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Spectral Studies of Conformational Change at the Active Site of Mutant O-acetylserine Sulfhydrylase-A (C43S)

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Abstract: The cysteine 43, potentially important in the activity of O-acetylserine sulfhydrylase (OASS) from *Salmonella typhimurium*, has been changed to serine. This mutant enzyme (C43S) has been studied in order to gain insight into the structural basis for the binding of inhibitor, substrate and product. UV-visible spectra of C43S exhibit the same spectral change in the presence of OAS as that observed with wild type enzyme, indicating C43S will form an α-aminoacrylate Schiff base intermediate. At pH 6.5, however, the deacetylase activity of C43S is much higher than wild type enzyme indicating that cysteine 43 plays a role in stabilizing the α-aminoacrylate intermediate. The fluoroscence spectrum of C43S exhibits a ratio of emission at 340 to 502 nm of 16.9, reflecting the lower fluorescence of PLP and indicating that the orientation of cofactor and tryptophan are different from that of the wild type enzyme. The emission spectrum of C43S in the presence of OAS gives two maxima at 340 and 535 nm. The 535 nm emission is attributed to the fluoroscence of the α-aminoacrylate intermediate. The visible circular dichroic spectrum was similar to wild type enzyme, but the negative effect observed at 530~550 nm and the molar ellipicity values for the mutant are decreased by about 50% compared to wild type enzyme. The circular dichroic and fluoroscence studies suggest binding of the cofactor is less asymmetric in C43S than in the wild type enzyme.
Key words: mechanism, O-acetylserine sulfhydrylase, spectral study.

O-Acetylserine sulfhydrylase (EC 4.2.99.8; O-acetylserine(thiol)-lyase) is a key enzyme in the synthesis of cysteine in enteric bacteria. The biosynthetic pathway for L-cysteine consists of two enzymatic reactions as illustrated below:

L-serine + acetyl-CoA → O-acetyl-L-serine + CoA (1)
O-acetyl-L-serine + sulfide → L-cysteine + acetate (2)

The first reaction is catalyzed by the enzyme serine transacetylase and the second is catalyzed by O-acetylserine sulfhydrylase (OASS). The A (OASS-A) and B (OASS-B) isozymes are PLP-dependent and homodimeric with subunit MWs of 34450 (Levy and Danchin, 1988) and 27500 (Nakamula *et al.*, 1984), respectively. The gene for the A isozyme from *Salmonella trphimurium* has been sequenced (Byrne *et al.*, 1988). The second isozyme, O-acetylserine sulfhydrase-B, catalyzes the same reaction (Kredich, 1971; Hulanicka *et al.*, 1979). A large intracellular excess of the A isozyme over the B isozyme is required during aerobic growth (Filutowicz, 1982).

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OASS-A has been shown to have a ping pong kinetic mechanism. The first half reaction requires the β-elimination of acetate from OAS to generate an α-aminoacrylate intermediate in Schiff base with the active site PLP(Cook and Wedding, 1976). The Michael addition of sulfide to the α-aminoacrylate intermediate then occurs in the second half reaction to produce the final product L-cysteine. The ultraviolet-visible spectrum of OASS-A exhibits an absorption maximum at 412 nm, attributed to a protonated Schiff base between an active site lysine residue and the PLP(Cook and Wedding, 1976; Nalabolu *et al.*, 1992). Addition of OAS to the native enzyme results in the disappearance of absorbance at 412 nm and the appearance of new absorption maxima at 320 and 470 nm, indicative of the formation of a protonated Schiff base between PLP and α-aminoacrylate upon the β-elimination of acetate from OAS (Cook and Wedding, 1976; Schnackerz *et al.*, 1979; Nalabolu *et al.*, 1992).

The pH dependence of the ultraviolet-visible spectrum in the absence and presence of OAS indicates that the pK_a values for the Schiff base in the free enzyme and the α-aminoacrylate intermediate are greater than 10 (Nalabolu *et al.*, 1992). In addition, it has been

shown *via* ^{31}P NMR that the PLP phosphate group is dianionic over the pH range 6 to 10. A chemical mechanism for OASS-A from *Salmonella typhimurium* (Nalabolu *et al.*, 1994) has been proposed in which an active site general base (lysine 42) accepts a proton from the α-amine of the amino acid substrate to facilitate the nucleophilic attack on the C4′ carbon of the internal Schiff base. A proton from the α-carbon of the resulting external Schiff base is abstracted by ε-amino group of the active site lysine in the β-elimination of acetate from O-acetyl-L-serine (OAS). An unprotonated enzyme residue is also required to assist in the elimination of the acetate group when OAS is the amino acid substrate. 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.

OASS-A studies have shown that lysine 42 acts as the general base forms the internal Schiff base with PLP. Cysteine 43 is a potentially important active site residue for the reaction pathway because cysteine 43 has a polar character and will affects a geometry of lysine 42. Although many methods were mobilized to understand the chemical mechanism, little is known about the substrate, cofactor and enzyme functional groups in the active site. In this study we report a variety of spectral experiments with the mutant OASS-A in which serine replaces cysteine 43.

Materials and Methods

Chemicals and enzymes

O-Acetyl-L-serine, L-cysteine, L-serine and DTT were obtained from Sigma. All other reagents and chemicals were obtained from commercial sources and were of the highest quality available. OASS-A is a gene product of the *S. typhimurium* cysK gene, which has been previously isolated and cloned into pRSM40 (Byrne *et al.*, 1988). The serine-43 mutant was constructed by the method described by Colyer and Kredich (1994). Mutation was confirmed by DNA sequence analysis. Wild type OASS-A was purified by the method of Hara *et al.* (1990) as modified by Tai *et al.* (1993). The purification procedures for the mutant enzyme were essentially the same as those for the wild type enzyme. The enzyme preparation was judged 95% pure on the basis of SDS-PAGE. The protein concentration of the purified enzyme was determined from the enzyme absorbance at 412 nm using an extinction coefficient of 7,600 $M^{-1}\cdot cm^{-1}$ (Kredich *et al.*, 1969; Cook *et al.*, 1992). The activity of the enzyme is stable over the pH range of 5.5 to 10 (Hara *et al.*, 1990).

Enzyme assay

The disappearance of 5-thio-2-nitrobenzoate (TNB) as an alternative substrate was monitored at 412 nm, resulting from formation of S-(3-carboxy-4-nitrophenyl)-L-cysteine. Initial velocity data were collected using a Gilford 2600 spectrophotometer connected to a flatbed plotter. The pH of the reaction mixtures were determined with a Radiometer PHM 82 pH meter with a combined microelectrode before and after sufficient data were collected for determination of initial velocities. All experiments were carried out at 25℃ using a circulating water bath to maintain a constant temperature of the thermospacers in the cell compartment. Data were collected using a 1 cm light path cuvette. A typical assay in a final volume of 1 ml contained the following: Hepes, pH 7, 100 mM; OAS, 2 mM; TNB, 0.05 mM. The reaction was initiated with OASS. Initial rates were calculated using an ε_{412} of 13600 $M^{-1}\cdot cm^{-1}$ for TNB (Ellman, 1959). The initial velocity pattern was obtained by varying one reactant over a range of concentrations less than K_m and several fixed concentrations of the second reactant.

UV-Vis spectral studies

Absorbance spectra were measured on a Hewlett-Packard Model 8452A photodiode array spectrophotometer recording the absorbance at wavelengths from 240 to 540 nm using a light path of 1 cm. Measurements were made using 100 mM Mes for pH 6.5 and 100 mM Ches for pH 9.0. A typical assay consisted of 0.74 mg C43S, to which the amino acid was added. Each addition was performed in a separate cuvette and the absorbance spectrum of each reaction mixture was measured.

Fluorescence spectral studies

Fluorescence spectra were acquired on a Shimadzu RF5000U spectrofluorometer equipped with a water-jacketed sample compartment for maintenance of temperature in the sample cuvettes at 25℃. Spectra were taken of dilute samples contained in quartz cuvettes that can hold volumes as great as 4 ml. Excitation and emission slit widths were 5 nm for both emission and excitation spectra. Spectra of sample blanks, i.e., samples that contained all components except enzyme, were taken immediately prior to spectra of samples containing enzyme. Blank spectra were then subtracted from spectra of samples containing enzyme. Concentrations of solutes, substrates and ligands were chosen so as to minimize inner filter effects.

Circular dichroism spectral studies

Circular dichroic spectra were acquired at 25℃ on an Aviv 62DS circular dichroism spectrometer. Samples were contained in a quartz cuvette able to hold a volume as great as 0.5 ml. All far UV spectra were scan-

ned from 250 to 190 nm with a protein concentration of 70 mg/ml at intervals of 1nm with a 1.5 nm slit width and a 3s dwell time. All visible spectra were scanned from 550 to 300 nm with a protein concentration of 1.0 mg/ml at intervals of 2 nm with a 1.5 nm slit width and a 3s dwell time. The sample buffer was 20 mM KH_2PO_4, pH 6.5. Each spectrum is an average of three scans. The sample spectra were corrected by subtracting the appropriate buffer blanks. The digital data for the corrected far UV spectra were converted to mean residue ellipticity according to Eq. (1).

$$[q]=[q]_{obs}/10\{MRC\}1 \quad (1)$$

where [q] is the mean residue ellipticity in (deg)(cm^2)/dmol, $[q]_{obs}$ is ellipticity recorded by the instrument in millidegrees, MRC is the mean residue concentration of the enzyme estimated as the product of the number of amino acid residues and the protein concentration in dmol/ml and 1 is the path length in cm.

Results

UV-Visible spectral studies

The absorbance spectrum of the mutant enzyme (C 43S) was obtained over the wavelength range 280~500 nm. Addition of OAS to C43S results in changes in the absorbance spectrum as shown in Cook *et al.* (1992) (data not shown). The absorbance spectrum of the reaction of L-cysteine with C43S at pH 9.0 is shown in Fig. 1. There is no increase in absorbance at 470 nm or 320 nm, reflecting the elimination of SH- (Cook *et al.*, 1992; Cook and Wedding, 1976), but there is a noticeable shift of the absorbance max from 412 nm to 424 nm. The observed shift in l_{max} represents the formation of an external Schiff base with PLP. The absorbance spectrum of the reaction of serine with C43S at pH 9.0 is shown in Fig. 2. The reaction

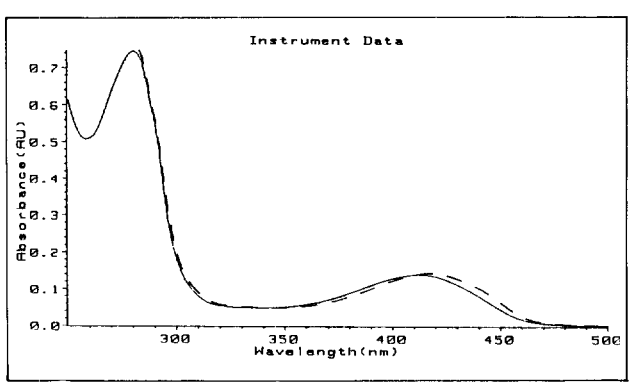

Fig. 1. The UV-Visible spectra of C43S obtained in the presence and absence of L-cysteine at pH 9 in 100 mM Ches. The solid line spectrum was recorded using 0.74 mg/ml C43S, and the dashed line spectrum was obtained following the addition of 3 mM L-cysteine.

of L-serine with C43S results in a decrease in the absorbance at 412 nm with a concomitant increase in absorbance at 320 nm. The observed changes reflect the formation of a geminal diamine intermediate. There is no observable increase in the absorbance at 470 nm indicating the lack of formation of the α-aminoacrylate intermediate resulting from the elimination of the β-hydroxyl group.

Fluorescence spectral studies

The emission spectrum of C43S excited at 298 nm shows two peaks at 340 and 502 nm (Fig. 3). The

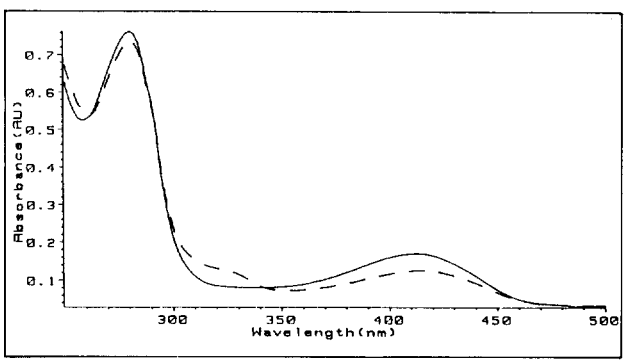

Fig. 2. The UV-Visible spectra of C43S obtained in the presence and absence of L-serine at pH 9 in 100 mM Ches. The solid line spectrum was obtained using 0.74 mg/ml C43S, and the dashed line spectrum was obtained following the addition of 30 mM L-serine.

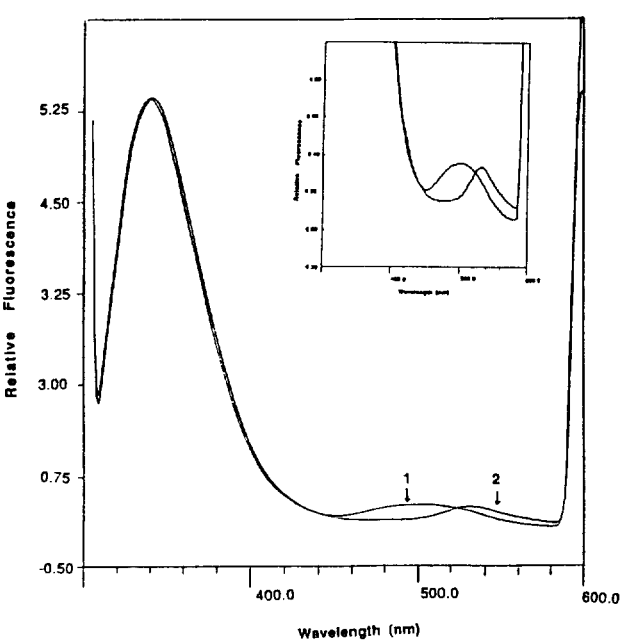

Fig. 3. Fluorescence emission spectra of C43S in the absence and presence of OAS at pH 6.5 in 100 mM phosphate buffer. Spectrum 1 was obtained using 250 μg/3 ml C43S. Spectrum 2 was obtained follwing the addition of 10 mM OAS. Excitation wavelength was 298 nm. The ordinate represents relative fluorescence intensity. While the abscissa represents emission wavelength.

peak of the shorter wavelength emission band at 340 nm is typical of tryptophan residues in proteins (Burstein *et al.*, 1973). The peak of the longer wavelength band at 502 nm is typical of emission from Schiff bases of pyridoxal phosphate. Either peak height or integrated area under the curve may be used as an estimator of the relative fluorescence of two peaks in a spectrum. The ratio of the height of the shorter wavelength peak to the height of the longer wavelength peak of C43S emission is 16.9. Addition of OAS to the enzyme results in a blue shift of the long wavelength band from a λ_{max} of 502 to 532 nm with no significant change in the 340 nm peak. Addition of L-serine or L-cysteine to C43S gives an identical blue shift in the λ_{max} of the long wavelength band as observed for the native enzyme (data not shown).

Circular dichroism spectroscopy

The far-UV spectrum for C43S exhibits a broad through from 225 to 205 nm with no significant difference in the presence and absence of OAS (data not shown). Also, no significant difference in the far UV CD spectrum is observed upon addition of either L-serine or L-cysteine (data not shown). The visible absorption spectrum for wild type enzyme exhibits a positive Cotton effect centered at 412 nm, as has been found for many PLP enzymes including D-serine dehydratase (Marceau *et al.*, 1988), which catalyze reactions very similar to that catalyzed by OASS-A. The induced CD spectra of the PLP cofactor has been obtained in the absence and presence of OAS, cysteine, and serine (Fig. 4). C43S displays a positive Cotton effect at 408 nm, identical with the wild type enzyme, but its molar ellipticity value was about 50% less compared to the wild type. Addition of L-cysteine to C43S gives a wavelength and intensity similar to the wild type. The presence of L-serine results in a similar wavelength, but with an intensity 50% that of C43S alone, negative a cotton effect at 500 nm and positive Cotton effect at 570 nm. The presence of OAS results in a negative Cotton effect at 470 nm, 395 nm and 540 nm.

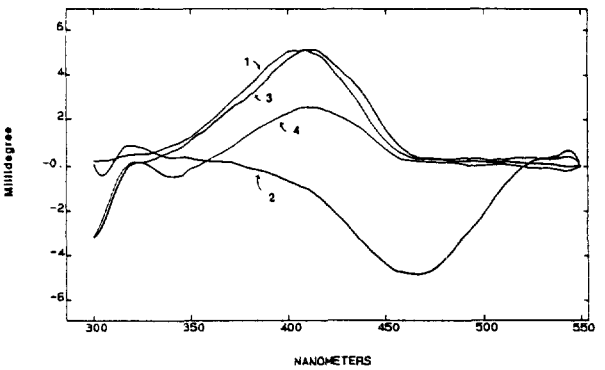

Fig. 4. Visible circular dichroic spectra of C43S in the absence and presence of OAS, L-serine and L-cysteine. Spectrum 1 was obtained using 75 μg C43S at pH 6.5 in 100 phosphate buffer. Spectrum 2 was obtained the addition of 10 mM OAS at pH 6.5 in 100 mM phosphate buffer. Spectrum 3 was obtained the addition of 1.5 mM L-cysteine at pH 9.0 in 100 mM boric acid. Spectrum 4 was obtained the addition of 15 mM L-Serine at pH 9.0 in 100 mM boric acid.

Disscusion

The protonated internal Schiff base of PLP and the ε-amino group of a lysine 42 of OASS in the presence of OAS undergo a transaldimination reaction to generate the external the Schiff base of OAS and PLP. The ε-aminoacrylate in Schiff base with the active site PLP is produced from the external Schiff base, and acetate is released to complete the first half of the reaction (Cook and Wedding, 1976; Nalabolu *et al.*, 1994). Cysteine 43, which is located next to lysine 42, may affect Shiff base formation of lysine 42 with PLP. The replacement of cysteine 43 by serine has been carried out to further understand the reaction pathway of OASS.

The spectrum of OASS-A shows a λ_{max} at 412 nm representing a hydrogen-bonded species in which the proton donor is the protonated Schiff base and the proton acceptor is the ionized phenolic oxygen at C-3 of PLP (Kallen *et al.*, 1985). The λ_{max} at 310 nm represents the unprotonated imine nitrogen for the internal aldimine (Cook *et al.*, 1992). The C43S enzyme in which cysteine 43 is replaced by serine displays the same UV-visible spectrum as wild type enzyme. The λ_{max} at 470 nm observed in the presence of OAS represents a hydrogen-bonded species in which the proton donor is the protonated form of the Schiff base of the α-amino of aminoacrylate intermediate and the proton acceptor is the ionized phenolic oxygen at C-3 of PLP (Cook and Wedding, 1976; Schnakerz *et al.*, 1979). In the presence of OAS, C43S displays the same spectrum as the wild type enzyme, indicating C43 S has the ability to convert OAS to the α-aminoacrylate Schiff base intermediate. The observed changes in absorbance at 470 nm at different concentrations of OAS were used to calculate a K_d of 3 mM for OAS at pH 6.9 with the wild type enzyme (Cook *et al.*, 1992). As the pH decreases the K_d increases an order of magnitude per pH unit. However, for C43S even at pH 6.5 the deacetylase activity is much higher than for wild type enzyme (i.e. disappearance at A_{470} vs. t).

Reaction of L-cysteine with C43S at pH 9 gives a slight shift in a λ_{max} from 412 to 424 nm same as wild type enzyme (schnackerz *et al.*, 1995).

Spectral data obtained with OASS-A and L-cysteine

are consistent with the formation of an external Schiff base with cysteine. No increase in the absorbance at 470 nm is observed, indicating the α-aminoacrylate intermediate is not formed, that is, the second half reaction catalyzed by OASS-A is for all intents and purposes, irreversible. Qualitatively identical results were obtained with OASS-A. Reaction of serine with C43S at pH 9 results in a decrease in the absorbance at 412 nm by about 73.5%. The latter results is the higher absorbance than wild type (62.5%).

Quantitatively different results were obtained with OASS-A. In addition, an increase in absorbance at 320 nm is observed, suggesting the presence of a gem-diamine intermediate. A gem-diamine intermediate has been identified in the serine hydroxymethyltransferase reaction that absorbs maximally at 343 nm (Schiirch, 1982).

McClure and Cook (1994) demonstrated that excitation of OASS at 290 nm gave emission bands at 337 nm due to intrinsic tryptophan fluorescence and at 500 nm attributed to energy transfer from an active site tryptophan to the protonated internal Schiff base. Peak heights are used as an estimator of the relative fluorescence of the two peaks in the spectrum. For C43S the ratio of emission at 340 to 502 nm is 16.9. This ratio is higher than that (8.8) observed in the wild type in the absence of substrates (McClure and Cook, 1994). By comparison, then, the PLP of C43S displays lower fluorescence. There is little change in the emission at 340 nm upon the binding of the substrate OAS. Since the 502 nm emission in holoenzyme appears to be the result of excitation of the PLP in the Schiff base with the active site lysine, the disappearance of this emission upon addition of OAS to the enzyme is expected. The kinetic mechanism for OASS-A proceeds *via* a ping pong kinetic mechanism in which OAS and the PLP Schiff base are converted to acetate and α-aminoacrylate in Schiff base with the active site PLP during the course of the first half reaction (Cook and Wedding, 1976; Tai *et al.*, 1993). The addition of OAS to enzyme causes the 412 nm absorbance of the PLP Schiff base to disappear and two new bands at 320 and 470 nm to appear. That is, the 412 nm absorbance band of the PLP Schiff base, which emits maximally at 502 nm, is no longer present when OAS is added to the enzyme. On the other hand, the 470 nm absorbance band, which is from the α-aminoacrylate intermediate, emits maximally at 532 nm.

The far UV circular dichroic spectrum for C43S displays no significant change in the presence and absence of OAS. By this overall similarity of the circular dichroic spectrum, addition of OAS to C43S appears to proceed without major reorganization of the secondary structure of C43S. This result indicates that the C43S mutant enzyme shows no gross change in protein structure. The visible circular dichroic spectrum for C43S shows a positive Cotton effect at 408 nm, the same as the wild type enzyme, but the molar ellipicity value is about 50% less than the wild type enzyme. The reduced cotton effect indicates that binding of the cofactor is less asymmetric. Addition of cysteine to C43S shows a shift to higher wavelength with no change in intensity similar to the wild type enzyme. These data suggest there are no significant conformational changes in the active site upon binding of L-cysteine to C43S. As has been shown for the addition of L-cysteine, addition of L-serine gives the same λ_{max} of maximal ellipticity. The molar ellipticity of the external Schiff base is reduced to about 50% compared to C43S. These data are also in agreement with a conformational change localized in the active site upon binding L-cysteine to C43S. In addition, there is the appearance of a second cofactor band located at 320nm attributed to the gem-diamine (Schnakerz *et al.*, 1994). In contrast, the presence of OAS results in a similar, but not identical Cotton effects as the wild type reactants. OAS causes a change in sign of the Cotton effect centered around the visible absorption band of the α-aminoacrylate intermediate in 470 nm, as is found in the UV-visible absorption spectrum of wild type enzyme.

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