A Change in the Internal Aldimine Lysine (K42) in *O*-Acetylserine Sulfhydrylase to Alanine Indicates Its Importance in Transimination and as a General Base Catalyst†

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ABSTRACT: *O*-Acetylserine sulfhydrylase (OASS) is a pyridoxal 5′-phosphate dependent enzyme that catalyzes a β-replacement reaction forming L-cysteine and acetate from *O*-acetyl-L-serine (OAS) and sulfide. The pyridoxal 5′-phosphate (PLP) is bound at the active site in Schiff base linkage with a lysine. In the present study, the Schiff base lysine was identified as lysine 42, and its role in the OASS reaction was determined by changing it to alanine using site-directed mutagenesis. K42A-OASS is isolated as an external aldimine with methionine or leucine and shows no reaction with the natural substrates. Apo-K42A-OASS can be reconstituted with PLP, suggesting that K42 is not necessary for cofactor binding and formation of the external Schiff base. The apo-K42A-OASS, reconstituted with PLP, shows slow formation of the external aldimine but does not form the α-aminoacrylate intermediate on addition of OAS, suggesting that K42 is involved in the abstraction of the α-proton in the β-elimination reaction. The external aldimines formed upon addition of L-Ala or L-Ser are stable and represent a tautomer that absorbs maximally at 420 nm, while L-Cys gives a tautomeric form of the external aldimine that absorbs at 330 nm, and is also seen in the overall reaction after addition of primary amines to the assay system. The use of a small primary amine such as ethylamine or bromoethylamine in the assay system leads to the initial formation of an internal (γ-thialysine) or external (ethylamine) aldimine followed by the slow formation of the α-aminoacrylate intermediate on addition of OAS. Activity could not be fully recovered, and only a single turnover is observed. Data suggest a significant rate enhancement resulting from the presence of K42 for transimination and general base catalysis.

Pyridoxal 5′-phosphate (PLP)[1] acts as a cofactor for many enzymes catalyzing a wide variety of reactions in the metabolism of amino acids, such as transamination, β-elimination, β,γ-replacement, and racemization. In all PLP-dependent enzymes, the carbonyl group of the coenzyme binds to an ϵ-amino group of a lysine residue in the active site, forming an internal aldimine. In the course of the catalytic reaction, the lysine may be involved in one or more functions including binding of PLP to the enzyme, formation and stabilization of intermediates, and/or release of products. The function of the lysine residue in Schiff base linkage has been studied in many of the enzymes that fall in either the α, β, or γ families of PLP-dependent enzymes (Alexander et al., 1994).

O-Acetylserine sulfhydrylase from *Salmonella typhimurium*, a member of the β-family of PLP-dependent enzymes (Kredich & Tomkins, 1966), catalyzes a β-replacement reaction forming L-cysteine and acetate from OAS and sulfide (Becker et al., 1969). A Bi-Bi ping-pong kinetic mechanism has been proposed for OASS-A (Cook & Wedding, 1976; Tai et al., 1993). A chemical mechanism has been postulated in which the unprotonated α-amine of the amino acid substrate attacks the C4′ carbon of the internal Schiff base (Tai et al., 1995). A proton from the α-carbon of the resulting external Schiff base is abstracted by the ϵ-amino group of the active site lysine in the β-elimination of acetate from OAS. A protonated enzyme residue is also required to assist in the elimination of the acetate leaving group to form α-aminoacrylate, acting to hold the acetyl group out of the plane of the PLP. After Michael addition of the nucleophilic substrate, a proton is donated to the α-carbon by the ϵ-amino group of the active site lysine to form the external Schiff base with product. The final product is then released, and the internal Schiff base with the active site lysine is re-formed.

In the present study, the lysine forming the Schiff base to PLP was identified using a combination of protein chemistry and molecular biology. In an attempt to investigate the functions of the Schiff base lysine, it was changed to an

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; OAS, *O*-acetyl-L-serine; OASS, *O*-acetylserine sulfhydrylase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; WT, wild type; Tris, tris(hydroxymethyl)-aminomethane; TNB, 5-thio-2-nitrobenzoate; $\alpha_2\beta_2$-TS, tryptophan synthase; AAT, aspartate aminotransferase.

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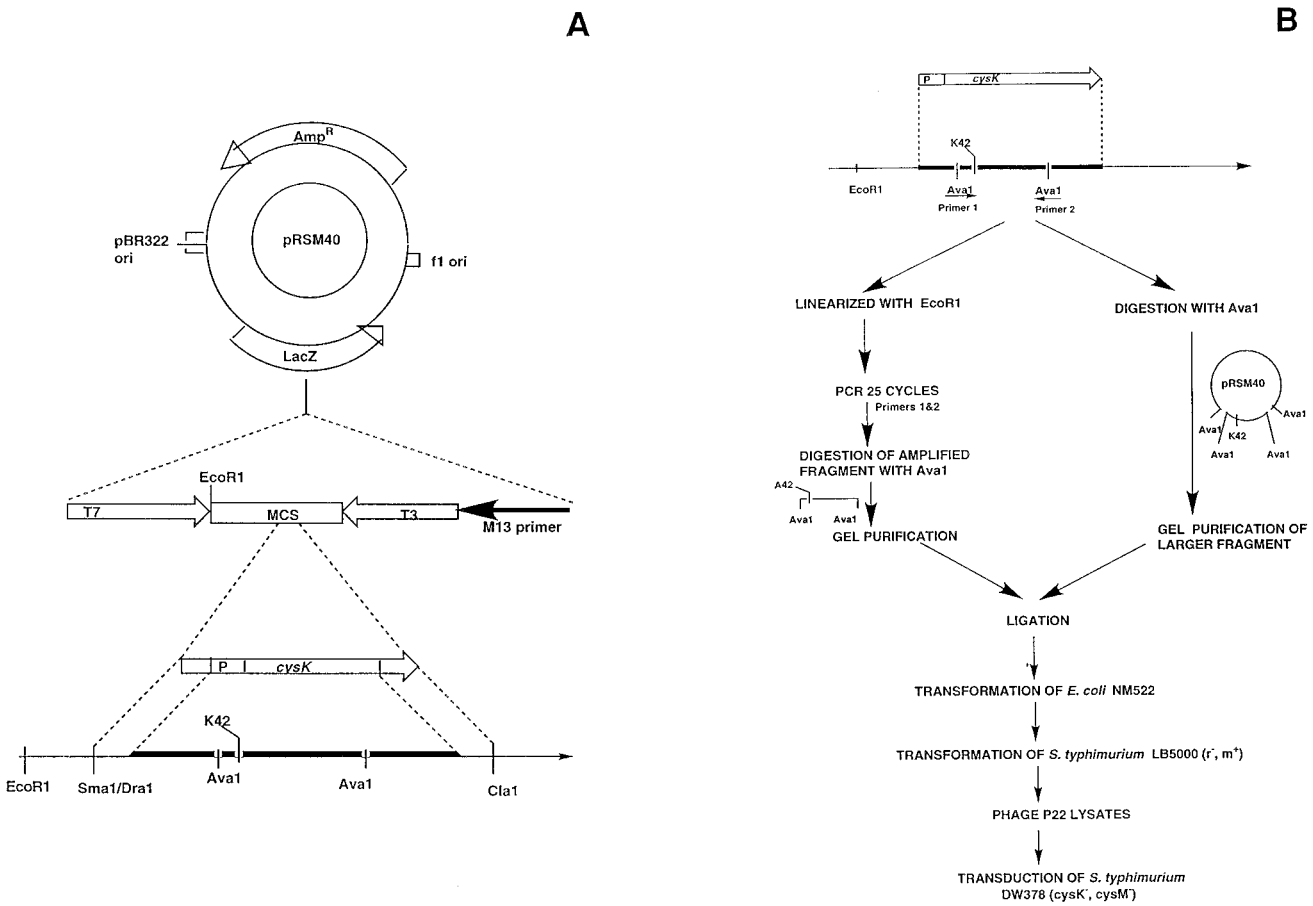

FIGURE 1: Procedure for site-directed mutagenesis. Panel A shows the map of the plasmid pRSM40 that carries the cysK gene. Panel B shows the procedure for mutagenesis with the help of PCR.

alanine using site-directed mutagenesis. Changes occurring in binding of PLP, catalysis, and binding and release of reactants, as well as possible structural differences at the active site in the overall reaction of the mutant enzyme have been determined.

MATERIALS AND METHODS

Chemicals. 2-Bromoethylamine, ethylamine, and guanidinium chloride were purchased from ICN. *O*-Acetyl-L-serine, PLP, L-alanine, L-cysteine, L-serine, Hepes, $NaBH_4$, pyridoxamine dihydrochloride, pyridoxal hydrochloride, α-*N*-carbobenzoxy-L-lysine, L-glutamic acid, and CNBr were purchased from Sigma. Endoproteinase Arg-C was from Boehringer Mannheim Biochemicals. Sodium [^{3}H]borohydride (100 mCi, 9.5 Ci/mmol) was purchased from Amersham.

Enzymes. Alkaline phosphatase and aspartate aminotransferase were obtained from Sigma.

Molecular Biology Reagents. Restriction enzymes were purchased from Promega or USB. The DNA sequencing was carried out using the kit from USB. For plasmid purification, the Nucleobond AX kit (The Nest Group, Inc.) was used. Oligonucleotides used for mutagenesis and sequencing were prepared using a Biosearch oligonucleotide synthesizer.

Bacterial Strains and Plasmids. The bacterial strains used in these experiments are *Escherichia coli* NM522 (*hsd*Δ*5*, Δ(*lac-pro*), [*F′*, *pro*$^+$, *laclqZ*Δ*M15*]), *Salmonella typhimurium* LB5000 (*metA metE551 trpD2 leu hsdLT hsdA hsdB* and m$^+$ for all three modification systems), and *Salmonella typhimurium* DW378 (*trpC109 cysK1772 cysM1770*). The plasmid pRSM40 contains the *S. typhimurium cysK* gene on a 1.48 *Cla*I−*Dra*I fragment which was inserted in pT7T3 19U that had been digested with *Cla*I and *Sma*I (Monroe & Kredich, 1990). The upstream end of *cysK* is nearest the *Sma*I/*Dra*I junction (Figure 1).

The plasmid, pRSM40, was linearized with *Eco*RI, and a 690 bp *Ava*I fragment containing the codon AAG for K42 was amplified using PCR. The oligonucleotide primers used were

AAG
|||
(1) 5′-AGTCGCGCAA**CCCGAG**CTTCAGCGTC**GC**GTGCCGTATCGG-3′

(2) 5′-AGGTTG**CCCGGG**ATGAAGCCTGCGC-3′

and primer 2 is complimentary to 5′-GCGCAGGCTTCAT-CCCGGGCAACCT-3′ in the DNA sequence of *cysK*.

In oligonucleotide 1, AAG encoding for lysine was changed as shown to GCG encoding for alanine. The sequence CCCGAG is the recognition site for *Ava*I. In oligonucleotide 2, the sequence CCCGGG is the recognition site for *Ava*I.

The protocol for mutagenesis is shown in Figure 1B. The ligated plasmids carrying the new GCG codon were transformed into *E. coli* NM522 using the $CaCl_2$ method (Sambrook et al., 1989) and purified using the Nucleobond AX kit. The complete gene was sequenced with a USB sequencing kit to confirm the mutation and to check for PCR errors. The mutant plasmids were transformed into *S.*

typhimurium LB5000 using a modified Hanahan method and stored as phage *P22* lysates (Monroe & Kredich, 1990). Since LB5000 is r^-m^+ for all three restriction−modification systems, the DNA obtained from this strain is not degraded in any other *S. typhimurium* strain.

Expression strains were constructed by transducing the *cysK*$^-$ *cysM*$^-$ strain (DW378) of *S. typhimurium* with phase *P22* lysates containing mutant plasmids. Briefly, 2 μL of the phage lysate was added to 100 μL of the DW378 culture grown overnight at 37 °C with good aeration. The transductants were selected on LB plates with 100 μg/mL ampicillin in an overnight incubation at 37 °C. The plasmid-containing strain was then allowed to grow for 18 h at 37 °C and 250 rpm on Vogel−Bonner medium E (Vogel & Bonner, 1956) supplemented with 0.5% glucose, 1% LB, 40 μM L-tryptophan, 500 μM reduced glutathione, and 100 μg/mL ampicillin. Reduced glutathione was the only sulfur source in the medium and was added to derepress the cysteine biosynthesis pathway (Kredich, 1971).

The mutant enzyme was purified by the method of Tai et al. (1993). UV−visible absorption spectra were recorded for each fraction of the Q-Sepharose and phenyl-Sepharose columns. The fractions with an $A_{280/424}$ of 5.0 were pooled and concentrated. The purity of the enzyme was further tested via SDS−PAGE.

Preparation and Reconstitution of Apo-K42A-OASS. The preparation of apo-K42A-OASS and its reconstitution were performed using the method of Schnackerz and Cook (1995) with the exception that OAS was not added prior to dialysis against 5 M guanidinium chloride.

Modification of C43 with Bromoethylamine. To modify K42-OASS with BEA, the holoenzyme was first incubated with 5 M guanidinium chloride in 50 mM phosphate buffer, pH 7.5, for 15 min, 40 mM 2-bromoethylamine was added to the apo-K42A-OASS, and the preparation was incubated for 14 h at room temperature. Reconstitution with PLP was carried out as described (Schnackerz & Cook, 1995).

Synthesis of ϵ-N-Pyridoxyl-L-lysine. For the preparation of ϵ-*N*-pyridoxyllysine, a mixture of 1.12 g of α-*N*-carbobenzoxy-L-lysine (4 mmol) and 0.81 g of pyridoxal hydrochloride (4 mmol) in 50 mL of CH_3OH was incubated with 0.8 g of sodium hydroxide (20 mmol) at 4 °C and stirred under a nitrogen atmosphere until the reaction was complete (30 min). Solid sodium borohydride was then added until the yellow color of the solution disappeared. The mixture was warmed to 25 °C, stirred for 1 h, brought to pH 6 with concentrated HCl, and evaporated to dryness *in vacuo*. The protecting group on the α-nitrogen was removed by refluxing with 6 N HCl for 2 h. After hydrolysis, the reaction mixture was evaporated to dryness. The residue was dissolved in H_2O and applied to a cation exchange column (1.5 × 25 cm, Dowex 50 × 8, H^+ form, 200−400 mesh, Sigma), which was washed with 200 mL of 1 N HCl and eluted with a 800 mL gradient of 1−5 N HCl. The fractions which absorb at 294 nm were combined and evaporated to dryness. Final purification of the ϵ-pyridoxyllysine was accomplished using a Beckman HPLC System Gold. Aliquots of the reaction mixture were loaded onto an SP-5PW cation exchange column (21.5 × 150 mm, Toso Haas) equilibrated with solvent A (H_2O, pH 2.5). The column was eluated by a 5 min linear gradient 0−7% solvent B (4 N NaCl, pH 2.5) at a flow rate of 5 mL/min followed by a 60 min gradient of 7−10% solvent B at the same flow rate. The pyridoxyllysine fractions were monitored at 294 nm, collected, and then desalted using a size exclusion column (1.5 × 80 cm, Sephadex G-10, Sigma).

Sodium [^{3}H]Borohydride Reduction of OASS-A. Twenty-four milligrams of OASS-A was reduced by the addition of 0.1 M $NaBH_4$ containing 100 mCi of sodium [^{3}H]borohydride in 20 mM Hepes, pH 8, until the yellow color disappeared. The reduced OASS-A was hydrolyzed in 6 N HCl, 110 °C, for 24 h. The tritiated pyridoxyllysine was purified as described above.

CNBr and Endo Arg-C Cleavage. Twenty-four milligrams of NaB^3H_4-reduced OASS-A was dialyzed 5 times against 1 L of 20 mM Hepes in order to remove excess NaB^3H_4. The reduced protein was digested in 70% trifluoroacetic acid with 205 mg of CNBr for 24 h, and the reaction was quenched by the addition of 10 volumes of H_2O and the mixture lyophilized. Lyophilized CNBr fragments were further digested at pH 8 for 2 days in 0.1 M NH_4HCO_3 and 0.1% SDS by endoproteinase Arg-C with a weight ratio of 20 to 1, OASS to Endo Arg-C.

Purification of the Pyridoxyl Peptide. The pyridoxyl peptide was isolated and purified using a Beckman System Gold HPLC with a Model 171 radioisotope detector. Mobile phases were as follows: solvent A consisted of 0.1% TFA in HPLC grade H_2O (Bio-Rad), while solvent B consisted of 0.1% TFA in HPLC grade CH_3CN. Aliquots of the digest were loaded onto a C_{18} reversed phase column (4.6 × 250 mm, Beckman) equilibrated with solvent A. The column was eluted with solvent A for 5 min followed by a 90 min linear gradient of 0−60% solvent B at a flow rate of 1 mL/min. Elution of peptide was simultaneously monitored using radioactivity and the absorbance at 215 nm.

Degradation of Pyridoxyllysine to Pyridoxamine. The pyridoxyllysine (20 mg) was dissolved in 0.5 mL of water at 4 °C. Argon was passed through the solution which was then treated with 0.06 mL of 1 N NaOH followed by 0.05 mL of 5% sodium hypochlorite. After 15 min, the solution was added dropwise to a flask of boiling water (15 mL) through which argon was bubbling. The solution was heated for 10 min, then cooled to 4 °C, neutralized with 0.1 N HCl, and evaporated to dryness in vacuo. The residue was dissolved in water and passed over a cation exchange HPLC column (21.5 × 150 mm, Toso Haas) using the same gradient and flow rate as used for the purification of pyridoxyllysine. The pyridoxamine was finally desalted using a size exclusion column (1.5 × 80 cm, Sephadex G-10, Sigma).

Stereochemical Analysis of [^{3}H]Pyridoxamine. In a typical analysis, 0.5 mg of the tritiated pyridoxamine was added to 0.3 mL of 20 mM Tris buffer, pH 8.1, and combined with 10 mL of 3.4 mM α-ketoglutarate and 10 mL of 0.15 M monosodium glutamate (Miles et al., 1982). The pH of the solution was adjusted to 8 with 1 M potassium carbonate, pH 8.7; 1.5 mg of apoasparate aminotransferase was added in a closed vessel and incubated for 3 days at 37 °C, and subsequently distilled to obtain the ^3H_2O.

Spectral Studies. UV−visible spectra were measured on a Hewlett-Packard, Model 8452A, photodiode array spectrophotometer. Spectra of apo-K42-OASS reconstituted with PLP in 10 mM Hepes, pH 7.5, and different concentrations of L-alanine, L-cysteine, *O*-acetyl-L-serine, or L-serine were recorded at wavelengths from 250 to 600 nm using a 1 cm light path. Both buffer and amino acid blanks were

subtracted from the spectra. Data were plotted using Cricket Graph III.

Fluorescence Studies. Fluorescence spectra of 2.5 μM mutant enzyme were obtained on a Shimadzu RF5000U spectrofluorometer in the absence and presence of amino acids at 25 °C. Excitation was at 298 nm, and the excitation slit widths were set at 5 nm. Emission was measured over the wavelength range 300−550 nm.

Circular Dichroism Studies. CD spectra were collected on an Aviv 62DS spectropolarimeter at 25 °C with a path length of 0.2 cm. Enzyme concentrations of 100 μg/mL and 4 mg/mL were used for far-UV and visible CD spectra, respectively. The buffer used for all spectra was 10 mM phosphate, pH 7.0, and a buffer blank was subtracted from each spectrum.

Enzyme Assays. The activity of PLP-reconstituted apo-K42A-OASS and the PLP-reconstituted bromoethylamine-modified apo-K42A-OASS was monitored using the following two assay methods. In the normal reaction, i.e., formation of L-cysteine from OAS and sulfide, the decrease in sulfide concentration was observed using a computer-assisted sulfide ion-selective electrode assay (Hara et al., 1990). In addition, the formation of *S*-(3-carboxy-4-nitrophenyl)-L-cysteine (Tai et al., 1993) was measured by monitoring the disappearance of TNB using a Gilford 2600 spectrophotometer at 412 nm (Ellman, 1959).

^{31}P NMR Spectroscopy. Fourier transform ^{31}P NMR spectra were collected at 121.497 MHz on a Bruker AM300 SWB superconducting spectrometer using a 10-mm multinuclear probehead with broadband ^{1}H decoupling. The NMR tube spinning at 15−20 Hz contained the sample (2 mL) and $^{2}H_2O$ (0.2 mL) as field/frequency lock and was maintained at 20 ± 0.1 °C using a thermostated continuous air flow. A spectral width of 2000 Hz was acquired in 8K data points with a pulse angle of 60°. The exponential line broadening used prior to Fourier transformation was 10 Hz. Protein samples were dissolved in 50 mM Mes or Hepes buffers containing 1 mM EDTA at the appropriate pH. Changes in pH were performed by dialysis against the desired buffer overnight. pH valves of the sample were determined before and after the NMR measurement. Positive chemical shifts in ppm are downfield changes with respect to 85% H_3PO_4.

RESULTS

Active Site Sequence. The main radiolabeled peptide from the elution profile of the Endo Arg-C/CNBr-cleaved OASS-A containing the active site lysine was subjected to solid phase sequencing using an Applied Biosystem gas phase protein sequencer. The peptide is nine amino acids long and contains >65% of the radioactivity in position 7. The sequence is as follows: AsnProSerPheSerValLys(Pxy)CysArg. The above sequence exactly matches residues 36−44 of that predicted based on the nucleotide sequence (Levy & Danchin, 1988; Byrne et al., 1988), but is not the one predicted based on the homology to other PLP enzymes (Levy & Danchin, 1988). The cysteine immediately C-terminal to the active lysine is the only one present in the polypeptide. The identification of the pyridoxyl peptide and the fact that the K42A mutation results in an inactive enzyme indicate that lysine-42 is the enzyme group covalently bound to the coenzyme PLP.

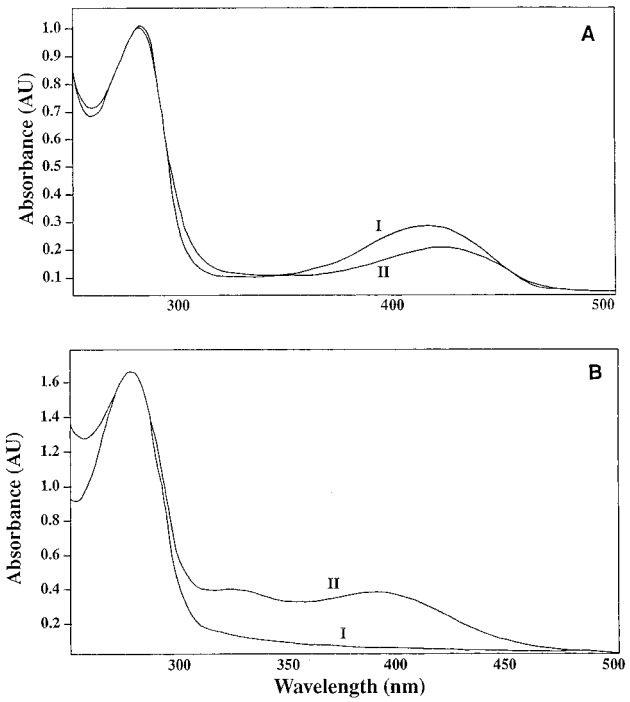

FIGURE 2: Panel A shows UV/visible spectra of OASS-A (I) and K42A-OASS (II) of equal concentration. Panel A shows UV/visible spectra of OASS-A (I) and K42A-OASS (II) of equal concentration. Panel B shows apo-K42A-OASS (I) and apo-K42A-OASS reconstituted with PLP (II).

Stereochemical Analysis at C4′. To determine which face of the internal Schiff base is reduced by sodium [^{3}H]-borohydride, the tritiated protein was hydrolyzed to [^{3}H]-pyridoxyllysine, which was subjected to hypochlorite oxidation to produce [^{3}H]pyridoxamine. Analysis of the tritium distribution between the two diastereotopic hydrogens at C4′ of pyridoxamine made use of the stereospecificity of apoaspartate aminotransferase for removal of the 4′-*pro-S* hydrogen (Dunathan et al., 1968). The tritium from the 4′-*pro-S* position is released into solution, while the tritium from the 4′-*pro-R* position is retained. The [4′-^{3}H]pyridoxamine generated by reduction of the internal Schiff base with sodium [^{3}H]borohydride retained most of its tritium after incubation with apoaspartate aminotransferase. Of the 30 000 cpm added to the apoaspartate aminotransferase incubation, 3280 cpm were released to solvent compared to 2245 for the minus apoenzyme control. These results agree with the hypothesis put forth by Dunathan (Dunathan, 1971; Dunathan & Voet, 1974) that a single surface (*re* face in this case) of the active site PLP is accessible to solvent and to borohydride.

Growth and Yield of the Mutant Protein. When cells were grown in shaker flasks overnight, the K42A mutant protein was obtained in a yield of 20 mg/25 g of wet cell paste. The mutant protein, as isolated, has λ_{max} values of 280 and 424 nm (Figure 2A) and a small amount of activity. Addition of $NaBH_4$ does not affect the absorbance at 424 nm, but eliminates all of the activity. Preparation of larger quantities of the K42A mutant was attempted by overnight growth in a 12 L fermenter with forced aeration. After a lag of several hours, growth was observed, and cells were harvested after 24 h. *O*-Acetylserine sulfhydrylase was isolated and found to be wild type OASS-A.

Spectral Properties. WT OASS-A, as isolated, shows an absorbance spectrum with two maxima, at 280 and 412 nm

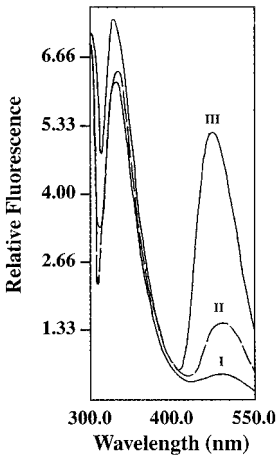

FIGURE 3: Fluorescence spectra of OASS-A (I), K42A-OASS (II), and addition of acetate to OASS-A (III). Each of these spectra was collected at 25 °C in 10 mM Hepes, pH 7.5, with an enzyme concentration of 2.5 μM for OASS-A and K42A-OASS.

(Figure 2A, I), the latter corresponding to the internal Schiff base (Cook et al., 1992). The ratio of $A_{280/424}$ is about 5.0 for the K42A mutant protein, compared to a value for $A_{280/412}$ of 3.4 for WT OASS-A. Addition of sodium borohydride alone has no effect on the 424 nm band of the K42A mutant protein as isolated, but in the presence of 5 M guanidinium chloride the absorbance at 424 nm disappears. The absorption spectrum of apo-K42A-OASS exhibits a single maximum at 280 nm (Figure 2B, I). Reconstitution of apo-K42A-OASS with PLP produces additional absorption bands at 330 and 390 nm (Figure 2B, II). The ratio of $A_{280}/A_{(330+390)}$ is around 5.0.

When excited at 298 nm, the fluorescence emission spectrum of WT OASS-A shows two maxima at 337 and 498 nm; the ratio of $A_{337/498}$ is 8.0 (Figure 3, I). The band at the shorter wavelength is a result of intrinsic tryptophan fluorescence whereas the band at the longer wavelength is due to delayed Schiff base fluorescence (McClure & Cook, 1994; Strambini et al., 1996). Excitation of the K42A mutant protein at 298 nm shows an emission spectrum, with maxima at 337 and 504 nm (Figure 3, II). The ratio for the K42A mutant ($A_{337/504}$) is around 4.5, which is a value between those observed for the WT enzyme in the absence and presence of acetate (Figure 3, III; McClure & Cook, 1994). Apo-K42A-OASS shows no emission around 500 nm (data not shown) as also seen for WT apo-OASS-A, and the emission at 337 nm is increased in the apo-K42A mutant, when compared to K42A-OASS.

The emission spectrum of apo-K42A-OASS reconstituted with PLP is not identical to that of K42A-OASS, when excited at 298 nm. The emission band at 500 nm has a lower intensity than that of K42A-OASS as isolated. When excited at 330 nm, the WT enzyme shows a major band with a λ_{max} of 484 nm and a weaker band with a λ_{max} of 362 nm (Strambini et al., 1996). Two bands are also observed in the K42A mutant reconstituted with PLP, but λ_{max} values appear at 444 and 387 nm, respectively (data not shown).

Circular dichroism spectra for K42A-OASS as isolated are identical to those of the WT enzyme in the far-UV (data not shown), suggesting essentially no gross change in the structure of the OASS as a result of the active site mutation. In the visible region, however, K42A-OASS shows a negative Cotton effect with λ_{max} at 424 nm and a molar

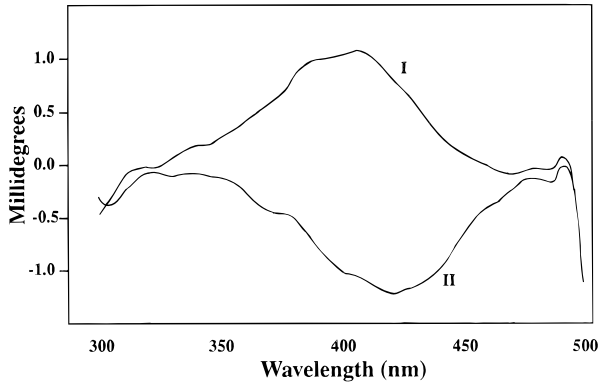

FIGURE 4: Visible circular dichroism spectra of OASS-A (I) and K42A-OASS (II). These spectra were taken at 25 °C with 10 mM phosphate buffer, pH 7.5. The enzyme concentration used was 500 μg for both the spectra.

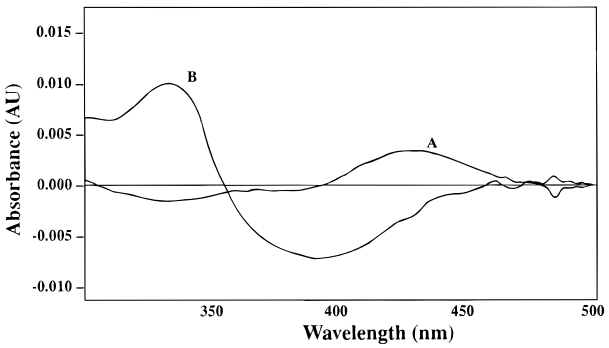

FIGURE 5: UV/visible difference spectra for external aldimine formation. Each of these spectra was taken at 25 °C with 1 mg of apo-K42A-OASS reconstituted with PLP in the absence and presence of 10 mM OAS (A) or 10 mM L-cysteine (B). Spectra are the difference of those in the presence and absence of amino acid. The reactions were carried out in 10 mM Hepes, pH 7.5.

ellipticity equal to that of the positive Cotton effect of the WT enzyme at 412 nm (Figure 4). Apoenzyme shows no ellipticity in the visible range whereas enzyme reconstituted with PLP shows positive ellipticity centered around 330 and 390−400 nm (data not shown).

The ^{31}P NMR spectra of K42A-OASS show a single resonance at 5.3 ppm in the pH range 6.5−8.5 (data not shown). This can be compared to a value of 5.2 ppm for the WT enzyme (Cook et al., 1992).

Formation of External Aldimine. Apo-K42A-OASS reconstituted with PLP exhibits spectral changes upon addition of amino acid reactants or analogs. Addition of 20 mM OAS to the reconstituted enzyme leads to a time-dependent decrease in the absorbance at 330 nm and a shift in the λ_{max} of the 390 nm band to 424 nm with a concomitant increase in the absorbance of the latter (Figure 5, A). The reaction is pH-independent. Formation of the external aldimine with OAS is complete in about 90 min. L-Alanine and L-serine produce similar changes over the same time period and show saturation at 10 mM concentration. L-Cysteine at a concentration of 15 mM produces an increase in the absorbance at 330 nm with a concomitant decrease at 390 nm (Figure 5, B). A first-order plot of the absorbance change for the formation of the L-Ala external Schiff base at 310 or 370 nm (wavelengths that produce maximum absorbance changes) vs time gives a maximum rate constant of 0.048 min^{-1}. Similar data for OAS, serine, and cysteine give first-order rate constants in the range of 0.035 or 0.04 min^{-1}. The

external aldimine species studied above are stable for at least 24 h. In the above experiments, no species absorbing at or near 470 nm, corresponding to the α-aminoacrylate intermediate, was formed (Cook et al., 1992), suggesting that the ϵ-amino group of lysine is important in the reaction.

Addition of any of the above amino acids to the reconstituted enzyme gives a decrease in the fluorescence at 337 nm and an increase in the fluorescence at 500 nm, with the λ_{max} at longer wavelength showing a blue shift with time (data not shown). The rates of the changes are similar to those obtained from UV/visible spectrophotometry. Formation of the external aldimine with OAS, L-Ala, or L-Cys was also studied using circular dichroism. Addition of OAS and L-Ala to the apoenzyme reconstituted with PLP shows a decrease in the ellipticity around 390 nm. Addition of L-Cys to the reconstituted apoenzyme, on the other hand, shows a prominent decrease in the ellipticity at 330 nm.

Formation of α*-Aminoacrylate*. Addition of OAS to the WT enzyme results in the formation of the α-aminoacrylate intermediate accompanied by an absorbance decrease at 412 nm and increases at 330 and 470 nm (Cook et al., 1992). The K42A mutant enzyme, when preincubated with 10 mM ethylamine (Figure 6A), shows formation of the external aldimine upon addition of OAS, followed by slow formation of the α-aminoacrylate intermediate absorbing at 330 and 470 nm (Figure 6B). A maximum first-order rate constant of 0.03 min^{-1} is obtained from the time dependence of the absorbance change at either 310 or 470 nm. Addition of sulfide results in an increase in absorbance at 330 nm and a decrease in the α-aminoacrylate species that absorbs at 470 nm (Figure 6C), giving a spectrum identical to that obtained upon addition of L-Cys to the PLP-reconstituted apo-K42A-OASS. Addition of an increasing concentration of ethylamine gave no change in the species at 330 nm. The resulting 330 nm species can be reduced by $NaBH_4$, giving an increase in the absorbance at 330 nm due to the presence of pyridoxylcysteine.

Circular dichroism experiments performed to study the formation of the external aldimine made use of the apoenzyme reconstituted with PLP after addition of 10 mM ethylamine. Addition of OAS to this enzyme species resulted in CD maxima around 330 and 430 nm with negative ellipticity. Addition of L-cysteine and L-alanine to the enzyme treated with 10 mM ethylamine showed no change in the spectrum.

Rates of L*-Cysteine Formation*. K42A-OASS as isolated and PLP-reconstituted apo-K42A-OASS do not catalyze either the overall reaction (formation of cysteine, with the natural substrates, OAS, and sulfide) the deacetylase activity (formation of pyruvate, ammonia, and acetate). As discussed above, a small amount of contaminating WT activity was detected in the K42A mutant enzyme as isolated, but it was eliminated by borohydride reduction with no change in the spectrum of the K42A mutant protein. Since the ϵ-amino group of lysine is absent in the K42A mutant enzyme, two different methods were attempted to restore activity, i.e., addition of ethylamine to the enzyme and chemical modification of C43 with bromoethylamine to form γ-thialysine at position 43. Addition of 10 mM ethylamine to apo-K42A-OASS reconstituted with PLP results in an absorbance shift from 394 to 398−400 nm, suggesting the formation of an external aldimine (Figure 6A). When apo-K42A-OASS reconstituted with PLP is preincubated with ethylamine, the

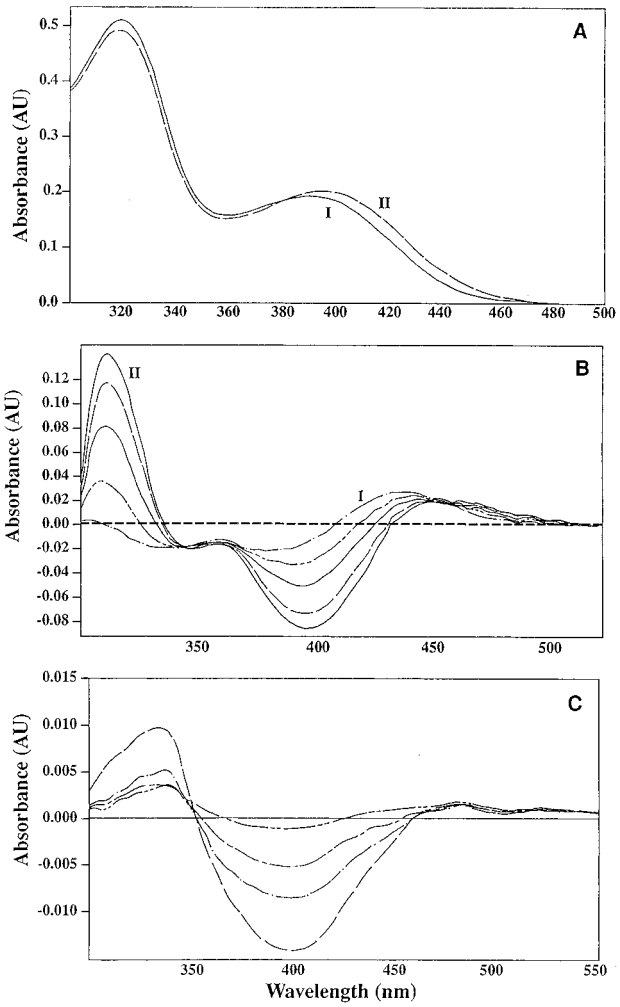

FIGURE 6: Panel A shows the UV/visible spectra of apo-K42A-OASS reconstituted with PLP (I) and formation of external aldimine in the presence of 10 mM ethylamine (II). The enzyme concentration used was 500 μg. Panels B and C show the difference between the spectrum in the presence of amino acid and that of free enzyme as a function of time. The reaction is that catalyzed by apo-K42A-OASS reconstituted with PLP in the presence of 10 mM ethylamine. Panel B shows, first, the formation of the external aldimine (I), followed by the formation of α-aminoacrylate (II) external Schiff base. Times are 5, 15, 30, 60, and 90 min from top to bottom at 400 nm. Panel C shows the formation of L-Cys after addition of sulfide to the reaction in panel B. Times are 5, 15, 30, and 60 min from top to bottom at 400 nm. The reaction was carried out at 25 °C with 10 mM OAS and 10 μM sulfide in 10 mM Hepes, pH 7.5. The enzyme concentration used for the reactions was 1 mg.

rate of α-aminoacrylate formation from OAS (first half-reaction) is slow compared to that with the wild type OASS-A. Addition of sulfide to the α-aminoacrylate intermediate causes a disappearance in the absorbance at 470 nm and at 400 nm with an increase in the absorbance at 330 nm resulting from the cysteine external Schiff base (Figure 6C). Using 9 μM apo-K42A-OASS reconstituted with PLP, a single turnover is observed with the catalytic cycle stopping at the L-cysteine external Schiff base. The maximum first-order rate of formation of the cysteine external Schiff base is $(3.0 \pm 0.4) \times 10^{-3}$ s^{-1}, 10^5-fold lower than V/E_t for the WT enzyme (Tai et al., 1995). Apparent K values for OAS and sulfide in the formation of the cysteine external Schiff base are 10 mM and 10 μM, respectively, compared to K_m values of 1 mM and 6 μM, respectively, measured for the rate of formation of cysteine by the WT enzyme. In the second case, C43, located next to the active site lysine, was

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MSKIYEDNSL TIGHTPLVRL NRIGNGRILA KVESRNPSFS VKCRIGANMI     50
WDAEKRGVLK PGVELVEPTN GNTGIALAYV AAARGYKLTL TMPETMSIER    100
RKLLKALGAN LVLTEGAKGM KGAIQKAEEI VASDPQKYLL LQQFSNPANP    150
EIHEKTTGPE IWEDTDGQVD VFISGVGTGG TLTGVTRYIK GTKGKTDLIT    200
VAVEPTDSPV IAQALAGEEI KPGPHKIQGI GAGFIPGNLD LKLIDKVVGI    250
TNEEAISTAR RLMEEEVFLA GISSGAAVAA ALKLQEDESF TNKNIVVILP    300
SSGERYLSTA LFADLFTEKE LQQ                                 323
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FIGURE 7: Deduced amino acid sequence of *O*-acetylserine sulfhydrylase A from *Salmonella typhimurium* (Bryne et al., 1988; Levy & Danchin, 1988).

modified with bromoethylamine, giving γ-thialysine which forms an internal Schiff base with an absorbance at 398 nm similar to that of K42A-OASS after addition of ethylamine. Reduction of the γ-thialysine OASS with sodium borohydride results in a bleaching of the 400 nm absorbance and an increase in the absorbance at 320 nm. Denaturation with 4 M guanidinium chloride and dialysis produce no change in the 320 nm absorbance. Again, a single turnover appears to occur, and rate data are essentially identical to those obtained with ethylamine. The alternative substrate, TNB, gave no discernible rate with the reconstituted mutant proteins.

DISCUSSION

Location of the Schiff Base Lysine. Based on the data obtained, a mixture of peptides was subjected to sequencing. The sequence of one of the peptides was readily acquired due to the constant yield per cycle. When compared to the amino acid sequence derived from the gene sequence, the radioactively labeled peptide is identical to positions 36−44 of the N-terminal sequence of OASS-A (Byrne et al., 1988) (Figure 7). Most of the radioactivity was found at position 42, indicating that this is the location of the Schiff base lysine.

K42A Mutant Enzyme. *(A) Growth*. Surprisingly, growth under conditions of forced aeration produced the wild type enzyme. However, the background strain, DW378, is *cysK*$^-$, *cysM*$^-$ as a result of point mutations, eliminating the activity of OASS-A and OASS-B (Dreyfuss & Monty, 1962). In addition, DW378 is a recombinant positive strain. As a result of the selective pressure of growth in the absence of cysteine, homologous recombination likely occurs between the *cysK* gene on the plasmid-carrying mutation K42A and the chromosomal *cysK* gene to produce the WT enzyme. As stated under Results, K42A mutant protein could be obtained in good yield only by overnight growth in shaker culture.

(B) Spectral Properties of K42A-OASS in the Absence of Amino Acids. The K42A mutant enzyme, as isolated, shows absorbance maxima at 280 and 424 nm, the latter corresponding to the external aldimine of PLP with a mixture of free methionine and leucine based on an amino acid analysis of isolated K42A that was heat-denatured and dialyzed. Similar spectra have been observed for the external Schiff bases of OASS with L-cysteine[3] or L-serine (Schnackerz et al., 1995). The external aldimine of K42A-OASS as isolated cannot be reduced with sodium borohydride under conditions normally used for the reduction of internal and/or external aldimines of PLP. Only in the presence of 5 M guanidinium chloride is the reduction with borohydride feasible, indicating that the external aldimine of K42A-OASS as isolated may be in a closed conformation. It is very difficult to remove PLP from K42A-OASS, even though the mutant enzyme lacks Lys-42, which in WT OASS-A binds PLP covalently via an internal aldimine linkage. The presence of 5 M guanidinium chloride is necessary to open the PLP binding site to facilitate resolution of the cofactor to produce apo-K42A-OASS. Reconstitution of the apoenzyme with PLP results in two absorption bands at 330 and 390 nm. Free PLP absorbs at 388 nm with a shoulder at 325 nm at neutral pH (Peterson & Sober, 1954). The spectrum of reconstituted apo K42A-OASS is very similar to that of free PLP at neutral pH, confirming the presence of the free aldehyde at the active site.

The fluorescence emission spectrum of WT OASS exhibits maxima at 337 and 500 nm when excited at 298 nm (McClure & Cook, 1994; Strambini et al., 1996). The 337 nm band results largely from intrinsic tryptophan fluorescence, while the 500 nm band results from delayed fluorescence of the internal Schiff base (Strambini et al., 1996). Addition of L-cysteine to WT OASS to form the external Schiff base[3] (or acetate which binds to the α-carboxylate subsite, mimicking cysteine) results in a significant enhancement of the long-wavelength band a blue shift in its λ_{max} to 490 nm (McClure & Cook, 1994; Schnackerz et al., 1995; Strambini et al., 1996). The fluorescence spectrum of K42A-OASS, as isolated, also shows maxima at 337 and 504 nm, when excited at 298 nm. The ratio of $A_{337/504}$ for K42A-OASS as isolated is about halfway between that of the WT OASS and the cysteine external Schiff base, suggesting the presence of about 50% occupancy of K42A with an external aldimine between PLP and a free amino acid (methionine and leucine as indicated under Results). Apo-K42A-OASS, on the other hand, shows fluorescence emission only at 337 nm, in agreement with results on WT apo-OASS (McClure & Cook, 1994). In apo-K42A-OASS, the fluorescence emission at 337 nm is increased when compared to K42A-OASS, likely the result of quenching upon binding of PLP to the apoenzyme, as is also observed for WT OASS and its apoenzyme (McClure & Cook, 1994; Strambini et al., 1996). Similar results are found for D-serine dehydratase (Schnackerz et al., 1973; Federiuk & Shafer, 1983), tryptophanase (Tokushige et al., 1980), and tryptophan synthase (Strambini et al., 1992). The emission band of PLP-reconstituted apo-K42A-OASS at 500 nm has a lower intensity than K42A-OASS, as isolated, when excited at 298 nm. Reconstituted apo-K42A-OASS exhibits two emission bands at 444 and 387 nm, respectively, when excited at 330 nm, qualitatively similar to WT OASS-A which shows maxima at 484 and 362 nm, respectively. For the WT OASS, which exists as an internal aldimine, the major band centered at 484 nm is characteristic of a ketoenamine tautomer, whereas the weaker band around 362 nm is typical for an enolimine tautomer (Strambini et al., 1996). In the case of the PLP-reconstituted

[2] The 418 nm tautomer observed for wild-type enzyme is actually that of *S*-(3-L-alanyl)-L-cysteine with the active site PLP, resulting from attack of the β-thiol of L-cysteine on the α-aminoacrylate intermediate (Woehl et al., 1996).

[3] The addition of L-cysteine actually results in a Schiff base between *S*-(3-L-alanyl)-L-cysteine (Woehl et al., 1996).

apo-K42A-OASS, the 444 nm band may represent the ketoenol form of free PLP, while the weaker 387 nm band may represent the enolaldehyde form of free PLP. The fluorescence data support the presence of an external aldimine in K42A-OASS as isolated.

The CD spectrum of K42A-OASS, as isolated, shows in the visible region a negative Cotton band with a maximum at 424 nm centered on the visible absorbance band. This can be compared to a visible CD band at 412 nm for the WT enzyme, also centered on its visible absorbance band (Schnackerz et al., 1995). Addition of L-cysteine to the WT enzyme causes a red shift in both the visible absorbance and CD bands. The visible CD band has a positive sign and is of equal intensity to that observed in the absence of L-cysteine (Schnackerz et al., 1995). The K42A-OASS as isolated exists as a mixture of methionine and leucine Schiff bases. Since these external Schiff bases have a visible Cotton band opposite in sign to that of the WT cysteine external Schiff base, there are two possible explanations. The difference in chemical structure of Met, Leu, and Cys is responsible for the opposite sign, or the opposite face of the external Schiff base interacts with the protein surface in the cysteine external Schiff base compared to the Met/Leu external Schiff bases. Since the K42A-OASS as isolated cannot be reduced, these possibilities cannot be distinguished at this time.[4] In the PLP-reconstituted apo-K42A-OASS, CD bands with positive ellipticity around 330 and 390−400 nm (centered on the visible absorbance bands) are observed. The ^{31}P signal of the internal aldimine of the WT OASS is 5.2 ppm, independent of pH (Cook et al., 1992), while that of the cysteine external Schiff base is shifted slightly downfield to 5.3 ppm (Schnackerz et al., 1995). The ^{31}P NMR spectra of K42A-OASS, as isolated, show a single resonance at 5.3 ppm, consistent with the occurrence of an external aldimine in K42A-OASS as isolated.

The Schiff base lysine has been replaced in three other PLP-dependent enzymes: K258A in aspartate aminotransferase (Toney & Kirsch, 1993), K145A in D-amino acid aminotransferase (Nishimura et al., 1991), and K87T in $\alpha_2\beta_2$-tryptophan synthase (Miles et al., 1989). The most closely related is K87T in $\alpha_2\beta_2$-tryptophan synthase which is isolated as an external aldimine with free L-serine. The spectral properties of the apo-K87T-tryptophan synthase reconstituted with PLP are identical to those of the apo-K42A-OASS reconstituted with PLP. The Schiff base lysine mutants of the aminotransferases, however, are isolated with the free aldehyde of PLP bound.

(C) Spectral Properties of K42A-OASS in the Presence of Amino Acids. The external Schiff base forms slowly upon addition of OAS, L-Cys, L-Ser, or L-Ala to the PLP-reconstituted mutant enzyme. The final equilibrium mixture of species is similar to that observed for the WT OASS in the case of L-Ser, that is a mixture of tautomers with λ_{max} values at 330 and 418 nm, but differs in the case of L-Cys with the 330 nm tautomer observed for K42A-OASS compared to a 418 nm tautomer observed for the wild type enzyme (Schnackerz et al., 1995). The slow formation of the external Schiff base was also observed for K87T $\alpha_2\beta_2$-TS (Lu et al., 1993) and K258A-AAT (Toney & Kirsch, 1993).

(D) Regeneration of Activity. All amino acids tested, OAS, L-cysteine, L-serine, and L-alanine, with PLP-reconstituted apo-K42A-OASS form an external Schiff base slowly (0.04 min^{-1}) compared to WT OASS (700−1000 s^{-1}; Woehl et al., 1996). Cordes and Jencks (1962) demonstrated that the rate constants for reactions in solution of imines of PLP with semicarbazide are greater than those for the parent aldehyde alone with semicarbazide. The present results are consistent with the suggestion of Cordes and Jencks that the rate constant for formation of the extenral aldimine from the WT OASS internal aldimine should be greater than that for enzyme-bound free aldehyde of PLP. Thus, significant rate enhancement is realized as a result of K42 allowing transimination to occur in the catalytic cycle.

Addition of 10 mM ethylamine to reconstituted apo-K42A-OASS in the presence of OAS shows the formation of external aldimine with λ_{max} at 398 nm, followed by the slow formation of the α-aminoacrylate intermediate. Addition of sulfide results in an increase in the absorbance at 330 nm and a decrease in the α-aminoacrylate intermediate, giving a spectrum identical to that obtained upon addition of cysteine to reconstituted apo-K42A-OASS. Similar spectral changes in the presence of OAS were observed for K42A-OASS modified at position 43 to a γ-thialysine.[5] The restored activity is 10^5-fold lower than that of WT enzyme, and only one turnover is carried out in both half-reactions. The spectral changes observed suggest that the reaction is terminated with the formation of the cysteine external Schiff base and the cysteine cannot be displaced, a prerequisite to start a new cycle. Data are consistent with the above conclusion that K42 is important for transimination, not only to form the external Schiff base, for example, with OAS, but also to form the internal Schiff base and release the amino acid product, for example, cysteine. The lack of ability of ethylamine to displace cysteine may be entropic, since ethylamine lacks the advantage of being locked into place,[6] or may be geometric resulting from ethylamine being bound at the active site in such a way that it is unable to carry out the displacement effectively. Planas and Kirsch (1991) have suggested that the decreased basicity of the γ-thialysine group in K258γ-thialysine-AAT is considered to be principally responsible for the 14-fold lower V_{max} value compared to WT AAT. The 10^5-fold lower activity of the C43-γ-thialysine derivative of K42A is likely a result of geometric considerations, since C43 is one amino acid removed from the normal Schiff base lysine position.[5]

In conclusion, lysine-42 of OASS-A facilitates the formation and dissociation of the OAS and L-cysteine external Schiff bases, allowing more facile transimination. It also participates as a general base in the first half-reaction, abstracting the α-proton of OAS, and as a general acid in the second half-reaction, donating a proton to the α-carbon.

[4] The three-dimensional structure of OASS-A has recently been solved (P. Burkhard, E. Hohenester, G. S. J. Rao, P. F. Cook, and J. N. Jansonius, unpublished results), and crystallization trials are now in progress on the K42A-OASS as isolated.

[5] The observed restoration of activity upon bromoethylamine treatment may also result from the presence of bromoethylamine acting in the same manner as ethylamine. The similarity in kinetic data obtained for apo-K42A-OASS reconstituted with PLP in the presence of ethylamine or after bromoethylamine treatment is consistent with this alternate interpretation. In addition, the crystal structure of WT OASS suggests C43 is behind the active site, inaccessible to bromoethylamine.

[6] We thank the reviewer for this suggestion.

REFERENCES

Alexander, F. W., Sandmeier, E., Mehta, P. K., & Christen, P. (1994) *Eur. J. Biochem. 219*, 953−960.

Becker, M. A., Kredich, N. M., & Tomkins, G. M. (1969) *J. Biol. Chem. 244*, 2418−2427.

Byrne, C. R., Monroe, R. S., Ward, K. A., & Kredich, N. M. (1988) *J. Bacteriol. 170*, 3150−3157.

Cook, P. F., & Wedding, R. T. (1976) *J. Biol. Chem. 251*, 2033−2029.

Cook, P. F., Hara, S., Nalabolu, S. R., & Schnackerz, K. D. (1992) *Biochemistry 31*, 2298−2303.

Cordes, E. H., & Jencks, W. P. (1962) *J. Am. Chem. Soc. 84*, 832−837.

Dreyfuss, J., & Monty, K. J. (1962) *J. Biol. Chem. 238*, 1019−1024.

Dunathan, H. C. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol. 35*, 79−134.

Dunathan, H. C., & Voet, J. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3888−3891.

Dunathan, H. C., Davis, L., Kury, P. G., & Kaplan, M. (1968) *Biochemistry 7*, 4532−4537.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.

Federiuk, C. S., & Shafer, J. A. (1983) *J. Biol. Chem. 258(9)*, 5372−5378.

Hara, S., Payne, M. A. Schnackerz, K. D., & Cook, P. F. (1990) *Protein Expression Purif. 1*, 70−76.

Kredich, N. M. (1971) *J. Biol. Chem. 246*, 3474−3484.

Kredich, N. M., & Tomkins, G. M. (1966) *J. Biol. Chem. 241*, 4955−4965.

Levy, S., & Danchin, A. (1988) *Mol. Microbiol. 2*, 777−783.

Lu, Z., Nagata, S., McPhie, P., & Miles, E. W. (1993) *J. Biol. Chem. 268*, 8727−8734.

McClure, G. D., Jr., & Cook, P. F. (1994) *Biochemistry 33*, 1674−1683.

Miles, E. W., Hauch, D. R., & Floss, H. G. (1982) *J. Biol. Chem. 257*, 14203−14210.

Miles, E. W., Kawasaki, H., Ahmed, S. A., Morita, H., Morita, H., & Nagata, S. (1989) *J. Biol. Chem. 264*, 6280−6287.

Monroe, R. S., & Kredich, N. M. (1990) *J. Bacteriol. 172(12)*, 6919−6929.

Nishimura, K., Tanazawa, K., Yoshimura, T., Esaki, N., Futaki, S., Manning, J. M., & Soda, K. (1991) *Biochemistry 30*, 4072−4077.

Peterson, E. A., & Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169.

Planas, A., & Kirsch, J. F. (1991) *Biochemistry 30*(*33*), 8268−8278.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp 1.82−1.84, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schnackerz, K. D., & Cook, P. F. (1995) *Arch. Biochem. Biophys. 324*, 71−77.

Schnackerz, K. D., Erlich, J. H., Wipf, A., & Wirths, G. G. (1973) *Z. Physiol. Chem. 354*, 1241.

Schnackerz, K. D., Tai, C.-H., Simmons, J. W., III, Jacobson, T. M., Rao, G. S. J., & Cook, P. F. (1995) *Biochemistry 34*, 12151−12160.

Strambini, G., Cioni, P., Peracchi, A., & Mozzarelli, A. (1992) *Biochemistry 31*, 7527−7534.

Strambini, G., Cioni, P., & Cook, P. F. (1996) *Biochemistry 35*, 8392−8400.

Tai, C.-H., Nalabolu, S. N., Jacobson, T. M., Minter, D. E., & Cook, P. F. (1993) *Biochemistry 32*, 6433−6442.

Tai, C.-H., Nalabolu, S. R., Simmons, J. W., Jacobson, T. M., & Cook, P. F. (1995) *Biochemistry 34*, 12311−12325.

Tokushige, M., Iinuma, K., Yamamoto, M., & Nishijima, Y. (1980) *Biochem. Biophys. Res. Commun. 96*, 863−869.

Toney, M. D., & Kirsch, J. F. (1993) *Biochemistry 32*, 1471−1479.

Vogel, H. J., & Bonner, D. M. (1956), *J. Biol. Chem. 218*, 97−106.

Woehl, E. U., Tai, C.-H., Dunn, M. F., & Cook, P. F. (1996) *Biochemistry 35*, 4776−4783.

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