The dcluster-type ferredoxins from the thermoacidophilic archaea such as *Thermoplasma acidophilum* and *Sulfolobus* sp. are known to contain an unusually long extension of unknown function in the N-terminal region. Recent x-ray structural analysis of the *Sulfolobus* ferredoxin has revealed the presence of a novel zinc center, which is coordinated by three histidine ligand residues in the N-terminal region and one aspartate in the ferredoxin core domain. We report here the quantitative metal analyses together with electron paramagnetic resonance and resonance Raman spectra of *T. acidophilum* ferredoxin, demonstrating the presence of a novel zinc center in addition to one [3Fe-4S] and one [4Fe-4S] cluster (Fe/Zn = 6.8 mol/mol). A phylogenetic tree constructed for several archaeal monocloner and dcluster-type ferredoxins suggests that the zinc-containing ferredoxins of *T. acidophilum* and *Sulfolobus* sp. form an independent subgroup, which is more distantly related to the ferredoxins from the hyperthermophiles than those from the methanogenic archaia, indicating the existence of a novel group of ferredoxins, namely, a "zinc-containing ferredoxin family" in the thermoacidophilic archaea. Inspection of the N-terminal extension regions of the archaean zinc-containing ferredoxins suggested strict conservation of three histidine and one aspartate residues as possible ligands to the novel zinc center.

Archaea (archaeacteria) represent deep and short lineages of the universal phylogenetic tree, comprising the third independent domain of life (1–3). They grow under various extreme environments, and contain a variety of unique electron transfer proteins, which are currently under extensive investigation (4–7). One of the characteristic features in the archaean central metabolic pathways is the involvement of small iron-sulfur (FeS) proteins called ferredoxins in several key steps, where NAD(P)⁺ usually substitutes in some bacteria (eubacteria) and euarya (eukariotes) (5, 8–10). This is also the case in several strictly aerobic archaia, including *Sulfolobus* sp. strain 7, which contains nearly complete sets of the membrane-bound proteins for aerobic respiration (7, 11–16), indicating that the central metabolic pathways of aerobic archaia are probably more closely related to the ferredoxin-linked fermentative pathways of anaerobic archaia and bacteria than is the case for the aerobic bacteria and euarya (e.g. see Iwasaki et al. (7)).

In earlier studies, Oesterhelt and coworkers (17, 18) have established that ferredoxins from the aerobic, thermoacidophilic archaia function as an effective electron acceptor of the cognate coenzyme A-acylating 2-oxoacid:ferredoxin oxidoreductases, the key enzymes of the archaean oxidative tricarboxylic acid cycle and pyruvate oxidation (8). Later, these ferredoxins were found to contain an unusually long N-terminal extension region of unknown function, which was not detected in the bacterial type ferredoxins from other sources (10, 18–22). This probably represents a unique evolutionary event, but it has not been pursued further.

Quite recently, T. Fujii, N. Tanaka, T. Oshima, and coworkers have determined the 2.0 Å resolution crystal structure of a dcluster type ferredoxin from *Sulfolobus* sp. strain 7 (optimal growth conditions, pH 2.5–3 and 80°C) (10, 11) with the R-factor of 17.9% by multiple isomorphous replacement method (23). This led to an unexpected finding that, in addition to two FeS clusters, a tightly bound zinc atom is coordinated by His16, His19, His34, and Asp78 in a tetragonal fashion, in the boundary between the N-terminal extension region (containing three histidine ligand residues) and the pseudo-2-fold symmetrical "ferredoxin core-fold" portion (containing one aspartate ligand residue), fixing these together. Thus, the *Sulfolobus* sp. ferredoxin appears to be the first example that inherently contains an additional metal, i.e. Zn²⁺, besides the iron atoms. The tetragonal ligation of the novel zinc center in the *Sulfolobus* sp. ferredoxin is similar to those of other structurally unrelated zinc-containing proteins, e.g. adenosine deaminase and carbonic anhydrase in which a zinc center is coordinated by three histidine residues and a water molecule (24, 25).

In order to investigate a possibility of the presence of an additional metal center in other ferredoxins containing the long N-terminal extension region, we have performed chemical and spectroscopic characterization of an another example of a bacterial type ferredoxin with a long N-terminal extension region from a thermoacidophilic archaean, *Thermoplasma acidophilum* (optimal growth conditions, pH 1.8 and 56°C) (26). In this study, we report that *T. acidophilum* ferredoxin (19) is in fact a 7Fe ferredoxin containing a zinc center, as in the case of *Sulfolobus* sp. ferredoxin. In addition, the amino acid sequence

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alignment and phylogenetic tree analyses of several archael ferredoxins provide strong evidence that ferredoxins with a long N-terminal extension region derived from several thermoacidophilic archaea (10, 18–22) form a novel separated group, namely, a “zinc-containing ferredoxin” family. The possible zinc-binding ligand residues are strictly conserved in the archael zinc-containing ferredoxins.

EXPERIMENTAL PROCEDURES

Materials—DEAE-Sepharose Fast Flow and Sephadex G-50 gels were purchased from Pharmacia Biotech Inc. Water was purified by the Milli-Q purification system (Millipore). Other chemicals used in this study were of analytical grade.

Organism, Cell Culture, and Protein Preparation—T. acidophilum strain HO-62 cells, originally isolated from hot sulfur springs at Owakudani sulfataric field in Hakone, Japan, were cultivated at pH 1.8 and at 56 °C as described by Yasuda et al. (26). The Thermoplasma ferredoxin was purified essentially as outlined by Kerscher et al. (18), using a DEAE-Sepharose Fast Flow column (Pharmacia) connected to a Pharmacia fast protein liquid chromatography and a Sephadex G-50 column chromatography. Purified ferredoxin had a purity index ($A_{420}/A_{280}$) of 0.53 (18) and showed a single band on 20% analytical polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate.

Sulfolobus sp. strain 7 cells, originally isolated from Beppu hot springs, Japan, were cultivated aerobically and chemoheterotrophically at 75–80°C and at pH 1.8, and the archaeal ferredoxin was routinely purified as described previously (10, 23). The cognate 2-oxoacid:ferredoxin oxidoreductase has been purified as described elsewhere (10, 27).

Analytical Methods—Absorption spectra were recorded as described previously (10). Electron paramagnetic resonance measurements were carried out 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, or a JEOL JES-PE3XG spectrometer equipped with an Air Products model LTR-3-110 Heli-Tran cryostat system, in which temperature was monitored with a Scientific Instruments series 5500 temperature indicator/controller. Spin concentrations were estimated by double integration, with 0.1 and 1 mM Cu−

Matrix-assisted laser desorption ionization-time of flight mass spectrometry of the purified protein gave an average mass (M + H)$^+$ of ~15960 (data not shown), which is significantly close to the value estimated from the primary structure of T. acidophilum apoferrodoxin (15963 Da) (19). These data suggest that the ferredoxin purified from T. acidophilum strain HO-62 is essentially identical to that previously reported by Kerscher and co-workers (18, 19) and contains a long N-terminal extension region. Metal content analysis by inductively coupled plasma atomic emission spectrometry showed that the purified ferredoxin contained a tightly bound zinc atom (6.8 Fe/Zn (mol/mol)). The following metals were not detected: molybdenum, cobalt, nickel, and copper. In conjunction with the primary structural evidence suggesting two sets of FeS cluster-binding motifs (19), this indicates that T. acidophilum ferredoxin contains a unique zinc center beside two FeS clusters (see below).

The properties of the FeS clusters in T. acidophilum ferredoxin were investigated by X-band EPR spectroscopy (Fig. 1). The EPR spectrum at 8.2 K of the isolated T. acidophilum ferredoxin elicited a sharp g = 2.02 signal (~1.1 spin/mol), which is characteristic of a [3Fe-4S]$^1$ cluster (Fig. 1, trace A). Upon reduction by excess sodium dithionite at pH 6.8, the relative intensity of this EPR signal decreased by ~85%, indicating the partial reduction of the [3Fe-4S] cluster, but no additional EPR signal could be detected (data not shown). Because the decrease of the A$_{420}$nm peak in the optical spectrum of the purified protein was ~20% under the conditions, these data suggest the presence of a lower potential [4Fe-4S] cluster that is not readily reduced by dithionite, in addition to a [3Fe-4S] cluster. This was further supported by the EPR spectra at 8.2 K of T. acidophilum ferredoxin anaerobically reduced by excess dithionite at pH 9.3 (Fig. 1, traces B and C). The [3Fe-4S] cluster was fully reduced, thus giving rise to a very broad low field resonance at g ~ 11, which is characteristic of the reduced S = 2 [3Fe-4S]$^1$ cluster (Fig. 1, trace C). In addition, a rhombic EPR signal at g$_{x,y,z}$ = 2.06, 1.94, and 1.88

RESULTS AND DISCUSSION

Purification and Characterization of T. acidophilum Dicluster Ferredoxin—The x-ray crystal structure of the dicluster ferredoxin from Sulfolobus sp. strain 7 at 2.0-Å resolution has demonstrated the presence of a novel zinc center which is ligated by His$^{16}$, His$^{19}$, His$^{34}$, and Asp$^{78}$ in a tetragonal coordination, in the boundary between the N-terminal extension and the FeS cluster-binding core regions, fixing these together.

The metal content analysis of the isolated Sulfolobus sp. ferredoxin in solution also showed the presence of a tightly bound zinc atom, in a ratio of ~6.4–6.9 Fe/Zn (mol/mol; data not shown). The zinc atom could not be removed by dialysis against buffer containing 5 mM EDTA. These data suggest that the Sulfolobus sp. ferredoxin inherently contains an additional metal binding site specific to zinc atom, beside two FeS clusters. For comparison, we have also purified a 7Fe ferredoxin from a thermophilic bacterium Thermus thermophilus strain HB8 (30) using the same buffer system: The purified ferredoxin gave an average mass (M + H)$^+$ of ~8684 by matrix-assisted laser desorption ionization-time of flight mass spectrometry, being consistent with the value estimated from the primary structure (8687 Da) (30), and contained no zinc atom.

In order to investigate whether the zinc center is unique to the Sulfolobus sp. ferredoxin, another bacterial-type ferredoxin with a long N-terminal extension has been purified to an electrophoretically homogeneous state from a thermophilic archaean, T. acidophilum strain HO-62 (26), according to the guidelines of Kerscher et al. (18). The purified ferredoxin of T. acidophilum strain HO-62 had a purity index ($A_{420}/A_{280}$) of 0.53, and showed the optical properties identical to those reported previously by Kerscher et al. (18) (data not shown). The N-terminal 15 amino acid residues of the purified ferredoxin (VKLLELDKFJKPKPEIDE) were completely identical to that reported previously by Wakabayashi et al. (19). Moreover, the matrix-assisted laser desorption ionization-time of flight mass spectrometry of the purified protein gave an average mass (M + H)$^+$ of ~15960 (data not shown), which is significantly close to the value estimated from the primary structure of T. acidophilum apoferrodoxin (15963 Da) (19). These data suggest that the ferredoxin purified from T. acidophilum strain HO-62 is essentially identical to that previously reported by Kerscher and co-workers (18, 19) and contains a long N-terminal extension region. Metal content analysis by inductively coupled plasma atomic emission spectrometry showed that the purified ferredoxin contained a tightly bound zinc atom (6.8 Fe/Zn (mol/mol)). The following metals were not detected: molybdenum, cobalt, nickel, and copper. In conjunction with the primary structural evidence suggesting two sets of FeS cluster-binding motifs (19), this indicates that T. acidophilum ferredoxin contains a unique zinc center beside two FeS clusters (see below).

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3 T. Iwasaki and T. Oshima, unpublished results.
attributed to the reduced $S = \frac{1}{2}$ $[4Fe-4S]^{1+}$ cluster, was detected together with additional wings on the high and low filed sides of the main EPR signal ($g = 2.13$ and $1.78$) which are due to the magnetic interactions with the reduced $S = 2$ $[3Fe-4S]^{0}$ cluster (Fig. 1, trace B). These properties are characteristic of those of conventional reduced 7Fe ferredoxins.

The properties of the oxidized FeS clusters of $T$. acidophilum ferredoxin were further investigated by the low temperature resonance Raman spectroscopy, which was performed at 77 K using 488.0 nm and 457.9 nm Ar$^+$ ion laser excitation (Fig. 2, traces A and B). On the basis of extensive assignments by Johnson and co-workers (31, 32), two weak bands at 334 cm$^{-1}$ and $-358$ cm$^{-1}$, whose signal relative intensities were enhanced upon 457.9 nm Ar$^+$ ion laser excitation (Fig. 2, trace B), were assigned to be associated primarily with Fe-S bridging mode and Fe-S terminal mode, respectively, of a conventional biological $[4Fe-4S]^{2+}$ cluster with the $D_{2d}$ structure. On the other hand, the $[3Fe-4S]^{1+}$ cluster exhibited three bands associated primarily with Fe-S bridging modes at 265 cm$^{-1}$, $-288$ cm$^{-1}$, and $347$ cm$^{-1}$, and at least two bands associated primarily with Fe-S terminal modes at 369 cm$^{-1}$ and $387$ cm$^{-1}$ (31, 32). Thus, although 7Fe ferredoxin of $T$. acidophilum strain HO-62 contains a zinc center, the EPR and resonance Raman spectral properties are very similar to those of conventional 7Fe ferredoxins reported earlier (31), clearly demonstrating the existence of common $[3Fe-4S]$ and $[4Fe-4S]$ core structures.

Conservation of Zinc-binding Ligand Residues in Ferredoxins from Several Thermoacidophilic Archaea—Of all amino acid sequences of the bacterial type ferredoxins reported to date, only four ferredoxins, all of which have been derived from thermoacidophilic archaea, are known to possess a long N-terminus.
terminal extension region (10, 18–22). Fig. 3 shows the amino acid sequence alignment of the N-terminal extension region of these ferredoxins. The N-terminal extension region of archaeal ferredoxins from Sulfolobus sp. strain 7 (10),\(^2\) S. acidocaldarius (20, 22), and Desulfolofulbus ambivalens (21) are considerably homologous to each other, leaving that from T. acidophilum to be less homologous (19). We have found, however, by careful amino acid sequence alignment that all four ligand residues which have been identified as ligands to the tetragonal zinc center in Sulfolobus sp. ferredoxin (His\(^{18}\), His\(^{19}\), His\(^{34}\), and Asp\(^{78}\)\(^1\)) are strictly conserved among these ferredoxins (Fig. 3). Thus, these archaeal ferredoxins show the overall amino acid sequence homology. Since both the purified Sulfolobus and Thermoplasma dicluster ferredoxins contain a stoichiometric amount of a zinc atom as mentioned in the preceding section, we suggest that a bacterial-type ferredoxin containing a long N-terminal extension region with the consensus three histidine motif (Fig. 3) would bind a zinc ion, beside one or two conventional FeS clusters. A tentative model of possible metal-binding ligand residues of T. acidophilum zinc-containing 7Fe ferredoxin is schematically presented in Fig. 4, in light of the three-dimensional structure of the Sulfolobus sp. ferredoxin.\(^1\) It should be remembered that this is a putative schematic drawing, and the detailed characterization of the coordination environments of the zinc center of T. acidophilum ferredoxin would require the extended x-ray absorption fine structure or the x-ray crystallography analyses. However, at this stage limited availability of the archaeal ferredoxin, due largely to extremely slow growth and poor yield of T. acidophilum strain HO-62 cells (26), has made such analyses practically difficult.

Phylogenetic Tree Analysis of Archaeal Zinc-containing Ferredoxins—The bacterial type ferredoxins investigated so far possess conserved FeS cluster binding motifs and overall homologies at least at the primary structural level (33–35). Using the amino acid sequences of eight archaeal monocluster and dicluster ferredoxins (from Sulfolobus sp. strain 7 (accession nos. PC2290 and D78179),\(^2\) S. acidocaldarius (accession no. P00219), T. acidophilum (accession no. P00218), Methanococcus thermolithotrophicus (accession no. P21305), Methanosarcina thermoeutropha (barkeri) (accession no. P00202), Methanosarcina thermolithotrophicus (accession no. A42960), Thermococcus litoralis (accession no. P29604), and Pyrococcus furiosus (accession no. X79502) and a 4Fe ferredoxin from the hyperthermophilic bacterium Thermotoga maritima (accession no. X82178), putative phylogenetic relations were estimated, taking the positions of conserved cysteine ligand residues and charged and hydrophobic amino acid residues into account (Fig. 5). Some of these ferredoxins have been shown to serve at least as an electron acceptor of the cognate 2-oxoacid:ferredoxin oxidoreductases (10, 18, 21, 27, 36, 37). Both distant geometry and parsimony analyses of the amino acid sequence alignment gave essentially identical topologies of the trees.\(^5\)

The three-dimensional structures of all bacterial-type ferredoxins contain a monomethyllysine residue at position 29 (20).

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\(^1\) T. Oshima, unpublished result. Although not shown in the figure, both Sulfolobus sp. and S. acidocaldarius ferredoxins contain a monomethyllysine residue at position 29 (20).

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\(^2\) S. acidocaldarius strain DSM 639 ferredoxin (20, 22); D. ambivalens ferredoxin (21); T. acidophilum ferredoxin (19) (T. Iwasaki and T. Oshima, unpublished result). Although not shown in the figure, both Sulfolobus sp. and S. acidocaldarius ferredoxins contain a monomethyllysine residue at position 29 (20).

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\(^3\) T. Suzuki, T. Iwasaki, and T. Oshima, unpublished data. The details of the amino acid sequence alignment of archaeal ferredoxins are available upon request.
doxins so far determined exhibit a remarkable pseudo-2-fold symmetry, indicating that a putative initial gene duplication event might have taken place in the earliest stage of the molecular evolution of a primordial bacterial type ferredoxin (10, 38, 39). The basic concepts of this have been generally accepted, and the extreme cases may be emphasized for the unusual polyferredoxins typically found in certain methanogenic archaea (40) (not considered in the phylogenetic tree shown in Fig. 5). The pseudo-2-fold symmetry of the three-dimensional structures of both the archaeal and bacterial ferredoxins suggests that a putative initial gene duplication event might have occurred long before the divergence of the Archaea and the Bacteria domains (1, 2). The archaeal ferredoxin tree shown in Fig. 5 clearly suggests their monophyletic origin, and, in a supposed first evolutionary event, divergence of the monocluster and dicluster ferredoxins occurred prior to the divergence of the archaeal genera.

Intriguingly, the ferredoxins containing a long N-terminal extension region and a zinc center phylogenetically form a separate group, and they are supposed to be evolved from a common ancestor. Thus, the phylogenetic analysis clearly suggests that they can be classified as a novel, separate group, namely, the “zinc-containing ferredoxin” family. This is supported from our findings that the overall topologies of the calculated trees are essentially identical whether or not information of the long N-terminal extension and the central additional loop regions of the zinc-containing ferredoxins is taken into account for the analysis (data not shown).

Another interesting feature of the archaeal ferredoxin tree (Fig. 5) is that it unexpectedly well reflects the differences in the growth conditions of the archaeal species, rather than their phylogenetic relationships based on the universal phylogenetic tree calculated on the basis of the 16S rRNA base sequences (2). Thus, ferredoxins from hyperthermophiles, methanogens, and thermoacidophiles are clustered together in separate groups as the monocluster type, the dicluster type, and the novel zinc-containing dicluster type, respectively. Moreover, the zinc-containing ferredoxins have been found only in the “fast clock” archaea (such as Sulfolobales and Thermoplasma) (3), while ferredoxins from the “slow clock” hyperthermophilic archaea and bacteria (3) are primarily the monocluster ferredoxins. Given the physiological importance of ferredoxins in the archaeal central metabolisms (5, 8–10, 18), these observations seem to be somewhat meaningful (e.g. see Darimont and Sterner (39)), although further discussion should await comparison at the base sequence level in order to preclude a possibility of lateral gene transfer event. One possibility is that the molecular evolution of the archaeal ferredoxins might have largely been affected by environmental pressure which should have made considerable influences on the central and energy metabolisms as well as the growth of the cells. The closer relationship between the zinc-containing ferredoxins of thermoacidophilic archaea and the dicluster ferredoxins of methanogens in the archaeal ferredoxin tree also raises an interesting question of whether methanogens contain a putative zinc-binding polypeptide of ~50–100 amino acids long, which could potentially interact with the cognate ferredoxin. However, preliminary search against the complete genome sequence of the methanogenic archaeon Methanococcus jannaschii (41) failed to identify the presence of such a sequence.

A specific role of the novel zinc center of the zinc-containing ferredoxin family is currently unclear, besides its obvious structural contribution. It is very unlikely that ferredoxin itself serves as a zinc sensor or a zinc storage protein in vivo, because the bound zinc center is not released upon dialysis against buffer containing 5 mM EDTA. Nevertheless, formation of a mature ferredoxin molecule should be correlated to intracellular levels of both Zn²⁺ and Fe³⁺ ions at least at the translation level, thus raising an interesting possibility that the ferredoxin-dependent central metabolism of the fast clock archaea (17, 18) could be potentially regulated by the availability of zinc. In this connection, a zinc-containing ferredoxin identified in this study may be useful as a simple model for more complex, mixed-metal metalloenzymes, to investigate how different kinds of metal ions are inserted into distinct metal binding sites of the same apoprotein to give mature protein conformation. Moreover, since the redox partners of the zinc-containing ferredoxins are known (10, 18–22), it will be tempting to construct mutant proteins with modified N-terminal extension region by protein engineering and to determine the effects on the protein stability and redox activity.

**Conclusion**—Since the first discovery of a bacterial type ferredoxin by Mortenson et al. in 1962 (42), we have recognized for the first time the existence of a novel ferredoxin family inherently containing a zinc center besides conventional FeS clusters, in several thermoacidophilic archaea. These ferredoxins, namely, zinc-containing ferredoxins, can be characterized by a long N-terminal extension region with a consensus of three histidine residues, which probably function as parts of ligands to a zinc center. Chemical and spectroscopic analyses of one such example from *T. acidophilum* have shown that the purified ferredoxin contains one zinc center, one [3Fe-4S] cluster, and one [4Fe-4S] cluster, as in the case of *Sulfolobus sp.* ferredoxin.7 The presence of a unique zinc center in these ferredoxins might represent a unique evolutionary event in early thermoacidophilic archaea.

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