Tris-HCl and two fractions were obtained, one containing rubredoxin and another containing flavodoxin (in 200 and 430 ml, respectively).

Rubredoxin. The volume of the fraction containing rubredoxin was brought up to 450 ml with distilled water and the rubredoxin was adsorbed onto another DEAE-cellulose column (3.5 × 10 cm). The protein was eluted with a discontinuous gradient of Tris-HCl and the rubredoxin (eluted at 0.35 M) was collected in a volume of 260 ml. The protein was directly applied to a silica column (Baker) (2.5 × 27 cm) equilibrated with 0.35 M Tris-HCl. Rubredoxin was not adsorbed and was eluted in a volume of 220 ml. It was then adsorbed onto a calcinated alumina column (2.5 × 10 cm) equilibrated with 0.35 M Tris-HCl; the column was washed with the same buffer and the protein was eluted with 20 mM phosphate buffer in a volume of 50 ml. The protein was judged to be pure from polyacrylamide gel analysis and isoelectric focussing (pI = 3.1). The ratio $A_{280}/A_{490} = 2.55$ is identical to other rubredoxins isolated from Desulphovibriones [23].

Flavodoxin. The fraction containing flavodoxin obtained after the second DEAE-cellulose column was brought to 900 ml with distilled water. It was adsorbed on another DEAE-cellulose column (2.5 × 10 cm) and immediately eluted with 0.5 M Tris-HCl buffer in a final volume of 15 ml. No gradient could be accomplished since reversible dissociation of the prostetic group was observed during the gradient elution. The flavodoxin was then passed through a Sephacryl S-200, Pharmacia Chemical Co., superfine column (5 × 100 cm). The main band of flavodoxin was made 0.3 M in NaCl and passed through a silica column (Baker) (2.5 × 20 cm) equilibrated with 0.3 M NaCl. The eluted flavodoxin was judged to be pure both from its spectrum ($A_{280}/A_{456} = 4.34$) and from polyacrylamide gel electrophoresis.

Ferredoxin. During the purification of ferredoxin we observed that the ferredoxin was very unstable (see Discussion).

Physiological activity

The results of the coupling activity of the flavodoxin and rubredoxin between hydrogenase and sulphite reduction are reported on Fig. 1.

Rubredoxin was inactive in the reaction but flavodoxin completely restored the activity of the crude extract. It was not possible to try the coupling activity experiments with ferredoxin due to the instability of the protein.

Electronic spectral data

In Table II we report the molar extinction coefficients of the principal absorption bands of *D. salexigens* rubredoxin and flavodoxin. The spectra are identical to other homologous proteins isolated from *Desulphovibrio* spp. [9,23].

Amino acid composition and molecular weight

Hydrolysis of several samples of rubredoxin with 6 N HCl under vacuum at

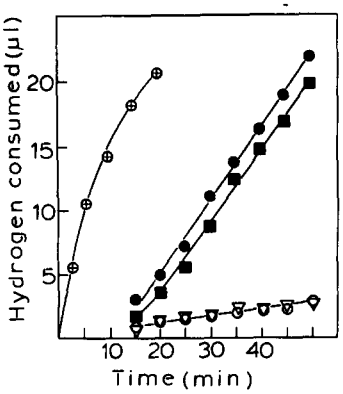

Fig. 1. The sulfite reduction pathway and the coupling effect of flavodoxin and rubredoxin in *D. salexigens* extracts. The reaction mixture contained: reductase preparation (22 mg protein) and pure hydrogenase (400 μg protein). The values of hydrogen uptake were measured: •, in the initial system, crude extract (control); ⊕, in the sulfite reductase-containing extract devoided of electron carriers plus methylviologen (100 nmol); ■, plus 100 nmol flavodoxin; ▽, plus 100 nmol rubredoxin, ○, and without added electron carriers. The scale for the observed stimulation in the presence of methylviologen must be multiplied by a factor of five.

110°C for 20 h followed by quantitative amino acid analysis, gave the amino acid content listed in Table III. The amino acid compositions of the other three *Desulphovibrio* spp. rubredoxins already reported are added for comparison. Acidic amino acids were preponderant and all the rubredoxins were devoid of histidine and arginine residues. All the rubredoxins contained 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 tetrahedral arrangement to the sulfur of cysteinyl residues. After filtration on a Sephadex G-50 column a molecular weight of 6000 was obtained. Calculations of molecular weight from the amino acid composition gave a value of 6149 including one atom of iron per molecule.

The amino acid composition of *D. salexigens* flavodoxin is presented in Table III. The protein contained 138 amino acids and was devoid of histidine like almost all the flavodoxins. The minimum molecular weight was calculated

TABLE II

MOLAR EXTINCTION COEFFICIENTS OF *D. SALEXIGENS* RUBREDOXIN AND FLAVODOXIN

Protein	λ (nm)	ε (M^{-1} · cm)
Rubredoxin (oxidized form)	280	15980
	355 (shoulder)	6980
	376	7750
	493	6270
	575 (shoulder)	2790
Flavodoxin (oxidized form)	273	48880
	374	10050
	456	11260

TABLE III

AMINO ACID COMPOSITION OF *D. SALEXIGENS* RUBREDOXIN AND FLAVODOXIN. COMPARISON WITH OTHER *DESULPHOVIBRIO* sp. HOMOLOGOUS PROTEINS

n.d., not determined.

Protein *Desulphovibrio* sp.	Rubredoxin			Flavodoxin		
	D. salexigens	*D. gigas*	*D. vulgaris*	*D. salexigens*	*D. gigas*	*D. vulgaris*
Lys	3	6	4	10	8	4
His	0	0	0	0	0	1
Arg	0	0	0	2	3	7
Trp	n.d.	1	1	n.d.	1	2
Asp	8	8	7	20	17	19
Thr	2	2	3	8	9	7
Ser	1	2	2	9	8	8
Glu	7	4	3	19	18	16
Pro	5	5	6	4	6	3
Gly	6	5	6	13	14–15	18
Ala	3	4	4	9	15	17
Cys (half)	4	4	4	3	5	4
Val	2	3	5	10	16	9
Met	1	1	1	1–2	2	1
Ile	0	2	0	9	5	9
Leu	2–3	1	1	10	14	12
Tyr	2	3	3	5	5	5
Phe	2	2	2	5	3	6
Total	48–49 *	53	52	137–138 *	149–150	148

* Without Trp.

to be 17 178. The value estimated by filtration on a Sephadex G-50 column was 16 500.

Discussion

The proteins reported in this work (a rubredoxin and a flavodoxin) together with the cytochrome c_3 (M_r 13 000) isolated by Drucker et al. [15] are the only electron carrier proteins purified so far from *D. salexigens*. The presence of desulfoviridin, a cytochrome reducible by ascorbic acid (*c*-553 type, as the one isolated from *D. vulgaris* [27]), an acidic cytochrome c_3 (M_r 26 000 type, as the one present in *D. gigas* [28]) and a 'blue protein' containing molybdenum (Le Gall, J., unpublished results) have also been shown to be present but their purification has not been completed yet. A similar 'blue protein' containing molybdenum and iron-sulfur centers was recently isolated from *D. africanus* [29]. The ferredoxin present in this organism is quite labile and sensitive towards oxygen, with consequent decrease of optical absorption at 405 nm, even in the presence of an argon atmosphere. No reactivity experiments could be performed and no iron content could be estimated. A second ferredoxin observed by Bruschi et al. [2] in *D. desulphuricans* (Norway 4) was shown to be unstable and could not be characterized. A two cluster (4 Fe, 4 S) ferredoxin isolated from the sulfur-reducing bacterium *Desulphuromonas*

acetoxidans showed also a rapid destruction of the chromophore by oxygen and storage of the protein under argon results in the bleaching of the active center [30]. So the proteins isolated from *D. salexigens* belong to the class of homologous proteins present in Desulfovibriones. The presence of unstable ferredoxins seems to be a common factor among these organisms, but high stable ferredoxin forms are present in *D. gigas* [31], *D. desulphuricans* (Norway 4) [2] and *D. vulgaris* (our unpublished results). Rubredoxin does not differ essentially from other *Desulphovibrio* spp. rubredoxins [23—25] in molecular weight, number of cysteines/molecule and amino acid composition. However, the protein was not totally reduced by ascorbic acid, in contrast to *D. gigas* rubredoxin. Also noteworthy is the strong interaction that we observe between rubredoxin and flavodoxin during the purification steps. Rubredoxin is easily separated in the first column of DE-52 in *D. gigas* and *D. vulgaris* purification steps. Some of the properties of *D. salexigens* flavodoxin differ from other *Desulphovibrio* spp. flavodoxins [9,26]. Purification steps using alumina or DEAE-cellulose (DE-52) reversibly dissociated the prostetic group (FMN) suggesting a greater lability of the prostetic group towards the apoprotein than for other flavodoxins isolated from desulfovibriones. An attempt to obtain the semiquinone [9] in deaerated solutions was not successful and only a small percentage of semiquinone form was obtained. Oxidation-reduction studies of *Desulphovibrio* rubredoxin and flavodoxin are under study by potentiometric titration followed by EPR measurements and comparison of mid-point redox potential values may indicate the degree of homology between these proteins. Flavodoxin is active in the sulfite reductase system of *D. gigas* [32] and *D. vulgaris* [33] as well as in the pyruvate dehydrogenase system [32]. Ferredoxin can replace flavodoxin in the above pathways [32,34] and the redox couples involved when these proteins mediate the indicated metabolic pathways have been discussed elsehwere [35]. In *D. salexigens*, flavodoxin mediates the electron transport in the reduction of sulfite by hydrogen in the presence of hydrogenase. Full stimulation of the activity of the enzymatic extract devoid of electron carriers could be observed from 50 nmol of flavodoxin (in the conditions of the assay, as shown in Fig. 1). A normal saturation curve of the activity versus concentration of the carrier could be obtained. Due to instability of the ferredoxin from this organism the participation of this electron carrier in the indicated pathway could not be tested.

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