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Mutagenesis of Arginine-186 Located on a Helix Interacting with Pyridoxal 5'-Phosphate in *O*-Acetylserine Sulfhydrylase

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Abstract: We mutated Arg-186, which was located in the middle of a helix interacting with the phosphate group of PLP in OASS, to study effects on catalytic properties. The Arg-186 to Pro mutation completely inactivated the enzyme due to a loss of PLP. In contrast, R186L OASS retained one PLP molecule per subunit. The replacement of Arg-186 with Leu accelerated the reaction with OAS by 1.8-fold and the aminoacrylate intermediate of R186L OASS was converted to the internal Schiff base with azide or thiosulfate faster than that of the wild type by 1.5 and 1.3-fold, respectively. Although the changes in catalytic properties were moderate, the loss of guanidino group might have resulted in the reorganization of active site structure.

Key words: *O*-acetylserine sulfhydrylase, cystathionine β-synthase, pyridoxal 5'-phosphate

INTRODUCTION

The sulfur-containing amino acids (i.e. cysteine, cystathionine, homocysteine, methionine) are metabolically linked by trans-sulfuration and reverse trans-sulfuration pathways (Amadasi *et al*., 2007). In bacteria and higher plants, the biosynthesis of cysteine proceeds via two-step enzymatic reactions. Serine acetyltransferase (SAT) is responsible for the first step, which is the conversion of serine and acetyl CoA into *O*-acetylserine (OAS) and *O*-acetylserine sulfhydrylase (OASS) is involved in the second step, which is the transformation of OAS and sulfide into cysteine by a β-replacement reaction (Kredich, 1996; Hell, 1997). OASS, a pyridoxal-5'-phosphate (PLP) dependent enzyme, from *Salmonella typhimurium* (*S. typhimurium*) has been extensively investigated and kinetic studies indicate the double displacement mechanism (Cook and Wedding, 1976; Tai *et al*., 1993). In the first half reaction, an internal Schiff base reacts with OAS to form an aminoacrylate intermediate. Then the addition of sulfide produces cysteine and regenerates the internal Schiff base in the second half reaction (Scheme 1).

There are two OASS isozymes, A and B, which are expressed in a variety of bacteria under aerobic and anaerobic conditions, respectively. The crystal structures of both isozymes from *S. typhimurium* were solved as dimeric forms with one PLP molecule per subunit (Burkhard *et al*., 1998; Chattopadhyay *et al*., 2007). Although the overall sequence identity of isozyme A and B is only 45%, common active site residues appear to be involved in interactions with PLP. The C4' carbon of PLP is covalently bound to the ε-amino group of Lys-41 in OASS-A from *S. typhimurium* (Fig. 1). The O3' oxygen of PLP is within hydrogen-bonding distance to the carboxamide nitrogen of Asn-71 as well as the imine nitrogen of the internal Schiff base and the N1 nitrogen of pyridine ring interacts with γ-oxygen of Ser-272. The phosphate moiety of PLP is anchored appropriately by hydrogen bonding interactions between the non-ester phosphate oxygen atoms and main-chain NH groups of a glycine

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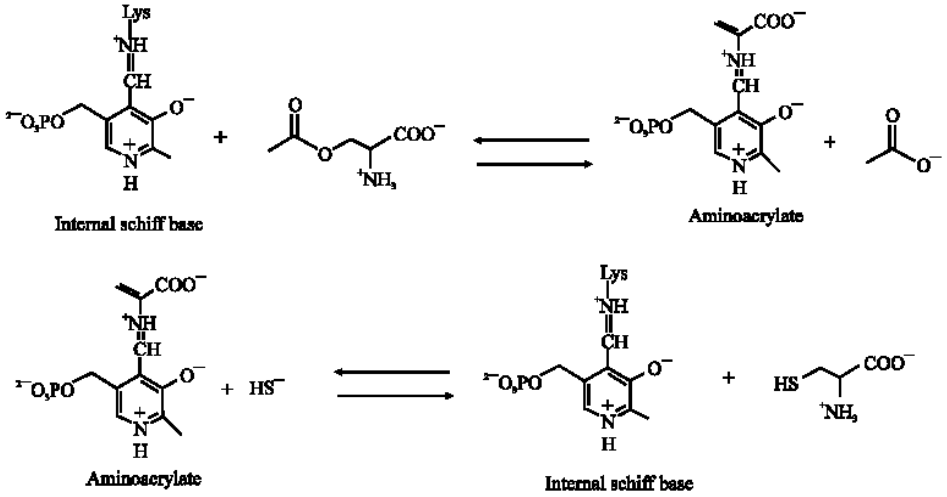

Scheme 1: Mechanism for synthesis of cysteine by OASS

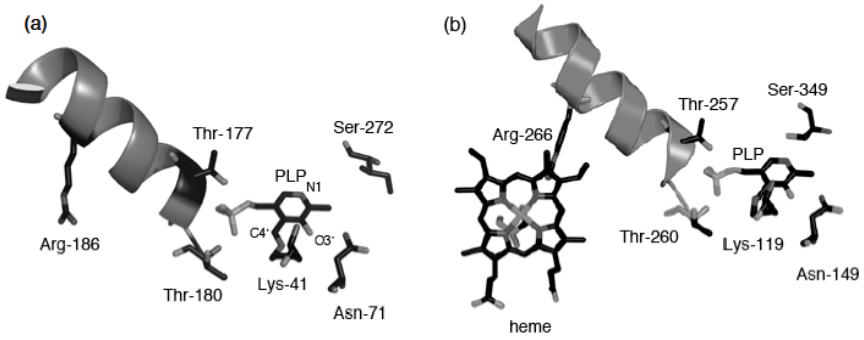

Fig. 1: The active site of (a) OASS-A from *S. typhimurium* and (b) cystathionine β-synthase (CBS) from human. Although the crystal structure of OASS-A from *E. coli* is not available at the moment, 97% of its amino acid residues are in common to those in OASS-A from *S. typhimurium* and the arginine residues (i.e., Arg-186 in OASS and Arg-266 in CBS) are conserved in both enzymes

rich region (G179-T180-L181-T182-G183) as well as β-OH groups of Thr-177 and Thr-180. Hence mutations in the glycine rich loop induced perturbations of the active site (Marceau *et al*., 1990). Since the glycine rich region exists at the N-terminus of the α-helix consisting of residues 179-191, it appears that the helical structure must be appropriately aligned to keep PLP in the proper position.

Human cystathionine β-synthase (CBS), a unique PLP-dependent hemoenzyme catalyzing the condensation of serine and homocysteine, also retains the corresponding helical structure (residues 259-271) (Fig. 1) (Meier *et al*., 2001). It was recently proposed that structural changes in the heme vicinity might be sensed by Arg-266 and relayed to the active site through the helix (Puranik *et al*., 2006; Ozaki *et al*., 2008). Although OASS does not have heme as a prosthetic group, the corresponding arginine residues (i.e., Arg-186 in OASS and Arg-266 in CBS) are conserved. In order to consider a role of the arginine residue on the helix, we performed mutagenesis of Arg-186 in OASS-A and examined the influence on catalytic properties. We report here that the replacement of Arg-186 with Pro

generated a PLP-free inactive enzyme. In contrast, R186L OASS-A retained one PLP molecule per subunit; however, the kinetics of the mutant slightly differed from those of the wild type enzyme.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Wako Chemicals (Osaka, Japan) , Nakalai Tesque (Kyoto, Japan), Takara Bio (Otsu, Japan) and Sigma-Aldrich (St. Louis, MO). GST Trap FF and Mono Q column were available from GE Healthcare Bioscience. DE52 was obtained from Whatmann. Absorption spectra were recorded on a Shimadzu UV-1200 or a MultiSpec 1500. Fluorescence spectra were measured on a Shimadzu RF-1500. Rapid-Scanning stopped-flow experiments were performed on a Unisoku RSP-1000.

Enzyme Expression and Purification

The gene encoding OASS-A was amplified by PCR and inserted into pGEX 4T-2 vector (GE Healthcare Bioscience) using the EcoRI and SalI restriction sites. The Arg-186 to Pro and Leu mutations were introduced by a PCR based technique and confirmed with a DNA sequencer. The plasmid was transformed into BL21 *E. coli* cells to obtain OASS-A as glutathione S-transferase (GST) fusion protein.

Typical expression and purification procedures are described as follows. Cells containing pGEX 4T-2/OASS-A plasmid were grown in Luria broth medium at 30°C to A_{600} of 0.5. The expression was induced with isopropyl-1-thio-β-D-galactopyranoside (0.1 mM) and the cells were allowed to grow for an additional 24 h. The harvested cells (about 35 g of wet weight obtained from 8 L of culture) were resuspended in 100 mL of 50 mM Tris buffer, pH 8, containing 10 mM EDTA, 15 mM β-mercaptoethanol, 0.05 mM N^{α}-p-tosyl-L-lysine chloromethylketone (TLCK), 1 mg L^{-1} leupeptin, 1 mg L^{-1} aprotinin and 1 mg L^{-1} pepstatin. Twenty milligram of lysozyme and PLP were added, the suspension was stirred for 4 h and then centrifuged to remove cell debris.

The supernatant was loaded on a DE52 anion exchange column. The column was washed with 50 mM Tris buffer, pH 8, containing 10 mM EDTA and 15 mM β-mercaptoethanol. The bound proteins were eluted with a linear gradient ranging from 0 to 0.5 M NaCl in 50 mM Tris buffer, pH 8, containing 10 mM EDTA and 15 mM β-mercaptoethanol. Fractions containing OASS-A were pooled, concentrated and then applied on a GSTrap 4B (GE Healthcare Bioscience). The column was washed with PBS buffer, pH 7.4, containing 140 mM of NaCl, 2.7 mM of KCl, 10 mM of Na_2HPO_4 and 1.8 mM of KH_2PO_4. The fusion protein was eluted with 50 mM Tris, pH 8, buffer containing 20 mM reduced glutathione and treated with thrombin at 4°C for 12 h. The protein solution was dialyzed with 15 mM potassium phosphate, pH 7.2 and applied on a DE 52 column. A linear gradient ranging from 15 to 300 mM potassium phosphate, pH 7.2, allowed us to separate OASS-A from the N-terminal GST tag and thrombin. The purity of OASS-A was estimated by SDS-PAGE analysis and the protein concentration was determined by Bradford (1976) method.

Determination of PLP Contents

PLP was measured fluorometrically by a previously published procedure (Srivastava and Beutler, 1973). In short, the enzyme solution (1.5 μM of OASS-A in 5 mL 0.1 M potassium phosphate buffer, pH 7.2) was treated with 5 mM hydroxylamine at 4°C for 24 h to remove PLP from the enzyme. The fluorescence emission of the PLP oxime was detected at 446 nm when the sample was excited at 353 nm. A standard curve was generated using PLP samples of known concentrations.

Rapid-Scanning Stopped-Flow Experiments

The reaction of OASS-A (10 μM) with OAS (0.1-8 mM) was performed in 50 mM phosphate buffer, pH 7, at 25°C and spectral changes were analyzed using Specfit (Spectrum Software Associates). Time course curves were best fitted by a single exponential equation to calculate k_{obs} values. We used the reaction mechanism of IS + OAS → AA + acetate, where IS is the internal Schiff base and AA is the aminoacrylate intermediate. The second order rate constant (k) was determined to be the slope of a liner region of k_{obs} versus [OAS] plot.

In order to study the second half of reaction, azide or thiosulfate (0.25-6.25 mM) was added to the preformed aminoacrylate intermediate by mixing OASS-A (10 μM) and OAS (400 μM) in 50 mM phosphate buffer, pH 7, at 25°C. We used the reaction mechanism of AA + Nu → IS + P, where Nu is nucleophile and P is product. The values of k_{obs} were determined by fitting time course curves using a single exponential equation.

RESULTS

Expression and Purification of OASS

The wild type and the mutant enzymes were expressed in a glutathione S-transferase (GST) fusion expression system and purified by a combination of glutathione sepharose and anion exchange columns. Deletion of the N-terminal GST tag afforded OASS-A with a molecular mass of 35 kDa on the basis of SDS-PAGE gel. Absorption spectra of wild type and R186L OASS-A were essentially identical and exhibited absorption maximum at 412 nm due to PLP covalently bound to the ε-amine of Lys-41 (Fig. 2). The measurements of fluorescence emission confirmed that the enzymes retained one molecule of PLP per subunit. On the other hand, the R186P mutant did not show an absorption band at 412 nm. The introduction of a proline residue in the helix (residues 179-191) interacting with the phosphate group of PLP appeared to alter the active site environment and decreased the affinity to PLP.

Reaction of the Internal Aldimine with O-Acetylserine

Absorption spectral changes upon mixing of R186L OASS-A and OAS indicated the conversion of the internal Schiff base (λ_{max} = 412 nm) to the aminoacrylate intermediate (λ_{max} = 470 nm) with isosbestic point at 429 nm and the rate of aminoacrylate generation was identical to that of disappearance of internal Schiff base (Fig. 3A). Similar spectral changes were observed in the reaction

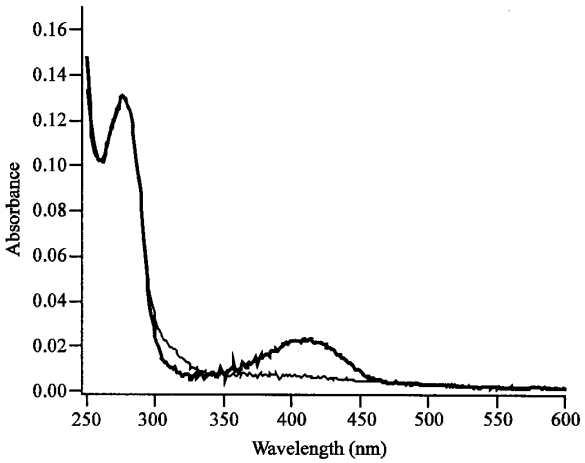

Fig. 2: Absorption spectra of R186L (thick line) and R186P (thin line) OASS

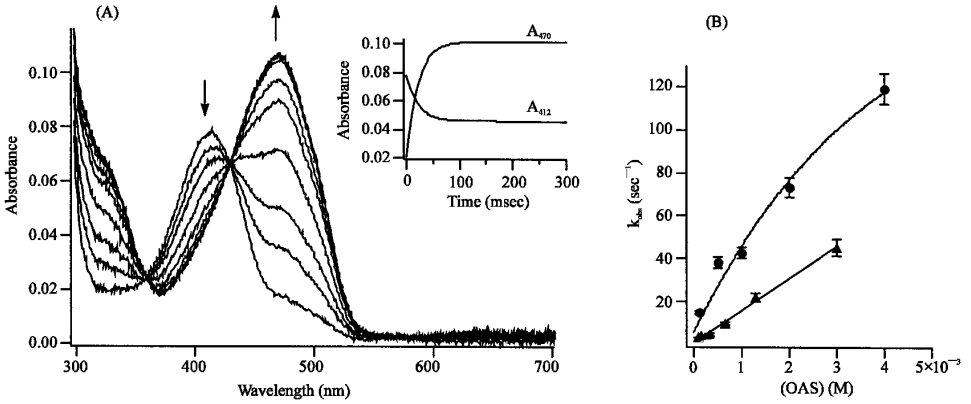

Fig. 3: (A) Rapid-scanning stopped-flow spectra measured upon reaction of R186L OASS-A and OAS. The spectra at 0, 5, 10, 20, 35, 50, 100, 200 and 300 ms after mixing are shown. The inset indicates absorbance changes at 412 and 470 nm versus time and (B) Dependence of the k_{obs} values for aminoacrylate formation on the concentrations of OAS. Triangle and circle indicate wild type and R186L OASS-A, respectively

of the wild type enzyme with OAS. The external aldimine with OAS was not accumulated in the reaction mixture because the elimination of acetate, a good leaving group, proceeded rapidly. The rate of aminoacrylate formation was almost linearly dependent on the concentration of OAS, but the complete saturation of rate was not reached (Fig. 3B). The second order rate constant (k) of the R186L mutant was (2.7±0.3) ×-10^4 M^{-1} sec^{-1}, which was 1.8-fold greater than that of the wild type (i.e., (1.5±0.1) ×-10^4 M^{-1} sec^{-1}). Our observations indicated that the Arg-186 to Leu mutation moderately altered the rate of aminoacrylate formation.

Reaction of the Aminoacrylate Intermediate with Nucleophiles

When the preformed aminoacrylate of wild type or R186L OASS-A was mixed with sulfide, the intermediate was simultaneously transformed into the internal Schiff base within a dead time of our stopped-flow apparatus. In addition, we did not observe the aminoacrylate intermediate in the reaction mixture under the steady state condition. Thus, the rate-determining step of cysteine synthesis by OASS-A appeared to be the aminoacrylate formation, which is the first half of reaction (Scheme 1).

In order to examine if the replacement of Arg-186 with Leu alters the rate for the second half reaction, azide and thiosulfate are utilized as alternative substrates. Since the alternative nucleophiles reacted with the aminoacrylate intermediate slower than sodium sulfide, we could determine the k_{obs} values of the second half reaction (Fig. 4A). Spectral changes indicated that the external aldimine with the product was not significantly accumulated in the reaction mixtures. Comparisons of the k_{obs} values indicated that (1) the aminoacrylate intermediate reacted with azide by approximately two orders of magnitude faster than thiosulfate and (2) the aminoacrylate of R186L OASS-A reacted with azide and thiosulfate faster than the wild type enzyme by approximately 1.5-fold and 1.3-fold, respectively, when the k_{obs} values reach the saturation (Fig. 4B-C). Present observations indicated that the Arg-186 to Leu mutation influenced not only the first half (i.e., the aminoacrylate intermediate formation) but also the second half (i.e. the reaction of aminoacrylate intermediate with nucleophiles) of the catalytic cycle although changes in rate were moderate.

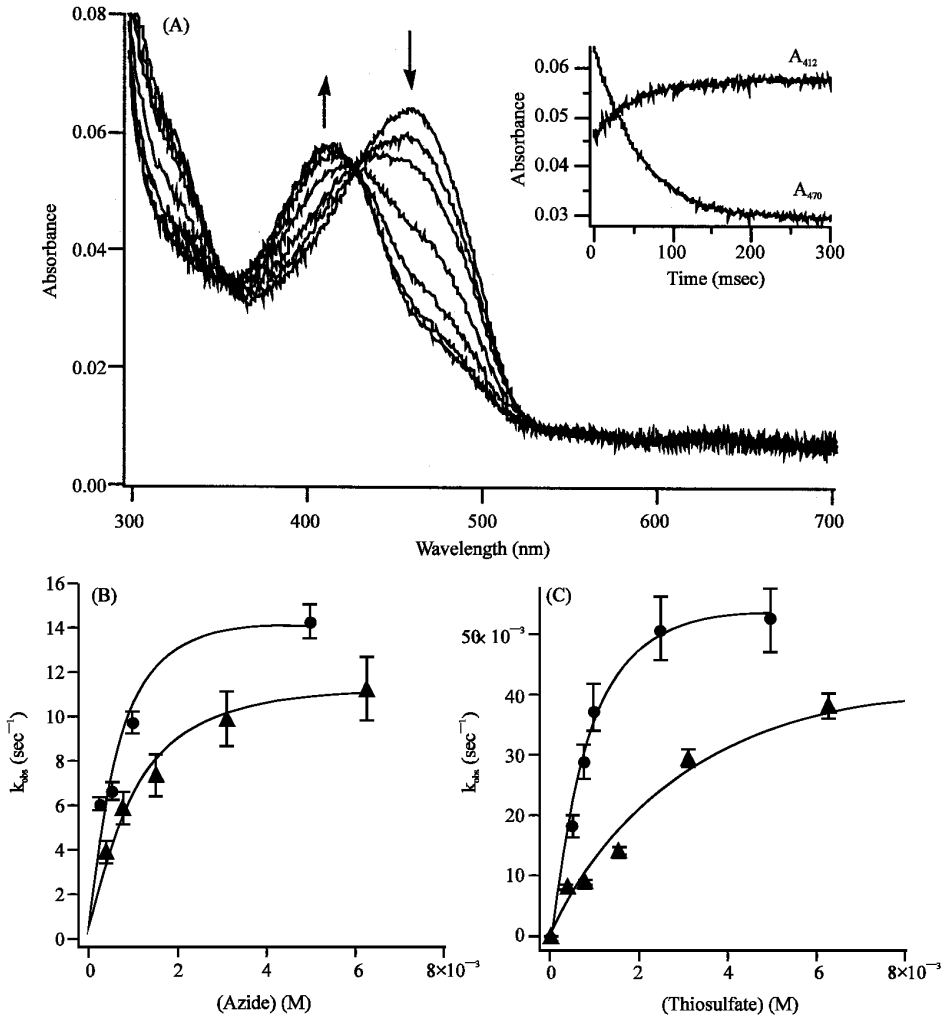

Fig. 4: (A) Rapid-scanning stopped-flow spectra measured upon reaction of the aminoacrylate intermediate of R186L OASS-A and azide. The spectra at 0, 10, 20, 50, 100, 200 and 300 ms after mixing are shown. The inset indicates absorbance changes at 412 and 470 nm *versus* time. Dependence of the k_{obs} values for aminoacrylate disappearance on the concentration of (B) azide and (C) thiosulfate. Triangle and circle indicate wild type and R186L OASS-A, respectively

DISCUSSION

An important finding in this study is that the replacement of Arg-186 with Pro in OASS-A of *E. coli* produced a PLP-free inactive enzyme. Arg-186 is present in the middle of the helix followed by a glycine rich region (i.e., G179-T180-L181-T182-G183) making electrostatic interactions with the phosphate group of PLP (Fig. 1). Since the destabilization of proteins by introducing proline residues in the middle of α-helices is commonly observed (Pakula and Sauer, 1989), we suppose that the R186P mutation might have disrupted the helical structure consisting of residues 179-191 and decreased the affinity to PLP.

In contrast, the substitution of Arg-186 with Leu did not decrease the PLP content and the mutant retained one PLP molecule per subunit as the wild type enzyme does. The 1.8-fold enhancement in the rate of aminoacrylate formation was observed by the R187L mutation and the aminoacrylate intermediate reacted with azide or thiosulfate faster than that of wild type OASS-A. Present results indicate that the replacement of Arg-186 with Leu moderately influences catalytic properties although a reason of moderate changes in catalysis remains to be clarified. It is interesting to note that the wild type as well as the R186L mutant enzyme reacted with azide faster than thiosulfate although the nucleophilic constants of azide and thiosulfate are 4.0 and 6.4, respectively (Swain and Scott, 1953). Steric effects might be important in the reaction of aminoacrylate with nucleophiles.

In summary, we mutated Arg-186 in OASS-A of *E. coli* and found that the R186P mutant lost PLP in the active site. On the contrary, the replacement of Arg-186 with Leu did not influence the PLP content in the enzyme. The R186L mutant reacted with OAS faster than the wild type and the mutation slightly accelerated the conversion of the aminoacrylate intermediate to the internal Schiff base in the presence of azide or thiosulfate. Present observations suggest that the manipulation of helical structure interacting with the phosphate group of PLP in OASS-A influences the catalytic properties.

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