The dicluster-type ferredoxin is a key electron carrier in the cytoplasm of the aerobic and thermoacidophilic archaeon, *Sulfolobus* sp. strain 7, and contains 1 separate and 7 cysteine residues as possible ligands to two FeS clusters. The optical, electron paramagnetic resonance (EPR), and cyclic voltammetric studies suggest the presence of one each of [3Fe-4S]'+pO (-280 mV) and [4Fe-4S]P'+ (-530 mV) clusters in the purified *Sulfolobus* ferredoxin, and the lower potential [3Fe-4S] cluster was scarcely reducible by excess dithionite even at pH 9. While the *Sulfolobus* ferredoxin has been known to function as an electron acceptor of 2-oxoacidferredoxin oxidoreductase, the functional significance and evolutionary implications of the [3Fe-4S] center in dicluster-type ferredoxins are discussed.

The biological significance of [3Fe-4S] cluster in iron-sulfur proteins has been a matter of discussion since its discovery (1–3). While this type of cluster is present in a variety of electron transfer proteins including small and acid iron-sulfur proteins called ferredoxins (Fds) (4), most of studies have been focused on the cluster conversion behavior (from [4Fe-4S] to [3Fe-4S] cluster) due to the oxidative damage in *vitro*; the best characterized is the case of aconitase, whose [4Fe-4S] cluster can be converted to the inactivated [3Fe-4S] form (1, 5). On the other hand, Fee and co-workers (3, 6) carried out the extensive comparative studies on 7Fe-type Fds from *Azotobacter vinelandii* and *Thermus thermophilus* and showed that the presence of [3Fe-4S] cluster in both Fds is not due to the oxidative damage. In the cases of the succinate dehydrogenase and fumarate reductase complexes, a [3Fe-4S] cluster (center S-3) is shown to be involved in the catalytic function (9–11). Thus, not all of the [3Fe-4S] clusters widely spread in nature represent a converted form of FeS cluster due to the oxidative damage.

*Sulfolobus* sp. strain 7 (formerly *Sulfolobus acidocaldarius* strain 7) is a typical thermoacidophilic archaeon (archaeabacterium) isolated from Beppu hot springs, Kyushu, Japan. It has at least two major redox systems; one is the membrane-bound aerobic respiratory chain, and the other is the cytoplasmic Fd-dependent redox system. Both systems are coupled at the different sites of the archaeal "oxidative" tricarboxylic acid cycle, and facilitate downstream electron transfer from the physiologically important intermediate metabolites. Our final goal is to elucidate the details of these redox systems of this particular archaeon in molecular details and to discuss it from the biochemical and evolutionary points of view. As an initial step, we have recently purified all constituents of the succinate-oxidizing aerobic respiratory system of *Sulfolobus*, which could reconstitute a cyanide-sensitive electron transport chain *in vitro*. We now turn our attention to the *Sulfolobus* Fd-dependent redox system, because none of the aerobic archaeal Fd-dependent redox systems has been investigated in details while the physiological roles of Fds are more closely linked to the central metabolisms of archaea than in the cases of aerobic respiratory (eu-)bacteria or eukarya (12, 13). For this purpose, we have previously isolated and crystallized a dicluster-type Fd, which is present in a large quantity in the archaeal cytoplasm (14), and more recently cloned and sequenced the gene encoding the protein. The deduced amino acid sequence of the protein contains 7 cysteine residues, which are sufficient in number as potential ligands to hold two FeS clusters, as in the case of the structural homologue from *S. acidocaldarius* strain DSM 639 (15). Thus, two parts of the sequence, namely Cys(35)

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*This investigation was supported in part by grants-in-aid from the Ministry of Education, Science and Culture of Japan and by a grant from the Biodesign Research Program of RIKEN. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This investigation was supported in part by grants-in-aid from the Ministry of Education, Science and Culture of Japan and by a grant from the Biodesign Research Program of RIKEN. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

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1. T. Iwasaki, T. Wakagi, K. Matsuura, and T. Oshima, manuscript in preparation (preliminary results have been presented at the *International Symposium on Flavins and Flavoproteins* (Nagoya, 1993)).

2. *The organism had been isolated from Beppu hot springs, Kyushu, Japan, originally named *S. acidocaldarius* strain 7, but was recently redesignated tentatively as "*Sulfolobus* sp. strain 7" due to a small difference in 16 S rRNA base sequences of strain 7 and *S. acidocaldarius* type strain DSM 639. The preliminary 16 S rRNA sequence analysis suggests that the isolate is a novel species belonging to the genus *Sulfolobus*.*

3. T. Iwasaki, T. Wakagi, K. Matsuura, and T. Oshima, manuscript in preparation (preliminary results have been presented at the *International Workshop on Molecular Biology and Biotechnology of Extremophiles and Archaeabacteria* (Wako-shi, 1993)).
Pro and Cys\(^{89}\)-Ile-Phe-Cys\(^{90}\)-Met-Ala-Cys\(^{91}\), provide a typical binding motif for one [4Fe-4S] cluster, and the other two parts, namely Cys\(^{92}\)-Leu-Ala-Asp\(^{89}\)-Gly-Ser-Cys\(^{91}\) and Cys\(^{91}\)-Pro, provide a second binding motif, respectively. Interestingly, the latter motif contains aspartate residue (number 48) in a position normally for cysteine (4, 15, 16). These, together with the quantitative iron and amino acid composition analyses indicating the presence of 7–8 mol of Fe/mol of protein, suggested that the Sulfolobus Fd is probably of the dicluster-type (14, 15, 17). However, the types of the FeS centers in the purified protein remain to be characterized.

In Sulfolobus, Fd is known to serve as an electron acceptor of a 2-oxoacid:Fd oxidoreductase (17), which is distinct from the well known 2-oxoacid dehydrogenase complexes from mitochondria and most of aerobic respiratory prokaryotes in many respects (12, 15). This enzyme has been purified recently from Sulfolobus sp. strain 7 and appears to be similar to those from Halobacterium salinarum (14–22). Since the Halobacterial Fd is a monocluster plant-type functioning as a single electron carrier (23–26), while the Sulfolobus Fd protein has been proposed as a dicluster bacterial-type (15, 17), it is of interest to study whether both FeS clusters of the latter protein participate in electron transfer from 2-oxoacid:Fd oxidoreductase. In this study, we have carried out the preliminary spectroscopic and cyclic voltammetric characterization of Fd from Sulfolobus sp. strain 7, and show that it is in fact a 7Fe-containing dicluster-type. In addition, evidence is presented that only the [3Fe-4S]\(^{+}\) center (–280 mV versus normal hydrogen electrode) of the Sulfolobus Fd is reducible during the steady-state turnover of the purified 2-oxoacid:Fd oxidoreductase. The functional significance of the [3Fe-4S] cluster in the archael dicluster-type Fd is demonstrated for the first time, and its evolutionary implication is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—DEAE-Sephasel, Superose-6, and Sephadex G-50 were purchased from Pharmacia LKB Biotechnology Inc, hydroxylapatite HTP from Bio-Rad, and HW-55C and phenyl-Toyopearl 6550M from Tosoh Corp., respectively. Tobramycin was purchased from Sigma, and MES, PIPES, and HEPES were from Nakarai Tesque. Water was purified by the Milli-Q purification system (Millipore). Other chemicals mentioned in this study were of analytical grade.

**Protein Preparation**—Sulfolobus sp. strain 7 was cultivated aerobically and chemoheterotrophically at pH 2.5–3 and 75–80 °C as described previously (27, 28), and was harvested in the stationary phase of growth and stored at –80 °C until use. The cells were suspended in 10 mM Tris-C1 buffer, pH 7.5, containing 0.5 mM phenylmethylsulfonyl fluoride and 1 mM EDTA and disrupted with a French press (Otake Works, Tokyo, Japan) at 1500 kg/cm\(^2\) twice, and the membrane fraction was removed by ultracentrifugation with a Beckman 45Ti rotor at 130,000 \(\times\) g for 100 min at 15 °C; the supernatant thus obtained contained negligible amounts of cytochrome and was used as the cyto- solic fraction.

A bacterial-type Fd present in the cytoplasm was purified by a DEAE-Sephasel (Pharmacia Biotech Inc.), a hydrophobic HW-55C (Tosoh Corp.), and a Sephadex G-50 gel filtration column chromatography to an electrophoretically homogeneous state, by following the patterns on polyacrylamide gel electrophoresis in the presence of SDS and denaturant at 280, 408, and 450 nm of each fraction at different purification steps as described elsewhere (14), and was stored at –80 °C. These modifications of the original procedure by Kerscher et al. (17), used for the purification of S. acidocaldarius Fd, provided a reproducibly crystallizable Fd; the purified Fd gave a single band on 20% analytical gel electrophoresis and had a purity index (A\(405\)/A\(280\)) of 0.70 (14). Approximately 30–40 mg of purified material could be routinely obtained from about 100 g (wet weight) of the cells.

Purification of a 2-oxoacid:Fd oxidoreductase was also carried out also from the cytoplasmic fraction of Sulfolobus sp. strain 7, as will be described elsewhere. In summary, the enzyme was purified by conventional column chromatographies (using DEAE-Sephasel (Pharmacia Biotech Inc.), hydroxylapatite HTP (Bio-Rad), and phenyl-Toyopearl 6550M columns (Tosho)) and preparative gel density gradient centrifugation, and the purified enzyme was stored at –80 °C until use. Approximately 6–8 mg of pure enzyme could be obtained from about 100 g (wet weight) of the cells.

**Measurement of Enzymatic Activity**—The 2-oxoacid:Fd oxidoreductase activities were monitored with a horse heart cytochrome c reduction assay using the Sulfolobus purified dicluster Fd as an intermediate electron acceptor (17), except that the reaction was initiated by addition of the purified enzyme. The assay was performed at 50 °C in 10 mM potassium phosphate buffer, pH 6.8.

**Analytical Methods**—Absorption spectra were recorded with a Hitachi U-3210 spectrophotometer equipped with a thermoelectric cell holder. EPR measurements were carried out using a JEOL JES-REX10 spectrometer equipped with a 1500 G Instruments model LTR-3 Bell-Drum cryostat system, in which temperature was monitored with a Scientific Instruments series 5500 temperature indicator/controller. Spin concentrations were estimated by double integration, with Cu-EDTA as a standard (29).

Electrochemical measurements were made using a Hago Electrochemical System potentialstat model 315A. DC cyclic voltammetry was carried out with the extensively degassed protein solution, typically 1 ml of 70–100 μM Fd from Sulfolobus sp. strain 7, contained in a caged cell at room temperature and under continuous flow of O\(_2\)-free N\(_2\) gas, with a three-electrode configuration as described by Armstrong et al. (30). The pyrolytic graphite "edge" electrode was polished and cleaned before measurements. An aminoglycoside, tobramycin (Sigma), was added as aliquots from 100 mM stock solutions (adjusted at pH 7.0) to promote electrochemical response of the protein, which was buffered by a mixture of MES, PIPES, and HEPES (each 5 mM) in 100 mM NaCl. All redox potentials quoted in this paper are versus normal hydrogen electrode.

Protein was measured by the BCA assay (Pierce) with bovine serum albumin as a standard and by dividing the results by 1.48 for calibration (see "Results and Discussion"). Metal content analysis was carried out by inductively coupled plasma atomic emission spectrometry with a Seiko SPS 1500 VR instrument.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to Laemmli (31) on 13 or 15% gels after treating proteins with 2% SDS in the presence or absence of 2% mercaptoethanol at 90 °C for 5 min, and proteins were visualized by Coomassie Brilliant Blue staining.

**RESULTS AND DISCUSSION**

**Optical and EPR Characterization of the Sulfolobus Ferredoxin**—A dicluster-type ferredoxin is present in a large quantity in chemoheterotrophically grown Sulfolobus sp. strain 7. It has been reported as an [3Fe-4S] cluster type in the case of S. acidocaldarius strain DSM 639 (17), although no detailed study was carried out for the assignment of the cluster type.

Fd purified from Sulfolobus sp. strain 7 has an apparent molecular weight of 12,000 on a calibrated size exclusion fast protein liquid chromatography in the presence of 1.0 M NaCl, in agreement with the previous reports (14, 17). However, the average iron content of the purified materials (4.73 mol of Fe/mol of Fd) on the basis of the BCA protein assay and inductively coupled plasma atomic emission spectrometry was unexpectedly low, and the subsequent quantitative analysis indicated the overestimation of the amount of protein by BCA assay method with BSA (bovine serum albumin) as a standard, as reported by others (17); this has been also experienced in other FeS proteins, including the bacterial-type Fds (16, 32–35). We therefore used the protein concentration by the BCA assay method using BSA as a standard and by dividing the result by 1.48 for calibration throughout this study. The presence of two FeS centers in the Sulfolobus Fd has been indicated from its deduced primary sequence (17) and the preliminary cyclic voltammetric analysis in the presence of 2 mM tobramycin as a promoter described below. In the following studies, we used the pH value of 6.8 throughout the experiments, because the value is very close to the physiological pH of Sulfolobus (44).

Fig. 1 shows the optical absorption spectra of purified Sulfolobus Fd. It exhibits maxima at 281 and 408 nm (A\(408\)/A\(280\) = 0.70) and a shoulder at 310 nm in the air-oxidized state (as...
prepared; trace A). Anaerobic addition of excess sodium dithionite in 40 mM potassium phosphate buffer, pH 6.8 (A and B), and after the adjustment of pH at 9 with a small amount of 2 M Tris base and the base-line correction (C): ΔA408 = -19-20% for dithionite-reduced ferredoxin at pH 6.8 (B), and ΔA408 = 30% at pH 9 (C), respectively; the protein concentration, 0.93 mg/ml; a purity index of the sample (A408/A281), 0.70.

Fig. 2 shows the effect of temperature on the X-band EPR spectra of air-oxidized ferredoxin from the Sulfolobus Dicluster Fd. It elicits EPR signals, which give rise to a sharp peak at g = 2.03, a broad trough around g = 1.94 with an extended tail toward the high field, and a distinct shoulder at g = 1.98 (at 11 K or below; traces A and B). This spectrum could not be observed clearly at temperatures above 15 K (trace C), indicating the extremely rapid spin relaxation of this center. These EPR properties are very similar to those of the [3Fe-4S]1+ cluster of the dicluster Fds from T. thermophilus and A. vinelandii (7, 8, 36), and quantitation of the signal using Cu-EDTA as a standard under the non-saturating conditions resulted 0.9–1.1 spin/mol Fd. No other EPR signal could be detected in the lower field region under the conditions (data not shown). These data clearly suggest the presence of at least one [3Fe-4S]1+ cluster in the Sulfolobus Fd.

Fig. 3 shows the reduction behavior of the purified Fd monitored by the X-band EPR spectroscopy at 10 K, under the same conditions as described in Fig. 1. Upon anaerobic addition of excess sodium dithionite at pH 6.8, the EPR signals derived from the oxidized [3Fe-4S]1+ cluster (trace A) were almost abolished, while no other new signal was observed at 10 K (trace B). Quantitation of the remaining S = 1/2 resonance resulted in ~0.08 spin/mol Fd, indicating the bulk (~92%) of the [3Fe-4S] cluster was reduced under the conditions, where ~20% reduction of the 408 nm absorbance occurred (Fig. 1, trace B). Further anaerobic reduction of the purified Sulfolobus Fd at pH 9, where ~30% of the 408 nm absorbance occurred (Fig. 1, trace C), resulted in a slight alternation of the overall EPR line shapes in addition to the appearance of several new signals (g = 2.09, 2.03, 2.01, 1.99, 1.92, and 1.86; Fig. 3, trace C). The complex spectrum, though not very clearly, resembles those of highly reduced 7Fe Fds (7, 8, 30, 34, 36) exhibiting a spin-spin interaction between two FeS clusters. Quantitation of the remaining S = 1/2 resonance resulted only 0.04 spin/mol Fd, indicating that the extremely low potential [4Fe-4S] cluster is probably diamagnetic (in the S = 0 ground state) even under the conditions (see below).

Cyclic Voltammetry—Fig. 4 shows the preliminary cyclic voltammogram of the dicluster-type Fd from Sulfolobus sp. strain
7 in the presence of 2 mM tobramycin as a promoter. Well-defined quasi-reversible waves (not observed in buffer alone) are obtained only in the presence of an aminoglycoside, tobramycin, as an electrode promoter (typically 1–3 mM), as in the cases of the 7Fe Fds from _Azotobacter chroococcum_ (30) and _Desulfovibrio africanus_ (Fd III) (37–39), though even in the presence of aminoglycoside, the _Sulfolobus_ Fd shows a poorer electrode response than the cases of these bacterial Fds, and thus slightly higher concentrations of protein were required.

The parameters obtained from Fig. 4 are: $E_{1/2} = -280 \pm 10$ mV, and the cathodic to anodic peak potential separation $\Delta E_p$ (scan rate 40 mV s$^{-1}$, pH 7.0) = 60–70 mV for couple “A”; $E_{1/2} = -550 \pm 10$ mV, and $\Delta E_p$ (scan rate 40 mV s$^{-1}$, pH 7.0) = 60–70 mV for couple “B”; and $E_{1/2} = -690 \pm 10$ mV, and $\Delta E_p$ (scan rate 40 mV s$^{-1}$, pH 7.0) = 40 mV, for couple “C”, respectively. Couples A and B are of similar intensity to each other, and have the reproducible and almost constant $\Delta E_p$ values (60–70 mV) over the scan-rate range 3–200 mV s$^{-1}$. In addition, a linear relationship between the anodic-peak current and the square root of the potential scan rate at least up to 100 mV s$^{-1}$ suggests that these electrode processes are quasi-reversible and effectively diffusion-controlled (data not shown).

Couple C has a slightly smaller intensity than those of couples A and B and has almost constant $\Delta E_p$ values (30–40 mV) up to 100 mV s$^{-1}$, where the electrode process is quasi-reversible and effectively diffusion-controlled, in contrast to the case of _D. africanus_ Fd III (37). On the other hand, all of these couples (especially couple C) show the increments of $\Delta E_p$ and of the intensity of both cathodic and anodic waves at scan rates above 100 mV s$^{-1}$ (especially at 200–400 mV s$^{-1}$), indicating the occurrences of increased relative current contributions from adsorbed protein under these conditions.

Thus, the preliminary cyclic voltammetric studies suggest the presence of two reducible centers with $E_{1/2}$ values at pH 6.8–7.0 of $-280 \pm 10$ mV and $-530 \pm 10$ mV, at room temperature (Table I). A further reduction process is observed at $E_{1/2}$ values at pH 6.8–7.0 of $-690 \pm 10$ mV, which can be assigned to be the further two-electron reduction process of the one-electron reduced [3Fe-4S]$^0$ cluster like the cases of other 7Fe Fds (30, 37, 39, 40) (in the cases of _A. vinelandii_ and _D. africanus_ Fds (39, 40), the products have been assumed to be a strong base, which binds protons possibly at the bridging sulfide ligands. These data, in conjunction with the optical and EPR studies, suggest that the _Sulfolobus_ dicluster Fd is in fact a 7Fe type similar to those from _A. vinelandii_ (40–42), _T. thermophilus_ (8, 16, 36), and _Streptomyces griseus_ (34, 43), in that it has one each of the dithionite-reducible [3Fe-4S]$^{2-}$ ($-280$ mV) and the dithionite-unreducible extremely low potential [4Fe-4S]$^{2+}$ clusters ($-530$ mV). The midpoint redox potentials of the FeS clusters of the _Sulfolobus_ Fd resemble most closely to those of the corresponding centers of _T. thermophilus_ Fd (36) (see Table I). The midpoint redox potentials of the [4Fe-4S]$^{2+}$ clusters of these 7Fe Fds are considerably lower, therefore requiring either the photochemical (6, 34) or direct electrochemical reduction method (30, 37) for complete reduction. On the other hand, the [4Fe-4S]$^{2+}$ clusters of 7Fe Fds from _D. africanus_ (Fd III; see Refs. 37 and 38) and _Bacillus subtilis_ (32) are readily reducible by dithionite, indicating that there are large differences among the redox properties of [4Fe-4S] centers of prokaryotic 7Fe Fds, in spite of their similarities at the primary structure level.

**Functional Importance of the [3Fe-4S] Center of the Sulfolobus Ferredoxin**—Kerscher et al. (17) previously reported a preliminary survey indicating the presence of a 2-oxoacid:Fd oxidoreductase activity in thermoacidophilic archaea including _S. acidocaldarius_ and _Thermoplasm acidophilum_. This activity was recently purified from _Sulfolobus_ sp. strain 7 to an apparent homogeneity using the cognate Fd as an electron acceptor. As in the case of _H. salinarium_ pyruvate:Fd oxidoreductase (20, 21), the purified _Sulfolobus_ enzyme consisted of two non-identical subunits and exhibited a broad substrate specificity toward 2-oxoacid (with the specific activities of $-69$ units/mg in the case of 2-oxoglutarate ($K_a = 870$ mK), and of $-39$ units/mg in the case of pyruvate ($K_a = 250$ mK), respectively); the details of the molecular properties of the _Sulfolobus_ enzyme will be reported elsewhere.

Using the purified 2-oxoacid:Fd oxidoreductase and the dicluster Fd of _Sulfolobus_, an in vitro 2-oxoacid-dependent Fd-reducing system was constructed to test whether one or both of the FeS clusters of the Fd were reduced during the steady-state turnover of the enzyme. This system consists of 56 mK Fd and 8.6 mK of the purified enzyme in 20 mM potassium phosphate buffer, pH 6.8 (the value close to the reported intracellular pH of _Sulfolobus_; Ref. 44), in the presence of 2 mM 2-oxoglutarate and 100 mK coenzyme A. It should be mentioned that Fd is not reoxidized under the anaerobic conditions due to the absence of any electron acceptor in the _in vitro_ system, whereas oxygen reoxidizes Fd when tested aerobically. On the other hand, the archaeal 2-oxoacid:Fd oxidoreductase catalyzes the steady-state turnover in either conditions, due to the presence of excess amounts of both Fd (56 mK) and 2-oxoglutarate (2 mK) over enzyme in the system. One typical result is shown in Fig. 5, which indicates the maximal reduction of the 406 nm absorbance of the dicluster Fd to be $-19$–$20\%$ under the experimental conditions even when tested anaerobically at 50 °C. The enzymatically reduced spectra of Fd were neither altered at least for 6 h at 50–60 °C, nor affected by further addition of the enzyme or substrates (data not shown). Furthermore, when the reaction was performed anaerobically in an EPR tube at 56 °C for 5 h, the resulting spectrum at 10 K was completely EPR silent in the $g = 2$ region, indicating that the [3Fe-4S] cluster was almost fully reduced under the conditions (data not shown). These data, together with the spectroscopic properties of the _Sulfolobus_ Fd (see Fig. 1 and 3), clearly suggest that only the dithionite-reducible [3Fe-4S] cluster is reduced during the steady-state turnover of the enzyme, while the bulk of the
[3Fe-4S] Cluster of the Sulfolobus Dicluster Ferredoxin

Table 1: Midpoint redox potentials of FeS clusters of several bacterial-type ferredoxins

| Couplet            | Azotobacter vinelandii Fd | Azotobacter chroococcum | Desulfovibrio africanus Fd III | Thermus thermophilus | Sulfolobus sp. strain 7<sup>a</sup> | Pyrococcus furiosus<sup>b</sup> |
|--------------------|----------------------------|--------------------------|--------------------------------|----------------------|---------------------------------|----------------------------------|
| [3Fe-4S]<sup>1+0</sup> | -430 mV                    | -460 mV                  | -140 mV                        | -250 mV              | -280 mV (Absent)                |                                  |
| [4Fe-4S]<sup>2+0</sup> | -647 mV                    | -645 mV                  | -410 mV                        | -530 mV              | -530 mV                         | -345 mV (at pH 8)               |
| **Couple C**       | (~ -0.8 V at pH 8.4)       | (~ -0.8 V at pH 8.3)     | (~ -0.75 V at pH 7.45)         | (~ -0.69 V at pH 7.00) |                                 |                                  |

| Cyclic | Cyclic | Cyclic | EPR | Cyclic | EPR |
|--------|--------|--------|-----|--------|-----|
| voltammetry | voltammetry | voltammetry | EPR | voltammetry | EPR |
| (40)<sup>c</sup> | (30) | (37) | (36) | This work | (33, 50) |

<sup>a</sup> Archaeal Fds.
<sup>b</sup> Monocluster-type [4Fe-4S] Fd; ---, not reported.
<sup>c</sup> Reference.

The primary structure of the dicluster Fd of Sulfolobus sp. strain 7 indicated the overall homology (87% identity) to that of *S. acidocaldarius* Fd (15). The alignment of the primary structures of several Azotobacter-type Fds with that of the Sulfolobus Fd indicated the strict conservation of 7 cysteine residues<sup>5</sup> (see Fig. 6, bottom), all of which have been assigned as ligands to the FeS clusters at least in the case of A. vinelandii Fd I (41, 42). These data indicate that the [3Fe-4S]<sup>1+0</sup> cluster of the Sulfolobus Fd corresponds to the cluster I, and the low potential [4Fe-4S]<sup>2+0</sup> cluster to the cluster II, respectively (Fig. 6).

This is very intriguing in conjunction with the reduction of the Sulfolobus Fd enzymatically with 2-oxoacid:ferredoxin oxidoreductase at 50 °C. The Sulfolobus Fd in 30 mM potassium phosphate buffer, pH 6.8 (solid line) was reduced by 8.6 µg/ml of 2-oxoacid:Fd oxidoreductase in the presence of 2 mM 2-oxoglutarate and 100 µM coenzyme A, either aerobically for 4 h (∆A<sub>900</sub> ~14%; ---) or anaerobically for 4 h (∆A<sub>900</sub> ~19.5% (no further reduction was achieved); - - -).

The bacterial-type Fds on the basis of their comparative studies on the three-dimensional (three-dimensional) structures of various bacterial-type Fds, as illustrated in Fig. 6 (top). In their model, early dicluster Fds have been derived from a putative common ancestor of bacterial-type Fds with a single FeS cluster as a result of a gene duplication event (4); the polypeptide backbone structures of such dicluster-type Fds might be similar to those of the 8Fe-type dicluster Fds (designated as the “clostridial-type”), in that they exhibit a marked two-fold symmetry as exemplified by *Peptococcus aerogenes* Fd (49). The 7Fe-type dicluster Fds (“Azotobacter-type”) have been also derived from early dicluster-type Fds, but have a distorted two-fold symmetry due to the insertions of several loops. On the other hand, the three-dimensional structures of the “monocluster-type” Fds known so far exhibit a remarkable pseudo-two-fold symmetry in spite of the presence of only a single [3Fe-4S] or [4Fe-4S] cluster at the position corresponding to the “cluster I” site of the dicluster-type Fds; Fukuyama et al. (48) therefore proposed that this might be due to the loss of the [4Fe-4S] cluster at the “cluster II” site and the subsequent insertion of an α-helix structure and a disulfide bond into the corresponding site for stabilization of the protein. Thus, cluster I, which corresponds to a [3Fe-4S] center in *Desulfovibrio gigas* and Azotobacter-type Fds, is strictly conserved in all bacterial-type Fds, while the cluster II is missing (replaced by an α-helix) in the monocluster-type Fds (Fig. 6).

**Evolutionary Implication**—Fukuyama et al. (see Ref. 48, and references therein) proposed a possible evolutionary scheme of...
Sulfolobus 7Fe Fd is also capable of undergoing two-electron reduction in a reversible manner in vitro as indicated by the cyclic voltammetric analysis (Fig. 4).

These data indicate the following hypotheses on the evolution of the bacterial-type Fds from their dicluster prototype. (i) The cluster I has been conserved because of the functional importance. (ii) The cluster II may have lost its original redox role in the early stage of the evolution; the presence of a single [4Fe-4S] cluster in the Fd of the hyperthermophilic archaean Pyrococcus furiosus (33, 50, 51) (see also Fig. 6, bottom) indicates that this may have taken place even before the divergence of the Archaea and Bacteria domains (52, 53). (iii) This possibly allowed the replacement of the cluster II by structural polypeptides in some Fds in the later stage (cf. Fig. 6 and Ref. 4). If these are indeed the cases, the cluster II of the dicluster-type Fds may possibly represent an "evolutionary relic," since one can easily speculate that the extremely low potential natural of cluster II (cf. Table I) is functionally no more necessary after adaptation to the oxidative atmosphere on the ancient earth during the evolution.

Although our hypotheses require more discriminations for further proofs, it should be added that the presence of such evolutionary relics has also been reported for certain chromophores of the bacterial photosynthetic reaction centers (64) and the heme group of succinate dehydrogenase complexes (11), and presumably such prosthetic groups may be found in some other redox proteins.

Acknowledgments—We thank Dr. N. Wakiya (Tokyo Institute of Technology) for the metal content analysis by the ICP atomic emission spectrometry, and M. Sakurai for preparing the computer graphics presented in Fig. 6.

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