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# Isolation and characterization of a rubredoxin and a flavodoxin from *Desulfovibrio desulfuricans* Berre-Eau

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A rubredoxin and a flavodoxin have been purified and characterized from soluble extracts of a sulfate-reducing bacterium able to grow with $N_2$ as the only nitrogen source: *Desulfovibrio desulfuricans* strain Berre-Eau. These two electron carriers have characteristics similar to homologous proteins found in other *Desulfovibrio* species (molecular mass, absorption spectrum, extinction coefficient and amino acid composition). In contrast to rubredoxin, flavodoxin mediates electron transfer in the reduction of sulfite to sulfide and in hydrogen evolution from pyruvate, when in the presence of hydrogenase

Rubredoxin; Flavodoxin; Electron transfer; Sulfate reduction; (*Desulfovibrio*)

## 1. INTRODUCTION

The sulfate-reducing bacteria are strict anaerobic microorganisms with an oxidative metabolism based on the utilization of sulfate and other sulfur anions as terminal electron acceptors [1,2]. The dissimilatory sulfate-reducers *Desulfovibrio desulfuricans* strains Berre-Eau and Berre-Sol have been isolated from enrichment cultures with $N_2$ as sole nitrogen source [3]. Recently, several strains and species of sulfate-reducing bacteria of genera *Desulfovibrio* and *Desulfotomaculum* were shown to be able to grow while fixing $N_2$ [4–6].

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*Abbreviations:* FMN, flavin adenine mononucleotide; HPLC, high-pressure liquid chromatography; PITC, phenylisothiocyanate; $\epsilon_M$, molar extinction coefficient

A rubredoxin and a ferredoxin with one [4Fe-4S] center have been isolated from *D. desulfuricans* Berre-Sol [7,8], but no study has been reported on the electron carrier system of *D. desulfuricans* Berre-Eau. Rubredoxins are the simplest and the smallest iron-sulfur proteins and have been isolated from 7 *Desulfovibrio* species [7,9–14] and one strain of sulfur-reducing bacteria *Desulfuromonas acetoxidans* [15]. Flavodoxins are a class of low-$M_r$ proteins containing FMN as prosthetic group which are not found in all of the *Desulfovibrio* species [13,16,17].

Here, we report on the purification and characterization of a rubredoxin and a flavodoxin from *D. desulfuricans* Berre-Eau.

## 2. MATERIALS AND METHODS

*D. desulfuricans* Berre-Eau (NCIB 8387) was grown at 37°C on the lactate-sulfate medium of Starkey [18], under non-nitrogen-fixing conditions. Wet cells (600 g) were suspended in 10 mM

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Tris-HCl buffer (pH 7.6) and ruptured by passing twice through a French press at 7000 lb/inch$^2$. The extract was centrifuged at 20000 × *g* for 45 min and the supernatant constituted the crude cell extract. Purification of proteins was performed from the soluble fraction by conventional chromatographic procedures; the rubredoxin was purified in four steps (DEAE-cellulose, DEAE-Biogel A, hydroxyapatite and DEAE-cellulose) and the flavodoxin in three (DEAE-cellulose, DEAE-Biogel A, and hydroxyapatite).

Protein homogeneities were checked by electrophoresis on 7% polyacrylamide gels with Tris-HCl glycine buffer at pH 8.9 [19]. The molecular masses of rubredoxin and flavodoxin were estimated by gel filtration on a Sephadex G-50 column (1.5 × 105 cm) according to Whitaker [20]. UV-visible absorption spectra were recorded using a Beckman model 35 spectrophotometer. EPR spectra were carried out on a Bruker ER-200 tt spectrometer equipped with an Oxford Instruments continuous helium flow cryostat interfaced to a Nicolett 1180 computer. Protein concentrations were determined by the method of Lowry et al. [21] with bovine serum albumin as standard.

Amino acid analyses were performed on an LKB amino acid analyzer. The protein samples were hydrolyzed under vacuum in 6 N HCl at 113°C for 24 h. The analysis of amino acid hydrolysates was performed after PITC derivatization and HPLC separation on a reversed-phase column $C_{18}$. The values of the molar extinction coefficients were calculated using protein concentrations determined from amino acid analysis of an aliquot of a protein solution for which the absorbance had been previously determined. As described in [22], manometric assays were utilized to determine the physiological activity of the two proteins following respectively at 37°C the reduction of fresh sodium

Table 1

Amino acid composition of rubredoxins isolated from 8 *Desulfovibrio* species

| | *D. salexigens* | *D. gigas* | *D. vulgaris* Hildenborough | *D. africanus* | *D. desulfuricans* Norway 4 | *D. desulfuricans* ATCC 27774 | *D. desulfuricans* Berre-Sol | *D. desulfuricans* Berre-Eau |
|---|---|---|---|---|---|---|---|---|
| Lys | 3 | 6 | 4 | 4 | 5 | 2 | 4 | 3 |
| His | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Arg | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Trp | n.d. | 1 | 1 | 3 | n.d. | 1 | 1 | 1 |
| Asp | 8 | 8 | 7 | 9 | 13 | 8 | 7 | 6 |
| Thr | 2 | 2 | 3 | 1 | 4 | 0 | 2 | 2 |
| Ser | 1 | 2 | 2 | 2 | 0 | 2 | 2 | 3 |
| Glu | 7 | 4 | 3 | 5 | 5 | 5 | 8 | 5 |
| Pro | 5 | 5 | 6 | 6 | 5 | 5 | 6–7 | 7 |
| Gly | 6 | 5 | 6 | 5 | 7 | 2 | 6 | 5 |
| Ala | 3 | 4 | 4 | 2 | 5 | 2 | 6 | 5 |
| Cys (half) | 4 | 4 | 4 | 4 | 4 | 5 | 4 | 4 |
| Val | 2 | 3 | 5 | 6 | 6 | 5 | 5 | 4 |
| Met | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Ile | 0 | 2 | 0 | 1 | 0 | 0 | 2 | 2 |
| Leu | 2–3 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| Tyr | 2 | 3 | 3 | 3 | 4 | 3 | 3 | 3 |
| Phe | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 2 |
| Total | 48–49 | 53 | 52 | 54 | 62 | 45 | 60–61 | 53 |
| References | [13] | [25] | [10] | [12] | [11] | [28] | [7] | this work |

n.d., not determined

sulfite (4 $\mu$mol) by hydrogen and $H_2$ production from sodium pyruvate (30 $\mu$mol). Pure periplasmic *D. gigas* hydrogenase was added in all cases to the enzymatic systems to ensure excess of this activity.

## 3. RESULTS AND DISCUSSION

The molecular mass of the rubredoxin from *D. desulfuricans* Berre-Eau was estimated to be 5700 Da by gel filtration. The nearest minimum molecular mass as determined by amino acid analysis was found to be 5691 Da for a total of 53 residues. The calculation of this value is based on the presence of one methionine residue and by adding one tryptophan. These two residues are present in such an amount in all the rubredoxins for which the amino acid sequences have been determined. The amino acid composition of this rubredoxin is shown in table 1 and compared with seven other rubredoxins from *Desulfovibrio* species. Acidic amino acids are predominant in these proteins and all the rubredoxins isolated so far lack arginine. The absorption spectrum of the oxidized *D. desulfuricans* Berre-Eau rubredoxin, shown in fig.1, is similar to the optical spectra

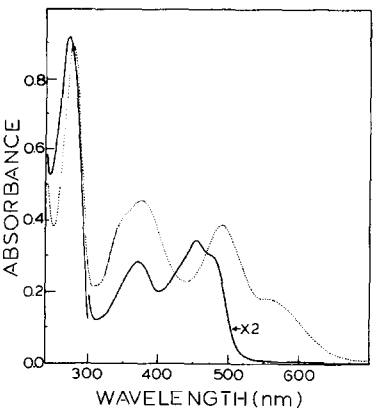


Fig.1. Absorption spectra of oxidized *D. desulfuricans* Berre-Eau flavodoxin (——) and rubredoxin (···). The protein concentrations for rubredoxin and flavodoxin were 50.8 and 34.9 $\mu$M, respectively.

Table 2

Amino acid composition of 4 flavodoxins from *Desulfovibrio* species

| | *D. gigas* | *D. vulgaris* Hildenborough | *D. salexigens* | *D. desulfuricans* Berre-Eau |
|---|---|---|---|---|
| Lys | 8 | 4 | 10 | 6 |
| His | 0 | 1 | 0 | 0 |
| Arg | 3 | 7 | 2 | 4 |
| Trp | 1 | 2 | n.d. | n.d. |
| Asp | 17 | 19 | 20 | 20 |
| Thr | 9 | 7 | 8 | 8 |
| Ser | 8 | 8 | 9 | 9 |
| Glu | 18 | 16 | 19 | 18 |
| Pro | 6 | 3 | 4 | 5 |
| Gly | 14–15 | 18 | 13 | 19 |
| Ala | 15 | 17 | 9 | 15 |
| Cys(half) | 5 | 4 | 3 | 7 |
| Val | 16 | 9 | 10 | 10 |
| Met | 2 | 1 | 1–2 | 2 |
| Ile | 5 | 9 | 9 | 6 |
| Leu | 14 | 12 | 10 | 14 |
| Tyr | 5 | 5 | 5 | 4 |
| Phe | 3 | 6 | 5 | 4 |
| Total | 149–150 | 148 | 137–138 | 151 |
| References | [17] | [17] | [13] | this work |

n.d., not determined

reported for rubredoxins from different *Desulfovibrio* species. The absorption maxima were at 278, 378 and 491 nm with molar extinction coefficients of 18521, 8396 and 6976 $M^{-1} \cdot cm^{-1}$, respectively (an error of ± 9% is estimated). Again, these values are similar to those obtained with other rubredoxins from *Desulfovibrio* and *Dm. acetoxidans* [9–15]. As for the *D. gigas* rubredoxin, the protein from *D. desulfuricans* Berre-Eau is completely reduced by ascorbate at pH 7.6. In contrast, the rubredoxins from *D. salexigens* British Guiana [13] and *D. vulgaris* Hildenborough (unpublished) are only partly reduced under the same conditions.

The oxidized form of rubredoxin shows an EPR spectrum which does not differ from that reported for other sulfate- and sulfur-reducing organisms [23]. High-spin ferric resonances were observed at *g* values 4.3 and 9.4 (below 20 K).

The amino acid composition of *D. desulfuricans* Berre-Eau flavodoxin is presented in table 2. The protein contains 151 residues, with a rather large proportion of acidic amino acids and is devoid of histidine, like almost all the flavodoxins (table 2). The minimum molecular mass was calculated to be 15801 Da and the value estimated by gel filtration was 15400 Da.

The absorption spectrum of *D. desulfuricans* Berre-Eau oxidized flavodoxin is shown in fig.1. The absorption maxima were at 274, 374 and 456 nm with molar extinction coefficients of 45835, 8460 and 10035 (an error of ± 10% is estimated) respectively and a shoulder is noticeable at 480 nm, identical to that obtained for *D. gigas* flavodoxin [16].

The results of the coupling effect of *D. desulfuricans* Berre-Eau rubredoxin and flavodoxin on sulfite reductase and pyruvate dehydrogenase activities are reported in table 3. Rubredoxin was inactive both in the coupling between hydrogenase and sulfite reductase and in the phosphoroclastic reaction. When compared with the endogenous activity of the control using acidic electron carrier-free extracts, the system containing the *D. desulfuricans* Berre-Eau flavodoxin exhibited significant stimulations for both sulfite reduction and $H_2$ evolution from pyruvate (table 3). There is an evident lack of specificity of *D. desulfuricans* Berre-Eau extract for the electron carrier isolated from the same strain mainly in the phosphoroclastic reaction. However, *D. vulgaris* Hildenborough and *Clostridium pasteurianum* flavodoxins are also active in the coupling between the pyruvate dehydrogenase and the hydrogenase (table 3).

Table 3

Coupling activity of *D. desulfuricans* Berre-Eau flavodoxin in the sulfite reductase activity and in the phosphoroclastic reaction

| Enzymatic extract | Sulfite reductase activity[a] | Phosphoroclastic reaction[b] |
|---|---|---|
| Crude extract | 9.30 | 7.90 |
| DEAE-cellulose extract | 1.10 | 1.35 |
| + *D. desulfuricans* Berre-Eau flavodoxin | 6.45 | 6.70 |
| + *D. vulgaris* Hildenborough flavodoxin | 4.95 | 6.10 |
| + *C. pasteurianum* flavodoxin | n.d. | 5.45 |
| + methyl viologen | 10.2 | n.d. |

[a] $H_2$ consumed in 20 min under the assay conditions
[b] $H_2$ evolved in 12 min under the assay conditions

Enzymatic activities were determined as described [22]. The crude extract and DEAE-cellulose-treated extract, prepared as in [22], contained 23 mg protein. Flavodoxins, 30 nmol (saturation conditions); methyl viologen, 100 nmol; n.d., not determined

The function of electron transfer proteins from *Desulfovibrio* species is far from being understood [24]. Here, we have reported the purification and characterization of a rubredoxin and a flavodoxin from *D. desulfuricans* Berre-Eau grown on a lactate-sulfate medium under non-nitrogen-fixing conditions. We have also isolated other proteins from this strain: two ferredoxins, a molybdenum-containing iron-sulfur protein, a low redox potential tetraheme cytochrome $c_3$ and a monohemic cytochrome *c*-553 [25]. As for other rubredoxins from strict anaerobic bacteria the physiological function of *D. desulfuricans* Berre-Eau rubredoxin is still unknown. The relatively high redox potential of *Desulfovibrio* rubredoxins (around 0 mV) [26] makes it difficult to place this electron carrier in the frame of the physiological reactions occurring during the metabolism of sulfate-reducing bacteria.

In the sulfate reducers of the genus *Desulfovibrio* flavodoxin may replace ferredoxin as electron carrier in both the pyruvate dehydrogenase system and sulfite reductase reaction [27]. The flavodoxin from *D. desulfuricans* Berre-Eau is also able, in the presence of hydrogenase, to mediate electron transport in the reduction of sulfite and in $H_2$ evolution from pyruvate. It will be interesting to test the specificity of the two ferredoxins from *D. desulfuricans* Berre-Eau in these two metabolic pathways.

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