We heterologously overproduced a hyperthermostable archaeal low potential (E_m = −62 mV) Rieske-type ferredoxin (ARF) from Sulfolobus solfataricus strain P-1 and its variants in Escherichia coli to examine the influence of ligand substitutions on the properties of the [2Fe-2S] cluster. While two cysteine ligand residues (Cys42 and Cys61) are essential for the cluster assembly and/or stability, the contributions of the two histidine ligands to the cluster assembly in the archaeal Rieske-type ferredoxin appear to be equivalent as indicated by much higher stability of the His^{64} → Cys variant (H64C) than the His^{44} → Cys variant (H44C). The x-ray absorption and resonance Raman spectra of the H64C variant firmly established the formation of a novel, oxidized [2Fe-2S] cluster with one histidine and three cysteine ligands in the archaeal Rieske-type protein moiety. Comparative resonance Raman features of the wild-type, natural abundance and uniformly ^{15}N-labeled ARF and its H64C variant showed significant mixing of the Fe-S and Fe-N stretching characters for an oxidized biological [2Fe-2S] cluster with partial histidine ligation.

Proteins containing Rieske-type [2Fe-2S] clusters are widespread in nature from hyperthermophilic Archaea and Bacteria to Eukarya and play critical electron transfer roles in various pathways such as aerobic respiration, photosynthesis, and bio-

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Ligand Mutagenesis of Archaeal Rieske-type Ferredoxin

EXPERIMENTAL PROCEDURES

E. coli strain DH5α and strain HB101 (TaKaRa) used for cloning were grown in LB or terrific broth medium with 50 μg/ml ampicillin when required. Plasmids pGEMT and pGEM3Zf+ (Promega) were used for cloning and sequencing. The expression vectors pET28a and pTrC99A were purchased from Novagen and Amerham Biosciences, respectively. DNA was manipulated by standard procedures (33). Water was purified with a Milli-Q purification system (Millipore). Sulredoxin was purified from the soluble fraction of S. tokodaii strain 7 (formerly Sulfolobus sp. strain 7; JCM 10545T (34)) as described previously (28, 35). Other chemicals mentioned in this study were of analytical grade.

The arf gene coding for the hypothetical ARF (orf c69009; DDBJ accession number AB047031 (27)) of S. solfataricus strain P-1 (DSM 1616T) was cloned and sequenced as follows. The PCR was carried out to obtain a partial genomic fragment encoding the arf gene using S. solfataricus strain P-1 genomic DNA and the following oligonucleotide primers (designed based on the genomic DNA sequence data available for strain P-2 (36)): P1-4 primer, 5′-CCC CCT TCC AGT CAG AG-3′; and P1-4 primer, 5′-CCC CCC CGG ATC TCT AAA TTT GTA T-3′. The determined nucleotide sequence (DDBJ accession number AB047031) completely matched the gene sequence reported for the hypothetical ORF c69009 of S. solfataricus strain P-2 (36). This gene is located shortly after another hypothetical ORF (orf60088) of unknown function, although it is apparently not a part of a multicomponent oxygenase gene cluster (36). The nucleotide sequences of these flanking regions were also confirmed for both strands with new sets of PCR primers designed for amplification of the whole arf-coding region and the flanking sequences (DDBJ accession number AB047031).

The PCR product with expected length of 327 bp as amplified above was subcloned into an NdeI BamHI site in pET28a vector (Novagen), and the nucleotide sequence was determined with vector-specific T7 promoter and T7 terminator. The resultant expression vector was named pET28aARF. It contains the pET28a vector-derived leader sequence to facilitate efficient transcription of archaeal genes in E. coli so as to produce a recombinant protein with the N-terminal hexahistidine tag for rapid protein purification. The hexahistidine tag is attached away from the cluster-binding subdomain of ARF and apparently does not disturb its redox site as judged by X-ray crystallography (Amersham Biosciences). The purified recombinant holoprotein was overproduced by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 24 h at 30 °C in the presence of natural abundance 15N-labeled holoprotein was overproduced by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 24 h at 30 °C. The cells were pelleted by centrifugation and stored at −80 °C until use.

Purification of each recombinant holoprotein having a hexahistidine tag at the N terminus was performed as described previously for the S. tokodaii recombinant ShcCu iron-sulfur protein (39) except that the heat treatment step (at 65 °C for 20–30 min) was omitted for three variants, C425, C615, and H44C. When required, the recombinant holoprotein was further purified by Superdex G-75 gel filtration chromatography (Amersham Biosciences). The purified recombinant holoprotein was stored at either 4 °C or −80 °C until use.

Absorption spectra were recorded with a Hitachi U3210 spectrophoto- meter or a Beckman DU-7400 spectrophotometer equipped with a ther- moelectric cell holder. Visible-near UV CD spectra were recorded with a JASCO J720 spectropolarimeter with 0.5-cm cells. EPR measurements were performed by using a JEOL JEX-REX3X spectrometer equipped with an ES-CT470 Heli-Tran cryostat system and a Scientific Instruments digital temperature indicator/controller Model 9650. Spin concentrations of purified recombinant proteins were estimated by double integration with Cu-EDTA (0.1 mM) and S. tokodaii sulredoxin (0.05 mM) as standards. RR measurements were recorded with a Bruker 750M Raman spectrometer fitted with a Spectrum-One 2048 × 512 charge-coupled device camera and a Spectra-Physics 2017 Ar+ laser (output, 500 milliwatts) by collecting 45° backscattering off the surface of a frozen sample in a glass cylindrical cell with sintered glass cap (39). The slit width of the spectrometer was 80 μm, and a multiscan signal-averaging technique was used to improve the signal-to-noise ratio. The spectral data were processed using KaleidaGraph Version 3.05 (Abelbeck Software).

Purified recombinant proteins were concentrated by pressure filtration with an Amicon YM-10 membrane. Further concentration was achieved by placing the samples under a stream of dry argon gas. The resultant samples (~1 ml), containing 30% (v/v) glycerol, were frozen in 2.4-mm-cuvette surfaces with thiopeptide crystals using a Mylar tape front window for XAS studies (27). X-ray absorption spectra at the iron K-edge were recorded at 10 K at Stanford Synchrotron Radiation Laboratory, beamline 7-3, with the SPEAR storage ring operating at 3.0 GeV and 60–100 mA. A Si(220) double crystal monochromator (with one crystal detuned to 50% reflected intensity for harmonic rejection) and a phosphor-resolving 30-element germanium detector (provided by the National Institutes of Health Biotechnology Research Resource) were used for data collection. Energies were calibrated using an internal iron foil standard, assigning the first inflection point to 7113.3 eV. All other data collection parameters were as reported previously (40, 41).

The intensities and energies for the 1s→3d pre-edge features of the iron K-edge XAS data were quantified using the program EDG_FIT. All spectra were fit over the range 7100–7165 eV. Pseudo-Voigt line shapes of a fixed 1:1 ratio of Lorentzian to Gaussian contribution were used to model pre-edge features and successfully reproduced the spectra. Func-

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The Active Site Features, Heterologous Overexpression, and Characterization of a Novel Archaeal Rieske-type Ferredoxin from *S. solfataricus* Strain P—I—Fig. 1 shows the multiple sequence alignment of the cluster binding site of high and low potential Rieske-type proteins. The deduced amino acid sequence of the hypothetical protein c06009 contains 108 amino acids (with a molecular mass of 12,509 Da and pI of 8.14) and lacks any transmembrane helical region (DDBJ accession number AB047031). The putative cluster binding sequence of the hypothetical protein c06009 is characteristic of those observed for oxygenase-associated Rieske-type ferredoxins (1, 3) with the consensus motif, -Cys-Xaa-His-∥-Gly-Xaa₅-Val/Ile₇-Asx-Cys-Xaa₅- (Leu)-His-, where the two cysteines and two histidines (Cysₓ², Cysₓ⁴, Hisₓ⁴, and Hisₓ⁴) are predicted as ligands to a [2Fe-2S] cluster based on the sequence homology (Fig. 1). No extra histidines or cysteines exist in the entire amino acid sequence (DDBJ accession number AB047031). Thus, this archaeal protein does not contain any solvent-exposed disulfide linkage, unlike the high potential Rieske proteins found as part of aerobic respiratory chain and photosynthesis, and appears to be suitable for the ligand substitution studies.

Fig. 1. Multiple sequence alignment of the cluster binding sites of the selected low potential Rieske-type ferredoxins, low potential Rieske-type protein domains of terminal oxidases, and high potential Rieske proteins. The cluster binding motif of *S. solfataricus* ARF is characteristic of Rieske-type ferredoxins involved in bacterial multicopper oxidases and lacks two conserved cysteine residues that serve as the solvent-exposed disulfide linkage in high potential, respiratory Rieske proteins. Accession numbers are as follows: *S. solfataricus* ARF (hypothetical ORF c06009), CAA669492, AB047031; *P. putida* strain ML2 bedB, AAA17780; *Burkholderia cepacia* Rieske Fdh (bfhP), P37332; *Burkholderia* sp. dntAb, AAB09765; *Xanthobacter* sp. xamoC, CAA09913; *Pseudomonas* sp. naphthalene 1.2-dioxygenase (NDO), P23094; *B. cepacia* 2,4,5-trichlorophenoxyacetic acid monoxygenase (TMO), AAB39767; *S. tokodaii* sulredoxin, AB022585; *S. solfataricus* probable sulredoxin, hypothetical ORF c45-010; *Bos taurus* (bovine) mitochondria, P08067; *Rhodobacter capsulatus*, A29336; *Spinacia oleracea* (spinach) chloroplast, S00454; *Chlamydomonas reinhardtii* chloroplast, P13572; *Saccharomyces cerevisiae* mitochondria, P08067; *Rhodobacter capsulatus*, A92396; *Spinacia oleracea* (spinach) chloroplast, S00454; *Chlamydomonas reinhardtii* chloroplast, P49728; *Synechococcus* sp., P26292; *Chlorobium limicola*, S38460, G46136; *Thermus thermophilus* chloroplast, P49728.

Starting from dithionite-reduced protein by injecting small aliquots of air in a stepwise manner (as described in Ref. 47) using a specially designed gas-tight syringe. All redox potentials quoted in this report are versus those from a standard hydrogen electrode.

RESULTS AND DISCUSSION

The absence of any disulfide linkage in the wild-type ARF suggests that its remarkable stability must be found in a small number of highly conserved residues, e.g., charge clusters, networks of hydrogen bonds, optimization of packing, hydrophobic interactions, and charge-charge interactions on the surface of proteins that often do not obey any obvious traffic rules in hyperthermophilic enzymes (for reviews, see Refs. 72 and 73). We are attempting to determine the origin on the basis of the crystal structure of the wild-type ARF. Another possible thermodynamic mechanism stabilizing the archaeal protein may come from a very slow unfolding rate as reported for several hyperthermophilic enzymes (74–76).
We heterologously overproduced the arf gene product with a hexahistidine tag at the N terminus in the E. coli BL21-Codon-Plus(DE3)-RIL strain to characterize the Rieske-type cluster in S. solfataricus ARF in greater detail. The purified recombinant protein, named ARF, was water-soluble and had a dark purple color. The visible absorption spectrum of the oxidized recombinant ARF showed an absorption maximum at 464 nm and a shoulder at 580 nm, characteristic of a Rieske-type [2Fe-2S] protein (1, 26, 48) (Fig. 2A).

In the visible-near UV CD spectrum in the 350–500 nm region, the oxidized ARF (Fig. 2B, solid line) showed a deep trough at 378 nm and at least two peaks between 400 and 510 nm (at 419 and 490 nm). These visible CD features did not change at least up to pH 10.2 (data not shown). The dithionite-reduced protein (Fig. 2B, dashed line) showed a deep trough at 391 nm, a dominant peak at 464 nm, and a shallow trough at 505 nm. These CD spectral features are more similar to those of bacterial low potential Rieske-type ferredoxins (such as BedB from Pseudomonas putida ML2 (23) and BphF from Burkholderia sp. strain LB400 (26)) than to those of high potential Rieske proteins (such as those from bovine mitochondria (49, 50) and T. thermophilus (48) and archaeal sulredoxin from S. tokodaii (29)); the reduced forms of the latter proteins show at least three positive bands between 400 and 510 nm at neutral pH.

The dithionite-reduced ARF elicited an EPR spectrum with an anisotropic line shape characterized by a rhombic g tensor with principal values of $g_1 = 2.02$, $g_2 = 1.90$, $g_3 = 1.81$ ($g_{av} = 1.91$) (concentration typically 0.9–1.0 spin/mol) (Fig. 3A), which was optimally observed around 15 K. The principal values for ARF fit well within the typical interval of spectral characteristics reported for Rieske-type [2Fe-2S] clusters (1, 3). On the
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Fig. 3. X-band EPR spectra of dithionite-reduced forms of the wild-type ARF at 15 K (A) and the H64C variant at 25 K (B). Microwave power, 1.0 milliwatt; modulation amplitude, 0.63 millitesla (mT); variable gain. The g values are indicated in the figure.

other hand, the air-oxidized ARF was EPR-silent and did not show a g = 4.3 EPR signal in significant amount (data not shown). These results clearly indicate the assembly of a single Rieske-type [2Fe-2S] cluster in the recombinant ARF holoprotein produced in E. coli without any requirement of in vitro reconstitution procedures.

The potentiometric titration curve of the wild-type ARF at pH 7.0, as monitored by visible absorption spectroscopy (in triplicate), could be fit with a simple n = 1 Nernst equation, giving the $E_{1/2}$ of $-62 \pm 7$ mV (Fig. 2A, inset). This value is in the range of those of the low potential Rieske-type [2Fe-2S] clusters in a diverse group of bacterial multimponent terminal oxygenases and soluble Rieske-type ferredoxins ($-150$ to $-50$ mV) (1, 3, 8, 9, 16–27) and is significantly more positive than typical four-cysteine ferredoxins involved in the cytochrome P-450 monooxygenase system such as adrenodoxin ($-270$ mV (54)). Thus, S. solfataricus ARF represents the first archaeal homolog of bacterial low potential Rieske-type ferredoxins, sharing similar cluster surroundings as judged by CD and EPR spectra (Figs. 2B and 3A). Interestingly the cluster reduction potential of ARF is significantly more negative than that of S. tokodaii sulredoxin, another archaeal water-soluble Rieske protein ($E_{1/2\text{, low pH}} + 190$ mV) (28, 29), suggesting operation of multiple soluble Rieske-type proteins in the Sulfolobus redox system; ARF likely plays a redox role in an unknown electron transfer sequence, perhaps involved in biodegradation of alkene or aromatic compounds at high temperature.

Resonance Raman Characterization of the Oxidized Rieske-type Cluster in ARF—Low temperature RR spectroscopy is a sensitive probe for the microenvironment of biological [2Fe-2S] clusters (52–54). Earlier RR studies on the pH dependence of the oxidized forms of archaeal and bacterial high potential Rieske proteins (as models for components of proton-translocating respiratory complexes) (17, 20, 29) have been considered to be the primarily spectroscopic supporting source for the common view to assign the $pK_{\text{ox}}$ near $-8$, which influences the $E_{1/2}$ and the visible CD spectrum, to one of the terminal histidinyl ligands (1–4). Although this is chemically reasonable and likely in light of available crystal structural information (9, 11), actual experimental evidence for deprotonation of the liganding histidine imidazole groups is very weak because (i) no rigorous assignment of Fe-N stretching frequencies has been made in the RR spectra of these high potential Rieske proteins and (ii) the variations of vibrational modes with respect to those of low potential Rieske-type proteins, which do not show redox-linked ionization in the physiological pH range (1, 3), have remained poorly understood.

The availability of the heterologous overexpression system for ARF and archaeal sulredoxin (28–30) (see “Experimental Procedures”) has allowed us to address these important issues by utilizing a uniform $^{15}$N-labeling technique and by characterizing the structural differences in the coordination environment of their oxidized Rieske-type [2Fe-2S] clusters in detail by RR spectroscopy (Fig. 4A and C). In both cases, at least eight bands are observed in the 240–450 cm$^{-1}$ region. There are three to four additional bands in the 280–320 cm$^{-1}$ region whose peak positions are too weak to be determined with accuracy (not labeled in the figure). These spectra suggest a lower symmetry around the Rieske-type [2Fe-2S] cluster core than that around the biological [2Fe-2S]$^{2+}$ clusters with complete cysteynil ligation that typically exhibit six or seven bands under similar conditions (17, 20, 29, 39, 53–56). The major differences in the RR spectra of ARF and sulredoxin are found in the 240–400 cm$^{-1}$ region; interestingly the overall RR features of oxidized ARF are more similar to those reported for the low potential Rieske-type cluster in bacterial phthalate dioxygenase (17, 20), which is in line with our recent iron K-edge XAS analysis showing the structural similarity in the coordination environment of the Rieske-type clusters in ARF and bacterial anthranilate dioxygenase (27) (Table 1).

As an initial step toward understanding the RR spectral difference of the wild-type ARF and sulredoxin, we prepared the uniformly $^{15}$N-labeled wild-type ARF and sulredoxin using $^{15}$NH$_4$Cl as a sole nitrogen source in the growth media of E. coli. Successful incorporation of the Rieske-type cluster was detected for both proteins when the cells were grown at 30 °C in the modified M9 minimal salt medium containing $^{15}$NH$_4$Cl (see “Experimental Procedures”). The purified $^{15}$N-labeled wild-type ARF and sulredoxin showed the same visible CD and x-band EPR characteristics as the corresponding natural abundance, wild-type proteins (data not shown). The 77 K RR spectra of $^{15}$N-labeled sulredoxin and ARF (Fig. 4B and D) showed the expected mass-dependent downshifts by 1–2 cm$^{-1}$ for most vibrational modes in the 240–390 cm$^{-1}$ region. This demonstrates the extensive kinetic mixing of the Fe-S and Fe-N$_{\text{imid}}$ stretching characters in the 240–390 cm$^{-1}$ region, which is expected if the Fe-S and Fe-N$_{\text{imid}}$ stretching vibrations are extensively coupled with other vibrations in the same cluster environment in the molecule involving complicated deformational displacements in the polypeptide backbone (57–60). In the 390–440 cm$^{-1}$ region, a contribution of Fe-S stretching characters becomes dominant, showing no significant mass-dependent downshift with the uniformly $^{15}$N-labeled proteins (Fig. 4B and D).

In conjunction with recent structural information of Rieske and Rieske-type proteins (6, 7, 9, 10), it is expected that the excited state of electrons localized around the (N$_{\text{imid}}$)$_2$Fe(Se)$_2$ portion in the Rieske-type [2Fe-2S] cluster contributes to the higher frequency region in the UV-visible spectra than those around the (S)$_2$Fe(Se)$_2$ moiety, essentially being off resonance in the RR spectra upon excitation in the visible region. Thus, overall contributions of the Fe-N$_{\text{imid}}$ vibrations to the RR spec-

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6 For Rieske-type [2Fe-2S] proteins, the EXAFS is a measure of the average coordination environment of both iron atoms in the cluster (27). One iron has a coordination sphere including four sulfur atoms and a distant iron, while the other iron has a sphere that includes two sulfur atoms, two nitrogen atoms, and a distant iron (6–10). Thus, the average coordination environment in the wild-type ARF is three sulfur atoms, one nitrogen atom, and a distant iron (see Table I, Fit 1).

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tra of Rieske and Rieske-type proteins are weaker than those of the Fe-Sb and/or Fe-St vibrations (although they do influence the RR characteristics). This is consistent with the present RR results (Fig. 4). In contrast to the previous interpretation of the RR spectra of high potential Rieske proteins (17, 20, 29), our preliminary normal coordinate analysis (on the mass group approximation for hydrogen atom) suggests that an anomalously low frequency band at 260–270 cm$^{-1}$, which is characteristic for the RR spectra of Rieske and Rieske-type proteins, has a dominant contribution from Fe-Sb vibrations and little from Fe-N imid vibrations. However, it also shows a mass-dependent downshift by 2 cm$^{-1}$ with the uniformly $^{15}$N-labeled proteins (Fig. 4, B and D), suggesting the coupling of Fe-Sb vibrations with other complicated vibrational modes involving additional amides in the polypeptide backbone that extend beyond the liganding residues. A similar situation is observed for the 391 cm$^{-1}$ band in sulredoxin with a dominant contribution from Fe-Sb vibrations, which is also coupled with other vibrational modes in the backbone involving Fe-Nimid stretches albeit less significant in the RR spectra. Reexamination of the 270 and 391 cm$^{-1}$ bands in the RR spectra of sulredoxin between pH 5 and 10.6 showed no detectable pH-dependent change (data not shown). This indicates a negligible mass-dependent shift (estimated to be $-0.2$ cm$^{-1}$, which cannot be resolved in the present RR spectra with accuracy) upon protonation/deprotonation of $\text{N}^\delta$ of the Fe-N imid stretching vibrations that are extensively coupled to the dominant Fe-Sb and/or Fe-St vibrational modes and confirms no marginal structural rearrangement of the immediate cluster environment of the oxidized high potential Rieske protein at different pH values at least up to 10.6.

Based on these results, we conclude that the major RR spec-

**Fig. 4.** Low temperature resonance Raman spectra of the oxidized high potential Rieske center in natural abundance (A) and uniformly $^{15}$N-labeled (B) S. tokodaii sulredoxin and the oxidized low potential Rieske-type [2Fe-2S] cluster in the natural abundance (C) and uniformly $^{15}$N-labeled (D) wild-type ARF. The spectra were obtained at 77 K using 488.0 nm Ar$^+$ laser excitation. An asterisk indicates an ice mode from buffer.

**TABLE I**

| Sample (filename (k range), $\Delta k^3 \chi$) | Fit | Shell | $R_{\text{m}}$ | $\sigma_{\text{m}}^2$ | $\Delta E_0$ | $f^*$ |
|-----------------------------------------------|-----|-------|----------------|----------------|--------------|------|
| Wild-type ARF, oxidized (FFO0A ($\Delta k = 2–13.5$ Å$^{-1}$), $\Delta k^3 \chi = 13.35$) | 1   | Fe-S$_2$ | 2.20 | 0.0048 | -6.88 | 0.063 |
|                                                |     | Fe-N$_i$ | 1.95 | 0.0007 |         |       |
|                                                |     | Fe-Fe$_1$ | 2.67 | 0.0060 |         |       |
| H64C, oxidized (FSFCA ($\Delta k = 2–13.5$ Å$^{-1}$), $\Delta k^3 \chi = 14.71$) | 2   | Fe-S$_i$ | 2.24 | 0.0035 | 2.35 | 0.082 |
|                                                |     | Fe-Fe | 2.73 | 0.0039 |         |       |
|                                                | 3   | Fe-S$_{2,i}$ | 2.24 | 0.0044 | 2.12 | 0.084 |
|                                                |     | Fe-Fe | 2.73 | 0.0038 |         |       |
|                                                | 4   | Fe-S$_{2,5}$ | 2.24 | 0.0042 | 3.42 | 0.080 |
|                                                |     | Fe-N$_{2,5}$ | 2.25 | 0.0010 |         |       |
|                                                |     | Fe-Fe | 2.73 | 0.0033 |         |       |

*a $f^*$ is defined as $f^* = \left( \sum (k^3 \chi_{0}^{\text{obs}} - k^3 \chi_{0}^{\text{calc}})^2 / N \right)^{1/2} / \left( \sum (k^3 \chi_{0}^{\text{max}})_{\text{max}} - (k^3 \chi_{0}^{\text{min}})_{\text{min}} \right)^{1/2}$.
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Identification of Individual Ligand Residues to the Rieske-type Cluster in ARF by Site-directed Mutagenesis—We individually replaced each histidine ligand residue (His14 and His64) by cysteine and each cysteine ligand residue (Cys42 and Cys61) by serine to investigate the influence of each ligand substitution on the archaeal low potential Rieske-type [2Fe-2S]2 cluster. For the cysteine → serine substitutions, the purified site-directed variants, C42S and C61S, respectively, showed little cluster assembly although the heat treatment step was omitted from the purification (data not shown). These results indicate that both cysteine ligand residues, Cys42 and Cys61, play crucial roles in the Rieske-type cluster assembly and/or stability of ARF.

For the histidine → cysteine substitutions, a novel [2Fe-2S]2 cluster was incorporated into H64C, whereas a cluster in H44C was very unstable (Fig. 2C). This indicates that two histidine ligands, His14 and His64, located in the cluster binding loops of ARF are apparently inequivalent with more structural freedom allowed around His64 to adapt to the [2Fe-2S]2 cluster assembly.5

The optical spectra of the purified H64C showed an intense positive peak at 457 nm in the visible CD spectrum (Fig. 2D). The visible-near UV absorption and CD features of the air-oxidized protein are more similar to those of regular [2Fe-2S]2 clusters with complete cysteinyl ligation (61, 62) than that of the Rieske-type cluster in ARF (Fig. 2B), although the positive CD peak is red-shifted by 27 nm as compared with the equivalent positive peak in spinach ferredoxin (61).

The dithionite-reduced form of highly concentrated H64C samples showed a novel near axial EPR signal at $g \approx 2.00$ and 1.92, which was best observed at 25–30 K and is attributed to an $S = 1/2$ [2Fe-2S]2 cluster (Fig. 3B). However, a comparison of the total signal intensity of the EPR absorbance signals from the H64C variant and wild-type ARF indicated that the average intensity of the spectrum from the H64C variant (from three independent preparations) was less than $\sim 5\%$ of that from the wild-type ARF (Fig. 3A). A similar observation has been reported for a Clostridium pasteurianum rubredoxin variant within which a [2Fe-2S]22+ cluster is assembled by site-directed mutagenesis (63). Dithionite-treated H64C samples gradually gave featureless absorption (Supplemental Fig. S-1A) and CD spectra (data not shown) under anaerobic conditions, with occasional formation of aggregation and apo-protein precipitation, as reported previously for the dithionite-treated recombinant SdhC iron-sulfur protein of the S. tokodaii respiratory complex II (39) that also causes complete degradation of the [2Fe-2S]2 cluster, releasing Fe2+ from the polypeptide chain into solution (64). Thus, unlike the wild-type ARF, the reduced [2Fe-2S]2 cluster in the H64C variant is very unstable and undergoes irreversible cluster breakdown upon dithionite treatment, confirming the EPR results (Fig. 3).

In the wild-type ARF, dithionite treatment causes an increase in the average Fe-histidine ligand bond length and the Fe–Fe distance, suggesting small expansion of the cluster core dimensions upon reduction (27). A similar structural expansion of the core dimensions and accompanying structural alterations of the immediate cluster surroundings may trigger the irreversible breakdown of the dithionite-treated cluster in the H64C variant.

Although the instability of the dithionite-treated cluster in H64C makes accurate determination of its midpoint redox potential impractical, this was estimated by comparing the initial point of reduction of the protein with that of reference dye with known redox potential by using the xanthine/xanthine oxidoreductase-mediated reduction method described by Massey (44). This method is based on the rapid equilibration of catalytically generated reducing equivalents between the cluster of the protein and a dye of known reduction potential in the presence of a low concentration of benzyl viologen or methyl viologen ($\sim 2 \mu M$). Reduction of H64C started only after reduction of benzyl viologen at pH 8.5 ($\sim 359 \text{ mV (44)}$) but before reduction of methyl viologen ($\sim 436 \text{ mV}$) (Supplemental Fig. S-1B and Table S-I), indicating that the reduction potential is in the range of $\sim 359$ to $\sim 436 \text{ mV}$. In contrast, the reduction potential of the wild-type ARF ($E_{\text{m,9}} = \sim 52 \pm 4 \text{ mV}$) was obtained by using indigo disulfonate as reference dye at pH 5.9 ($\sim 54 \text{ mV (44)}$) by the same method (Supplemental Fig. S-1C and Table S-I). The latter value was also confirmed by reductive titrations starting from oxidized protein with dithionite and oxidative titrations starting from dithionite-reduced protein under anaerobic conditions (data not shown). These results indicate that the relative reduction potential of the H64C variant is lower by at least 300 mV than that of the wild-type ARF and likely in the range of those of low potential-plant-type ferredoxins (approximately $\sim 400 \text{ mV}$) (1, 3).

Probing the Novel [2Fe-2S]2 Cluster Surroundings in the Oxidized H64C Variant—The [2Fe-2S]2 cluster assembled in H64C is expected to have one histidine and three cysteine ligand residues (see Fig. 1) and was stable as isolated in the oxidized form, allowing a more detailed structural analysis. The iron K-edge x-ray absorption spectrum for the oxidized recombinant H64C variant was significantly different from the previously reported spectrum for the wild-type ARF (27) in both edge position and edge shape as shown in Fig. 5A. A shift to lower edge energy is observed for the H64C variant as would be expected with an increasing number of S-donor ligands. The relatively weaker and broader edge peak of H64C also suggests more sulfur and less nitrogen ligands on average around the iron atoms (65). However, the integrated peak areas (0.203 eV for the wild-type ARF and 0.286 eV for H64C) for the $1s \to 3d$ transition at $\sim 7113 \text{ eV}$ fall in the range expected for tetrahedral compounds (66, 67).

The Fourier transforms of iron EXAFS for both wild-type ARF and H64C variant display two peaks (Fig. 5B).6 For the H64C variant, the stronger first Fourier transform peak clearly indicates that there are more sulfur scatterers in the first coordination shell of the [2Fe-2S]2 cluster as expected from edge analysis. Curve fitting analysis reveals that the average Fe–S and Fe–Fe interaction distances in H64C are slightly expanded by 0.05 and 0.06 Å, respectively, as compared with those in the wild-type ARF (Table I). Thus, replacement of the His64 ligand by cysteine in ARF results in a small XAS-detectable expansion of the [2Fe-2S]2 cluster core dimensions, which may contribute to the instability of this novel cluster.

It is difficult to discuss the Fe–N bond distance in H64C by XAS because the Fe–N contribution at 1.95 Å in the Fourier transform spectra of the oxidized wild-type ARF6 (see Fig. 5B) is already small as discussed in our previous studies (27). As expected, the inclusion of the Fe–N shell did not improve the fits for H64C (Table I; the Fe–N interaction distance is artifactually similar to the dominant Fe–S distance). The coordination...
environment of the [2Fe-2S] cluster in the oxidized H64C variant was therefore addressed by low temperature RR spectroscopy (Fig. 6A). The 77 K RR spectrum of H64C shows the presence of at least eight bands in the 240–450 cm\(^{-1}\) region as in the case of the wild-type ARF (Fig. 6B). This is consistent with a relatively small contribution of the Fe-Nimid\(^3\) stretches to the RR spectrum via extensive coupling to the dominant Fe-Sb and/or Fe-St vibrations (Fig. 4). In the RR spectra of biological [2Fe-2S]\(^2+\) clusters with one serinate ligand introduced by site-directed mutagenesis (55, 56) and those of bacterial biotin synthase (68) and IscU iron-sulfur protein (69) that have been interpreted in terms of partial non-cysteinyl ligation, the B3u\(^t\) modes with predominantly Fe-St stretching characters occur at anomalously high frequencies in the range 289–302 cm\(^{-1}\) compared with 281–291 cm\(^{-1}\) for clusters in the plant- and vertebrate-type [2Fe-2S] ferredoxins with complete cysteinyl ligation (53, 54). Notably the overall RR characteristics in the 250–450 cm\(^{-1}\) region of the oxidized [2Fe-2S] center in H64C are distinctly different from these cases, showing an anomalously low frequency band at 258 cm\(^{-1}\); the equivalent band in Rieske and Rieske-type proteins at 260–270 cm\(^{-1}\) (Fig. 4) has a dominant contribution from the Fe-Sb vibrations that are also coupled with other complicated vibrational modes involving additional amides in the polypeptide backbone that extend beyond the liganding residues.\(^7\)

As compared with the RR spectra of the wild-type ARF, most bands in the H64C RR spectra occur at lower frequencies. Such downshifts of vibrational modes would not be expected for a simple Nimid\(^3\) → S ligand substitution as judged by the large effective mass of an imidazole group; rather they most likely result from a variety of structural changes that include weakening of Fe-S bonds, consistent with the small XAS-detectable expansion of the [2Fe-2S] cluster core dimensions in H64C (Fig. 5 and Table I). Taken together, the present RR results provide evidence for the presence of a histidine ligand (His\(^{44}\)) to the oxidized [2Fe-2S] cluster in H64C variant. The marked RR spectral changes in the 240–390 cm\(^{-1}\) region upon replacement of His\(^{64}\) by cysteine are also consistent with the extensive kinematic mixing of the Fe-Nimid and dominant Fe-Sb and/or Fe-St stretching characters for the oxidized biological [2Fe-2S] cluster with partial histidine coordination (Figs. 4 and 6).

**Conclusion**—By using a hyperthermostable, low potential Rieske-type ferredoxin from *S. solfataricus* strain P-1 having the canonical cluster binding motif, -Cys\(^{42}\)-Xaa-His\(^{44}\)-/Cys\(^{51}\)-Xaa-Leu-His\(^{64}\)-, we were able to characterize the influence of substitution of one of its histidine ligands on the properties of a Rieske-type [2Fe-2S] cluster in greater details, which was not possible in earlier studies with mesophilic enzymes due to protein instability (31, 32).\(^5\) In general, the four ligand residues

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**Fig. 5.** Iron K-edge XAS (A) and \(k^3\)-weighted EXAFS (inset) and sulfur phase-corrected Fourier transforms (B) (over \(k = 2–13\) Å\(^{-1}\)) of the oxidized wild-type ARF (dashed line) and the H64C variant (solid line). The inset in A expands the 1s → 3d transition. The vertical dashed line in Fourier transform is positioned to illustrate the shift to longer distance of the shoulder of the first shell peak, which represents the Fe-Nimid scattering pair for the oxidized wild-type ARF (dashed line). FT, Fourier transform.
to the Rieske-type cluster in ARF contribute significantly to its stability and/or cluster insertion, although one of the histidine ligands, His44, appears to have some structural flexibility. The present RR and XAS evidence demonstrate the formation of a slightly expanded, novel low potential [2Fe-2S] cluster with one histidine and three cysteine ligands (His44, Cys58, Cys61, and Cys64, respectively) in the oxidized H64C variant. Instability of its reduced cluster may explain why the “three cysteines-plus-one histidine” type coordination is rarely seen in biological iron-sulfur enzymes; for the Rieske-type protein modules, the stability of the [2Fe-2S] cluster is apparently optimized by the “two histidines-plus-two cysteines” ligations, which might have been of early evolutionary origin considering also the wide distribution of the Rieske and Rieske-type clusters in all three domains of life (i.e. Archaea, Bacteria, and Eukarya (70, 71)). In this protein family, the electronegativity of the terminal ligands (histidine versus sulfur) as well as the overall charge of the cluster significantly contributes to higher reduction potentials of the clusters than those in regular plant-type ferredoxins, allowing their distinct physiological functions throughout the modular evolution.

Our uniform 15N-labeling and site-directed mutagenesis studies on ARF also prove the extensive kinematic mixing of the Fe-Nmid vibrations with the dominant Fe-S and/or Fe-S stretching characters of the immediate surroundings of a [2Fe-2S] cluster with partial histidine ligation in the 240–390 cm\(^{-1}\) region. Thus, the Fe-Nmid Fe-S stretching vibrations and bond angle bending displacements are probably extensively coupled in the polypeptide backbone and widely spread into the molecule around the Rieske-type [2Fe-2S] cluster as reported previously for blue copper proteins and rubredoxin (57–59). Importantly the liganding histidine imidazole groups do not behave as simple point groups in the high and low potential Rieske-type [2Fe-2S] system, and the previous tentative assignments and interpretations of pH dependence in the RR features of high potential Rieske proteins (17, 29) appear to require substantial revision. The present results provide a useful spectroscopic fingerprint for other biological [2Fe-2S] protein systems with partial histidine ligation and would be helpful for deeper RR analysis of a high potential Rieske [2Fe-2S] protein found as a part of cytchrome bc, complex in the aerobic respiratory chain, one of whose histidine ligands is directly involved in the substrate binding and oxidation at the quinol-oxidizing Q\(_0\) site (13–15).

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