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ISOLATION AND CHARACTERIZATION OF TWO RUBREDOXINS FROM *CLOSTRIDIUM THERMOACETICUM*

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Summary

Two rubredoxins with similar molecular weights (about 6000) have been purified from *Clostridium thermoaceticum*, a thermophile and strict anaerobe. They exhibit minor differences in several properties like elution pattern from DEAE-cellulose column, isoelectric point, amino acid composition, absorption and EPR spectra and redox potential. Their chemical and physical properties are similar to those of other rubredoxins from anaerobic microorganisms.

Introduction

Clostridium thermoaceticum, a strictly anaerobic and thermophilic microorganism, ferments sugars with acetate as the almost exclusive product. Part of the acetate is synthesized from CO_2 which serves as the acceptor of electrons generated during the fermentation [1,2]. It has been suggested that *C. thermoaceticum* may have electron transport phosphorylation in connection with the reduction of CO_2 to acetate [2]. This suggestion is based on exceptionally high growth yields [3] and on the presence of electron carriers such as cytochrome *b* and menaquinone [4] in *C. thermoaceticum*. Further support for the formation of energy during the reduction of CO_2 to acetate is the recent isolation of *Acetobacterium woodii* [5]. This organism grows autotrophically and generates energy by reducing CO_2 to acetate by the use of molecular hydrogen [6]. Enzyme studies indicate that in *A. woodii* acetate is synthesized from CO_2

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via a pathway similar to that found in *C. thermoaceticum* [7].

During fermentations of glucose and other sugars by *C. thermoaceticum* electrons are generated in the form of NADH and reduced ferredoxin. However, the reduction of CO_2 to acetate requires NADPH and most likely a reduced flavin. Therefore, a transfer must occur of electrons from NADH and reduced ferredoxin to NADP and a flavin [8]. A ferredoxin in *C. thermoaceticum* containing only one (4 Fe—4 S) cluster has been described [9] and a reduced ferredoxin-NADP reductase has been demonstrated [9,10]. In our continuing effort to elucidate the electron transfer processes in *C. thermoacticum* we have found that it contains two different rubredoxins. In this report we describe the purification and a number of physical and chemical properties of the two rubredoxins.

Materials and Methods

C. thermoaceticum (DSM 521) was grown at 58°C under 100% CO_2 on a glucose medium described previously [9]. Cells were stored at −20°C for several weeks until being used.

Purification of rubredoxin 1 and rubredoxin 2

The purification was performed at 4°C and with Tris-HCl buffer at pH 7.6. The results of a purification are summarized in Table I.

Preparation of cell-free extract. 1 kg of frozen cells were thawed and suspended in 1 l of 0.05 M Tris-HCl. The suspension was passed twice through a Manton-Gaulin homogenizer. The pH of the homogenized suspension was adjusted to 7.6 with NH_4OH. After addition of 1 mg each of deoxyribonuclease and ribonuclease the suspension was left for 15 min before it was centrifuged at 40 000 × *g* for 40 min to obtain a dark-brown supernatant extract.

Step I. DEAE-cellulose, column 1. The extract was applied to a Whatman DE-23 cellulose column (3 × 23 cm) previously equilibrated with 0.05 M Tris-HCl. A dark band of adsorbed proteins formed at the top of the column which was washed with 500 ml of 0.05 M Tris-HCl. The proteins were subsequently eluted with a linear NaCl gradient consisting of 1.8 l of 0.05 M Tris-HCl containing 0.1 M NaCl and 1.8 l of 0.05 M Tris-HCl with 0.7 M NaCl. Fractions of 18 ml were collected. Those fractions having an absorbance at 490 nm higher than 0.05 and which eluted between 0.2 M and 0.25 M NaCl, contained the two rubredoxins.

Step II. Sephadex G-75 column. The rubredoxin fractions from step I were combined and the solution was concentrated to about 20 ml by ultrafiltration using a Diaflo membrane UM 05 (Amicon Corporation, Lexington, MA). The solution was applied to a Sephadex G-75 column (2.5 × 85 cm) (Pharmacia Fine Chemicals), which had been equilibrated with 0.05 M Tris-HCl. The column was eluted with the same buffer and fractions of 10 ml were collected. A brownish-yellow band eluted first. It was followed by a red band, and a slower moving bright-yellow band. The red-colored band containing the rubredoxins, eluted in fractions 33—38 which were combined for the next step.

Step III. DEAE-cellulose, column 2. The rubredoxins of step II were adsorbed on a Whatman DE-23 column (2 × 36 cm) previously equilibrated

with 0.05 M Tris-HCl. The column was first washed with 150 ml of 0.05 M Tris-HCl, and then elution was performed with a linear NaCl gradient consisting of 500 ml of 0.05 M Tris-HCl, 0.2 M NaCl and 500 ml of 0.05 M Tris-HCl, 0.4 M NaCl. Fractions of 5 ml were collected. The adsorbed rubredoxins, which initially formed a red band on the top portion of the column, split into two red bands during the elution. The fast-moving band, designated rubredoxin 2, was eluted at NaCl concentrations between 0.25 M and 0.3 M. The slower moving band, designated rubredoxin 1 was eluted between 0.36 M and 0.39 M NaCl.

Step IV. Ultrogel ACA-54 column. Two columns (1.5 × 85 cm) of Ultrogel ACA-54 were connected in tandem and equilibrated with 0.1 M Tris-HCl. Rubredoxin 1 and rubredoxin 2 were individually concentrated by ultrafiltration using Diaflow membrane UM 05, and separately applied to the columns. The columns were eluted with 0.1 M Tris-HCl and fractions of 2 ml were collected. Fractions containing rubredoxin were scanned between 700 nm to 240 nm using a Beckman Acta CV spectrophotometer and ratios of A_{280}/A_{490} were calculated. Rubredoxin 1 fractions with A_{280}/A_{490} values of 2.78—2.25 were combined. The combined fractions had a ratio of 2.56. Rubredoxin 2 fractions with A_{280}/A_{490} values between 5.63 and 3.67 were combined to give a fraction with the ratio of 4.08.

Step V. Sephadex G-50 column. The rubredoxins were further purified by passing the concentrated fractions from step IV through a superfine Sephadex G-50 column (1.5 × 85 cm) with 0.05 M Tris-HCl, containing 0.1 M NaCl as solvent. The purification of rubredoxin 1 obtained in this step was marginal (the A_{280}/A_{490} ratio decreased from 2.56 to 2.25) and the step was normally not carried out. However, the purification of rubredoxin 2 was substantial (the A_{280}/A_{490} ratio decreased from 4.08 to 3.25).

Analytical procedures

Absorption spectra were recorded at room temperature on a Beckman Spectrophotometer Acta CV. Molecular weight determinations were done by gel filtration on the superfine Sephadex G-50 column of purification step V according to the method of Andrew [11] or by calculation from sedimentation equilibria. Sedimentation equilibrium and sedimentation velocity experiments were done with a Beckman Spinco Model E. The partial specific volume was determined according to Edelstein and Schachman [12]. A synthetic boundary cell was used in the sedimentation velocity experiments [13]. Analytical polyacrylamide gel discontinuous electrophoresis was performed according to the methods of Brewer and Ashworth [14] and Gabriel [15]. Isoelectric points were determined by using a LKB 8101 electrofocusing column (110 ml) according to the Instruction Manual supplied by LKB Products Inc. Amino acid analyses were performed with Beckman Model 120C automatic amino acid analyzer on salt-free protein samples hydrolyzed in evacuated and sealed tubes with 6 N HCl for 24, 48 and 72 h. Cystine and cysteine were determined as cysteic acid by oxidation with performic acid prior to acid hydrolysis [16]. Tryptophan was estimated after hydrolysis in the presence of 2% thioglycolic acid [17]. Iron was determined according to the method of Massey [18] and inorganic sulfide according to Siegel [19]. Protein concentrations were deter-

mined according to Lowry et al. [20] using human serum albumin as a standard or calculated on the basis of amino acid compositions and molecular weights [21]. EPR spectroscopy was performed as previously described [22]. EPR conditions are given in figure legends. Redox potentials at 25°C of oxidized, anaerobic rubredoxins 1 and 2 were determined using the microcoulometric method of Watt [23] with the rubredoxin dissolved in 0.05 M Tris-HCl, pH 7.6, and 0.1 M NaCl.

Results

Purification of rubredoxin 1 and rubredoxin 2

Since a biochemical assay method for anaerobic type of rubredoxin does not exist [24], the purification of the rubredoxins was followed by the characteristic absorption at 490 nm, the line shapes of the spectra, and the ratio of A_{280}/A_{490}.

As is shown in Table I the two rubredoxins in *C. thermoaceticum* copurified through step II. However, they were clearly separated in step III as is demonstrated in Fig. 1. From several preparations, it was found that the ratio of the amounts of rubredoxin 1 to rubredoxin 2 was fairly constant; it appeared to be about 3 to 1. The purification procedure outlined in this paper for the rubredoxins may not be the most efficient. The procedure is designed to effectively utilize the cell material for preparation of many enzymes and other proteins from *C. thermoaceticum* [25]. Thus the protein fraction, which does not adsorb on the DEAE-cellulose column column 1 (step I) contains the tetrahydrofolate-dependent enzymes, that are involved in the synthesis of acetate from CO_2. From step I ferredoxin [9] and other acidic proteins are also recovered.

Properties of rubredoxin 1 and rubredoxin 2

The properties, as discussed below, of rubredoxin 1 and rubredoxin 2 are listed in Table II.

Gel electrophoresis and isoelectric focusing. The purified rubredoxins were

TABLE I

PURIFICATION OF RUBREDOXINS 1 AND 2 FROM 934 g OF WET CELLS OF *C. THERMOACETICUM*

Purification steps	Protein (mg)	Volume (ml)	Total A_{490} (A_{490} X volume)	A_{280}/A_{490}
I DEAE-cellulose column 1	1463	585	102.4	20
II Sephadex G-75 column	210	50	36.1	11.5
III DEAE-cellulose column 2				
Rubredoxin 1	35	125	25.5	2.99
Rubredoxin 2	25.5	100	5.2	10.77
IV Ultrogel ACA-54 column				
Rubredoxin 1	18.7	15.5	16.2	2.56
Rubredoxin 2	6.2	14	3.9	4.08
V Sephadex G-50 column				
Rubredoxin 2	2.8	5	2.4	3.24

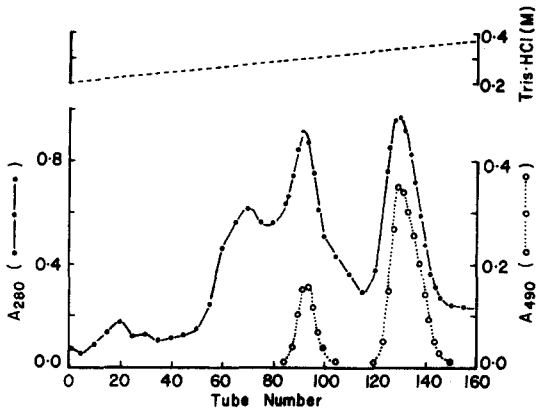

Fig. 1. Separation of rubredoxins 1 and 2 on a DEAE-cellulose column (step III). Fractions of 5 ml were collected and analyzed for absorbance at 280 nm and 490 nm. Fractions 86—105 and 122—146 were pooled separately; they were disignated as rubredoxin 2 and rubredoxin 1, respectively.

examined in a 7.5% polyacrylamide gel electrophoresis system. Individually as well as in a mixture they moved as a single red band which had a mobility close to that of the tracking dye. Staining for protein revealed only one band which coincided with the red rubredoxin band. In a 15% polyacrylamide gel electrophoresis system a mixture of rubredoxin 1 and rubredoxin 2 separated as they moved toward the anode with different R_F values of 0.94 and 0.85, respectively.

Rubredoxin 1 and rubredoxin 2 were combined and electrofocused in a LKB 8101 column (pH range 2.5—4). Two sharp red bands, which were very close together, formed at the top of the column after about 1 h of electrofocusing. The bands then migrated toward the anode (at the bottom of the column).

TABLE II

PROPERTIES OF RUBREDOXIN 1 AND RUBREDOXIN 2 FROM *C. THERMOACETICUM*

n.d., not determined.

Porperties	Rubredoxin 1	Rubredoxin 2
Molecular weight		
Gel filtration	7 400	6 000
Sedimentation equilibrium	6 165 ± 341	5 781
Amino acid analysis	6 709	6 095
Partial specific volume (cm^3/g)	0.68—0.70	0.71 **
$s_{20,w}$ (Svedberg units)	1.0875 *	n.d.
Isoelectric point	3.07	3.32
Redox potential (mV)	−27	+20
Iron content (gatom/mol protein)	0.938	0.855
Inorganic sulfur (gatom/mol protein)	0	0
Molar extinction coefficient ($M^{-1} \cdot cm^{-1}$)		
At 490 nm	6 280	5 150
At 375 nm	7 150	5 990
At 280 nm	14 750	16 700

* Approximately 5 mg/ml of protein was used in synthetic boundary ultracentrifugation.

** Calculated from amino acid composition.

After 48 h of electrofocusing, the p*I* values measured for rubredoxin 1 and rubredoxin 2 were 3.07 and 3.32, respectively. The p*I* values obtained for the rubredoxins are somewhat uncertain because the proteins moved continuously toward the anode during the experiment. However, the different charge-bearing properties of the rubredoxins were clearly demonstrated.

Molecular weights. Molecular weights of 7400 for rubredoxin 1 and of 6000 for rubredoxin 2 were obtained by gel filtration on superfine Sephadex G-50. From sedimentation equilibrium experiments the partial specific volume for rubredoxin 1 was found to be 0.68—0.70 cm^3/g. The average molecular weight calculated from these data, was 6165 ± 341. The partial specific volume for rubredoxin 2 was not determined but by using a value of 0.71 cm^3/g, calculated from the amino acid composition, a molecular weight of 5681 was obtained. From a sedimentation velocity experiment a $s_{20,w}$ value of 1.0875 S was found for rubredoxin 1. A $s_{20,w}$ value for rubredoxin 2 was not obtained.

Amino acid compositions. Results of amino acid analyses are given in Table III. Both rubredoxins, identically with rubredoxins isolated from other anaerobic bacteria [24,26], are devoid of histidine and arginine. Rubredoxin 1 in comparison with rubredoxin 2 contains two additional glutamic acid residues and two less lysine residues. This may account for the more acidic behavior of rubredoxin 1 on DEAE-cellulose column and the difference in p*I* values of the two rubredoxins. Four cysteine residues were found in rubredoxin 2, and six in rubredoxin 1. The latter number of cysteine residues is unusually high. All other anaerobic rubredoxins, so far examined, contain only four cysteine residues, which are involved in binding the sole iron atom found in these rubredoxins.

TABLE III

AMINO ACID COMPOSITION OF RUBREDOXINS 1 AND 2 FROM *C. THERMOACETICUM*

	Residues found per molecule of	
	Rubredoxin 1	Rubredoxin 2
Tryptophan	1	2
Lysine	2	4
Histidine	0	0
Arginine	0	0
Aspartate	7	7
Threonine	3	2
Serine	2	1
Glutamate	7	5
Proline	9	6
Glycine	6	9
Alanine	6	5
Cysteine	6	4
Valine	3	2
Methionine	1	1
Isoleucine	1	1
Leucine	2	3
Tyrosine	3	2
Phenylalanine	2	2
Total residues	61	56

Iron content. Iron analyses revealed that rubredoxin 1 contains 0.938 mol of iron and rubredoxin 2, 0.855 mol of iron per mol of protein. It should be mentioned that rubredoxin 2 of several preparations contained less iron than the amount given. We believe this is due to a loss of iron during the purification procedure. If so it is an indication that rubredoxin 2 is less stable than rubredoxin 1, with wich iron analyses always gave close to one. Inorganic sulfide could not be demonstrated in the rubredoxins. We believe, that our data indicate, that each rubredoxin contains 1 g atom of iron per mol of protein.

Absorption spectra. The absorption spectra of rubredoxins 1 and 2 in the oxidized forms are shown in Fig. 2. The spectra are typical for rubredoxins from anaerobes [24]. Both rubredoxins exhibit absorption maxima at 275, 375 and 490 nm, and a shoulder around 585 nm. The molar coefficients are given in Table II. Although the spectra of two rubredoxin are very much alike, there are some differences particularly in the 350 nm region and at 275 nm. The larger absorption at 275 nm by rubredoxin 2 in comparison to rubredoxin 1 is a reflection of higher tryptophan content in the former. On reduction either by sodium dithionite or by NADH or NADPH together with a catalytic amount of a rubredoxin reductase isolated from *C. thermoaceticum* [8,27] both rubredoxins are completely bleached (spectra not shown), which is evidenced by the disappearance of the 490 nm absorption peak.

EPR spectroscopy. The oxidizied state of rubredoxins 1 and 2 at 12 K are shown in Fig. 3. Both rubredoxins show narrow EPR absorption at $g = 9.34$ and 4.32 and broader absorptions at $g = 4.74$ and below $g = 4.30$ due to high-spin ferric ion in a rhombic field. Rubredoxin 1 differs from rubredoxin 2 in subtle line shapes but not in g values. EPR double integrations for both rubredoxins according to Aasa and Vanngard [28] using Cu(II)-EDTA as standard recovered 95% of the EPR spins compared to the chemically determined iron contents. It is clear that rubredoxin I which differes in several criteria form rubredoxin 2 also expresses subtle differences in the oxidized ferric EPR spectrum. On reduction with either sodium dithionite or with NAD(P)H and the flavoprotein rubredoxin reductase [8,27] all EPR resonances for both

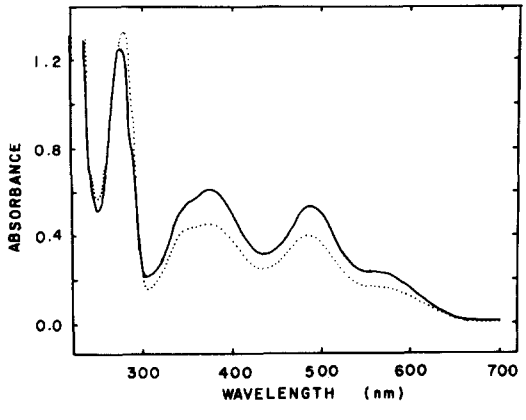

Fig. 2. Absorption spectra of oxidized rubredoxins 1 and 2 in 100 mM Tris-HCl, pH 7.6, at 25°C. ———, rubredoxin 1, 570 μg/ml. . . . . ., rubredoxin 2, 550 μg/ml.

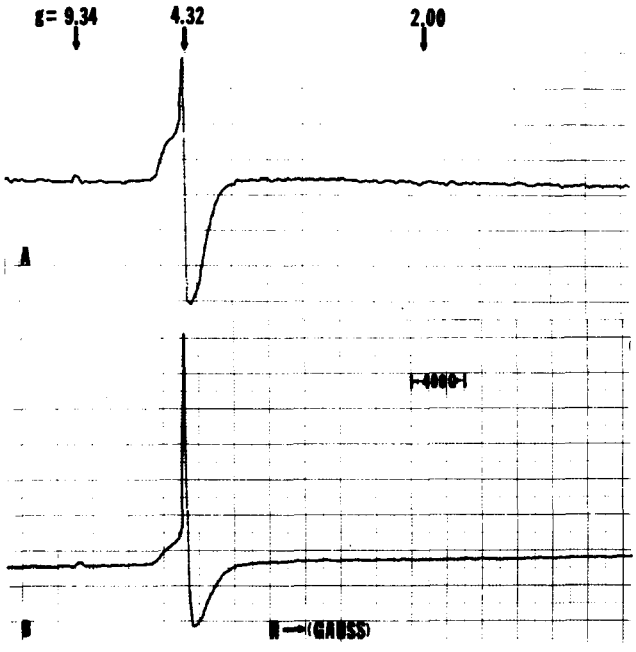

Fig. 3. EPR spectra of rubredoxin 1 (A) and rubredoxin 2 (B) in 100 mM Tris-HCl, pH 7.6, at 12 K. EPR conditions: modulation amplitude, 5.9 G; scanning rate, 2500 G/min; time constant, 0.1 s; microwave power, 2 mW; microwave frequency, 9.189 GHz; (in frequency matched EPR tubes) (A) rubredoxin 1, 6.7 mg/ml and (B) rubredoxin 2, 4.4 mg/ml.

rubredoxins completely disappear (not shown). Peisach et al. [29] have reported that the $g = 9$ signal arises from a ground-state transfer while that in the $g = 4.3$ region derived from an excited-state transition. The EPR resonances of the two rubredoxins in this study are strikingly similar to those reported for the rubredoxin from *Pseudomonas olevorans* [29].

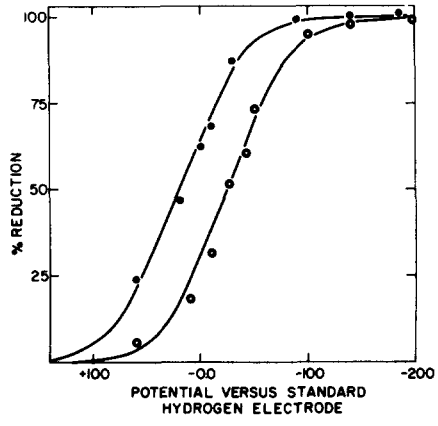

Fig. 4. Reductive titrations of anaerobic, oxidized rubredoxin 1 (○) and rubredoxin 2 (●) at pH 7.6 and 25° C. ———, theoretical curves calculated for $n = 1$ reactions with $E_2^\circ = +20$ mV and $E_1^\circ = -27$ mV for rubredoxin 2 and rubredoxin 1, respectively. ○ and ●, experimentally measured values at potentials relative to the standard hydrogen electrode ($E = 0$ and pH = 0).

Redox potentials. Fig. 4 shows the determinations of the redox potentials for rubredoxins 1 and 2. The values are −27 mV for rubredoxin 1 and 20 mV for rubredoxin 2, with reference to the standard hydrogen electrode. The *n* values obtained from the respective curves are both near 1.0 indicating a one-electron reduction of the proteins. The redox potentials are similar to those (−61 to +11 mV) reported for rubredoxins isolated from other sources [30–32].

Biological activity. Rubredoxins 1 and 2 were unable to replace ferredoxin in the pyruvate ferredoxin oxidoreductase system of *C. thermoaceticum* [9]. However, both rubredoxins accept electrons from NADH or NADPH in a reaction catalyzed by NAD(P)H-rubredoxin oxidoreductase which has been purified from *C. thermoaceticum* [8,27]. In this reaction the rate of reduction of rubredoxin 1 is faster than that of the reduction of rubredoxin 2. Besides serving as the electron acceptor in this reductase reaction, the role of the rubredoxins in the metabolism of *C. thermoaceticum* remains unknown.

Discussion

This report is the first of the presence of two different rubredoxins with similar molecular weights (about 6000) in an anaerobic bacterium. The presence of two rubredoxins, (1 Fe)- and (2 Fe)-rubredoxin, in *P. oleovorans* has been reported. These are derived from the same polypeptide chain, which has a molecular weight of 19 000, and which binds either 1 or 2 g atom(s) of iron. Furthermore, the (1 Fe)-rubredoxin is readily converted to the (2 Fe)-rubredoxin [33]. In contrast, rubredoxins 1 and 2 from *C. thermoaceticum* are two distinctive proteins. They have been found to be different in properties such as in elution pattern from a DEAE-cellulose column, mobility in polyacrylamide gel electrophoresis, isoelectric point, amino acid composition, absorption spectrum, EPR spectrum, redox potential, and activity in a NAD(P)H-rubredoxin reductase-catalyzed reaction.

The chemical and physical properties of the rubredoxins from *C. thermoaceticum* are similar to those of other anaerobic rubredoxins [26,30,31,34–38]. They contain 1 g atom of iron and four cysteine residues (in rubredoxin 1, possibly six residues), providing one iron-binding site. They undergo one-electron redox reactions. They have no histidine, arginine and inorganic sulfide. Their molecular weights and spectral properties (visible, ultraviolet and EPR) resemble those of rubredoxins of other anaerobic bacteria.

Similar to rubredoxins from other anaerobic microorganisms, a specific physiological role for the rubredoxins in *C. thermoaceticum* has not been found. In *Clostridium pasteurianum*, rubredoxin is reduced by H_2 in the presence of hydrogenase of this organism [30]. Since *C. thermoaceticum* lacks hydrogenase, rubredoxins 1 and 2 are not expected to share a role in this reaction. Rubredoxin has been reported to be reduced by NADPH and spinach ferredoxin-NADP reductase [24]. A NADH-rubredoxin reductase has been purified from *Desulfovibrio gigas* [39]. Similarly a rubredoxin reductase has been purified from *C. thermoaceticum* [8,27]. It catalyzes the electron transfer from NADH or NADPH to oxidized form of rubredoxin 1 and 2. However, how the reduced rubredoxins are further metabolized has not been established.

In the aerobe *P. oleovorans*, reduced rubredoxin, functions in an ω-hydroxylation reaction involving fatty acids and hydrocarbons [33]. A similar role has been demonstrated for rubredoxin in *Acinobacter calcoaceticus* [40].

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