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Purification and Characterization of Cytochrome c_3, Ferredoxin, and Rubredoxin Isolated from *Desulfovibrio desulfuricans* Norway

MIREILLE BRUSCHI,* CLAUDE E. HATCHIKIAN, LUDMILLA A. GOLOVLEVA,[1] AND JEAN LE GALL

Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, 13274 Marseille Cedex 2, France

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Different electron carriers of the non-desulfoviridin-containing, sulfate-reducing bacterium *Desulfovibrio desulfuricans* (Norway strain) have been studied. Two nonheme iron proteins, ferredoxin and rubredoxin, have been purified. This ferredoxin contains four atoms of non-heme iron and acid-labile sulfur and six residues of cysteine per molecule. Its amino acid composition suggests that it is homologous with the other *Desulfovibrio* ferredoxins. The rubredoxin is also an acidic protein of 6,000 molecular weight and contains one atom of iron and four cysteine residues per molecule. The amino acid composition and molecular weight of the cytochrome c_3 from *D. desulfuricans* (strain Norway 4) are reported. Its spectral properties are very similar to those of the other cytochromes c_3 (molecular weight, 13,000) of *Desulfovibrio* and show that it contains four hemes per molecule. This cytochrome has a very low redox potential and acts as a carrier in the coupling of hydrogenase and thiosulfate reductase in extracts of *Desulfovibrio gigas* and *Desulfovibrio desulfuricans* (Norway strain) in contrast to *D. gigas* cytochrome c_3 (molecular weight, 13,000). A comparison of the activities of the cytochrome c_3 (molecular weight, 13,000) of *D. gigas* and that of *D. desulfuricans* in this reaction suggests that these homologous proteins can have different specificity in the electron transfer chain of these bacteria.

The classification of sulfate-reducing bacteria within the genus *Desulfovibrio* is based upon several properties: absence of sporulation, desoxyribonucleic acid composition, growth on certain carbon sources, and, particularly, presence of desulfoviridin and cytochrome c_3 (molecular weight [MW], 13,000) (22).

The Norway strain of *Desulfovibrio desulfuricans* (19), although in most respects typically a *Desulfovibrio*, is characterized by an absence of desulfoviridin. It has recently been demonstrated by Jin-Po Lee et al. (14) that another pigment, desulforubidin, replaces desulfoviridin as a sulfite reductase for this organism. It was thus of taxonomic and biochemical interest to investigate the electron carrier content of the Norway strain to see if other differences could be found between the latter and other strains of *Desulfovibrio* such as *D. vulgaris*, strain Hildenborough, *D. gigas*, and *D. desulfuricans* strain E1 Agheila Z, all known to contain desulfoviridin as a sulfite reductase.

[1] Present address: Institute of Biochemistry and Physiology of Microorganisms, Academy of Sciences of U.S.S.R., Moscow, U.S.S.R.

In this work we report the purification from the Norway strain of several electron carrier proteins, together with some of their physicochemical properties and their reactivity toward thiosulfate reduction.

MATERIALS AND METHODS

Organism and growth conditions. *D. desulfuricans* (strain Norway 4, National Collection of Industrial Bacteria no. 8310) was grown in a lactate-sulfate medium and harvested as published previously (16).

Enzyme assays. A manometric assay was utilized for determination of the biological activity of the purified electron carriers utilizing the reduction of thiosulfate with H_2. The main compartment of each manometric vessel contained 0.1 M phosphate buffer (pH 7.0), the carrier, hydrogenase, and the reductase preparation in a final volume of 3.0 ml. A 15-μmol amount of sodium thiosulfate, freshly prepared, was added from a side arm after preincubation of the flask for 30 min under H_2 at 37°C. The center well contained 0.1 ml of 10% $CdCl_2$ and 0.1 ml of 10 N NaOH. Thiosulfate reductase activity was measured by following the initial rates of hydrogen utilization. The thiosulfate reductase-hydrogenase-

containing extracts, devoid of electron carriers, were prepared from *D. gigas* and *D. desulfuricans* (Norway strain) as already reported (11).

However, as the hydrogenase activity of *D. desulfuricans* appeared to be very sensitive to ammonium sulfate fractionation, pure hydrogenase prepared from *D. gigas* by the method of Bell (G.R. Bell, Ph.D. thesis, University of Georgia, Athens, 1973) and exhibiting a specific activity of 30 μmol of H_2 consumed/min per mg was added in all cases to the systems to insure an excess of this activity. Ferredoxin and cytochromes c_3 (MW 13,000) and c_3 (MW 26,000) from *D. gigas*, used to study the electron transfer between hydrogenase and thiosulfate reductase, were purified as described previously (4, 15, 16).

Amino acid analysis. Protein samples were hydrolyzed for 18, 20, and 48 h at 110°C in 6.0 M HCl in evacuated, sealed tubes according to the method of Moore and Stein (20). The average was calculated from at least five analyses. The amino acid composition was determined with a Beckman Multichrom amino acid analyzer. The values for serine and threonine have been corrected after extrapolation to zero hydrolysis time. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs (12).

Molar extinction coefficients. The molar extinction coefficients of the proteins were obtained by measuring the values of the optical densities of their absorption maxima of a solution of known protein concentration. The molarity of the used solutions was calculated from amino acid analysis.

Iron determination. Non-heme (ferrous iron) was determined by the *o*-phenanthroline method of Harvey et al. (10), as modified by Lovenberg et al. (17). The absorption of the complex of the ferrous iron and *o*-phenanthroline was measured at 512 nm.

Inorganic sulfide determination. The inorganic sulfide content of ferredoxin was determined by using an adaptation of the method of Fogo and Popowski (8) by Lovenberg et al. (17) A 1.3-ml amount of 1% zinc acetate and 0.05 ml of 12% sodium hydroxide were added to the sample to be analyzed. A 0.25-ml portion of 0.5% *N,N*-dimethylenephenylenediamine hydrochloride and 0.05 ml of 0.023 M ferric chloride were then added to each tube. After 20 min, 0.85 ml of water was added, and the absorbance was determined at 670 nm (A_{670}). The tubes were stoppered and open only for the time necessary for each addition of reagents.

Molecular weight determination. The molecular weights were estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, using the procedure of Weber and Osborn (27), and by molecular filtration on a Sephadex column.

Spectral studies. The visible and ultraviolet spectra were determined with a Cary 14 spectrophotometer. A spectrophotometric method was also used for the monitoring of the purification of the electron carrier proteins, using a specific absorption band: 552 nm for cytochrome c_3, 390 nm for ferredoxin, and 490 nm for rubredoxin.

RESULTS

Protein purification. (i) Preparation of extracts. In these protein fractionation procedures, unless otherwise noted, all buffers were at pH 7.6 and all operations were performed at +4°C.

For preparation of the crude extract, 50 mM tris(hydroxymethyl)aminomethane (Tris)hydrochloride was added to 1,069 g (wet weight) of bacterial paste to bring the volume to 1,600 ml. After addition of a few deoxyribonuclease crystals, the cell suspension was passed twice through a Gaulin homogenizer. The resulting extract was centrifuged for 2 h at 39,000 × *g*, and the pellet was discarded.

(ii) Cytochromes. The clear extract was stirred overnight with 200 ml of silica gel, and the gel, to which the cytochromes were adsorbed, was separated by decantation. The decanted extract was saved for acidic protein purification (see below). The silica gel was washed several times with 10 volumes of 10 mM Tris-hydrochloride buffer until the supernatant was free of protein. The cytochromes were then eluted from the gel with 1 M K_2HPO_4, giving 650 ml of extract, which was dialyzed overnight against 10 liters of distilled water. The cytochromes were then adsorbed on a calcinated alumina column (4 by 14 cm) equilibrated with 10 mM Tris-hydrochloride buffer. The column was washed with 100 ml of the same buffer, and the protein was eluted with 1 M potassium phosphate buffer, giving 110 ml of extract.

After dialysis against 10 liters of distilled water, the cytochrome fraction was lyophilized, resuspended in 50 ml of 10 mM Tris-hydrochloride buffer, and placed on a Sephadex G-75 column (4 by 200 cm). Three cytochrome bands were separated: the first (with the largest molecular weight) was not purified further; the second contained cytochrome c_3; and the third (with the smallest molecular weight) contained a cytochrome presenting a "split α" band upon reduction with ascorbic acid. This cytochrome was called cytochrome $c_{555(550)}$ by analogy with the cytochrome found by Shioi et al. (24) in *Chloropseudomonas ethylica*.

(iii) Purification of cytochrome c_3. The cytochrome c_3 fraction from the G-75 column had a volume of 394 ml and a purity coefficient, defined as (A_{553} [red.] − A_{570} [red.])/A_{280} [ox.], of 3.03. The cytochrome was adsorbed on an Amberlite CG-50 type II column (4 by 15 cm) equilibrated with 10 mM Tris-hydrochloride buffer and eluted stepwise with 50 ml each of 50, 100, 200, 300, and 500 mM Tris-hydrochloride buffer. The cytochrome was eluted in a 110-ml fraction,

and its purity coefficient was 3.10. Finally, the cytochrome was filtered through a G-50 Sephadex column (5 by 200 cm) equilibrated with 10 mM Tris-hydrochloride buffer. The cytochrome-containing fraction had a volume of 360 ml, and the purity coefficient was 3.30, higher than any coefficient for cytochrome c_3 found so far. After dialysis against 20 liters of distilled water, the cytochrome was lyophilized. The yield was equal to 550 mg of cytochrome c_3, judged to be pure by polyacrylamide gel electrophoresis.

(iv) Purification of the non-heme iron proteins. A settled volume of diethylaminoethyl (DEAE)-cellulose, equal to 300 ml of DEAE-cellulose, was added to the extract obtained after the silica gel treatment used to remove the cytochromes, and the mixture was stirred overnight. The DEAE-cellulose was then decanted and washed several times with 10 mM Tris-hydrochloride buffer. The adsorbed acidic proteins were eluted with 1 M Tris-hydrochloride buffer, giving a volume of 480 ml. This extract was dialyzed against 10 liters of distilled water and placed on a DEAE-cellulose column (4 by 30 cm) equilibrated with 10 mM Tris-hydrochloride. The column was washed with 200 ml of 150 mM Tris-hydrochloride, and the protein was eluted with 1 M Tris-hydrochloride in a volume of 120 ml. The volume of the extract was brought to 1,200 ml by addition of distilled water, and the proteins were adsorbed on a second DEAE-cellulose column (4 by 25 cm) and eluted with a linear gradiant (2,000 ml) from 200 mM to 1 M Tris-hydrochloride. The first colored protein to be eluted was found to be rubredoxin, and two other bands of more acidic proteins had the typical absorption spectrum of ferredoxin. In contrast to all *Desulfovibrio* species we have studied so far, no flavodoxin band was found, and the ferredoxin content of the extract was clearly higher in the Norway strain than in the others.

(a) Purification of the rubredoxin. After the second DEAE-cellulose column, the rubredoxin was contained in a volume of 160 ml. The volume of the extract was brought to 350 ml with distilled water, and the rubredoxin was adsorbed on a DEAE-cellulose column (4 by 30 cm). The proteins were eluted with a linear gradiant (1,200 ml) of 250 to 500 mM Tris-hydrochloride, and the rubredoxin was collected in a volume of 220 ml. The extract was directly applied to a silica gel column (2.5 by 30 cm) equilibrated with 350 mM Tris-hydrochloride. The rubredoxin migrated slowly in the column and was eluted in a volume of 180 ml. It was then adsorbed on a calcinated alumina column (2.5 by 10 cm) equilibrated with 350 mM Tris-hydrochloride; the column was washed with 50 ml of the same buffer, and the rubredoxin was eluted with 10 mM Tris-hydrochloride in a volume of 30 ml. The protein was judged to be pure both from its spectrum ($A_{280}/A_{490} = 2.35$, identical to other rubredoxins from desulfovibriones) and from polyacrylamide gel analysis; it was then dialyzed against 10 liters of distilled water and lyophilized, giving a yield of 45 mg. Some of its spectral characteristics are reported in Table 1.

(b) Purification of ferredoxin. The ferredoxin is acidic and was eluted with 500 mM Tris-hydrochloride (in a volume of 175 ml) from the second DEAE-cellulose column. The ferredoxin fraction was placed on a calcinated alumina column (5 by 25 cm), equilibrated with 500 mM Tris-hydrochloride, in order to remove a contaminant with a strong absorption at 260 nm. The ferredoxin was collected in the same buffer in a volume of 170 ml, and the volume was brought to 250 ml with distilled water. The ferredoxin was then adsorbed on a DEAE-cellulose column (4 by 25 cm) and eluted with a linear gradiant (800 ml) from 350 to 550 mM Tris-hydrochloride. It was collected in a volume of 200 ml and placed on a calcinated alumina column (2 by 15 cm), equilibrated with 500 mM Tris-hydrochloride, in order to remove the last traces of the 260-nm contaminant, and was collected in a volume of 195 ml. The volume was brought to 240 ml with distilled water, and the ferredoxin was adsorbed on a DEAE-Sephadex column (2 by 25 cm) equilibrated with 400 mM Tris-hydrochloride. The protein was eluted with a linear gradiant (300 ml) from 400 to 600 mM Tris-hydrochloride and collected in a volume of 74 ml. It was dialyzed against 2 liters of

TABLE 1. *Molar extinction coefficients of rubredoxin, ferredoxin, and cytochrome c_3 from D. desulfuricans Norway*

Protein[a]	λ/nm	ε/mol per cm
Rubredoxin (ox.)	490	6,900
	375.5	8,142
	279	15,870
Ferredoxin (ox.)	390	17,500
	305	20,588
Cytochrome c_3 (ox.)	531.5	50,132
	408.5	530,918
	351	118,737
Cytochrome c_3 (red.)	552	128,481
	523	662,46
	418.5	891,944

[a] ox., Oxidized; red., reduced.

distilled water, concentrated, and precipitated by dialysis against 500 ml of saturated ammonium sulfate solution. The precipitate was collected by centrifugation, washed in saturated ammonium sulfate, and finally resuspended in 10 ml of the same solution. The precipitate was dissolved by the addition of a few drops of distilled water, and a few crystals of ammonium sulfate were added to the solution. Crystals were formed in a few hours. This crystallization process was repeated twice. A small fraction of the crystallized ferredoxin was then dialyzed against distilled water, and the protein was judged to be pure from its spectrum (ratio $A_{305}/A_{390} = 0.84$, identical to ferredoxins from other desulfovibriones) and by polyacrylamide gel analysis. The ferredoxin was usually kept in a crystalline form in ammonium sulfate solution and dialyzed against distilled water just prior to its utilization. The yield was 55 mg of pure ferredoxin.

The absorption spectrum of ferredoxin shows two peaks, one at 305 nm and the other at 390 nm, with a shoulder at 290 nm. The spectral properties are similar to those of the *D. gigas* ferredoxin (15). The molar extinction coefficient is 17,500 at 390 nm (see Table 1).

(v) Coupling activity of different electron carriers between hydrogenase and thiosulfate reductase. The coupling activities between hydrogenase and thiosulfate reductase of cytochrome c_3 and ferredoxin from *D. desulfuricans* and *D. gigas* were tested using reductase preparations devoid of electron carriers from both microorganisms. The concentrations were chosen to give maximal activity. In addition, the activity of *D. gigas* cytochrome c_3 (MW 26,000) in this reaction was compared with that obtained with the above-mentioned electron carriers. Cytochrome c_3 from *D. desulfuricans* is an efficient carrier in this reaction with enzymatic preparation from the same organism, whereas *D. gigas* cytochrome c_3 is inactive with the *D. desulfuricans* thiosulfate reductase and with *D. gigas* gives only a slight stimulation of thiosulfate reductase independent of the concentration, as already reported (11) (Fig. 1 and 2). The activity exhibited by cytochrome c_3 from *D. desulfuricans* increases with increased cytochrome concentrations, and the maximum activity is reached with 24 nmol of cytochromes, using the same amount of reductase as in the experiment reported in Fig. 1. Both ferredoxins from *D. desulfuricans* and *Desulfovibrio gigas* are carriers in the coupling between hydrogenase and thiosulfate reductase from *D. gigas*; however, the two ferredoxins are less efficient with the enzymatic preparation from *D. desulfuricans*. Finally, as already reported (4), cytochrome c_3 (MW 26,000) from *D. gigas* gives a strong stimulation with both enzymatic preparations of the same magnitude, on a molar basis, as that obtained with methyl viologen.

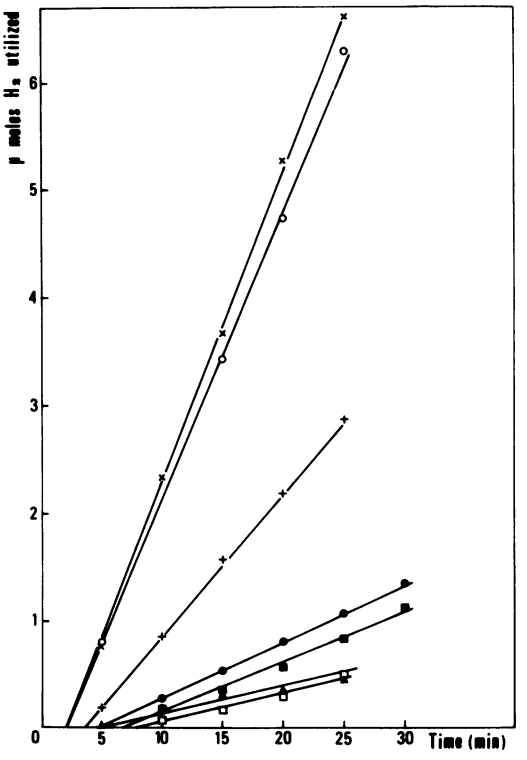

FIG. 1. *Reduction of thiosulfate by H_2 in the presence of various electron carriers with D. desulfuricans enzymatic preparation. The reaction mixture contained all the reactants indicated in Materials and Methods. Each flask contained enzymatic preparation (31 mg) and hydrogenase (400 μg). Symbols:* (▲) *control without added electron carrier;* (○) *plus methyl viologen (100 nmol); plus D. desulfuricans cytochrome c_3 (20 nmol);* (●) *plus D. desulfuricans ferredoxin (100 nmol); plus D. gigas cytochrome c_3 (MW 13,000) (20 nmol);* (■) *plus D. gigas ferredoxin (100 nmol);* (×) *plus D. gigas cytochrome c_3 (MW 26,000) (10 nmol).*

Amino acid compositions. The amino acid values were determined from the average of several acid hydrolysates. Corrections were made, when necessary, for the degradation or slow release of certain residues. The number of residues per mole of protein was determined by the mole ratio method.

(i) Ferredoxin. The amino acid composition and iron sulfide content of *D. desulfuricans* Norway ferredoxin are presented in Table 2. The protein contains 54 amino acids and is

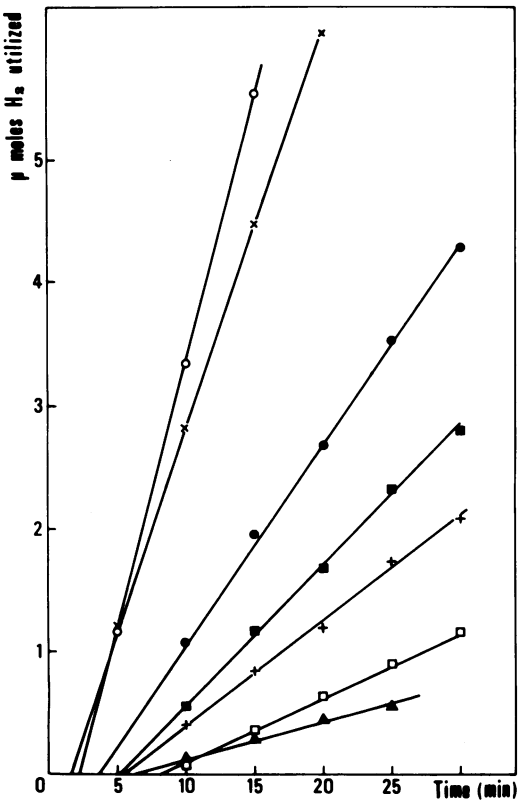

FIG. 2. *Reduction of thiosulfate by H_2 in the presence of various electron carriers with D. gigas enzymatic preparation. The reaction mixture contained all the reactants indicated in Materials and Methods. Each flask contained enzymatic preparation (22 mg of protein) and hydrogenase (400 μg). Symbols:* (▲) *control without added electron carrier;* (○) *plus methyl viologen (100 nmol);* (+) *plus D. desulfuricans cytochrome c_3 (30 nmol);* (●) *plus D. desulfuricans ferredoxin (100 nmol;* (□) *plus D. gigas cytochrome c_3 (MW 13,000) (30 nmol);* (■) *plus D. gigas ferredoxin (100 nmol);* (×) *plus cytochrome c_3 (MW 26,000) (10 nmol).*

devoid of histidine and tyrosine, like *D. gigas* ferredoxin (25). The minimum calculated molecular weight, including iron and sulfide, is 6,134. This value is in good agreement with the value estimated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate, by reference with the migration of *D. gigas* ferredoxin (MW 6,056).

The analytical results show the presence of four iron atoms, four sulfide groups, and six cysteine residues.

The amino acid composition of the *Desulfovibrio* ferredoxins *D. gigas* and *D. desulfuricans* Berre S (29) show identical contents of half-cystine, labile sulfur, and iron. All the *Desulfovibrio* ferredoxins have a low content of basic amino acid residues and aromatic residues.

(ii) Rubredoxin. Hydrolysis of several samples of rubredoxin with 6 N hydrochloric acid under vacuum at 110°C for 20 h, followed by quantitative amino acid analysis, showed the amino acid content listed in Table 3. The amino acid compositions of the three *Desulfovibrio* rubredoxins so far reported are added for comparison. Acidic amino acids are preponderant, and all the rubredoxins are devoid of histidine and arginine residues, which is consistent with chromatographic properties on DEAE-cellulose. All the rubredoxins contain four cysteine residues, one non-heme iron, and no acid-labile sulfide. Calculation of molecular weight from the amino acid composition gave a result of 6,728, including one atom of iron per molecule.

(iii) Cytochrome c_3. Cytochrome c_3, first described by Postgate (22), is characteristic of the genus *Desulfovibrio*. It was then proposed that it contains two hemes per molecule, but from amino acid sequence analysis (1, 3; R. P. Ambler, Biochem. J. 109:47P-48P) it became clear that cytochrome c_3 possesses four binding clusters for the hemes. Recent results of Yagi and Maruyama (28), Meyer et al. (18), and Dobson et al. (6) are consistent with the presence of four hemes per molecule. The amino acid composition of *D. desulfuricans* strain Norway cytochrome c_3 is presented in Table 4 and compared with that obtained for cytochromes c_3 from *D. vulgaris* strain Hildenborough, *D. gigas*, and *D. desulfuricans* strain El Agheila Z. The main characteristics of all cytochromes c_3 are a high number of cysteine residues (8), of histidine residues (6–10) and of lysine residues.

After filtration on a G-50 Sephadex column, a molecular weight of 16,000 was determined. This figure is higher that the value of 13,000 determined for other c_3-type cytochromes and renders difficult a nomenclature of this type of hemoprotein based on molecular weight. The molecular weight based on the amino acid composition is 16,096. These values are consistent and give an average molecular weight for *D. desulfuricans* strain Norway cytochrome c_3 of 16,000.

DISCUSSION

The non-heme iron proteins of *D. desulfuricans*, Norway strain, do not differ in molecular weight, iron, and acid-labile sulfur content or in the number of cysteine residues from the other characterized Desulfovibrio ferredoxins and rubredoxins. Like the other bacterial rubredoxins, the rubredoxin is devoid of histidine and arginine residues and possesses four cys-

TABLE 2. *Amino acid composition of D. desulfuricans Norway ferredoxin*[a] *and comparison with other Desulfovibrio sp.*

Amino acid	Amino acid residues/molecule			
	D. desulfuricans (Norway) ferredoxin		Ferredoxin	
	From analysis	Nearest integer	*D. gigas*[b]	*D. desulfuricans* Berre S[c]
Lysine	1.8	2	1	2
Histidine	0	0	0	1
Ammonia				
Arginine	0	0	1	0
Tryptophan	ND	ND	0	0
Aspartic acid	4.8	5	11	10
Threonine	2.6	3	0	3
Serine	2.5	3	3	2
Glutamic acid	11	11	9	11
Proline	2.6	3	4	3
Glycine	2	2	1	6
Alanine	6.8	7	6	2
Cystine (half)[d]	6	6	6	6
Valine	3	3	5	5
Methionine[d]	1.9	2	2	0
Isoleucine	4.8	5	5	4
Leucine	1	1	1	2
Tyrosine	0	0	0	0
Phenylalanine	0.8	1	1	0
Total residues		54	56	57
Non-heme iron[e]		4	4	4
Acid-labile sulfide[e]		4	4	4

[a] Amino acid analyses were carried out using general methods with 20-h hydrolysis (6 N HCl) at 110°C under vacuum. Abbreviation: ND, Not determined.

[b] From Travis et al. (25).

[c] From Zubieta et al. (29).

[d] Cysteine was determined as cysteic acid after performate oxidation of the protein sample according to the method of Hirs (12).

[e] Atoms per molecule.

teine residues, one non-heme iron, and no acid-labile sulfide. The ferredoxin is devoid of histidine and tyrosine like *D. gigas* ferredoxin and has four iron atoms, four sulfide groups, and six cysteine residues. It is noteworthy that two ferredoxins were detected in the Norway strain. This second ferredoxin is more acidic and can be separated from the other on a DEAE-cellulose column. We have already noted that, by comparison with other *Desulfovibrio* species, ferredoxin is more abundant, and this seemed to be related to the absence of flavodoxin in the Norway strain. However, during the purification, it became clear that the second ferredoxin was quite unstable. As a result, only small amounts of this ferredoxin were recovered in a pure state, just enough for purity tests and amino acid determination. No reactivity experiments could be performed.

In earlier publications (2, 5), we have reported the presence of three *c*-type cytochromes in *D. vulgaris* and *D. desulfuricans* (El Agheila Z), namely, c_{553}, c_3 (MW 13,000), and c_3 (MW 26,000). The latter contain eight hemes per molecule; an analogous cytochrome was not detected in the Norway 4 strain.

The data on the coupling activity of *D. desulfuricans* (Norway 4) cytochrome c_3, ferredoxin, and the homologous electron carriers of *D. gigas* between hydrogenase and thiosulfate reductase indicate that *D. desulfuricans* (Norway 4) cytochrome c_3 gives only a slight stimulation with the *D. gigas* reductase, as already reported (5). On the other hand, the ferredoxins of the two bacteria function as carriers in this reaction; however, these proteins are weakly active with the reductase preparation of *D. desulfuricans* (Norway 4). As described earlier (11), cytochrome c_3 (MW 26,000) from *D. gigas* appears to be the most efficient carrier in the

TABLE 3. *Amino acid composition of D. desulfuricans Norway rubredoxin[a] and comparison with other Desulfovibrio rubredoxins*

Amino acid	*D. desulfuricans* (Norway) rubredoxin		Rubredoxin		
	From analysis	Nearest integer	*D. gigas*[b]	*D. desulfuricans azotovorans*[c]	*D. vulgaris*[d]
Lysine	4.8	5	5	4	5
Histidine	0	0	0	0	0
Ammonia					
Arginine	0	0	0	0	0
Tryptophan	ND	ND	1	1	1
Aspartic acid	12.6	13	8	7	9
Threonine	3.5	4	2	2	2
Serine	0	0	4	2	4
Glutamic acid	5.2	5	5	8	5
Proline	5.1	5	5	6–7	7
Glycine	7.2	7	6	6	7
Alanine	5.0	5	5	6	6
Cystine (half)[e]	4	4	4	4	4
Valine	6.0	6	4	5	5
Methionine	0.82	1	1	1	1
Isoleucine	0	0	3	2	0
Leucine	0.94	1	2	0	1
Tyrosine	4.1	4	3	3	2
Phenylalanine	2.1	2	3	3	2
Total residues		63	61	60	61
Non-heme iron[f]		1	1	1	1
Acid-labile sulfide[f]		0	0	0	0

[a] Amino acid analyses were carried out using general methods with 20-h hydrolysis (6 N HCl) at 110°C under vacuum. ND, Not determined.

[b] From Laishley et al. (13).

[c] From Newman and Postgate (21).

[d] From Bruschi and Le Gall (4).

[e] Cysteine was determined as cysteic acid after performate oxidation of the protein sample according to the method of Hirs (12).

[f] Atoms per molecule.

coupling of hydrogenase with thiosulfate reductase. It should be emphasized that with our enzymatic preparation, devoid of electron carriers but containing thiosulfate and sulfite reductase activities, we used excess thiosulfate as substrate and measured only the reduction of thiosulfate, since sulfite is used only when all the thiosulfate has disappeared (11).

Wagner et al. (26) have reported the standard potential of thiosulfate reduction to sulfide and sulfite to be near −420 mV. This observation suggests that only a low-potential electron carrier could couple hydrogenase and thiosulfate reductase. The redox potential of *D. desulfuricans* cytochrome c_3 has been found to be in the vicinity of −500 mV (P. Bianco, personnal communication), a high electronegative value as compared with the potential (−250 mV) reported for *D. gigas* cytochrome c_3 (MW 13,000) (16). Thus, in *D. desulfuricans* (Norway 4), cytochrome c_3 is a good candidate for transferring electrons during thiosulfate reduction. However, in addition to the requirement for a low redox potential, the specificity of the electron carrier for hydrogenase and thiosulfate reductase is an important factor since ferredoxins from both bacteria, although extremely electronegative, give only a small stimulation at a concentration of 33.3 μM with the *D. desulfuricans* (Norway 4) reductase preparation. With this latter preparation the efficiency of *D. desulfuricans* (Norway 4) cytochrome c_3 in the coupling of hydrogenase with thiosulfate reductase is near that of *D. gigas* cytochrome c_3 (MW 26,000). In this case, a concentration of *D. desulfuricans* (Norway 4) cytochrome c_3 twice that of *D. gigas* cytochrome c_3 (MW 26,000) was used since, as already reported (5), the former cytochrome contains four hemes per molecule and the latter contains eight hemes. Then, in

TABLE 4. *Amino acid composition of D. desulfuricans Norway cytochrome c_3 (MW 13,000)*[a] *and comparison with other Desulfovibrio cytochromes*

Amino acid	*D. desulfuricans* (Norway) cytochrome c_3		Cytochrome c_3 (MW 13,000)		
	From analysis	Nearest integer	*D. vulgaris*[b]	*D. gigas*[c]	*D. desulfuricans* El Agheila Z[d]
Lysine	15.99	16	20	17	15
Histidine	7.2	7–8	9	8	8
Ammonia					
Arginine	1.9	2	1	0	1
Tryptophan	0	0	0	1	1
Aspartic acid	15.1	15	12	18	8
Threonine	9.7	10	5	5	5
Serine	5.6	6	6	6	8
Glutamic acid	10.1	10	5	4	6
Proline	7.8	8	4	4	6
Glycine	9.7	10	9	11	8
Alanine	12.8	13	10	9	13
Cystine (half)[e]	8	8	8	8	8
Valine	6.0	6	8	8	5
Methionine	0.8	1	3	0	4
Isoleucine	3.6	4	0	4	2
Leucine	4.8	5	2	42	0
Tyrosine	1.2	2	3	2	1
Phenylalanine	3.8	4	2		3
				111	
Total residues		127	107		102

[a] Amino acid analyses were carried out using general methods with 20-h hydrolysis (6 N HCl) at 110°C under vacuum.

[b] From Ambler (Biochem. J. 109:47P–48P, 1968).

[c] From Ambler et al. (1).

[d] From Ambler et al. (3).

[e] Cysteine was determined as cysteic acid after performate oxidation of the protein sample according to the method of Hirs (12).

D. desulfuricans (Norway 4), cytochrome c_3 replaces cytochrome c_3 (MW 26,000) as an electron carrier in the electron transport system of thiosulfate reduction. These observations suggest that cytochromes c_3, although homologous proteins, have different physiological roles in the sulfate-reducing bacteria, since ferredoxin is not essential for coupling between hydrogenase and thiosulfate reductase in *D. desulfuricans* (Norway 4). In *D. gigas*, ferredoxin, flavodoxin, or cytochrome c_3 (MW 26,000) is a carrier in the same reaction, when cytochrome c_3 (MW 13,000) is almost inactive (11).

It can then be postulated that an important structural modification took place in *D. desulfuricans* (Norway 4) cytochrome c_3, allowing the disappearance of flavodoxin and the exclusion of ferredoxin from the electron transfer chain between hydrogenase and thiosulfate reductase so that the ferredoxin could be utilized in some other part of the complex electron transfer system typical of the sulfate-reducing bacteria. Comparison of the primary structure of other cytochromes c_3 (3) shows that only 25% of the total residues remain unchanged. Such a variability is not common in cytochromes of the *c* type and supports the hypothesis of changes in the function of cytochromes c_3 in sulfate-reducing bacteria. The higher molecular weight of *D. desulfuricans* (Norway 4) cytochrome c_3 is also in favor of this change. Both primary and tertiary structure determinations, which are now in progress (6, 9), will provide more precise information concerning the structure/function relationships in this class of hemoproteins.

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