K30, H150, and H168 Are Essential Residues for Coordinating Pyridoxal 5′-Phosphate of *O*-Acetylserine Sulfhydrylase from *Acidithiobacillus ferrooxidans*

Chunli Zheng · Li Nie · Lin Qian · Zhilou Wang · Guizhen Liu · Jianshe Liu

Abstract *O*-acetylserine sulfhydrylase (OASS) is a key enzyme involved in the pathway of the cysteine biosynthesis. The gene of OASS from *Acidithiobacillus ferrooxidans* ATCC 23270 was cloned and expressed in *E. coli*, the soluble protein was purified by one-step affinity chromatography to apparent homogeneity. Colors and UV–vis scanning results of the recombinant protein confirmed that it was a pyridoxal 5′-phosphate (PLP)-containing protein. Sequence alignment and site-directed mutation of the enzyme revealed that the cofactor PLP is covalently bound in Schiff base linkage with K30, as well as the two residues H150 and H168 were the crucial residues for PLP binding and stabilization.

C. Zheng · L. Qian · Z. Wang · G. Liu · J. Liu (✉)
College of Environmental Science and Engineering, Donghua University, Shanghai 201620, People's Republic of China
e-mail: ljscsu@263.net

C. Zheng
e-mail: Zhengchunli1979@163.com

C. Zheng
School of Mathematics, Physics and Biological Engineering, Inner Mongolia University of Science and Technology, Baotou 014010, People's Republic of China

L. Nie
School of Environment and Energy, Inner Mongolia University of Science and Technology, Baotou 014010, People's Republic of China

Introduction

Acidithiobacillus ferrooxidans is one of the most studied bacteria that thrives in acidic mine drainage, which obtains all its energy and electron requirements from the oxidation of various forms of reduced sulfur and ferrous iron. However, how it uses to take up sulfur(sulfate) from its environment and how it is subsequently assimilated into organic compounds such as the amino acids methionine and cysteine, the mechanisms are not clear so far [1].

O-acetylserine sulfhdrylase (OASS) is a key enzyme involved in the pathway of the cysteine biosynthesis, which is thought to be involved in the global regulation of S-assimilation, as the substrates and products of OASS have been implicated in the regulation of gene expression of the sulfate transporter [2]. It is, therefore, of interest to investigate and identify the structural features that might contribute to OASS' unique functional properties.

The crystal structure of OASS from *Salmonella typhimurium* revealed that the enzyme was similar in structure to other OASS [3–5]. The active-site residues of OASS-A with the cofactor PLP covalently bound in Schiff base linkage with lysine residues. The phosphate group of the cofactor is bound at the N-terminus of helix 7, which interacts with the positive end of its dipole with the negative charge of the phosphate group. K41, k42, or k44 residues for OASS from other sources were bound to cofactor PLP to form Schiff base [6–8], but there are no reports for OASS from *A. ferrooxidans* so far.

In this study, the gene of OASS from *A. ferrooxidans* ATCC 23270 was cloned and successfully expressed in *E. coli*, finally purified by one-step affinity chromatography. As we know so far, this is the first report of expression in *E. coli* of OASS from *A. ferrooxidan*.

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. DH5-alpha competent cells, *E. coli* strain BL21 (DE3) competent cells were purchased from Invitrogen Life Technologies. The Plasmid Mini kit was purchased from Ambiogen Life Science Technology LTD. A gel extraction kit was obtained from OMEGA. *Taq* DNA polymerase, T4 DNA ligase, and restriction enzymes were purchased from MBI Fermentas of Germany. All other reagents were of research grade or better and were obtained from commercial sources.

Cloning of the OASS Gene from *A. ferrooxidans* ATCC 23270

This genomic DNA from *A. ferrooxidans* was used as a template for PCR reaction. The gene was amplified by PCR using primers that were designed to add six continuous histidine codons to the 5′ primer. The sequence of the forward primer was 5′-CGCGCGGATCCAGGAGGAAT TAAAATGAGAGGATCGCATCACCATCACCATCAC GGTGGCTGGAGAACTGGGTGGGCAATACC-3′, containing a *Bam*HI site (GGATCC), a ribosome binding site (AGGAGGA), codons for the amino acid sequence MRGSHHHHHH (start codon and hexahistag), and codons for amino acids 2–10 of mature OASS. The sequence of the reverse primer was 5′-CTGCAGGTCGACTTAGGCCGG AAAAACGCCCGTAGAGAGGTAGCG-3′, containing a *Sal*I site (GTCGAC), a stop anticodon (TTA), and anticodons for amino acids 2–11 of mature *cys*M. PCR amplification was performed using *Taq* DNA polymerase, and samples were subjected to 30 cycles of 45 s of denaturation at 95°C, 45 s of annealing at 62°C, and 2 min of elongation at 72°C in a Mastercycler Personal of Eppendorf Model made in Germany. The amplification products were analyzed by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide. The resulting PCR product was gel purified, double digested, and ligated into a pLM1 [9], expression vector resulting in the pLM1::*cys*M plasmid, then transformed *E. coli* strain BL21 (DE3) competent cells for expression purposes.

Construction of *A. ferrooxidans* OASS Mutant Plasmids

A QuikChange mutagenesis kit (Stratagene) was applied for constructing the mutant expression plasmids. The plasmid pLM1::*cys*M was used as a template for constructing mutant expression plasmids through PCR. The following primer and their antisense primer were synthesized to introduce the mutated sequence: K30A: 5′ TGCG GATTTTCGGA<u>GCT</u>CTGGAGGGGAACAA-3′, codon AAG for lysine (K) was changed to codon GCT for alanine (A); K41A: 5′ CCGCCGGTTCGGTC<u>GCT</u>GATCGTCCC GCCTT-3′, codon AAG for lysine (K) was changed to codon GCT for alanine (A); H150A: 5′ ATAATCCCGA GGCA<u>GCT</u>TACCTCGGGACCGG-3′, codon CAT for histidine (H) was changed to codon GCT for alanine (A); H168A: 5′ GCGGTACCGTGACC<u>GCT</u>TTCGTATCGTC-CAT-3′, codon CAT for histidine (H) was changed to codon GCT for alanine (A).

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 of Eppendorf Model made in Germany. *Dpn*I restriction enzyme was used to digest the parental super-coiled double-stranded DNA. The isolated mutant plasmids were then used to transform *E. coli* strain BL21(DE3) competent cells for expression purposes.

Expression and Purification of Recombinant OASS Wild-Type and Its Mutant Proteins in *E. coli*

E. coli strain BL21(DE3) cells with pLM1::*cys*M plasmid and the mutant plasmids were grown at 37°C in 500 ml of LB medium containing ampicillin (100 mg/l) to an OD_{600} of 0.6. Cells were incubated at room temperature with the addition of 0.5 mM IPTG overnight with shaking at 180 rpm. The cells were harvested by centrifugation and the cell pellet was washed with an equal volume of sterile water. The cells were again harvested by centrifugation, suspended in start buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl), incubated with 5 mg lysozyme at room temperature for half an hour, and then stored at −80°C for purification. The method of Bradford was used to determine the protein content with bovine serum albumin as the standard. The eluted fractions were analyzed by SDS–PAGE with 18% (v/v) acrylamide.

O-Acetylserine Sulfhdrylase (OASS) Activity Assays

The activity of OASS was determined according to the method of Demosthenis Chronis [10]. One unit of enzyme activity is defined as the conversion of 1 nM of OASs into cysteine per minute under the stated assay conditions. Assays were performed three times and each time was represented by two replications.
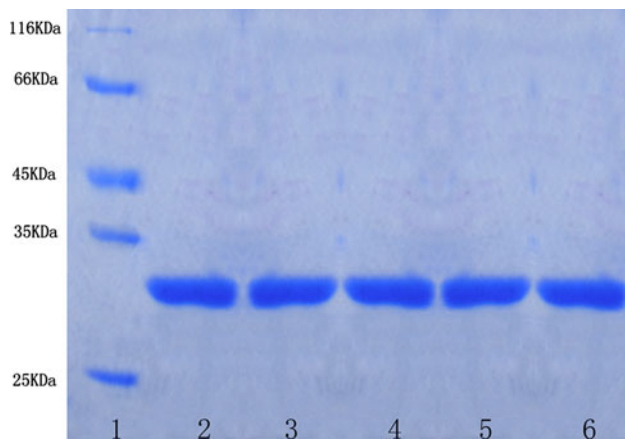
Fig. 1 Coomassie blued-stained SDS–PAGE of the purified OASS from *A. ferrooxidans* and its mutant proteins. *Lane 1* molecular mass standards, *lane 2* purified OASS wild-type, *lane 3* purified OASS mutant K30A, *lane 4* purified OASS mutant K41A, *lane 5* purified OASS mutant H150A, *lane 6* purified OASS mutant H168A
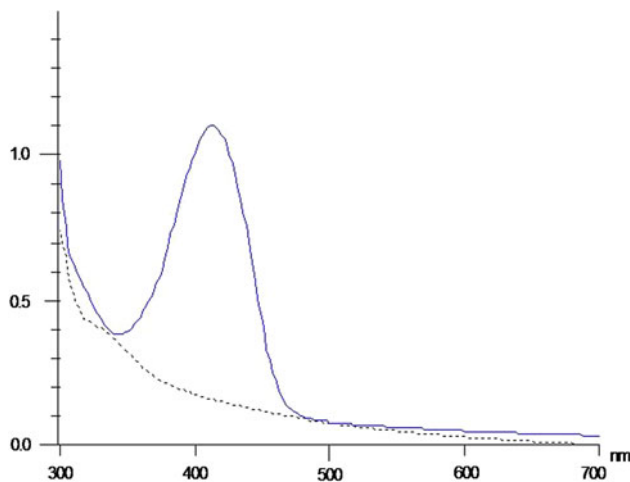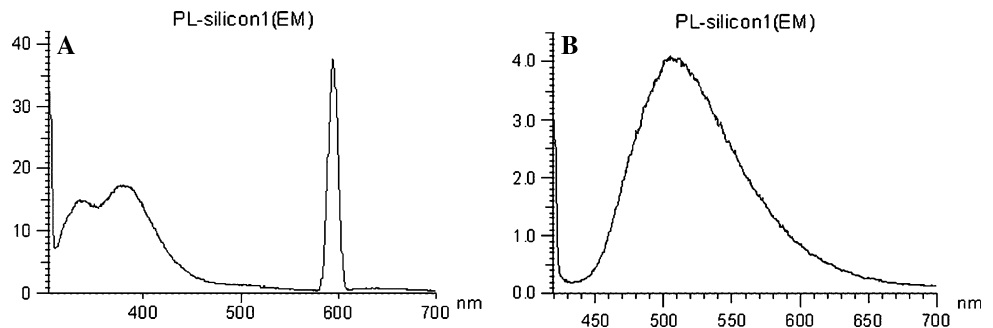

Fig. 2 UV–vis scanning of apo and holo*cys*M from *A. ferrooxidans*. *Solid line* indicates the holo*cys*M, *dashed line* indicates apo*cys*M without the PLP cofactor

UV-Scanning and Fluorescence of the OASS

UV–visible spectra and fluorescence were separately obtained on a Techcomp UV-2300 spectrophotometer and Hitachi FL Spectrophotometer. The temperature for measurement was maintained at 25°C.

Results and Discussion

Cloning of OASS Gene from *A. ferrooxidans*

PCR techniques were used to successfully add six continuous histidine residues to the N-terminal of the OASS from *A. ferrooxidans*. The transformant was grown in LB medium and the corresponding plasmid was isolated and re-transformed to *E. coli* BL21 (DE3) for expression. Mutant expression plasmids p*cys*M (K30A), p*cys*M (K41A), p*cys*M (H150A), and p*cys*M (H168A) were constructed, and their sequences were examined for the presence of directed mutations and the absence of PCR-generated random mutations by DNA sequencing.

Expression and Purification of OASS from *A. ferroodoxins*

The expressions of OASS from *A. ferrooxidans* and its mutant proteins in *E. coli* BL21 (DE3) were optimal with 0.5 mM IPTG concentrations at 25°C, nickel metal-affinity resin columns were used for single-step purifications of His-tagged *A. ferrooxidans* OASS wild-type and mutant proteins. The protein fractions were dialyzed against a 20 mM potassium phosphate buffer, pH 7.5, 5% glycerol, and 5 mM β-mercaptoethanol right after purification. The addition of 5% glycerol and 5 mM β-mercaptoethanol was essential for maintaining the long-term stability of the protein. The purified enzyme was observed to take on light-yellow color. The purity of the enzymes wild-type and mutant was examined by SDS–PAGE and single bands
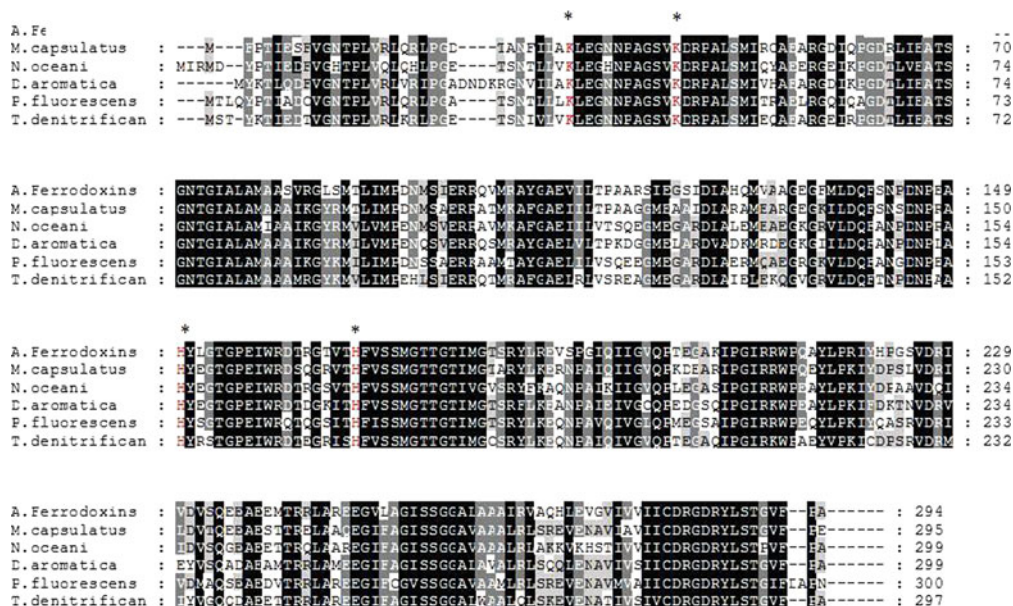
Fig. 3 Emission spectrum upon excitation at 298 nm and 412 nm (slitex = 5 nm, slitem = 5 nm) of OASS from *A. ferrooxidans*

Fig. 4 The sequence alignment of OASS from *A. ferrooxidans* and other sources. *A. ferrooxidans* from *A. ferrooxidans* ATCC 23270; *H. arsenicoxydans* from *Herminiimonas arsenicoxydans*; *A. vinosum* from *Allochromatium vinosum*; *H. halophila* SL1 from *Halorhodospira halophila* SL1; *B. cepacia* from *Burkholderia cepacia*. The four residues proposed to coordinate a PLP cofactor are marked in asterisk. The highly conserved residues are marked in *black*

corresponding to the 32 kDa proteins were observed with >95% purity (Fig. 1), which was in agreement with the deduced molecular mass of OASS.

UV-Scanning and Fluorescence of OASS

The UV–visible spectra of the recombinant OASS is shown in Fig. 2. It is very similar in spectrum the enzyme from other sources [11, 12], with an absorption band centered at 412 nm. This is indicative of a PLP molecule in an internal aldimine linkage with the active site lysine residue. Emission spectrum upon excitation at 298 nm and at 412 nm, which is shown in Fig. 3, with the direct emission at 344 nm of the aromatic residues and a longer wavelength emission at 509 nm [13–15], which was attributed to energy transfer from tryptophan to the enolimine tautomer of the internal aldimine.

Role of Conserved Residues of OASS in PLP Binding

Sequence alignment for OASS from various sources showed that the four residues of K30, K41, H150, and H168 were highly conserved (Fig. 4), which was proposed to be important for the PLP cofactor binding and stabilization. To confirm this, mutations for these four residues of OASS from *A. ferroxidans* were created, K30A, K41A, H150A, and H168A mutant plasmids were expressed in *E. coli* and then purified by affinity chromatography to homogeneity. Except K41A, the other three mutant

proteins are colorless after purification, and UV–Vis scanning of the mutant proteins showed that there were no absorptions between 300 and 500 nm (Fig. 5). The wild-type OASS has an enzyme activity of 5.0×10^4 U/mg, while the mutant proteins do not have. The results strongly suggested that the active-site residues of OASS and the cofactor PLP covalently bound in Schiff base linkage with
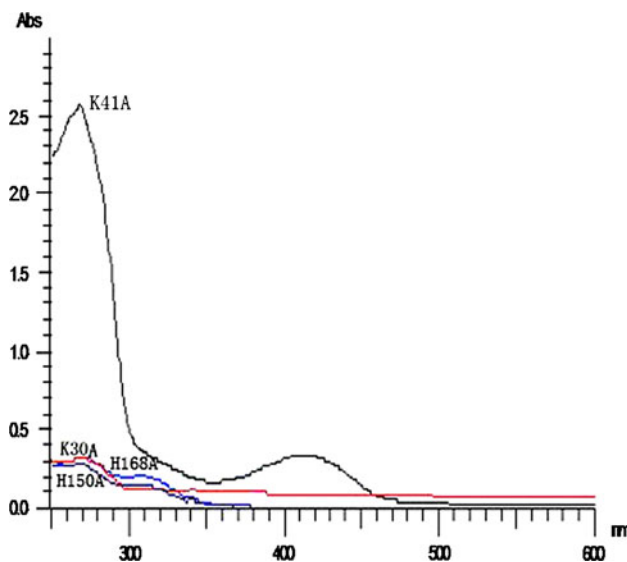

Fig. 5 UV–vis scanning of K30, H150, H168, and K41 OASS mutant enzymes

K30, as well as the two residues H150A and H168A were crucial residues for PLP binding and stabilization.

Summary

We reported here the first expression in *E. coli* of His-tagged *cys*M from *A. ferrooxidans*. The OASS was a PLP sequence dependant type enzyme. Sequence alignment and site-directed mutation of the enzyme revealed that K30, H150 and H168 were crucial residues for PLP binding. Analyses of the enzyme activity indicate that OASS can catalyze sulfide and OAS formation of cysteine.

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