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Sulredoxin: a Novel Iron-Sulfur Protein of the Thermoacidophilic Archaeon *Sulfolobus* sp. Strain 7 with a Rieske-Type [2Fe-2S] Center

TOSHIO IWASAKI,[1]* YASUHIRO ISOGAI,[2] TETSUTARO IIZUKA,[2] AND TAIRO OSHIMA[1]

Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Yokohama 226,[1] and Biophysical Chemistry Laboratory, The Institute of Physical and Chemical Research, Wako, Saitama 351-01,[2] Japan

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A novel pink [2Fe-2S] protein has been purified from the cytosol fraction of the thermoacidophilic archaeon *Sulfolobus* sp. strain 7 (originally named *Sulfolobus acidocaldarius* 7) and called "sulredoxin." Its absorption, circular dichroism, and electron paramagnetic resonance spectra suggest the presence of a Rieske-type [2Fe-2S] cluster (*g*-factors of 2.01, 1.91, and 1.79; average *g*- factor [g_{av}] = 1.90) which is remarkably similar to that of *Thermus thermophilus* respiratory Rieske FeS protein (J. A. Fee, K. L. Findling, T. Yoshida, R. Hille, G. E. Tarr, D. O. Hearshen, W. R. Dunham, E. P. Day, T. A. Kent, and E. Münck, J. Biol. Chem. 259:124–133, 1984) and distinctively different from those of the plant-type ferredoxins (g_{av} = 1.96). Sulredoxin, which is the first Rieske-type [2Fe-2S] protein isolated from an archaeal species, does not function as an electron acceptor of the cognate 2-oxoacid:ferredoxin oxidoreductase. Whether sulredoxin is derived from the archaeal membrane-bound respiratory Rieske-type FeS center (g_y = 1.91) is the subject of further investigation.

The plant-type [2Fe-2S] ferredoxins have midpoint redox potentials ranging from −230 to −450 mV (35) and serve as low-potential, single-electron carriers from the photosystem I reaction center to various ferredoxin-dependent oxidoreductases in cyanobacteria and chloroplasts of plants (21, 35). Their redox centers, namely, the plant ferredoxin-type [2Fe-2S] clusters, coordinate to the proteins by four sulfide ligands contributed by four cysteine residues and give a low-temperature electron paramagnetic resonance (EPR) signal only in the reduced state, with an average *g*-factor (g_{av}) of around 1.96 (28, 29). Essentially similar ferredoxins have also been isolated from the mammalian adrenal mitochondria (adrenodoxin), purple bacteria, and the extremely halophilic archaea, which participate in the cytochrome P-450-dependent steroid-hydroxylating system, nitrogen fixation system, and coenzyme A-acylating 2-oxoacid-oxidizing system, respectively (35).

Another class of the [2Fe-2S] cluster-containing FeS proteins comprises the respiratory Rieske proteins, which are an important constituent of the cytochrome *bc* (bc_1/b_6f) complex (respiratory complex III) of the aerobic respiratory and photosynthetic systems (28, 34). They have relatively high midpoint redox potentials ranging from −150 to +350 mV and elicit a characteristic EPR signal with an unusually low g_{av} of ~1.91 in the reduced state, as well as red-shifted visible spectra in the oxidized state. The [2Fe-2S] clusters of several ferredoxins involved in the bacterial dioxygenase systems also have spectral properties more similar to those of the Rieske-type FeS proteins than those of the plant-type ferredoxins (28). These spectral properties are due most likely to the asymmetrical ligand environments; extensive analyses of the Rieske-type [2Fe-2S] clusters of *Thermus thermophilus* respiratory Rieske protein (8) and *Pseudomonas cepacia* phthalate dioxygenase (3) suggested that one iron atom of the FeS cluster coordinated to the protein by two sulfide ligands contributed by two cysteine residues, while the other coordinated by two δ-nitrogens of the imidazole rings contributed by two histidine residues (5, 9, 13, 23).

Sulfolobus sp. strain 7 (originally named *Sulfolobus acidocaldarius* 7) is a typical aerobic and thermoacidophilic archaeon that grows optimally at pH 2.5 to 3 and at 75 to 80°C (36). Chemoheterotrophically grown cells acquire biological energy by aerobic respiration rather than simple fermentation (22, 36), and the aerobic respiratory chain, which is coupled to the oxidative tricarboxylic acid cycle at the level of succinate (17), contains the unusual membrane-bound terminal cytochrome oxidase system; it consists solely of *a*- and *b*-type but no *c*-type cytochromes (17, 36, 37), and the presence of a respiratory Rieske FeS protein or its analog has not been reported. The archaeon also contains a relatively large amount of ferredoxin in the cytoplasm (11), which is bacterium type as in the cases of a hyperthermophile (2, 6), a methanogen (33), and several extreme thermoacidophiles (18) in the *Archaea* domain. The *Sulfolobus* ferredoxin contains one [3Fe-4S] cluster and one [4Fe-4S] cluster and mediates electron transfer from 2-oxoacid:ferredoxin and NADPH:ferredoxin oxidoreductases (15). In contrast to the cases for the eukarya and bacteria, no [2Fe-2S]-type ferredoxin or its analog has been detected among the archaea (18), except for halobacteria (14, 20). Recent genetic evidence, however, suggests that *Halobacterium salinarium* ferredoxin may have been derived from halophilic cyanobacteria by means of lateral gene transfer (30).

In this communication, we report the purification of a novel ferredoxin-analogous pink FeS protein designated "sulredoxin" from the soluble fraction of *Sulfolobus* sp. strain 7. The subsequent characterization led to a very unexpected result in that the protein contains an unusual [2Fe-2S] center with spectral properties more similar to those of *T. thermophilus* Rieske FeS protein (8) than those of the conventional plant-type ferredoxins. In addition, we have detected a membrane-bound respiratory Rieske FeS center in *Sulfolobus* sp. strain 7, although it remains to be clarified whether sulredoxin may be derived from the membrane-bound Rieske protein.

Materials. DEAE-Sephacel and Sephadex G-50 were purchased from Pharmacia LKB Biotechnology Inc., and Butyl-

* Corresponding author. Mailing address: Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Yokohama 226, Japan. Phone: 81-45-924-5712. Fax: 81-45-924-5805.

Toyopearl 650M was purchased from Tosoh Corporation. The broad-range standard marker proteins and the 4 to 20% gradient Ready-Gels were purchased from Bio-Rad. Other chemicals used in this study were of analytical grade.

Organism, cell culture, and preparation of the cytosol and membrane fractions. *Sulfolobus* sp. strain 7 (originally named *S. acidocaldarius* 7, isolated from Beppu hot springs in Japan [36]), used in this study, was recently tentatively renamed *Sulfolobus* sp. strain 7 because of small differences in the 16S rRNA base sequences of strain 7 and *S. acidocaldarius* type strain DSM 639. The preliminary 16S rRNA sequence analysis suggests that the isolate is a novel species belonging to the genus *Sulfolobus*. The organism was cultivated aerobically and chemoheterotrophically at pH 2.5 to 3 and 75 to 80°C as described previously (36, 38) and was harvested in the late exponential phase of growth. The cells were suspended in 100 mM Tris-Cl (pH 7.3) containing 15 mM EDTA and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) at a final concentration of 4 ml/g [wet weight] of cells. The suspension was passed twice through a French pressure cell (Otake Works, Tokyo, Japan) at 1,500 kg/cm^2, and membranes were pelleted by ultracentrifugation with a Beckman 45Ti rotor at 130,000 $\times$ *g* for 100 min at 15°C. The precipitates were composed of two layers. The upper, soft layer was carefully collected and resuspended in 50 mM Tris-Cl (pH 7.3) containing 10 mM EDTA and 0.5 mM PMSF and then ultracentrifuged at 130,000 $\times$ *g* for 70 min at 15°C. The precipitate was suspended in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.0) containing 0.2 mM PMSF at 30 to 40 mg of protein per ml and was used as the crude membrane fraction. The supernatants were combined and used as the cytosol fraction for the following purification steps.

Purification of sulredoxin from *Sulfolobus* sp. strain 7. All steps were carried out at room temperature unless otherwise specified. The purification was monitored by examining three different absorption bands, at 280, 408, and 450 nm, and the patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purification of sulredoxin was initiated from the cytosol fraction containing a negligible amount of cytochrome, and no detergent was included at any step of the purification procedure. Water used in the following purification steps was purified by a Milli-Q purification system (Millipore).

(i) Step 1. The cytosol fraction was diluted threefold by addition of 30 mM Tris-Cl buffer (pH 7.3) containing 0.5 mM PMSF and was applied to a DEAE-Sephacel column (3.0 by 28 cm) which had been previously equilibrated with 30 mM Tris-Cl buffer (pH 7.3) containing 1 mM EDTA and 0.5 mM PMSF. The column was washed with 500 ml of the equilibration buffer, and the flowthrough fractions (800 ml) were combined. Then 194 g of solid ammonium sulfate was added to 800 ml of the combined fractions, and the mixture was gently degassed by aspirator while being gently stirred.

(ii) Step 2. The suspension was directly applied to a Butyl-Toyopearl 650M column (2.2 by 31.5 cm) that had been preequilibrated with 20 mM degassed potassium phosphate buffer (pH 6.0) containing 234 g of ammonium sulfate per liter (40% saturation). Then, the column was washed with 250 ml of the equilibration buffer, and an 800-ml linear gradient was run between 40 and 0% saturation of ammonium sulfate in 20 mM degassed potassium phosphate buffer (pH 6.0). The pink band eluted around 18% ammonium sulfate saturation was collected.

(iii) Step 3. The combined pink fraction was concentrated to ~3 ml by pressure filtration on an Amicon YM-10 membrane under N_2 gas at 4°C. It was then applied at 4°C to a Sephadex G-50 gel filtration column (2.5 by 75 cm) which had been preequilibrated with 80 mM potassium phosphate buffer (pH 6.8). The pink fractions, which showed a single band in SDS–20% PAGE, were combined. This material was used as purified sulredoxin and stored at −80°C until use.

Purification of other *Sulfolobus* proteins and enzymatic assays. The membrane-bound respiratory complex II (succinate dehydrogenase complex) of *Sulfolobus* sp. strain 7 was solubilized from the membrane fraction, purified to apparent homogeneity (17), and stored at −80°C until use.

Isolation of the 7Fe ferredoxin from the *Sulfolobus* cytosol fraction was carried out according to previously published procedures (11). Purified ferredoxin showed a single band on analytical 20% PAGE (in the absence of SDS) and was stored at −80°C until use.

A cognate 2-oxoacid:ferredoxin oxidoreductase was also purified from the cytosol fraction to apparent homogeneity by conventional methods using column chromatography and a glycerol density gradient centrifugation and was stored at −80°C until use; the details will be given elsewhere (16). The purified enzyme consists of two nonidentical subunits and is active in the 2-oxoacid- and *Sulfolobus* ferredoxin-dependent horse heart cytochrome *c* assay (69 U/mg with 4 mM 2-oxoglutarate and 50 μM coenzyme A; carried in 10 mM potassium phosphate buffer at pH 6.8 and 50°C, essentially as described by Kerscher et al. [18]).

The Fe^{2+}:cytochrome *c* oxidoreductase activity was tested at pH 6.8 and 3.5 as described by Fukumori et al. (12), except at 50°C. The enzyme assays were monitored with a Hitachi U-3210 spectrophotometer equipped with a thermoelectric cell holder.

Analytical methods. Absorption spectra were recorded either with a Hitachi U-3210 or a Beckman DU7400 spectrophotometer. Circular dichroism spectra were recorded at room temperature on a JASCO J-500C spectropolarimeter equipped with a JASCO data processor DP-50, in 0.5-cm-long cells. Low-temperature EPR measurements were made by using a JEOL JEX-RE1X spectrometer equipped with an Air Products model LTR-3 Heli-Tran cryostat system, in which temperature was monitored with a Scientific Instruments series 5500 temperature indicator-controller.

Quantitative amino acid analysis (the means of three determinations) was performed as described by Chang et al. (4, 25), with a Beckman dimethylaminoazobenzene sulfonyl amino acid analysis kit and Beckman System Gold. Trp and Cys residues were not determined. Protein was measured by the bicinchoninic acid assay (Pierce Chemical) with bovine serum albumin as a standard, and for purified sulredoxin, the results were divided by 2.8 for calibration (in conjunction with the quantitative amino acid analysis). Analytical ultracentrifugation was performed with a Beckman Optima XL-A analytical ultracentrifuge with a Beckman An-60Ti analytical rotor at 20,000 rpm at 20°C. Egg white lysozyme was used for calibration. Electrospray ionization mass spectra were measured in a Bruker REFLEX MALDI-TOF mass spectrometer at an accelerating potential of 20 kV.

The nonheme iron and total sulfur contents of the purified protein were determined by inductively coupled plasma atomic emission spectrometry with a Seiko SPS 1500 VR instrument. For sulfur content, $FeSO_4 \cdot 7H_2O$ (Wako Pure Chemicals) was used for calibration. The content of acid-labile sulfide (S^{2-}) was estimated essentially as described by Fee et al. (8), except that the initial denaturation step was performed anaerobically at 90°C for 20 min in the presence of 0.5 N NaOH, 0.15% (vol/vol) Triton X-100, and 2 mM EDTA because of the re-

markable stability of sulredoxin. This modification facilitated a marked improvement of the analysis.

SDS-PAGE was carried out with the Laemmli buffer system (24) on 4 to 20% gradient gels (Bio-Rad) after proteins were denatured under various conditions (see below), and the proteins were visualized by Coomassie brilliant blue staining.

N-terminal amino acid sequence analysis was carried out with a 470A protein sequencer (Applied Biosystems) after extensive dialysis of the purified protein against distilled water.

Molecular and spectroscopic properties of sulredoxin. "Sulredoxin" is a trivial name for a novel pink FeS protein identified and isolated from the cytosol fraction of *Sulfolobus* sp. strain 7. Approximately 4 to 5 mg of pure protein could be reproducibly obtained from ~60 g (wet weight) of the late-log-phase cells grown aerobically and chemoheterotrophically, indicating that it constitutes at least 0.05% of the total archaeal soluble proteins.

Figure 1A shows the patterns of purified sulredoxin on 4 to 20% gradient SDS-PAGE under various denaturation conditions. The protein migrates as a single broad band with an apparent molecular mass of ~170 kDa on SDS-PAGE after incubation in the presence of 2% SDS but in the absence of 2-mercaptoethanol at room temperature for 5 to 60 min (Fig. 1A, lane 2) and as a single 130-kDa band under the same conditions except in the presence of 1 to 2% 2-mercaptoethanol (lane 3). Complete denaturation was achieved upon heat treatment of the protein in the presence of 2% SDS and 2% 2-mercaptoethanol at 90°C for 15 min, as it appeared as a single 11-kDa band on SDS-PAGE (Fig. 1A, lane 5). Purified sulredoxin was also analyzed by electrospray ionization mass spectrometry after extensive dialysis against distilled water (Fig. 1B). It appears to consist of a single component with a mass of 12,155 Da (monomeric protein). Analytical ultracentrifugation analyses of purified sulredoxin in 100 mM potassium phosphate buffer (pH 7.6) containing 1.5 to 4.5 M guanidine hydrochloride suggested that the protein forms a very stable homodimer in the presence of 1.5 to 3.0 M guanidine hydrochloride, which dissociated into monomers in the presence of 4.5 M guanidine hydrochloride without release of chromophores (data not shown). These data indicate that purified sulredoxin consists of a single 12-kDa polypeptide and has a tendency to form an oligomeric structure in solution. The apparent size of the subunit is similar to those of ferredoxins (~10 to 15 kDa [28, 35]) and approximately half those of the respiratory Rieske FeS proteins (~20 to 25 kDa [9, 34]).

The metal analyses of purified sulredoxin by inductively coupled plasma atomic emission spectrometry showed the presence of tightly bound Fe which was not removable by dialysis for 2 days against 20 mM potassium phosphate buffer (pH 6.8) containing 1 mM EDTA. The following metals were not detected: Co, Mn, Mo, Ni, Se, W, and Zn. In conjunction with the quantitative amino acid analysis that allows the presence of one methionine per 12-kDa monomer (Table 1; the means from three determinations), the Fe and total S contents were determined to be 156 nmol of Fe per mg of protein (viz., 1.9 mol of Fe per mol of monomer) and 510 nmol of S per mg of protein (viz., 6.1 mol of S per mol of monomer). Purified sulredoxin also contained 1.7 mol of acid-labile sulfide per mol of monomer (viz., ~0.9 mol of acid-labile sulfide per mol of Fe; means from three different preparations), presumably indicating an S^{2-}/Fe ratio of 1 for the protein (due to the inherent difficulties in the analysis of highly stable FeS proteins in particular; see references 2 and 8). Thus, in conjunction with the quantitative amino acid analysis (Table 1), these data indicate that sulredoxin contains one [2Fe-2S] cluster per 12-kDa monomer, which has at least one noncysteinyl ligand (see below).

Figure 2 shows the optical spectra of purified sulredoxin. The spectrum of air-oxidized sulredoxin (as prepared) shows absorption maxima at 278, 342, and 443 nm and shoulders at 283, 495, and 575 nm, which are most similar to those of the [2Fe-2S] proteins with the Rieske-type FeS cluster (8, 28) and distinctively different from those of the bacterium-type ferredoxins or rubredoxins (27). Highly purified protein has purity indices A_{342}/A_{278} and A_{443}/A_{278} of 0.45 and 0.22, respectively. The extinction coefficient of oxidized sulredoxin at 443 nm which refers to the concentration of the [2Fe-2S] cluster, ε([2Fe-2S]) of 6.9 mM^{-1} cm^{-1} (mean from three different preparations), is similar to that at 458 nm for *T. thermophilus* Rieske protein (6.0 mM^{-1} cm^{-1}) (8). The optical spectrum was not affected either by dialysis against an EDTA-containing buffer or by addition of a small amount of $(NH_4)_2S_2O_8$ (not shown). When completely reduced by excess sodium dithionite, sulredoxin shows two shoulders, at 433 and 517 nm (Fig. 2). It is also readily reducible by sodium ascorbate in 1.5 min (Fig. 2), suggesting that the redox center has a reduction potential well above 0 V, as is the case for the respiratory Rieske-type FeS proteins (9, 34), and much higher than those of the [2Fe-2S] centers of the plant-type ferredoxins (~−230 to ~−450 mV [28, 35]).

Figure 3 shows the circular dichroism spectra of purified sulredoxin. The oxidized protein shows a shallow trough around 660 nm, a small peak at 590 nm, a weak shallow trough at 530 nm, at least three peaks between 400 and 510 nm (at 422, 458, and 486 nm), a deep trough at 380 nm, and two dominant peaks at 312 and 344 nm. The reduced protein shows a shallow trough between 700 and 800 nm, a small peak between 620 and 700 nm, a weak shallow trough around 690 nm, a small peak at 530 nm, a weak shallow trough at 504 nm, a dominant peak at 464 nm, and a deep trough at 390 nm. Notably, in agreement with the optical spectra in Fig. 2, these spectral properties are distinctively different from those of the ferredoxin-type [2Fe-2S] cluster (32) but rather similar to those of *T. thermophilus* Rieske protein (8).

Figure 4F shows the X-band EPR spectrum of purified sulredoxin at 10 K. While the air-oxidized form of sulredoxin as it is isolated is diamagnetic (EPR silent) and does not elicit any adventitious Fe^{3+} signal at a g of 4.3, indicating the absence of any rubredoxin-like Fe center (data not shown), the protein fully reduced by excess dithionite elicits a rhombic $S = 1/2$ resonance with principal $g_{x,y,z}$ values of 1.79, 1.91, and 2.01 (Fig. 4F), which could be readily detected at temperatures at least up to 70 K, as in the cases of some [2Fe-2S] clusters (8, 20, 29). In addition, its unusually low g_{av} value (1.90) is comparable to that of the Rieske-type [2Fe-2S] centers (g_{av} = ~1.91 [see references 9 and 26]), which is also supported by the analytical amino acid composition of sulredoxin (Table 1). The same EPR spectra were also obtained with the ascorbate-reduced protein (data not shown).

Thus, the redox center of sulredoxin consists of a pair of antiferromagnetically coupled ferric atoms which probably form a Rieske-type [2Fe-2S] cluster rather than the conventional plant-type ferredoxin. Although purified sulredoxin forms an oligomeric structure in solution (Fig. 1), the [2Fe-2S] clusters seem to be at least ~10 Å (~1.0 nm) apart, since no magnetic interaction was detected in the EPR spectra. On the other hand, the apparent size of its subunit is more similar to those of ferredoxins than those of respiratory Rieske proteins, indicating that sulredoxin has a feature which is chimeric between ferredoxin and respiratory Rieske FeS protein.

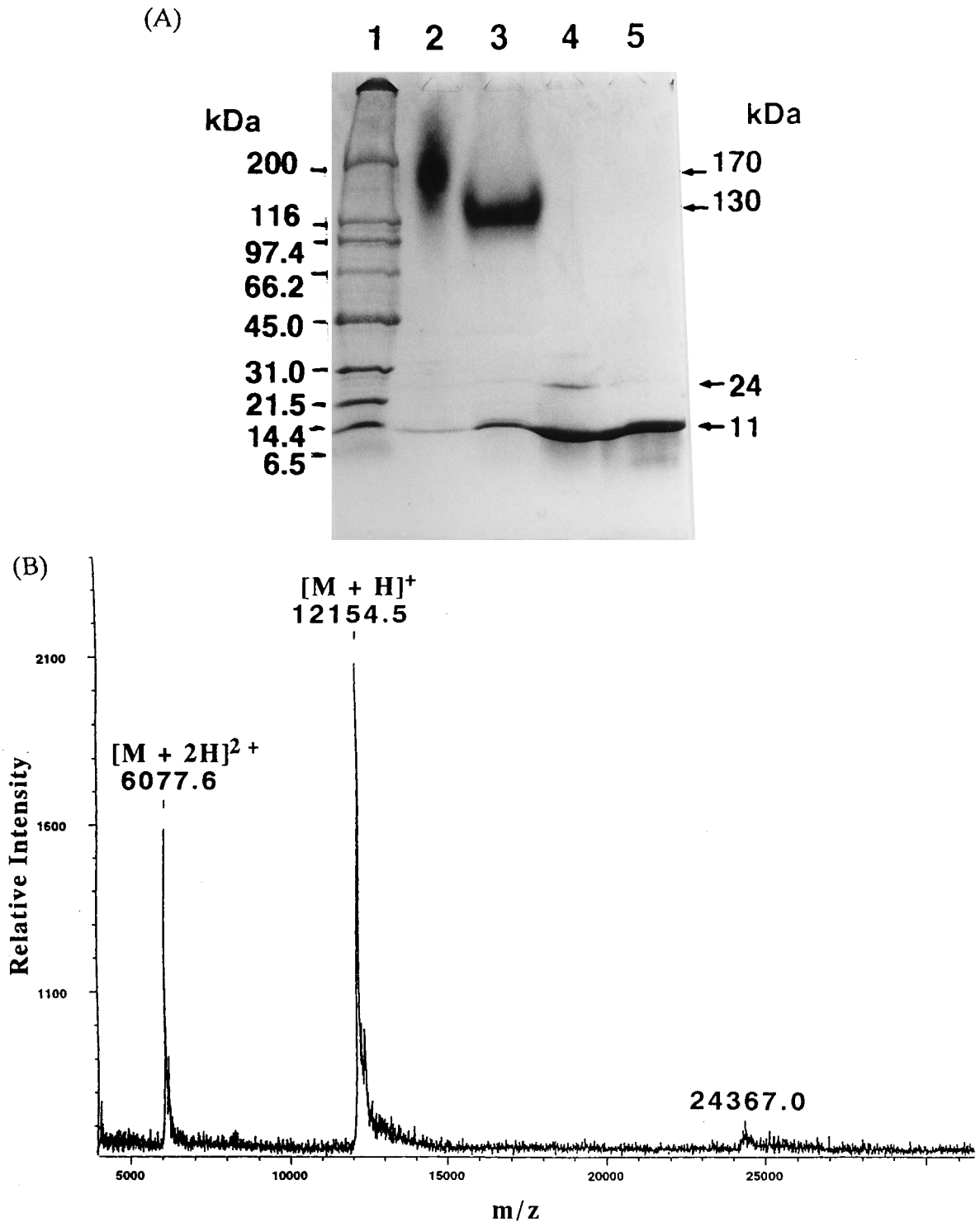

FIG. 1. (A) Gradient SDS-PAGE (4 to 20% polyacrylamide) and (B) electrospray ionization mass spectrum of purified sulredoxin from *Sulfolobus* sp. strain 7. (A) Purified sulredoxin was denatured under various conditions. Lane 1, broad-range standard marker proteins (Bio-Rad); lane 2, purified sulredoxin denatured in the presence of 2% SDS at room temperature for 30 min but in the absence of 2-mercaptoethanol; lane 3, the same as for lane 2 but in the presence of 2% 2-mercaptoethanol; lane 4, purified sulredoxin denatured in the presence of 2% SDS at 100°C for 10 min but in the absence of 2-mercaptoethanol; lane 5, the same as for lane 4 but in the presence of 2% 2-mercaptoethanol. Molecular masses are indicated (in kilodaltons). (B) Electrospray ionization mass spectrum of purified sulredoxin at an accelerating potential of 20 kV. Two signals corresponding to $[M + 2H]^{2+}$ and $[M + H]^{+}$ ions are indicated.

N-terminal amino acid sequence. To obtain more information on sulredoxin, the sequence of its N-terminal 21 amino acid residues was determined to be VWKR(T)ISAKALE(R) AKSAAVKV, where letters in parentheses represent ambiguous amino acid residues. However, the partial amino acid sequence search against the EMBL and Swiss-Prot amino acid sequence databases did not show any specific motif or considerable homology to any known sequence, including the Rieske

TABLE 1. Composition of sulredoxin from *Sulfolobus* sp. strain 7

Residue	mol/mol of protein
Asx	14.7
Glx	8.9
Ser	3.3
Thr	4.2
Arg	5.8
Gly	5.2
Ala	11.2
Pro	3.2
Val	8.0
Met	0.87
Ile	4.7
Leu	9.4
Phe	1.8
Lys	7.6
His	2.3
Tyr	0.57
Acid-labile S^{2-}	1.7
Fe	1.9
Total S (Met + Cys + S^{2-})	6.1

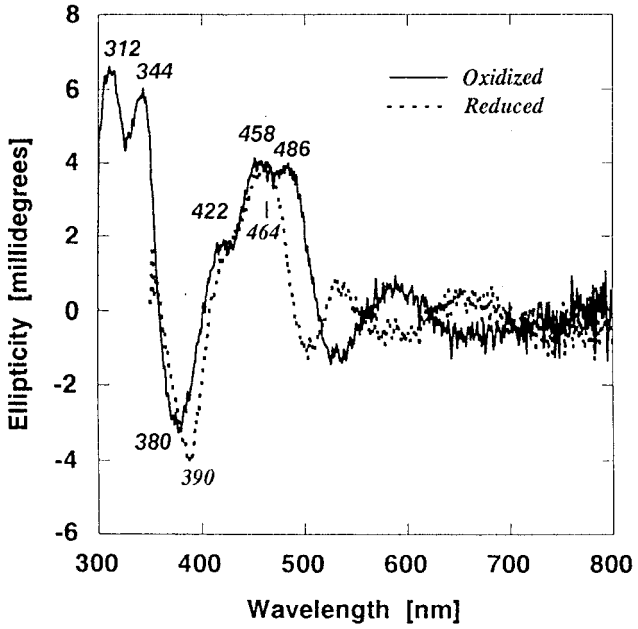

FIG. 3. Circular dichroism spectra of purified sulredoxin. The protein concentration was 0.31 mg/ml in 20 mM potassium phosphate buffer, pH 6.8. Conditions: cell length, 0.5 cm; time constant, 1 s; scan speed, 20 nm/min; number of scans, four.

FeS proteins and ferredoxins, except for 50% identity with residues 90 to 109 of an arginase encoded by an *Agrobacterium tumefaciens* Ti plasmid C58 gene (31) and 56% identity with residues 6 to 21 of the *ilvB* operon leader peptide of *Escherichia coli* (10).

Tests for biological function. Although sulredoxin was isolated from the soluble fraction of *Sulfolobus* sp. strain 7, it did not function as an electron acceptor of the archaeal 2-oxoacid: ferredoxin oxidoreductase, a key enzyme of the *Sulfolobus* oxidative tricarboxylic acid cycle (7, 16, 19), even at 50°C (see reference 15). It also did not show any Fe^{2+}:cytochrome *c* oxidoreductase activity at pH 3.5 to 6.8 (data not shown), in contrast to the *Thiobacillus ferrooxidans* soluble high-potential FeS protein with a ferrous ion-oxidizing activity (12).

Since the redox center of sulredoxin shows spectral properties very similar to those of the respiratory Rieske FeS proteins (8, 28), EPR analyses were carried out with several different batches of the isolated membranes of *Sulfolobus* sp. strain 7 to test for the presence of a membrane-bound Rieske-type FeS center. Typical results are shown in Fig. 4A to D. Upon addition of ascorbate, the *Sulfolobus* membranes elicited the EPR signal characteristic of the reduced respiratory Rieske FeS center ($g_{x,y}$ = ~1.73 and 1.91; Fig. 4A), whose lineshape, however, was slightly different from that of purified sulredoxin (Fig. 4F). The resonance at a g_y value of 1.91 was also observed reproducibly upon addition of respiratory substrates NADH (Fig. 4B) and succinate (Fig. 4C) at room temperature in the presence of cyanide as an inhibitor of the respiratory terminal

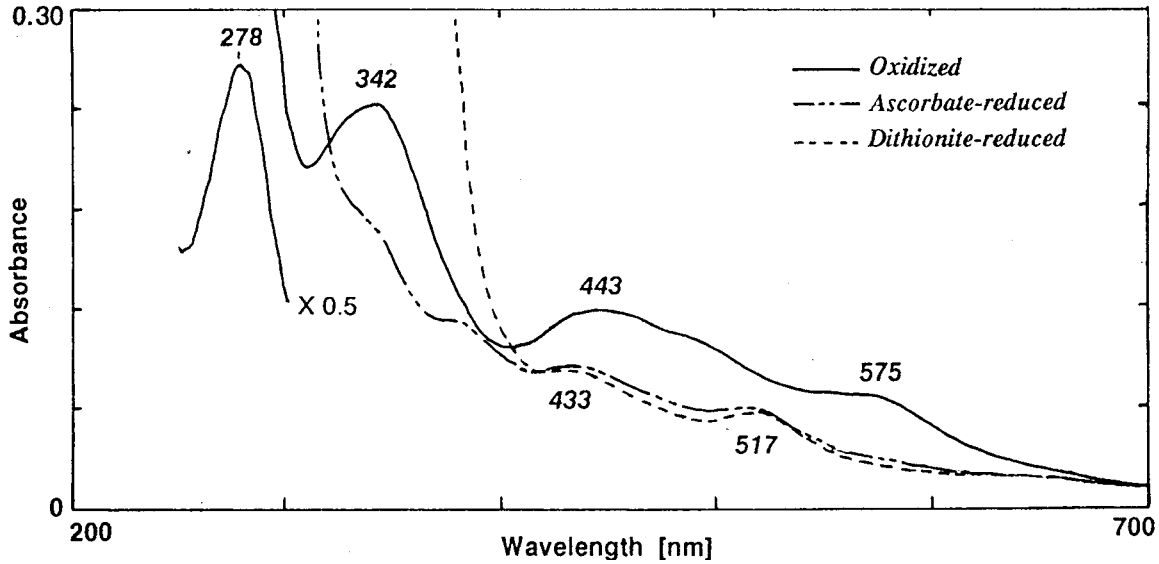

FIG. 2. UV-visible spectra of purified sulredoxin. The protein concentration was 0.21 mg/ml in 20 mM potassium phosphate buffer, pH 6.8.

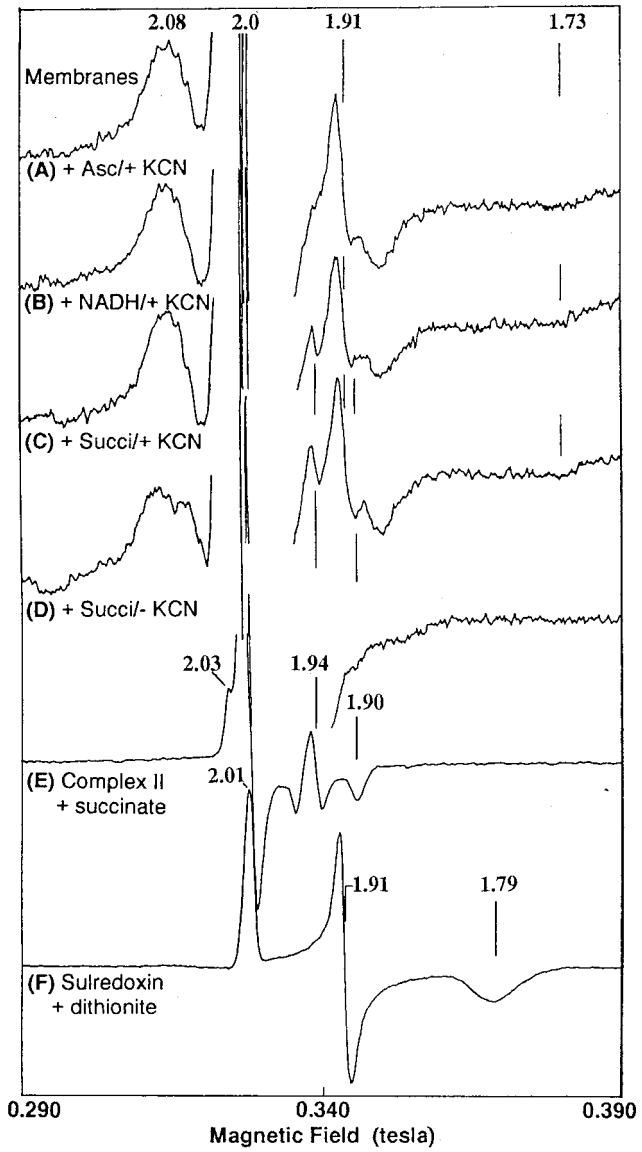

FIG. 4. EPR spectra of the reduced membrane-bound Rieske FeS center of *Sulfolobus* sp. strain 7. Approximately 40 mg of *Sulfolobus* membrane per ml was reduced in the presence of 2 mM cyanide at room temperature for 10 min by addition of 5 mM ascorbate (A), 2 mM NADH (B), and 10 mM succinate (C) and in the absence of cyanide by addition of 10 mM succinate (D). Subsequently, the samples were transferred to EPR tubes and frozen in liquid nitrogen. The EPR spectra of the center S-1 of purified *Sulfolobus* respiratory complex II (17) reduced by 5 mM succinate (E) and of dithionite-reduced purified sulredoxin (~0.21 mg/ml) (F) are also shown for comparison. Instrument settings: temperature, 10 K; microwave power, 0.4 mW except for panel F (1.0 mW); modulation amplitude, 7.9 G; gain, 2.0×10^3 (variable gain for panels E and F). *g* values are indicated in the figure. Asc, ascorbate; Succi, succinate.

oxidase complex (17), together with the signal at a *g* value of 1.94 derived from the partially reduced [2Fe-2S] cluster (center S-1) of the archaeal respiratory complex II (Fig. 4E) (17). On the other hand, the respiratory substrate-dependent reduction of the Rieske FeS center was not observed in the absence of cyanide (Fig. 4D). Other resonances at *g* values of 2.02 (the center S-3 of respiratory complex II [17]) and 2.0 (radical) were also affected by these treatments, whereas those overlapping at *g* values of 2.03 (presumably $[3Fe\text{-}4S]^+$ center of ferredoxin [15] or other unidentified FeS protein[s]) and ≈2.08 (presumably an adventitious copper), whose apparent intensities were considerably different depending on the batches of membranes and the extent to which the membranes were washed with buffer containing 5 mM EDTA, were not changed by the treatment (data not shown). These data clearly show the presence of a respiratory Rieske FeS center in the membranes of *Sulfolobus* sp. strain 7, as recently reported also for *S. acidocaldarius* DSM 639 ($g_{x,y,z}$ = 1.725, 1.890, 2.031; g_{av} = 1.88 [1]), which appears to be a constituent of the SoxCM oxidase complex (26). It thus seems plausible that the membrane-bound Rieske center newly detected in this study may also be a constituent of the terminal oxidase complex of *Sulfolobus* sp. strain 7 containing one *b*- and two *a*-type cytochromes (17).

Since the V-type ATPase and NADH dehydrogenase of *Sulfolobus* sp. strain 7 are only loosely bound to and partially released from the membrane upon disruption of the cells (22, 38), it is possible that sulredoxin may also be derived from the membrane-bound Rieske FeS center identified in this study. Alternatively, sulredoxin may be involved in a second (putative) cytoplasmic redox system that has not been identified yet, since the apparent size of its subunit is more similar to those of unusual [2Fe-2S]-type ferredoxins involved in bacterial nonheme iron dioxygenase systems (28) than to those of respiratory Rieske FeS proteins. Thus, determination of a physiological role for sulredoxin requires further investigation, and purification of the archaeal membrane-bound Rieske protein is under way.

Sulredoxin, identified and isolated from *Sulfolobus* sp. strain 7 in this study, is the first example of a Rieske-type [2Fe-2S] protein purified from the thermoacidophilic archaea, which also demonstrates that the Rieske-type [2Fe-2S] clusters are in fact widely spread in nature. Further studies comparing sulredoxin with the mitochondrial and bacterial respiratory Rieske FeS proteins (9, 34) and the unusual [2Fe-2S] ferredoxins of the bacterial dioxygenase system (28) in terms of primary structures and pH-dependent spectral and redox properties are under way.

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