J. Biochem. **142,** 577–586 (2007)
doi:10.1093/jb/mvm163

Characterization and Crystallization of an IscU-type Scaffold Protein with Bound [2Fe–2S] Cluster from the Hyperthermophile, *Aquifex aeolicus*

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Received July 5, 2007; accepted August 5, 2007; published online September 10, 2007

IscU plays a key role during iron–sulphur (Fe–S) cluster biosynthesis as a scaffold for the assembly of a nascent, highly labile Fe–S cluster. Here we report the characterization of an IscU-type protein (*Aa* IscU) from the hyperthermophilic bacterium *Aquifex aeolicus*. Unlike other known homologues of IscU, expression of *Aa* IscU in *Escherichia coli* has yielded an Fe–S cluster-containing holo-protein. Biochemical and spectroscopic studies of the wild-type *Aa* IscU and its Asp38-to-Ala substituted (D38A) variant molecule indicate that the holo-protein forms a trimer containing substoichiometric [2Fe–2S] cluster with its stability substantially increased by a D38A substitution. The [2Fe–2S] cluster was oxygen-labile and upon loss of the cluster, the resultant apo-form dissociated into a smaller species, a mixture of monomer and dimer with the dimer form predominating. Reddish-brown crystals of holo-*Aa* IscU-D38A were obtained under anaerobic conditions, that gave diffractions beyond 2.0 Å resolution with synchrotron radiation. The crystal belongs to the space group $P2_12_12$ with unit-cell parameters $a = 72.6$, $b = 122.3$, $c = 62.4$ Å, where the asymmetric unit contains three molecules of *Aa* IscU. Successful crystallization of holo-*Aa* IscU-D38A strongly suggests that the trimer association carrying substoichiometric [2Fe–2S] cluster represents a conformationally stable oligomeric state.

Key words: *Aquifex aeolicus*, crystallization, iron–sulphur cluster, IscU, trimer.

Abbreviations: Fe–S, iron–sulphur; SAXS, small-angle X-ray scattering; WT, wild-type.

Iron–sulphur (Fe–S) cluster-containing proteins are among the most abundant classes of metalloproteins in nature, and perform a variety of functions including electron transfer, redox and non-redox catalysis and regulation of gene expression (*1–4*). Biological assembly of Fe–S clusters and their insertion into apo-Fe–S proteins are mediated by three distinct enzyme systems called NIF, ISC and SUF (for recent reviews see 5–7), that are encoded in bacteria by the *nif* (*nifSU*), *isc* (*iscRSUA-hscBA-fdx-iscX*) and *suf* (*sufABCDSE*) operons, respectively (*8–13*). *In vivo* experiments have demonstrated interchangeability among the three systems (*14–16*), suggesting that they are all responsible for maturation of a wide variety of Fe–S proteins without strict specificity for apo-protein targets or Fe–S cluster types (either [2Fe–2S], [3Fe–4S] or [4Fe–4S]) to be assembled. The three systems share mechanistic similarity in their requirement for a cysteine desulphurase (sulphur donor) as well as the scaffold proteins in order to assemble transient Fe–S clusters, whereas other components play distinctive roles in their respective systems.

IscU and the N-terminal domain of NifU share high degree of sequence identity and play a central role in the ISC and NIF systems, respectively (*5*). The homologous protein called SufU is also encoded in the *suf*-related operons in several bacterial species including *Thermotoga maritima* and *Streptococcus pyogenes* (*15*). It is generally accepted that the IscU-type proteins serve as a scaffold for the biosynthesis of transient Fe–S clusters prior to its delivery to apo-protein targets. IscU and NifU interact with cysteine desulphurase (IscS and NifS, respectively) that catalyses sulphur abstraction from the substrate cysteine, thereby facilitating direct transfer of elemental sulphur between the proteins (*17–19*). Biochemical and spectroscopic studies indicated that the IscU-type proteins bind labile Fe–S cluster(s) by chemical or IscS/NifS-directed *in vitro* reconstitution, and the preformed Fe–S cluster can subsequently be transferred from the IscU-type proteins to a recipient apo-protein (*20–28*). In the conserved sequences of the IscU-type proteins, three invariant cysteine residues are essential for Fe–S cluster binding as well as *in vivo* scaffolding function (*29–31*).

Variation in oligomeric state has been reported in the IscU-type proteins from different organisms. For instance, *Escherichia coli* apo-IscU is a monomer or a S–S-bridged dimer (*18*, *19*, *27*), whereas

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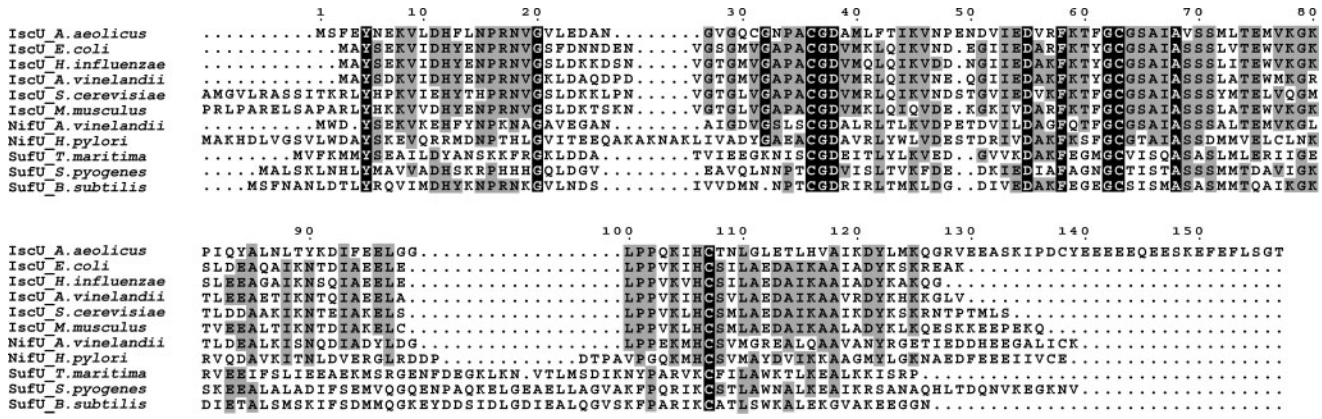

Fig. 1. **Multiple sequence alignment of the IscU homologs.** The invariant residues are highlighted in black and those highly conserved are in grey. Numbers refer to the IscU sequence from *A. aeolicus*. Only the N-terminal third of NifU, that is homologous to IscU/SufU, is shown. The figure was prepared with CLUSTAL W (*43*) and ESPript (*44*).

Azotobacter vinelandii IscU and *Schizosaccharomyces pombe* IscU are a dimer on which one or two [2Fe–2S] or a [4Fe–4S] cluster was reconstituted (*21*, *23*). Conversion of the oligomeric state was observed for *T. maritima* SufU, in which the apo-form eluted from a gel filtration column as a monomer, whereas the [2Fe–2S]-containing holo-form migrated as a dimer (*24*). In contrast, a monomeric [2Fe–2S] state was demonstrated for human IscU derivative that carries N-terminal His-tag and a D37A mutation (*30*). Recent NMR and crystallographic studies have revealed the tertiary structures of the monomeric apo-form of IscU and SufU from *Haemophilus influenzae* and *S. pyogenes*, respectively (*32*, *33*). In the highly conserved structures of the $\alpha+\beta$ core domain, three invariant cysteine residues are positioned at the solvent-exposed surface where a zinc ion is coordinated, suggesting the binding site for a nascent, labile Fe–S cluster. However, the exact location and coordination of the Fe–S cluster in either the monomer or oligomer have remained elusive, since no structural data have been available for the holo-form of this type of proteins.

Biochemical and structural studies of holo-IscU carrying the nascent Fe–S cluster can provide critical insight for understanding the molecular event underlying the Fe–S cluster biosynthesis, but have been proved to be challenging due to the intrinsic nature of instability. In this study, we have examined several homologues of IscU from different organisms and found that the protein from *Aquifex aeolicus* (*Aa* IscU) expressed in *E. coli* exceptionally accommodated a [2Fe–2S] cluster by itself. *Aquifex aeolicus* is the hyperthermophilic bacterium that can grow maximum at 95°C (*34*), and possesses *isc*-like genes for IscS, IscU, IscA and Fdx (*35*) but lacks *suf*- and *nif*-specific genes (*15*). Unlike NifU, *Aa* IscU is a single-domain protein sharing higher sequence identity with the bacterial and mitochondrial IscUs (about 50%) than SufUs (about 30%). Furthermore, *Aa* IscU has a highly conserved histidine at position 106 as well as the three invariant cysteines at 36, 63 and 107, but lacks about 20 amino acid insertion characteristic of the SufU sequences (Fig. 1). The [2Fe–2S] cluster of *Aa* IscU was still unstable but substantial stabilization was achieved by substitution of the Asp38 residue with Ala (D38A), which allowed us to rigorously study the molecular status of holo-form as well as its crystallization conditions. Here we report a novel trimer state of holo-*Aa* IscU-D38A that can be crystallized with bound [2Fe-2S] cluster.

MATERIALS AND METHODS

Cloning, Mutagenesis and Expression of A. aeolicus IscU—The gene for *iscU* (AQ_896) was amplified from *A. aeolicus* genomic DNA by PCR using the forward primer 5′-CATATGAGCTTCGAATACAATGAG-3′ (*Nde*I restriction site underlined) and the reverse primer 5′-GGATCCGAGCTCACGTTCCGGACAGGAATTCG-3′ (*Bam*HI site underlined). The PCR product was first cloned into the pCR2.1-TOPO vector (Invitrogen) by TA cloning method and then transferred into the *Nde*I/*Bam*HI site of the pET21a(+) vector (Novagen) to express the full-length *Aa* IscU protein (without a histidine tag). The truncated version at the C-terminus was amplified using the forward primer shown above and the reverse primer 5′-GGATCCGAGCTCAGTAGCAGTCGGGTATCT TG-3′ (for Δ140–157) or 5′-GGATCCGAGCTCATACTCT ACCCTGCTTCATGAG-3′ (for Δ130–157). The QuikChange technique (Stratagene) was employed for the D38A substitution using the primers, 5′-GTAACCCCGC TTGCGGCGCCGCAATGCTCTTTAC-3′ and 5′-GTAAA GAGCATTGCGGCGCCGCAAGCGGGGTTAC-3′, where the underlined positions indicate the mutations for D38A and a *Nar*I site. Cloning and mutagenesis results were confirmed by nucleotide sequencing of the entire coding region.

Escherichia coli C41(DE3) strain was used for protein expression. A 50 ml Terrific broth culture supplemented with 50 μg/ml ampicillin was grown overnight at 28°C. The pre-culture was used as an inoculum for a 5 l fermentation in Terrific broth supplemented with 50 μg/ml ampicillin and 0.1 mg/ml ferric ammonium citrate. Cells were grown at 28°C for 4 h prior to induction with 0.5 mM IPTG. The cells were harvested by centrifugation 20 h after the induction and stored at −80°C.

Protein Purification—Aa IscU-WT and the D38A mutant proteins were purified by the same procedure. The cell pellets were suspended in 50 mM Tris–HCl (pH 7.8) and 1 mM DTT and lysed by sonication on ice under a stream of nitrogen gas. The sonicated lysate was incubated at 70°C for 15 min under a nitrogen gas stream and centrifuged at 30,000*g* for 30 min at 4°C. All the following procedures were carried out in an anaerobic chamber (COY) containing 5% H_2 and 95% N_2. The clarified supernatant was loaded to two tandemly connected HiTrap Q HP 5 ml columns equipped with an ÄKTAprime chromatography system (GE Healthcare Bioscience) and proteins were eluted with a NaCl gradient from 0 to 1.0 M in 50 mM Tris–HCl (pH 7.8) at a flow rate of 1.0 ml/min. The fraction containing the target protein was concentrated, loaded on a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare Bioscience) and eluted with a buffer containing 150 mM NaCl and 50 mM Tris–HCl (pH 7.8) at a flow rate of 0.5 ml/min. At each step, the purity of the fractions was analysed by SDS–PAGE. The purified protein was concentrated with a VIVAPORE concentrator (VIVASCIENCE) and brought out from the anaerobic chamber in sealed tubes and immediately frozen in liquid nitrogen and stored at −80°C until use.

Analytical Methods—The concentration of the apo-*Aa* IscU was determined using a molar absorption coefficient at 280 nm of 7,700 $M^{-1}\,cm^{-1}$ that was calculated using ProtParam (http://au.expasy.org/tools/protparam.html). The concentration of the holo-protein was determined by the method of Bradford using apo-*Aa* IscU as a standard protein. UV-visible absorption spectra were recorded on a UV-3101PC (SHIMADZU) or a V-630BIO (JASCO) spectrometer. Protein-bound iron was determined after acid denaturation by the procedure described by Fish (*36*). Stability of the Fe–S cluster of *Aa* IscU-D38A was examined after exposure to air, in which the samples (0.5 mg/ml) were incubated in 150 mM NaCl and 50 mM Tris–HCl (pH 7.8) with gentle stirring at 4°C for several days and the UV-visible absorption spectra were recorded periodically.

Analytical gel filtration chromatography was carried out to determine the oligomeric states of WT and D38A mutant of *Aa* IscU. The as-isolated holo-protein solutions were supplemented with 1 mM DTT and incubated overnight in an anaerobic chamber at 4°C prior to chromatography. The colourless apo-forms were prepared by incubating in open-to-air tubes for 3 days at 4°C in a solution supplemented with 1 mM DTT and 10 mM EDTA, in which the disassembly of the Fe–S cluster was accelerated by EDTA. Aliquots of 100 µl samples were loaded on a Superdex 200 10/300 GL column (GE Healthcare Bioscience) equipped with an ÄKTAexplorer 10S chromatography system (GE Healthcare Bioscience), and eluted at a flow rate of 0.5 ml/min with the deoxygenated buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.8) and 1 mM DTT. A calibration was made using the standard proteins (gel filtration molecular weight marker kit, SIGMA-ALDRICH) including cytochrome *c*, carbonic anhydrase, albumin, alcohol dehydrogenase and β-amylase.

Small-angle X-Ray Scattering (SAXS) Experiments—SAXS measurements were carried out at BL-10C, Photon Factory, Tsukuba, Japan (*37*, *38*). The *Aa* IscU-D38A sample was dialysed and diluted in a solution containing 50 mM Tris–HCl, 150 mM NaCl and 3 mM DTT (pH 7.8) to a concentration varied from 0.5 to 14 mg/ml, and was put in a sample cell (1 mm path length). The wavelength of the X-ray was set to 1.488 Å with a Si monochromater. The scattering profiles were collected using a position sensitive proportional detector, PSPC, (RIGAKU). The temperature of the cell was kept at 20°C, and the exposure time was 10 min for each sample. To improve the signal-to-noise ratio at the lower protein concentration (<5 mg/ml), four sets of independent measurements were averaged. In a small angle region, a scattering intensity profile, $I(Q)$ can be approximated with the following equation.

$$I(Q) = I(0)\exp\left[-R_g^2\left(\frac{Q^2}{3}\right)\right], \quad Q = \frac{4\pi\sin\theta}{\lambda},$$

where $I(0)$ is scattering intensity at $\theta = 0$ and R_g is a radius of gyration (*39*, *40*). The slope and the Y-intersection of $\ln(I(Q))$ versus Q^2 plot, Guinier plot, give $-R_g^2/3$ and $\ln I(0)$, respectively. The concentration-normalized intensity at zero angle, $I(0)$/conc., is proportional to molecular weight of the sample. Using a standard protein solution, the apparent molecular weight of the sample can be determined. In this work, ovalbumin from chicken egg (43 kDa, Sigma A2512) dissolved in the same buffer was used as a standard solution.

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were performed with an XL-A analytical ultracentrifuge (Beckman-Coulter Inc.) at 20°C using double-sector standard centerpieces (1.2 cm) with quartz windows. Sample volumes of 140 µl were centrifuged at 16,000, 20,000 and 24,000 r.p.m. Data were collected in the radial step at 0.001 cm intervals and the average of sixteen measurements at each radial distance was calculated. Sedimentation equilibrium was ensured when replicate scans separated by ≥6 h were indistinguishable. The partial specific volume of the protein was assumed to be 0.73 cm^3/g and the density of the solvent 1.00 g/cm^3. For the analysis of the oligomeric states, non-linear least squares fitting using the following equation was performed to evaluate the population of the monomeric, dimeric and trimeric forms of apo-*Aa* IscU.

$$\begin{aligned} A(r) = {} & A_0(r_0)\exp\left[H(1-\bar{\nu}\rho)M_N\left(r^2-r_0^2\right)\right] \\ & + [A_0(r_0)]^2\exp\left[H(1-\bar{\nu}\rho)2M_N\left(r^2-r_0^2\right)+\ln[k_2]\right] \\ & + [A_0(r_0)]^3\exp\left[H(1-\bar{\nu}\rho)3M_N\left(r^2-r_0^2\right)+\ln[k_2k_3]\right]+B \end{aligned}$$

where $H = (\omega/2\pi)^2\,(2RT)^{-1}$; $\bar{\nu}$ is the partial specific volume of sample; ρ is the density of solvent; R the gas constant; T the absolute temperature; ω the rotor speed in r.p.m. units; A_0 the absorbance at a reference point, r_0; $A(r)$ the absorbance at a position of r cm from the rotor centre; B the baseline correction; and M_N is the molecular weight of the *Aa* IscU monomer. The non-linear least squares fittings were performed using Mathematica 3.0 (Wolfram Research Inc., Champaign, IL, USA) employing the Levenberg–Marquart algorithm.

Crystallization of Aa IscU-D38A and Preliminary X-ray Diffraction Study—Crystallization conditions were surveyed by the hanging-drop vapour-diffusion method in the anaerobic chamber at 20°C. Each droplet was prepared by mixing 1.0 µl of protein solution (in 50 mM Tris–HCl, pH 7.8, 150 mM NaCl, with varying protein concentration from 10 to 70 mg/ml) and 1.0 µl of the reservoir solution, and then equilibrated against 200 µl of the reservoir solution, in which the reservoir solutions were pre-equilibrated in the anaerobic chamber to remove residual O_2. The initial crystals of *Aa* IscU-D38A were obtained using a reservoir solution containing 1.6 M ammonium sulphate and 0.5 M LiCl (JBScreen Classic 6-B2) and the protein solution of 40 mg/ml. Additive solutions were screened by mixing 0.2 µl aliquot (Additive Screen kit, Hampton Research) with each drop containing 0.8 µl of the protein solution and 1.0 µl of the reservoir solution, where addition of 2.0 M NaCl solution (Additive Screen B6) significantly improved the quality. For further optimization, the micro-seeding method was also applied, in which *Aa* IscU-D38A crystals were crushed with a needle and the seed crystals were transferred into fresh drops. The *Aa* IscU-D38A crystals were soaked in a cryoprotectant solution containing 1.6 M ammonium sulphate, 0.5 M LiCl and 20% glycerol for a few seconds and then flash-cooled at 100 K. X-ray diffraction data were collected at 100 K on an ADSC Quantum 315 X-ray CCD detector using synchrotron radiation ($\lambda = 1.0000$ Å) at the BL41XU station of SPring-8 (Hyogo, Japan), and processed and scaled with the program HKL2000 suite (*41*).

RESULTS AND DISCUSSION

Expression, Purification and Stabilization of Holo-Aa IscU—While studying several IscU homologs from different organisms, we found that *E. coli* cells overexpressing IscU from the hyperthermophilic bacterium *A. aeolicus* (*Aa* IscU) were exceptionally dark brown, indicating that this protein harbours a relatively stable Fe–S cluster. The reddish-brown colour of the bacterial lysate survived the heat treatment step at 70°C, but gradually disappeared during the following chromatography under normal aerobic conditions. Since the Fe–S clusters of the known IscU homologues are notoriously sensitive against oxygen, the purification was carried out under anaerobic conditions including sonication and heat treatment under a stream of N_2 gas and the following chromatography steps in an anaerobic chamber. About 50 mg of reddish-brown *Aa* IscU was obtained from a litre of cell culture with high purity as judged by SDS–PAGE (Fig. 2). The UV-visible spectrum of *Aa* IscU showed absorbance peaks at 278, 321 and 410–460 nm characteristic of a [2Fe–2S] cluster (Fig. 3A). Although *Aa* IscU was purified as holo-protein, the overall holo concentration appeared to be low as judged by the spectroscopy (A_{415}/A_{278} ratio of 0.16). Upon exposure to air, the reddish-brown colour disappeared with a half-life of 2.3 h (Fig. 3B, inset), suggesting that the [2Fe–2S] cluster is unstable and might have been lost during the purification steps.

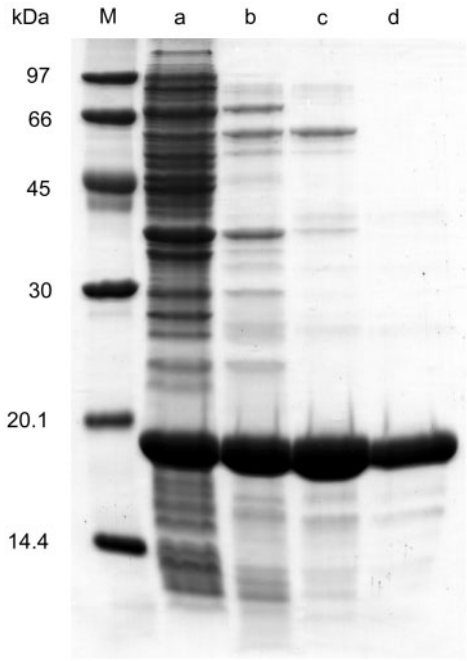

Fig. 2. **SDS–PAGE analysis of *Aa* IscU during purification.** Lane M, molecular size markers; lane a, the sonicated cell lysate; lane b, supernatant fraction after incubation at 70°C for 15 min; lane c, the peak fraction from HiTrap Q HP column; lane d, the peak fraction from a HiPrep 16/60 Sephacryl S-200 HR column.

Partial stabilization of the Fe–S cluster was previously demonstrated for *A. vinelandii* NifU by substitution of an invariant residue Asp37 by Ala (*20*), and also for human and *S. pombe* IscU as well as *T. maritima* SufU by the analogous mutations (*23–25*, *30*). Hence the corresponding site-directed mutation was introduced into *Aa* IscU and the resultant protein (*Aa* IscU-D38A) was purified to homogeneity by the anaerobic procedures described above. The D38A derivative showed reddish-brown colour as observed for the WT protein: noticeably, *Aa* IscU-D38A had higher absorption in the near UV-visible region with sharper, better defined peaks at 321 and 415–460 nm with A_{415}/A_{278} ratio of 0.32 (Fig. 3A), indicative of an intact [2Fe–2S] cluster. While the spectrum did not change in the anaerobic chamber, admission of air to the purified solutions resulted in gradual bleaching and decay of the UV-visible bands with a half-life of about 5–6 days (Fig. 3B). During the bleaching, the 6-day air-exposed *Aa* IscU-D38A had the absorption spectrum almost identical to the as-isolated WT protein, consistent with the low [2Fe–2S] content of *Aa* IscU-WT that originated from partial destruction during the purification steps. These results indicate that the [2Fe–2S] cluster of *Aa* IscU was more stable than IscUs from other sources, and that further stabilization was achieved by the D38A substitution.

Holo-Aa IscU Exists as a Trimer—The as-isolated *Aa* IscU-WT was separated into two peaks on a Superdex 200 gel filtration column. The larger species (~55 kDa) eluted with reddish-brown colour whereas the smaller one (~30 kDa) was colourless (Fig. 4), supporting the

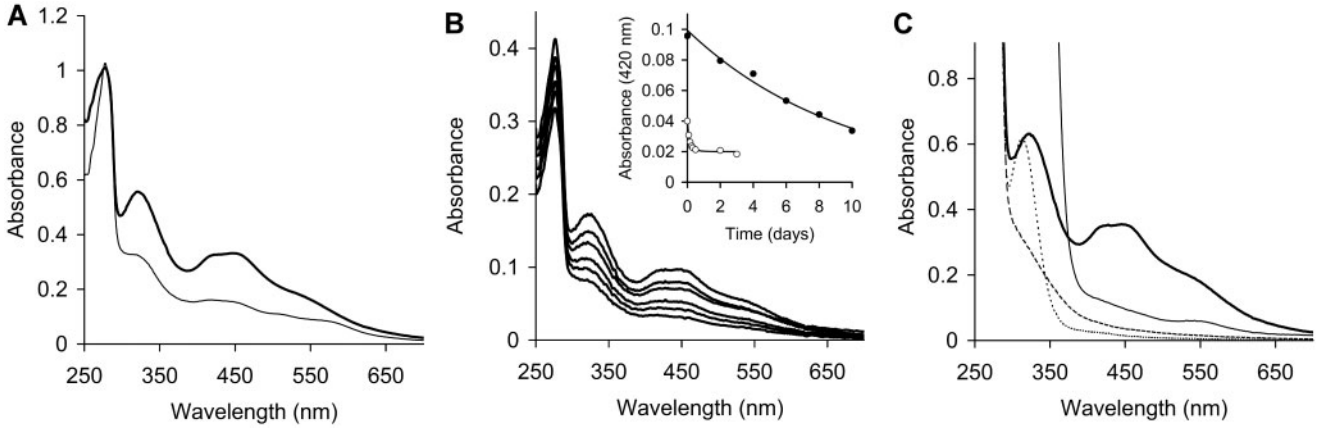

Fig. 3. **UV-visible absorption spectra.** (A) As-isolated samples of *Aa* IscU-WT (thin line) and its D38A derivative (thick line). The spectra were recorded in a solution containing 150 mM NaCl and 50 mM Tris–HCl (pH 7.8) and normalized to give peak absorbance at 280 nm of 1.0 to facilitate comparison. (B) Cluster degradation in the presence of oxygen. Spectra of holo-*Aa* IscU-D38A were recorded before (top trace) and after 2, 4, 6, 8 and 10 days of the exposure to air at 4°C. Inset: The absorbance at 420 nm was plotted for *Aa* IscU-WT (open circle) and the D38A derivative (filled circle) as a function of time after exposure to air. (C) Cluster degradation by dithionite. Spectra of holo-*Aa* IscU-D38A were recorded before and immediately after the addition of ~2 mM dithionite (thick and thin lines, respectively). The dithionite-bleached sample was further incubated in an open-to-air tube for 4h (dotted line) and 14h (broken line).

notion that the Fe–S cluster of this preparation was disassembled in part and the resultant apo-form was separated on the column. In contrast, *Aa* IscU-D38A migrated in the column predominantly as a [2Fe–2S] cluster-containing species that eluted at a position corresponding to the molecular mass of 55.0 ± 4.3 kDa (average ± SD of six experiments) with only a minor shoulder of colourless polypeptide. Since the molecular mass of 17.5 kDa is calculated from the amino acid sequence, the holo-*Aa* IscU most likely forms a trimer.

To further examine the oligomeric state of holo-*Aa* IscU-D38A, the SAXS measurements were performed. The values of $I(0)$/conc. and R_g^2 at various concentrations are shown in the Fig. 5A and B, respectively, where those of ovalbumin are superimposed for comparison. The values of $I(0)$/conc. and R_g^2 of ovalbumin linearly decrease with the increase of the concentration, as is caused by inter-particle interference. To eliminate the interference effect, the intrinsic values of $I(0)$/conc. and R_g^2 were obtained by extrapolation to zero-protein concentration using linear fitting. The resultant values of $I(0)$/conc. and R_g are 9900 ± 200 and 25.7 ± 0.4 Å, respectively. On the contrary, those of holo-*Aa* IscU-D38A rapidly increase up to 6 mg/ml, and reach almost constant values above the concentration. It can be interpreted that the characteristic curves reflect the oligomerization of *Aa* IscU-D38A. Because of the saturation above 6 mg/ml, a monodisperse oligomeric form can be assumed under the higher protein concentration. To evaluate the values of $I(0)$/conc. and R_g^2 of the oligomeric state at the zero-protein concentration, the 4 points above 6 mg/ml were fitted with a line, resulting in the values of $12{,}700 \pm 330$ and 570 ± 20 Å^2 (R_g, 23.9 ± 0.4 Å), respectively. Using the $I(0)$/conc. values of ovalbumin and *Aa* IscU-D38A at zero-protein concentration, the apparent molecular size was estimated to be 55.2 ± 2.0 kDa, which is nearly identical to that of a trimer (52.5 kDa). It should be noted that the apparent molecular weight even at 1 mg/ml is about 80% of that of a trimer, suggesting that the trimeric form would be a major species under physiological conditions.

To examine the oligomeric state of apo-form, the *Aa* IscU-WT and its D38A derivative were treated with oxygen in the presence of DTT and EDTA and the resultant colourless apo-proteins were subjected to gel filtration chromatography (Fig. 4C and D). When compared to the as-isolated holo-forms, increase in the smaller species was evident for the apo-forms with concomitant decrease in the larger species, suggesting dissociation of the trimer upon disassembly of the cluster. The smaller species eluted at positions corresponding to the molecular mass of 30 ± 0.5 kDa, and therefore, most likely represents a dimer form. Sedimentation equilibrium ultracentrifugation experiments of the apo-forms of *Aa* IscU-WT and the D38A derivative indicate that they exist in an equilibrium between the monomer and dimer where the trimer species was negligible. For instance, the data obtained at 24,000 r.p.m. were best fitted to the equation (see 'MATERIALS AND METHODS' section) with the ratio of 12% monomer and 88% dimer (Fig. 6). Taken together, the results suggest that *Aa* IscU undergoes interconversion of the oligomeric structures between holo-trimer and apo-dimer/monomer. Interconversion between holo-dimer and apo-monomer has been observed for *T. maritima* SufU (*24*), but not reported for any IscUs from other sources.

Fe–S Cluster Environment—In the UV-visible spectrum of *Aa* IscU-D38A (Fig. 3A), the extinction coefficient in the visible region ($\varepsilon_{420} = 3.3$–4.0 mM^{-1} cm^{-1}) suggests substoichiometric, approximately one or two [2Fe–2S] clusters per trimer based on the published values ($\varepsilon_{420} = 8$–11 mM^{-1} cm^{-1}) determined for well-characterized [2Fe–2S] ferredoxins (*42*). Analysis of protein-bound

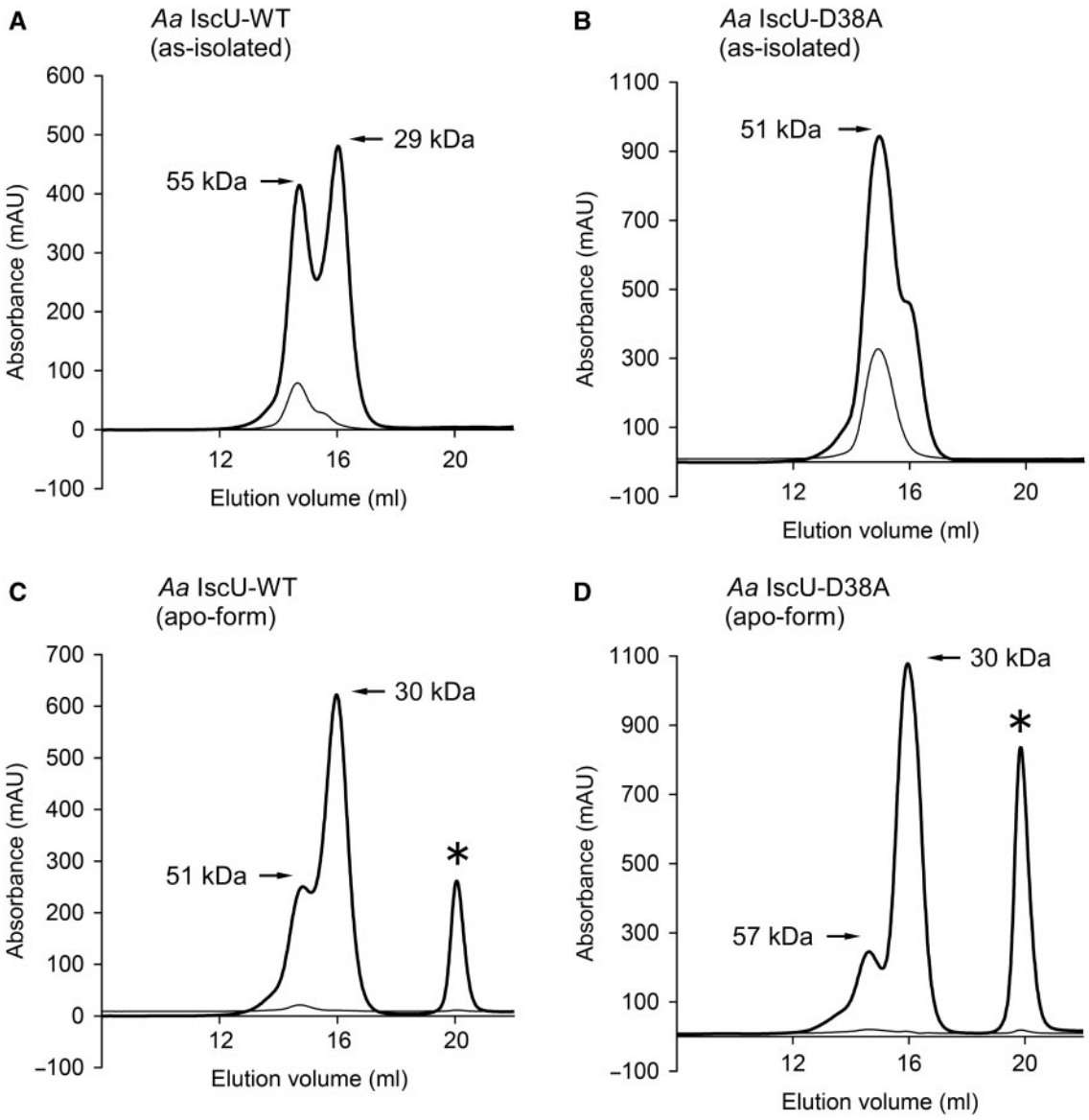

Fig. 4. **Conversion of oligomeric state of *Aa* IscU.** Gel filtration chromatography was carried out on a Superdex 200 10/300 GL column (GE Healthcare Bioscience) with deoxygenated buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.8 and 1 mM DTT) and eluates were monitored simultaneously by absorbance at 280 nm (thick line) and 415 nm (thin line). Prior to the chromatography, the as-isolated holo-proteins were supplemented with 1 mM DTT and anaerobically incubated for 16 h (A and B). Colourless apo-proteins were prepared by exposure to air at 4°C for 3 days in a solution containing 1 mM DTT and 10 mM EDTA (C and D). Each 100 μl sample with a concentration of 18 mg/ml for *Aa* IscU-WT and 27 mg/ml for the D38A derivative were loaded into the column. The peak fractions indicated by asterisks (in C and D) did not contain proteins as judged by SDS–PAGE, and probably represent the EDTA–iron chelate complex.

iron yielded 0.82 iron atoms per monomer which corresponds to 1.23 [2Fe–2S] cluster per homotrimeric *Aa* IscU-D38A. Reduction with dithionite resulted in an immediate bleaching of the visible absorption of *Aa* IscU-D38A (Fig. 3C). The subsequent air-oxidation did not restore the original spectrum, where the peak at 310 nm may represent the iron-bound form *en route* to cluster degradation and complete release. In a parallel experiment using *E. coli* Fdx, a [2Fe–2S] ferredoxin, almost complete recovery from the dithionite-reduced bleaching was observed within a few hours of air-oxidation (data not shown). The results suggest that the [2Fe–2S] cluster of *Aa* IscU is not redox-active but reductively labile, a common feature previously observed for IscUs from *A. vinelandii* and *Homo sapiens* (*22*, *30*). Further spectroscopic characterization of the cluster using electron paramagnetic resonance was not applicable because of the reductive lability of the cluster. *Aa* IscU has a strictly conserved histidine at position 106 as well as the three invariant cysteines at 36, 63 and 107, which were proposed to coordinate the Fe–S cluster. In addition, *Aa* IscU has a unique C-terminal extension of 28 amino acids compared

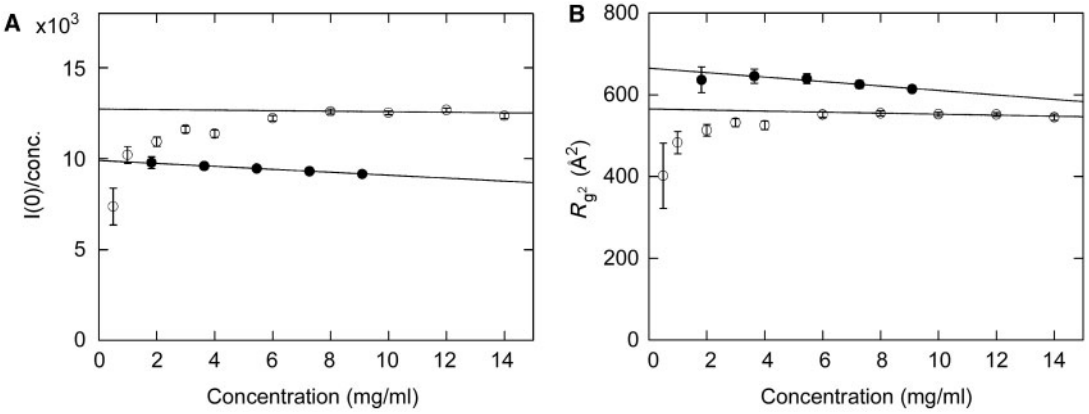

Fig. 5. **The SAXS analysis of the oligomeric state of holo-*Aa* IscU-D38A.** $I(0)$/conc. (A) and R_g^2 (B) were plotted against protein concentration (open circle). Those for ovalbumin are shown for comparison (filled circle).

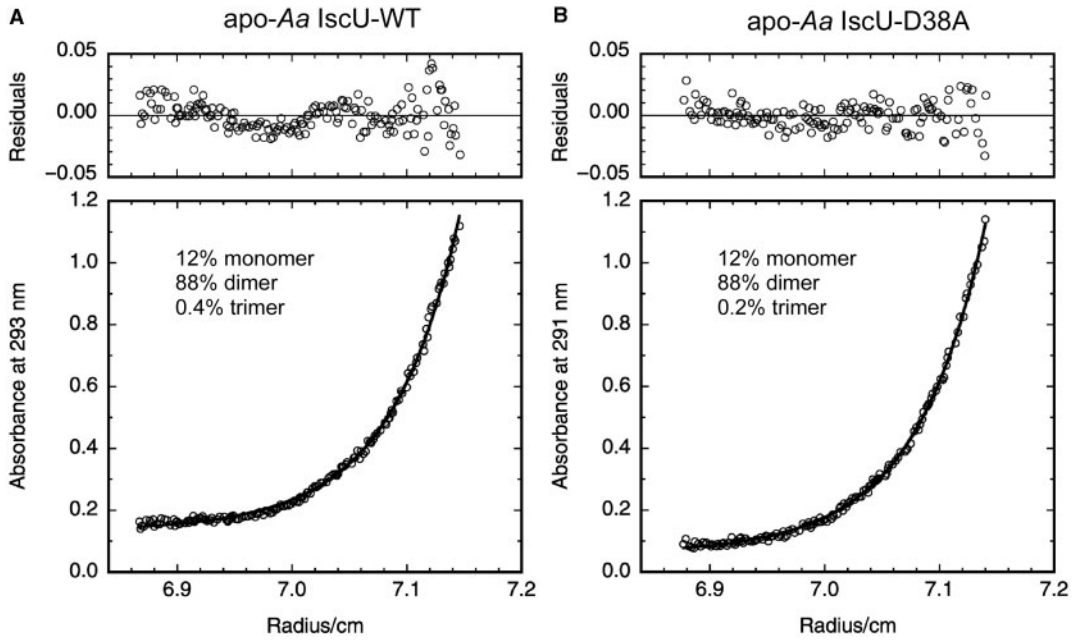

Fig. 6. **Sedimentation equilibrium profile of apo-*Aa* IscU.** Apo-*Aa* IscU-WT (A) and the D38A derivative (B) were prepared as described in Fig. 4 and dialysed against a solution containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.8) and 1 mM DTT. The data were recorded at 24,000 r.p.m. at 20°C. The protein concentration was 2.0 mg/ml. Lower panel: experimental absorbance as a function of the radial position and data fitting to the equation described in 'MATERIALS AND METHODS' section. Solid line indicates the result of non-linear least squares fitting employing monomer–dimer–trimer equilibrium model. Upper panel: Distribution of differences between experimental and calculated values.

with *E. coli* and *A. vinelandii* IscU, where 11 glutamate and one cysteine residues reside. To examine whether the observed stability of the [2Fe–2S] clusters is achieved by the characteristic C-terminal extension, truncated proteins were expressed and characterized. Removal of the 18 residues (Δ140–157) or 28 residues (Δ130–157) at the C-terminus did not significantly alter the expression and purification of the recombinant holo-proteins from *E. coli* as well as the UV-visible spectra (data not shown). Thus, it is unlikely that the C-terminal residues serve as a ligand of the [2Fe–2S] cluster or enhance its stability.

Crystallization and Preliminary X-ray Diffraction Analysis—The reddish-brown crystals of *Aa* IscU-D38A were obtained in the anaerobic chamber at 20°C. The initial crystals appeared under conditions of hanging-drop vapor-diffusion against a solution containing 1.6 M ammonium sulphate and 0.5 M LiCl, which corresponds to the JBScreen Classic 6-B2. However, the needle-like crystals were too small for the X-ray diffraction experiments. After extensive additive screening using micro-seeding method and fine-tuning of the protein concentration, we obtained plate-shaped reddish-brown crystals with typical dimensions of approximately

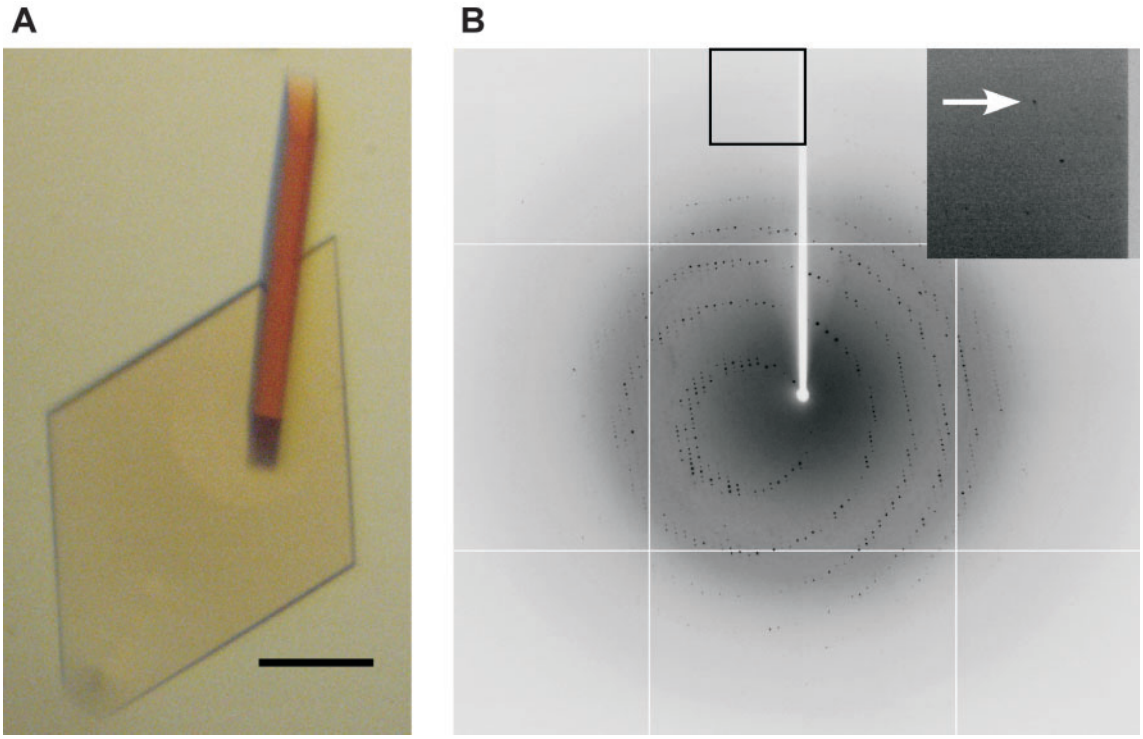

Fig. 7. **Crystallization of holo-*Aa* IscU-D38A.** (A) Typical crystals with dimensions of ~0.25 × 0.25 × 0.03 mm. The scale bar represents 0.1 mm. (B) Diffraction pattern of the holo-D38A *Aa* IscU crystal. The resolution of the spot indicated by the arrow is 1.96 Å.

0.25 × 0.25 × 0.03 mm (Fig. 7A). The crystals diffracted X-rays to beyond 2.0 Å resolution (Fig. 7B), and high-quality diffraction data were collected to 2.3 Å resolution. The crystal belongs to the orthorhombic system and the space group of $P2_12_12$ with unit-cell parameters $a = 72.6$, $b = 122.3$, $c = 62.4$ Å. The number of *Aa* IscU-D38A molecules was estimated to be three in asymmetric unit with the V_M value of 2.6 Å^3 Da^{-1}, which corresponds to a solvent content of 53%. Detailed crystal parameters and data-collection statistics are summarized in Table 1. Successful crystallization of holo-*Aa* IscU-D38A strongly suggests that the trimer association carrying substoichiometric [2Fe–2S] cluster represents a conformationally stable oligomeric state, rather than the deteriorated byproducts during its expression and purification. Efforts are now under way to determine the structure by the multi-wavelength anomalous dispersion method using iron in the cluster as the anomalous scattering atoms.

Fe–S Cluster Biosynthesis System in A. aeolicus—The three distinct systems NIF, ISC and SUF for the biosynthesis of Fe–S clusters are usually encoded in bacterial genomes as respective operons, whereas the related genes in *A. aeolicus* are scattered over several loci as in the case for other functionally grouped genes (*e.g.* biosynthetic pathways of amino acids) in this bacterium (*34*). Nonetheless, *A. aeolicus* IscS1, IscS2, IscU and IscA bear higher sequence homology to the respective counterparts involved in the NIF and ISC systems than to the components in the SUF system (Fig. 8). In addition, occurrence of [2Fe–2S] Fdx5 (*35*) and IscU rather than NifU (modular protein composed of three domains) makes it likely that the biosynthetic system in *A. aeolicus* is analogous to the ISC machinery. On the other hand, the bacterium possesses only one gene for Hsp70-type molecular chaperone and lacks HscB-type co-chaperone, suggesting that the ISC-specific chaperone system is absent. In accordance with this notion, a short, strictly conserved sequence within IscUs (LPPVK) involved in the interaction with HscA is substituted to 100-LPPQK-104 in *Aa* IscU sequence (Fig. 1). It should be noted, however, that recent genetic studies in *A. vinelandii* and *E. coli* have revealed that HscA and HscB are dispensable under low-oxygen concentration, despite the fact that they are the essential components of the ISC machinery under normal, aerobic growth conditions (*31* and Y.T. unpublished results). *Aquiflex aeolicus* is a deep-branching species in the bacterial lineage that grows at 80–95°C under

Table 1. **Data collection statistics.**

Space group	$P2_12_12$
Cell parameters (Å)	$a = 72.6$, $b = 122.3$, $c = 62.4$
Resolution range (Å)[a]	50–2.30 (2.38–2.30)
Measured reflections	186,307
Unique reflections	25,043
Redundancy[a]	7.4 (5.5)
Completeness (%)[a]	97.4 (90.1)
R_{merge} (%)[a,b]	5.8 (35.0)

[a]Values in parentheses are for the outermost shell. [b]$R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the average intensity over equivalent reflections.

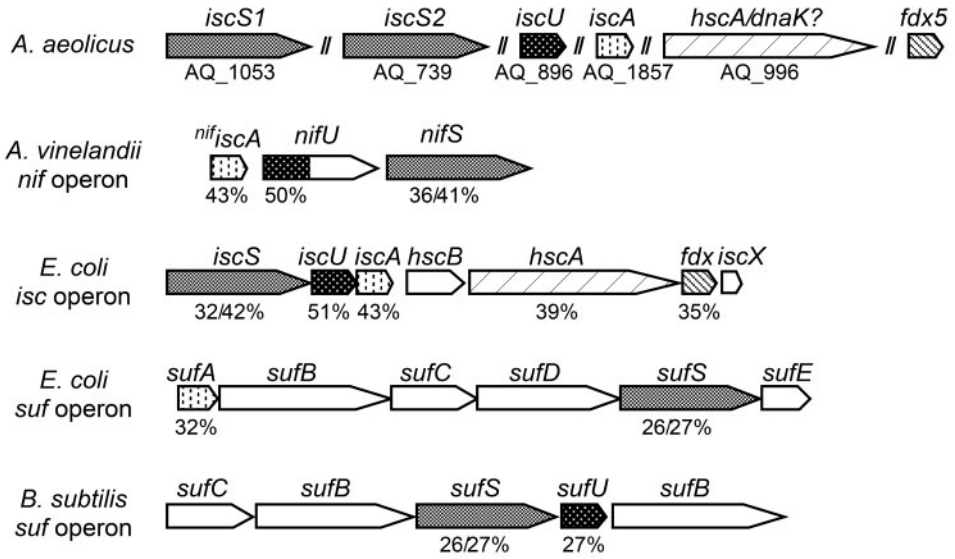

Fig. 8. ***Aquiflex aeolicus* genes encoding the ISC-like components.** *Aquiflex aeolicus* possesses two genes for cysteine desulfurase (designated IscS1 and IscS2), and one each for IscU, IscA, Fdx5 and HscA/DnaK, that are scattered over several loci in the chromosome. In contrast, the related genes are usually organized into the *nif*, *isc* and *suf* operons in other bacteria, where the amino acid identity is indicated in comparison with the corresponding homologue of *A. aeolicus*. NifS, IscS and SufS were compared with *A. aeolicus* IscS1/IscS2. The hatched region in NifU denotes the N-terminal domain.

oxygen-limited conditions (*34*). Accordingly, the lack of HscA/HscB chaperones and the relatively stable Fe–S cluster of *Aa* IscU shown in this study might be a consequence of this bacterium to adapt such extreme environments. It would be intriguing to study the structural basis of the scaffolding function of *Aa* IscU as well as its interaction with other ISC components, which will lead to a better understanding of the molecular mechanism involved in Fe–S cluster biosynthesis.

We thank Dr N. Shimizu and Dr M. Kawamoto (Japan Synchrotron Radiation Research Institute) for their aid with data collection using the synchrotron radiation at SPring-8 (Hyogo, Japan); Dr K. Wada (Osaka University, Japan) for his advices and valuable discussions; Dr R. Huber (Universität Regensburg, Germany) for the gift of the genomic DNA from *A. aeolicus*; H. Ohmori and N. Kaseda (Osaka University, Japan) for technical assistance. The synchrotron radiation experiments were performed with the approval of the JASRI (proposal nos. 2005B0984 and 2006A2727). This study was supported by a grant of the National Project on Protein Structural and Functional Analyses (to K.F.) and by a Grants-in-Aid for Scientific Research (17570112, 18054018 to Y.T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and also by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (to Y.S.).

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