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Asp97 is a Crucial Residue Involved in the Ligation of the [Fe_4S_4] Cluster of IscA from *Acidithiobacillus ferrooxidans*

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IscA was proposed to be involved in the iron-sulfur cluster assembly encoded by the *iscSUA* operon, but the role of IscA in the iron-sulfur cluster assembly still remains controversial. In our previous study, the IscA from *A. ferrooxidans* was successfully expressed in *Escherichia coli*, and purified to be a [Fe_4S_4]-cluster-containing protein. Cys35, Cys99, and Cys101 were important residues in ligating with the [Fe_4S_4] cluster. In this study, Asp97 was found to be another ligand for the iron-sulfur cluster binding according to site-directed mutagenesis results. Molecular modeling for the IscA also showed that Asp97 was a strong ligand with the [Fe_4S_4] cluster, which was in good agreement with the experimental results. Thus, the [Fe_4S_4] cluster in IscA from *A. ferrooxidans* was ligated by three cysteine residues and one aspartic acid.

Keywords: IscA, *Acidithiobacillus ferrooxidans*, mutation, iron-sulfur cluster, molecular modeling

Iron-sulfur proteins are widely distributed in nature and can be found in bacteria, fungi, plants, and mammals [2]. They exhibit diverse functions, which include electron transport, redox and nonredox catalysis, stabilization of proteins, DNA synthesis and repair, and sensing for regulatory processes [11, 14, 25]. Recent studies have revealed that a highly conserved gene cluster, *iscSUA-hscBA-fdx*, is essential for the general biogenesis of iron-sulfur proteins in bacteria [29, 31, 32, 38].

Three *isc* genes, *iscS*, *iscU*, and *iscA*, have been the targets of recent investigations. IscS and IscU are essential for cluster assembly, and have been previously purified and extensively characterized [1, 16, 20, 22, 26, 28, 33, 36]. The function of IscS is mainly in the production of sulfide from cysteine, resulting from a pyridoxal phosphate-dependent cysteine desulfurase activity [1, 16, 20]. The IscU is proposed to provide a scaffold for assembly of Fe-S clusters that can be subsequently used for maturation of apo Fe-S proteins [22, 26, 28, 33, 36].

The function of IscA in the biogenesis of iron-sulfur proteins remains controversial. It was proposed that IscA might function as an iron donor for iron-sulfur clusters synthesis, as deletion of IscA homologs resulted in the accumulation of iron in mitochondria and a deficiency of iron-sulfur proteins [10, 12]. Biochemical studies indicated that IscA could host a transient [Fe_2S_2] or [Fe_4S_4] cluster, but all the iron-sulfur clusters were assembled *in vitro* [15, 24, 34, 35]. The crystal structure of IscA suggests that it may exist as a tetramer, and that the IscA monomers are arranged as a dimer of $\alpha_1\alpha_2$ dimers about a central channel, with the conserved Cys35 located in this channel [3, 8]. Recently, a new report revealed that one partially exposed [2Fe-2S] cluster IscA from *Thermosynechococcus elongatus* was coordinated by two conformationally distinct IscA protomers, alpha and beta, with asymmetric cysteinyl ligation by Cys37, Cys101, Cys103 from alpha, and Cys103 from beta [21].

In our previous study, the IscA from *A. ferrooxidans* was expressed in *E. coli* and purified to be an iron-sulfur protein, which was capable of recruiting intracellular iron and sulfur and hosted a stable [Fe_4S_4] cluster, as shown in Fig. 1. Site-directed mutagenesis for the protein revealed that Cys35, Cys99, and Cys101 were ligating with the [Fe_4S_4] cluster [37]. According to the molecular modeling results for the IscA, Asp97 and Glu103 were in the vicinity

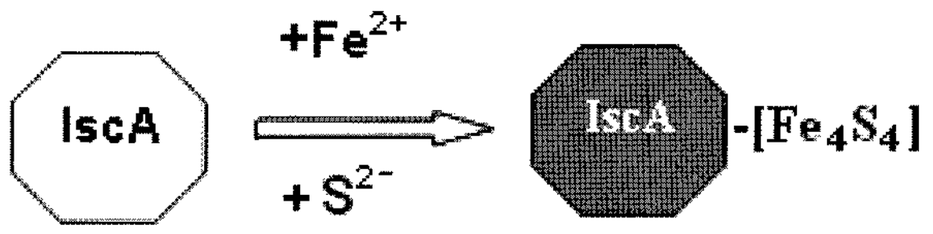

Fig. 1. Proposed mechanism for the iron-sulfur cluster assembly of IscA in *A. ferrooxidans*.

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of the iron-sulfur cluster and might be potential ligands for the $[Fe_4S_4]$ cluster in IscA.

In this study, Asp97 was found to be another ligand involved in the iron-sulfur cluster binding according to site-directed mutagenesis results, which was in good agreement with the molecular modeling result. Glu103 did not participate in iron-sulfur cluster binding.

MATERIALS AND METHODS

Materials

Acidithiobacillus ferrooxidans ATCC 23270 was obtained from the American Type Culture Collection. A HiTrap chelating metal affinity column was purchased from GE Healthcare Ltd. Top10 competent cells and *E. coli* strain BL21(DE3) competent cells came from Invitrogen Life Technologies. The Plasmid Mini kit, a gel extraction kit, and synthesized oligonucleotides were obtained from Sangon Company of Shanghai. *Taq* DNA polymerase, T4 DNA ligase, and restriction enzymes came from MBI Fermentas. All other reagents were of research grade or better and were obtained from commercial sources.

Construction of *A. ferrooxidans* IscA Mutant Plasmids

A QuikChange mutagenesis kit (Stratagene) was applied for constructing the mutant expression plasmids. The plasmid pLM1::ISCA was used as a template for constructing mutant expression plasmids through PCR reaction [37]. The following primer and their antisense primer were synthesized to introduce the mutated sequence:

D97A: 5'-AATCCCAACGTGAAGGCTGCCTGCGGTTGCGG-3', codon GAT for aspartic acid (D) was changed to codon GCT for alanine (A);
D97E: 5'-AATCCCAACGTGAAGGAAGCCTGCGGTTGCGG-3', codon GAT for aspartic acid (D) was changed to codon GAA for glutamic acid (E).
D97N: 5'-AATCCCAACGTGAAGAATGCCTGCGGTTGCGG-3', codon GAT for aspartic acid (D) was changed to codon AAT for asparagine (N).
D97S: 5'-AATCCCAACGTGAAGAGTGCCTGCGGTTGCGG-3', codon GAT for aspartic acid (D) was changed to codon AGT for serine (S).

PCR amplification was performed using *Pfu* DNA polymerase and samples were subjected to 13 cycles of 0.5 min of denaturation at 95°C, 1 min of annealing at 61°C, and 12 min of elongation at 72°C in a Mastercycler Personal (Eppendorf Model made in Germany). DpnI restriction enzyme was used to digest the parental supercoiled double-stranded DNA. The constructed mutant plasmids were transformed into TOP10 competent cells for screening purposes. The positive colonies with the mutant plasmids were identified by sequencing. The isolated mutant plasmids were then used to transform *E. coli* strain BL21(DE3) competent cells for expression purposes.

Expression and Purification of the IscA Mutant Proteins

Expression and purification procedures for IscA mutant proteins were identical to that of wild-type IscA [37]. The method of Bradford [4] was used to determine the protein content, with bovine serum albumin as the standard. The eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% of acrylamide according to Laemmli [17]. The gels were stained with Coomassie Brilliant Blue R-250.

UV-Vis Scanning and EPR Spectra

UV-visible spectra scanning was carried out at 25°C on a Hitachi UV-2800 spectrophotometer. The protein samples (20 μM) were prepared in 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4. X-band EPR spectra were recorded at 100 K on a JEOL JES-FE1XG spectrometer. Parameters for recording the EPR spectra were typically 15–30 mT/min sweep rate, 0.63 mT modulation amplitude, 9.154 GHz frequency, and 4 mW incident microwave power, and the sweep time was 2 min. The samples were diluted to 5 μM in 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4. The protein samples were prepared by incubating with 5 mM ammonium persulfate for 30 min.

Determination of Iron and Sulfide Content of IscA Mutant Proteins

Iron assays were performed by the colorimetric method [19], and the sulfide content was determined according to Siegel [27]. The samples were prepared in 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4.

Molecular Structure Modeling of IscA D97 Mutant Proteins

The preliminary models for the IscA D97A, D97E, D97N, and D97S mutant proteins were constructed using the approach of comparative protein modeling, applied by the Modeller module of Insight II software (Accelrys Software Inc.) running on Redhat Linux system. The template structure of the IscA from *E. coli* (PDB entry code 1S98) refined to 2.3 Å was used for modeling. During models generation, the structures were optimized according to CHARMM-derived stereochemical and nonbonded restraints, as well as statistical preferences for the Ramachandran plots and side-chain rotamers of different residue types. For all models, the optimal level parameters are set high and the cutoff radius parameters are set to 10 Å.

The initial model was improved by energy minimization (EM). After performing 200 steps of conjugate gradient (CG) minimization, the molecular dynamics (MD) simulation was then carried out to examine the quality of the modeled structure by performing 500 ps simulations at a constant temperature of 298 K. Finally, a conjugate gradient energy minimization of the full protein was performed until the root mean square gradient (RMSD) energy was lower than 0.001 kcal mol^{-1} $Å^{-1}$. All calculations were carried out using Discover_3 module of the Insight II system. The optimized structure was further checked with the ProStat module, and the structural features that differ significantly from the average values calculated from known proteins were then listed.

RESULTS AND DISCUSSION

Construction of the Mutant Plasmids

Mutant expression plasmids of pISCA(D97A), pISCA(D97E), pISCA(D97N), and pISCA(D97S) were constructed, and its sequences were verified for the presence of the directed

mutation and the absence of PCR-generated random mutations by DNA sequencing. The mutant expression plasmids were transformed into *E. coli* BL21(DE3) for expression.

Expression and Purification of IscA Mutant Proteins

Nickel metal-affinity resin column was used for single-step purifications of His-tagged IscA mutant proteins and all the mutant proteins were expressed as soluble proteins. The purified protein fractions were dialyzed against a 20 mM potassium phosphate buffer, pH 7.4, 5% glycerol, and 5 mM β-mercaptoethanol as soon as possible after the purification. The purities of IscA D97 mutant proteins were further examined by SDS-PAGE and single bands corresponding to the 9 kDa protein were observed with >95% purity when the purified proteins were boiled in Laemmli's sample buffer for 3 min, as shown in Fig. 2.

In our previous report, the iron-sulfur cluster was successfully incorporated in IscA in *E. coli*, which was capable of recruiting intracellular iron and sulfide and hosted a stable $[Fe_4S_4]$ cluster. After purification, the $[Fe_4S_4]$ cluster could still bind in the protein tightly [37]. The recombinant IscAs from *Escherichia coli*, *Azotobacter vinelandii*, and *Synechocystis* PCC 6803 have previously been produced in *E. coli*, and all the recombinant proteins were purified to be apoproteins without the iron-sulfur cluster [10, 24, 34, 35], but the $[Fe_2S_2]$ cluster or $[Fe_4S_4]$ cluster could be assembled in IscA *in vitro* in the presence of Fe^{2+} and sulfide.

The eluted IscA D97E mutant protein was observed to be brown proteins, indicating the iron-sulfur cluster is still bound to the protein after purification. The reduced D97E mutant protein exhibited a purity ratio (A_{280}/A_{380}) of 2.47,

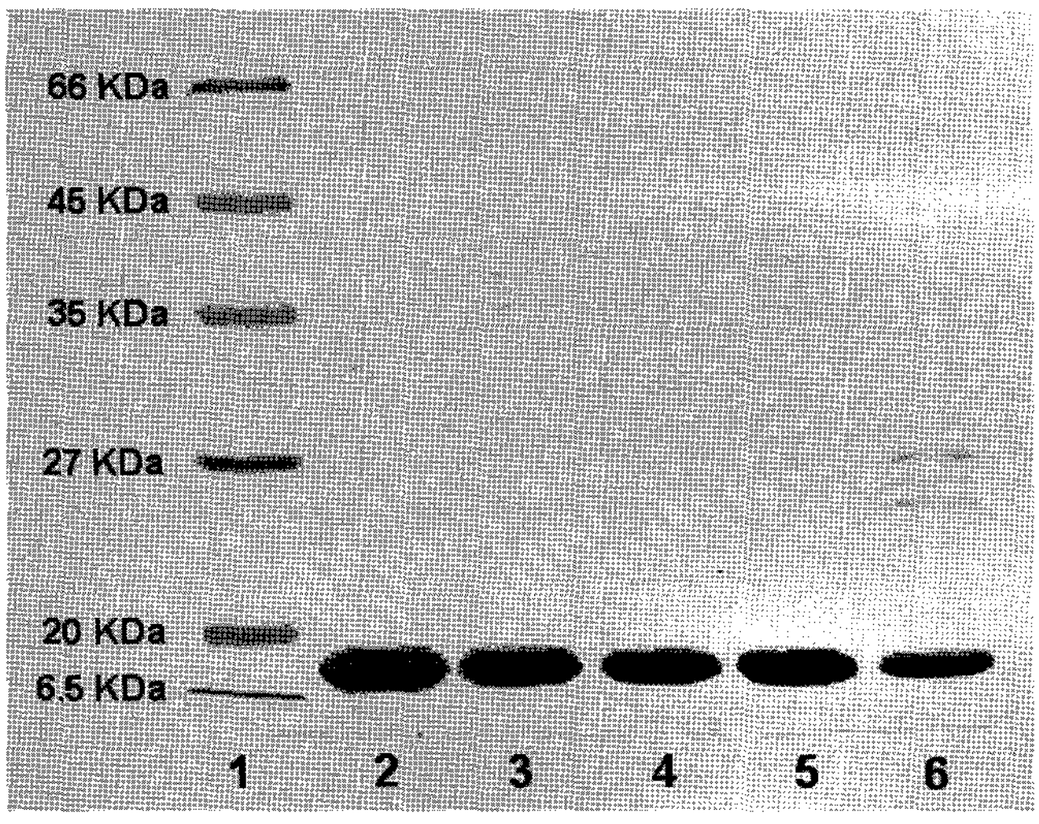

Fig. 2. Coomassie blue-stained SDS-PAGE of the purified IscA D97A mutant proteins.

Lane 1, molecular mass standards; lane 2, purified IscA wild type; lane 3, purified IscA D97A mutant protein; lane 4, purified IscA D97E mutant protein; lane 5, purified IscA D97N mutant protein; purified IscA D97S mutant protein.

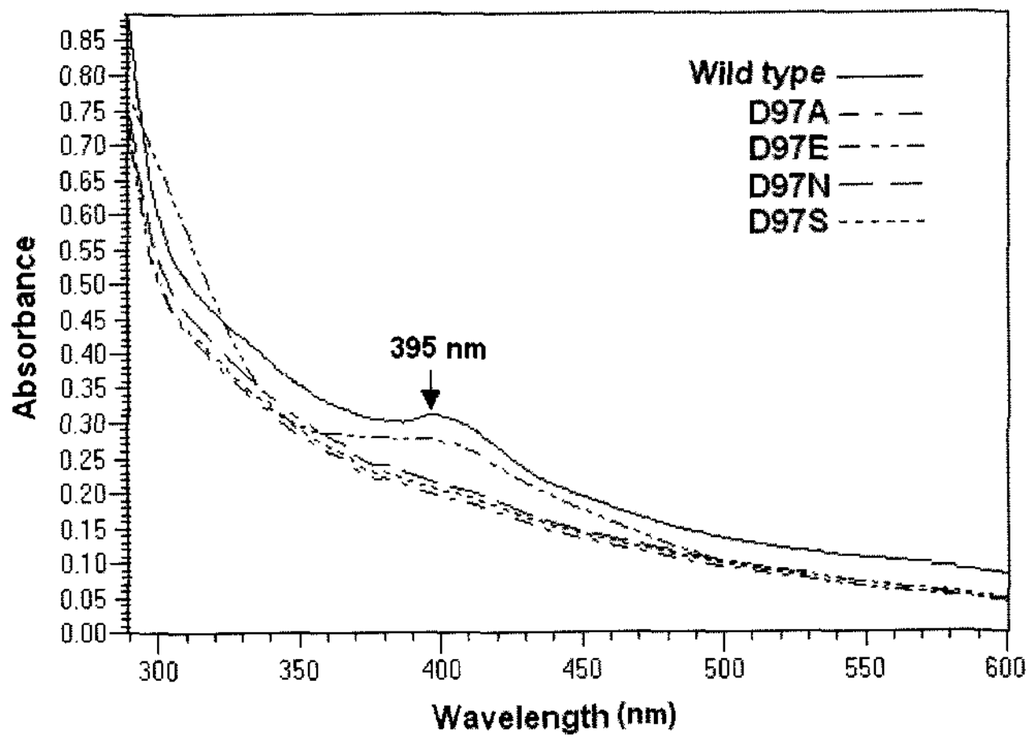

Fig. 3. UV-vis scanning of the D97A, D97E, D97N, and D97S mutant proteins of IscA from *A. ferrooxidans*.

which was comparable to 2.64 for the wild-type IscA [37]. The stability of the purified IscA D97E mutant protein was tested on the basis of its $[Fe_4S_4]$ cluster stabilization, and the mutant proteins were proved to be as highly stable as wild-type IscA. The protein could be stored at 4°C for one month without significant loss of the iron-sulfur cluster. To our surprise, the other three mutant proteins IscA D97A, D97N, and D97S had no colors after purification, and the purity ratios (A_{280}/A_{380}) for the three mutant proteins were all >10, indicating the loss of the $[Fe_4S_4]$ clusters.

UV Scanning of IscA D97 Mutant Proteins

The D97 mutant proteins were then subjected to UV-vis scanning, where the IscA D97E mutant protein after purification was observed to have the maximum visible absorption at 395 nm, as shown in Fig. 3, which was typical for proteins containing the $[Fe_4S_4]$ cluster [5–7, 10, 24, 34, 35, 37]. UV-vis

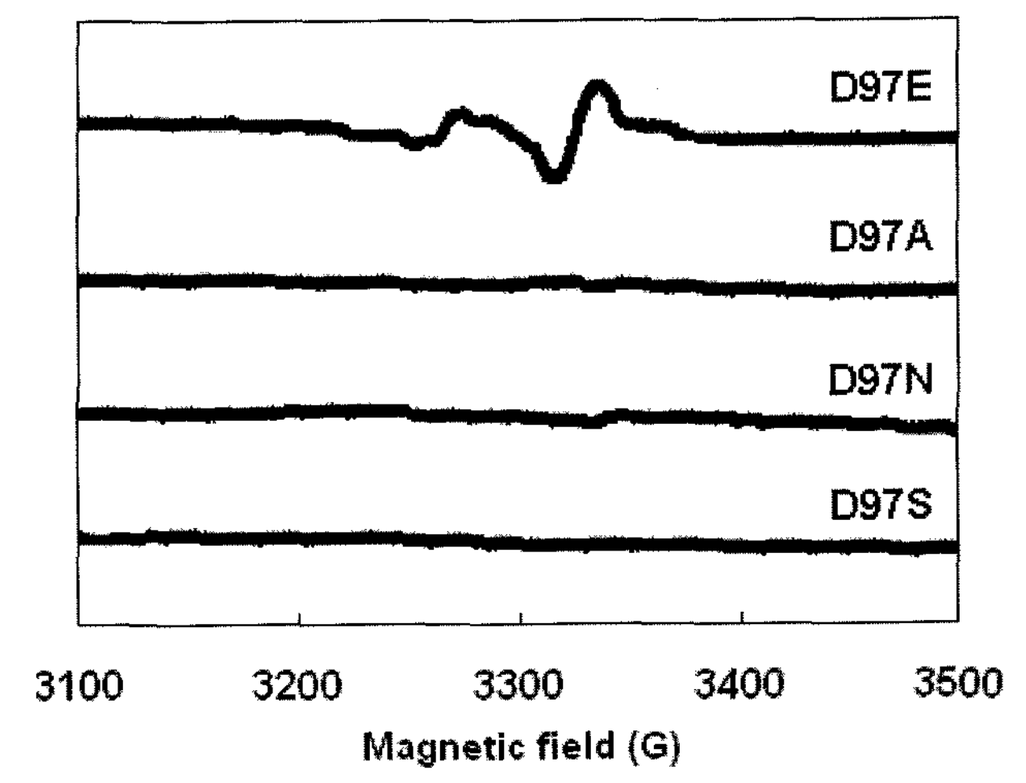

Fig. 4. EPR spectra of the D97A, D97E, D97N, and D97S mutant proteins of IscA from *A. ferrooxidans*.

scanning for the D97A, D97N, and D97S mutant proteins showed that there were no absorptions between 320–450 nm, indicating the absence of the $[Fe_4S_4]$ cluster in the mutant proteins.

EPR Spectra of IscA D97 Mutant Proteins

The EPR spectra of the purified IscA mutant proteins are shown in Fig. 4. The IscA D97E mutant protein in the oxidized state exhibited a typical EPR signal indicating the presence of the $[Fe_4S_4]^{3+}$ cluster; the oxidized state IscA D97E mutant protein gave an S=1/2 signal, and the g value was estimated to be 2.012. The iron-sulfur proteins of HiPIP from *A. ferrooxidans* ATCC 33020, *Rhodocyclus tenuis*, and the Iro protein from *A. ferrooxidans* also showed similar EPR signals [5–7]. The result further indicated that the $[Fe_4S_4]$ cluster was successfully incorporated in the IscA D97E mutant protein. The D97A, D97N, and D97S mutant proteins showed no EPR signals, indicating the absence of the iron-sulfur clusters in the proteins.

Iron and Sulfide Content of IscA D97 Mutant Proteins

The iron and sulfide contents of the IscA mutant proteins are shown in Table 1. The total iron content of the purified IscA D97E mutant protein was estimated to be 3.41± 0.09 μmol (μmol protein)$^{-1}$ by the colorimetric method, and the sulfide content for D97E was determined to be 3.37±0.10 μmol (μmol protein)$^{-1}$; the results were in good agreement with that of wild-type IscA. Thus, the IscA D97E mutant protein contained a single $[Fe_4S_4]$ cluster. The iron and sulfide contents for the IscA D97A, D97N, and D97S mutant proteins were all <1, indicating that the iron-sulfur cluster could not bind tightly in the proteins, and most of the $[Fe_4S_4]$ clusters were lost.

The sequence alignment for IscA from various sources showed that three cysteine residues of Cys35, Cys99, and Cys101 were highly conserved residues (Fig. 5), in our previous report, and the three conserved cysteines were ligating with the $[Fe_4S_4]$ cluster [37]. D97 was not a highly conserved residue, but conserved in *A. ferrooxidans*, *E. coli*, and *S. choleraesuis*, as shown in Fig. 5, which indicated that the aspartic acid of IscA from these sources might play a critical role for the iron-sulfur cluster binding.

Table 1. Iron and sulfur contents of IscA wild-type and D97 mutant proteins.

	Iron content/μmol (μmol protein)$^{-1}$	Sulfur content/μmol (μmol protein)$^{-1}$	Iron/sulfur
Wild type	3.77±0.10	3.80±0.09	0.99
D97A	<1	<1	NA
D97E	3.41±0.09	3.37±0.10	1.01
D97N	<1	<1	NA
D97S	<1	<1	NA

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A.ferrooxidans   MALTLSESAARQVRKSIAKRGKGLGIRIGVKTSGCSGLSYVMEFVDVPNPEDLVFPHDDV 60
E.coli           MSITLSDSAAARVNTFLANRGKGFGLRLGVRTSGCSGMAYVLEFVDEPTPEDIVFEDKGV 60
A.vinelandii     MAVTMTEAAAARHIRRSLDGRGKGEGIRLGVRTSGCSGLAYVLEFVDEVASEDQVFESHGV 60
P.viridiflava    MAISMTEAAANHVRRSLEGRGKGDGVRLGVRTTGCSGLAYVLEFVDEAASEDTVFEMHGV 60
S.cholerasesuis  MSITLSDSAAARVNTFLANRGKGFGLRLGVRTSGCSGMAYVLEFVDEPTAEDTVFEDKGV 60
X.nematophila    MSISLTESAAQRIVAFLDNRGKGVGLRLGVRTSGCSGMAYVLEFADVVNEEDQVFEDKGV 60
P.atrosepticum   MSISLSESAAQRVSAFIANRGKGLGLRLGVRTSGCSGMAYVLEFVDDLNDGDTVFEDKGV 60
                 *:::::::** ::   :  **** *:*:**:*:****::**:**.*     * **  ..*

A.ferrooxidans   NLFVDPKSLIYLDGTELDFTREGLNEGFRFNNPNVKSACGCGESFTT 107
E.coli           KVVVDGKSLQFLDGTQLDFVKEGLNEGFKFTNPNVKDECGCGESFHV 107
A.vinelandii     KVIVDPKSLVYLDGTELDFVREGLNEGFKFNNPNVRGECGCGESFNI 107
P.viridiflava    KVIIDPKSLVYLDGTELDFVREGLNEGFKFNNPNSRGECGCGESFNV 107
S.choleraesuis   KVVVDGKSLQFLDGTQLDFVKEGLNEGFKFSNPNVKDECGCGESFHV 107
X.nematophila    KVIVDGKSILYLDGTELDFVKEGLNEGFKFNNPNVSSECGCGESFHV 107
P.atrosepticum   KVIVDGKSLVYLDGTELDFVKEGLNEGFKFNNPNISGECGCGESFNV 107
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Fig. 5. The sequences alignment of IscA from *A. ferrooxidans* and other sources.
A. ferrooxidans: *Acidithiobacillus ferrooxidans* ATCC 23270; *E. coli*: *Escherichia coli*; *A. vinelandii*: *Azotobacter vinelandii*; *P. viridiflava*: *Pseudomonas viridiflava*; *S. choleraesuis*: *Salmonella choleraesuis*; *X. nematophila*: *Xenorhabdus nematophila*; *P. atrosepticum*: *Pectobacterium atrosepticum*. Residues conserved in all sequences are marked with *. Residues not conserved in all sequences but conserved in some sequences are marked with : or . based on the degree of conservation. The conserved cysteine residues are marked in red color, and the proposed aspartic acid involved in iron-sulfur cluster ligation is marked in green color. The sequence alignment of the template and target sequences was made with ClustalX version 1.8 [34].

Molecular Structure Modeling of IscA D97 Mutant Proteins

The IscA from *E. coli* was identified as the best structural template for modeling the molecular structure of IscA mutant proteins from *A. ferrooxidans*. This model was refined by MM optimization and MD simulation. The ProStat analysis results showed that there were no significant deviations for the calculated values of the bond lengths and angles of the total residues, which indicated that the modeled structures were reliable.

The modeled structures of the D97A, D97E, D97N, and D97S mutant proteins of IscA are shown in Fig. 6. We can see from the figure that mutations of Asp97 caused no significant geometry changes on the active site residues, so the $[Fe_4S_4]$ cluster loss in the mutant proteins was not due to conformation changes of the active site, as only the carboxyl group of Asp97 was changed, so the Fe-O (Asp97) ligating bond was crucial for iron-sulfur cluster binding, because when destroyed, the cluster was not stable in the active site, resulting in loss of the $[Fe_4S_4]$ cluster. For D97E mutation, the Fe-O (Glu97) ligating bond could still maintain the stability of the iron-sulfur cluster. The result indicated that the bond between the carboxyl group of Asp97 and iron was crucial for maintaining the stability of the metalloprotein. Removal of the carboxyl group of Asp97 resulted in protein inactivation.

The molecular modeling and experimental results showed that the sulfhydryl groups of Cys35, Cys99, and Cys101 were essential for the $[Fe_4S_4]$ cluster binding. The modeling

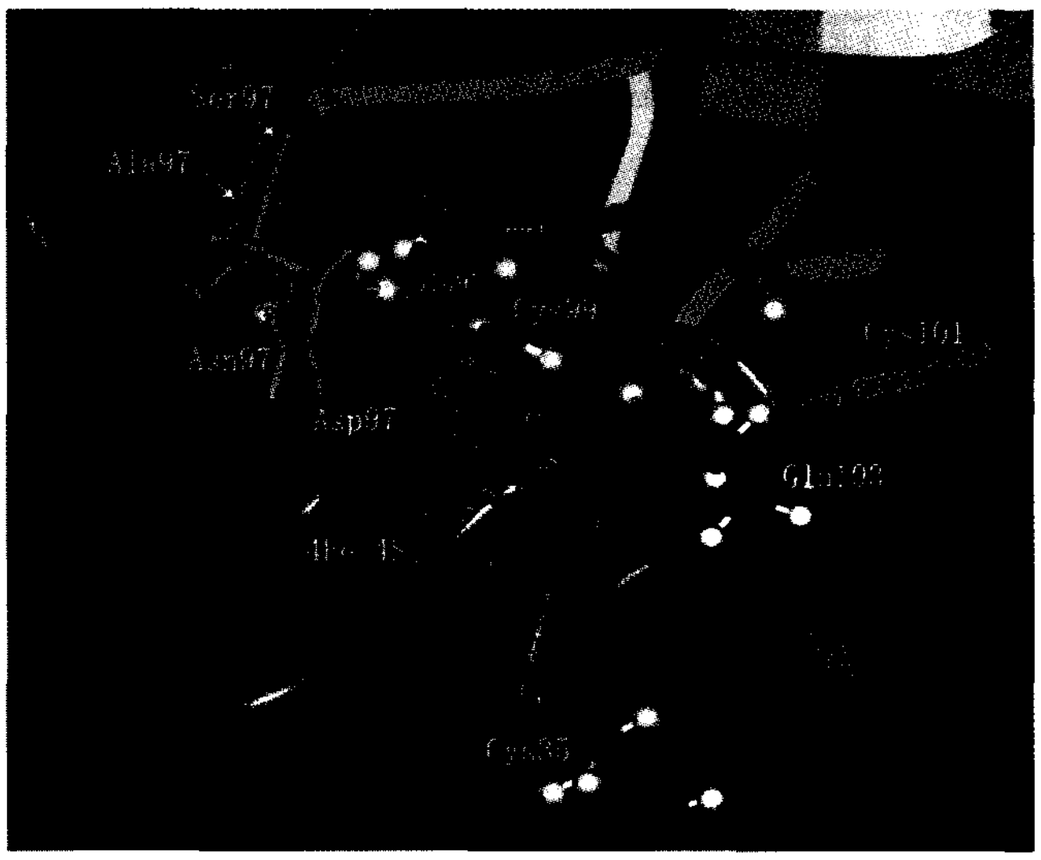

Fig. 6. The modeled structures of IscA D97 mutant proteins.
The structures of wild-type protein and the D97 mutant proteins were all superimposed. The [4Fe-4S] cluster is represented by the purple-yellow cube, which was ligated by the four residues of Cys35, Cys99, Cys101, and Asp97. The D97A, D97E, D97N, and D97S mutant proteins are represented by sticks in yellow, purple, blue, and red color, respectively.

results also indicated that Glu103 was in the vicinity of the iron-sulfur cluster and might be a potential ligand for the $[Fe_4S_4]$ cluster. Sequence alignment indicated that Glu103 was a conserved residue (Fig. 5), but the site-directed mutagenesis result showed that Glu103 was not involved in iron-sulfur cluster binding (data not shown).

It was reported that the $[Fe_2S_2]$ cluster of IscA from *E. coli* and *T. elongates* and the $[Fe_4S_4]$ cluster of HiPIP from *T. tepidum* and *C. purpuratum* were ligated by four cysteines [3, 8, 13, 21, 23]. The $[Fe_2S_2]$ cluster in Rieske-type proteins of the cytochrome bc1 complex was coordinated by the sulfur atoms of two cysteines and the imidazole ring nitrogen atoms of two histidines [9], and the $[Fe_2S_2]$ cluster in the iron-sulfur cluster assembly protein U was reported to be ligated by three cysteine residues and one histidine [18].

In summary, we report here that Asp97 was another ligand involved in the iron-sulfur cluster binding according to the site-directed mutagenesis results. The molecular modeling for the IscA also showed that Asp97 was a ligand with the $[Fe_4S_4]$ cluster, which was in good agreement with the experimental results. Thus, the $[Fe_4S_4]$ cluster in IscA from *A. ferrooxidans* was ligated by three cysteine residues and one aspartic acid.

Acknowledgments

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