Cysteine 42 Is Important for Maintaining an Integral Active Site for *O*-Acetylserine Sulfhydrylase Resulting in the Stabilization of the α-Aminoacrylate Intermediate†

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ABSTRACT: *O*-Acetylserine sulfhydrylase-A (OASS-A) is a pyridoxal 5′-phosphate (PLP) dependent enzyme from *Salmonella typhimurium* that catalyzes the β-replacement of acetate in *O*-acetyl-L-serine (OAS) by sulfide to give L-cysteine. The reaction occurs via a ping-pong kinetic mechanism in which α-aminoacrylate in Schiff base with the active site PLP is an intermediate [Cook, P. F., Hara, S., Nalabolu, S. R., and Schnackerz, K. D. (1992) *Biochemistry 31,* 2298−2303]. The sequence around the Schiff base lysine (K41) has been determined [Rege, V. D., Kredich, N. M., Tai, C.-H., Karsten, W. E., Schnackerz, K. D., & Cook, P. F. (1996) *Biochemistry 35,* 13485−13493], and the sole cysteine in the primary structure is immediately C-terminal to the lysine. In an effort to assess the role of C42, it has been changed to serine and alanine by site-directed mutagenesis. The mutant proteins are structurally nearly identical to the wild-type enzyme on the basis of UV−visible, fluorescence, far-UV and cofactor-induced CD, and ^{31}P NMR studies, but subtle structural differences are noted. Kinetic properties of both mutant proteins differ significantly from those of the wild-type enzyme. The C42S mutant exhibits a >50-fold increase in the OAS:acetate lyase activity and a 17-fold decrease in *V* for the cysteine synthesis compared to the wild-type enzyme, while decreases of >200-fold in the OAS:acetate lyase activity and a 30-fold decrease in *V* for the cysteine synthesis are found for the C42A mutant enzyme. In both cases, however, the pH dependence of kinetic parameters for cysteine synthesis and OAS:acetate lyase activity yield, within error, identical p*K* values. In the three-dimensional structure of OASS-A, cysteine 42 is located behind the cofactor, pointing away from the active site, toward the interior of the protein. The dramatic change in the OAS:acetate lyase activity of OASS-A in the C42S and C42A mutant proteins likely results from a localized movement of the serine hydroxyl (compared to the cysteine thiol) toward additional hydrophilic, hydrogen-bonding groups in C42S, or away from hydrophilic groups for C42A, repositioning structure around and including K41. Subtle movement of the ε-amino group of K41 may change the geometry for nucleophilic displacement of the amino acid from PLP, leading to changes in overall activity and stability of the α-aminoacrylate intermediate. Data indicate that single amino acid substitutions that yield only subtle changes in structure can produce large differences in reaction rates and overall mechanism.

The biosynthesis of L-cysteine in *Salmonella typhimurium* proceeds via the acetylation of L-serine catalyzed by serine transacetylase. *O*-Acetyl-L-serine is formed and converted in the presence of sulfide to L-cysteine by *O*-acetylserine sulfhydrylase (OASS;[1] EC 4.2.99.8). *O*-Acetylserine sulfhydrylase consists of two identical subunits each with 1 mol of PLP covalently bound to the protein in a Schiff base linkage (*1*). The native protein has a molecular mass of 68.9 kDa (*2*). As isolated, OASS has a visible λ_{max} of 412 nm, while the presence of OAS results in the formation of an α-aminoacrylate intermediate absorbing at 330 and 470 nm (*3*).

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[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TNB, 5-thio-2-nitrobenzoate; OAS, *O*-acetyl-L-serine; OASS, *O*-acetylserine sulfhydrylase; PLP, pyridoxal 5′-phosphate; *S*-CNP-L-cysteine, *S*-(3-carboxy-4-nitrophenyl)-L-cysteine.

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The kinetic mechanism of the sulfhydrylase is predominantly ping-pong (*4*, *5*). In the first half-reaction, OAS combines with the pyridoxal form of the enzyme to produce α-aminoacrylate in Schiff base linkage with the active site PLP, and sulfide adds to α-aminoacrylate to give cysteine in the second half-reaction (*1*, *3*, *4*).

The α-aminoacrylate intermediate is stable over the steady-state time scale but in the absence of sulfide does slowly disappear in a first-order process regenerating active free enzyme, pyruvate, and ammonia. The OASS-A thus has a OAS:acetate lyase activity (*3*). The OAS:acetate lyase activity is pH dependent, decreasing as the pH decreases, giving a p*K* of 8.2 assigned to the lysine that originally formed the internal Schiff base with PLP. The unprotonated form of the lysine displaces α-aminoacrylate by transimination, regenerating free enzyme and α-aminoacrylate, which decomposes nonenzymatically to give pyruvate and ammonia. The pH-independent first-order rate constant for the OAS:acetate lyase reaction is about 0.1 s^{-1}, 3 orders of magnitude lower than the turnover number for the conversion of OAS and sulfide to cysteine.

More recently, the active site lysine was identified as K41 by reduction of the internal Schiff base with borotritide, proteolysis, isolation, and sequencing of the radiolabeled peptide and by site-directed mutagenesis, changing K41 to Ala (*6*). The role proposed above for the active site Lys in the OAS:acetate lyase reaction has been confirmed. The sequence around K41 was also of interest in that the only cysteine in the primary sequence of OASS-A occurs immediately C-terminal to the lysine at position 41. In the present study, site-directed mutagenesis was used to change the cysteine to serine and alanine. Utilizing a battery of kinetic and spectral probes, it has been determined that cysteine 42 is required to stabilize the α-aminoacrylate intermediate.

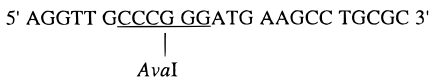
MATERIALS AND METHODS

Chemicals. The buffers Taps, Mops, and Ches were from Sigma, while Hepes and Mes were from Research Organics. L-Cysteine, L-serine, and OAS were obtained from Sigma. All restriction enzymes and T4 DNA ligase were from Promega. A DNA sequencing kit was from USB. *O*-Acetyl-L-serine-2-*d* was prepared according to Hwang et al. (*7*). All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

Enzyme. Cysteine 42 was replaced with serine and alanine using the technique of cassette mutagenesis via PCR. Plasmid pRSM40, containing a 1484 bp *Cla*I−*Dra*I fragment including the cysK coding region and promoter from pRSM28 (*2*) in the pT7T3 19U vector, was used to carry out the mutagenesis. Two *Ava*I sites at positions 108 (CCGAG) and 705 (CCCGGG) are found on either side of the TCG encoding for C42. Oligonucleotide primers were constructed with oligonucleotide 1 (5′ → 3′) containing the mutation and the *Ava*I site at position 108 and oligonucleotide 2 (3′ → 5′) containing the *Ava*I site at position 708. The codon for C42, TGC at positions 127−129, was changed to GCC, and TCC coding for Ala and Ser, respectively. The primers used are as follows compared to the WT sequence. Base substitutions and the *Ava*I site are underlined.

oligonucleotide 1

oligonucleotide 2



All remaining procedures were carried out as described previously (*6*). Mutant proteins were then isolated using the same methods as for the wild-type plasmid-containing strain (*5*, *8*). The cell mass from overnight growths and the amount of mutant proteins obtained were similar to those obtained from wild type.

Enzyme Assays. *O*-Acetylserine sulfhydrylase activity was monitored using TNB as the nucleophilic substrate; the intrinsic absorbance of TNB and its disappearance were monitored continually at 412 nm (ϵ_{412} 13 600 M^{-1} cm^{-1}) (*9*) using a recording spectrophotometer (*5*).

Initial Velocity Studies. Measurement of kinetic parameters was carried out according to Tai et al. (*5*, *10*). All assays were carried out in 100 mM Hepes, pH 7, and 25 °C. The pH dependence of kinetic parameters and the OAS:acetate lyase activity of the mutant enzyme were also carried out as previously described (*3*). Measurements were made using the following buffers at 100 mM concentration for the pH ranges indicated: Mes, 5.5−6.5; Mops, 7.0−7.5; Hepes, 8.0; Taps, 9.0; Ches, 10.0. All assay buffers used in these studies were titrated with KOH. The pH of the reaction was confirmed by measuring the pH of the reaction solution before and after measurements.

Deuterium Isotope Effects. Isotope effects were measured according to Hwang et al. (*7*). The isotope effects were measured in 100 mM Mes at pH 5.5. Nomenclature for isotope effects is that of Northrop (*11*) as modified by Cook and Cleland (*12*).

Spectral Measurements. All UV−visible, fluorescence, and CD (far-UV and visible) spectra were collected as previously described at 25 °C (*13*), while NMR spectra were measured at 20 °C according to Schnackerz and Cook (*14*). Specific conditions are provided when necessary in the legends to figures or in the text.

Data Processing. Data were fitted using the FORTRAN programs developed by Cleland (*15*). Individual saturation curves for TNB obtained as a function of pH were fitted using eq 1, while initial velocity patterns conforming to a ping-pong kinetic mechanism were fitted using eq 2, and those that intersect to the left of the *y*-axis were fitted using eq 3. Isotope effect data were fitted using eq 4.

$$v = VA/(K_a + A) \quad (1)$$

$$v = VAB/(K_aB + K_bA + AB) \quad (2)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (3)$$

$$v = VA/(K_a[1 + F_iE_{V/K}] + A[1 + F_iE_V]) \quad (4)$$

In eqs 1−3, v and V are initial and maximum velocities, respectively, A and B are reactant concentrations, and K_a and K_b are Michaelis constants for A and B, respectively. In eq 4, F_i reflects the fractional labeling with deuterium in OAS-2-*d*, while $E_{V/K}$ and E_V represent the isotope effect minus 1 on the kinetic parameters V/K and V, respectively. Data for the first-order plots of OAS:acetate lyase activity were fitted using the equation for a straight line, while the pH dependence of the first-order rate constant and log V/K_{TNB} were fitted using eq 5.

$$\log y = \log(C/[1 + H/K_1]) \quad (5)$$

In eq 5, y is the first-order rate constant for OAS:acetate lyase activity or the V/K for TNB at any pH, while C is its pH-independent value, H is the hydrogen ion concentration, and K_1 is the estimated pK value for a functional group in OASS.

RESULTS

Spectral Properties of the C42 Mutants of OASS-A. The C42 mutants of OASS-A are bright yellow-green in color and exhibit a UV−vis spectrum identical to that of the wild-type OASS-A (data not shown). The λ_{max} for the protonated internal Schiff base band is 412 nm, and the ratios of A_{280}/A_{412} are 3.4 and 4.2 for the C42S and C42A mutants, respectively, and the ϵ_{412} is ca. 7600 M^{-1} cm^{-1} for both proteins. Addition of 1 mM OAS results in no apparent change in the spectrum, but addition of 50 mM OAS gives a decrease in the absorbance at 280 and 412 nm, concomitant with the appearance of new bands at <250, 330, and 470 nm for the C42S mutant enzyme, indicating formation of an α-aminoacrylate intermediate identical to that of wild-type OASS-A (*3*) but requiring much higher concentrations of OAS. An ϵ_{470} of about 11 000 M^{-1} cm^{-1} is estimated for the α-aminoacrylate intermediate. Addition of 80 mM OAS to the C42A mutant enzyme gives changes that are qualitatively identical to those of WT OASS-A (data not shown). However, the long-wavelength band is broad and has a λ_{max} of 458 nm and an ϵ_{458} of 7600 M^{-1} cm^{-1}. Addition of 3 mM L-cysteine or 30 mM L-serine at pH 9 to C42S(or A)-OASS results in changes that are qualitatively identical to those of the wild-type enzyme (data not shown). Cysteine causes a shift in the λ_{max} from 412 to 418 nm, with a ratio for $\epsilon_{418}/\epsilon_{412}$ close to 1. Addition of 30−40 mM L-serine to C42S(or A)-OASS results in a decrease in the absorbance at 412 nm with appearance of new bands at 418 (for C42S) or 416 (for C42A) and 320 nm, respectively (data not shown). In all of the above cases, concentrations of the added amino acids were shown to be saturating.

The fluorescence emission spectra for C42S(or A)-OASS are also similar to those of native enzyme (*16*, *17*). The band for intrinsic tryptophan fluorescence (λ_{max}, 342 nm) and the long-wavelength band resulting from the cofactor (λ_{max} 500 nm) are both observed. The ratio of F_{342}/F_{500} is about 6 for C42S and 9 for C42A mutant proteins. Addition of 3−5 mM cysteine at pH 9 results in a blue shift of the λ_{max} and an enhancement of the long-wavelength band (λ_{max} 490 nm) such that F_{342}/F_{490} is 1.1 and 2.7 for C42S and C42A mutant enzymes, respectively. Addition of L-serine (30 mM) at pH 9 to the C42S mutant enzyme gives results similar to those obtained with L-cysteine with a F_{342}/F_{490} of 3. Addition of 50−80 mM OAS results in a disappearance of the long-

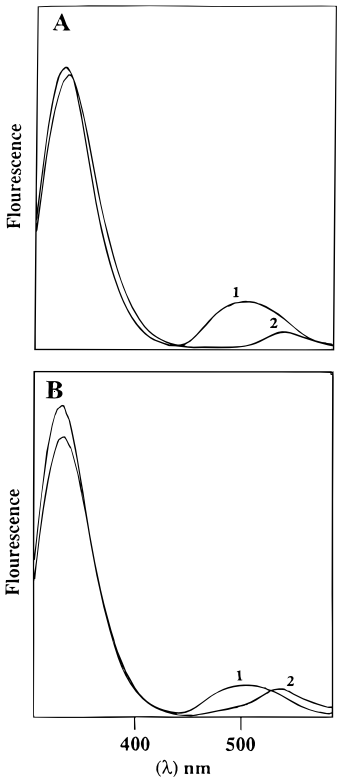


FIGURE 1: Fluorescence emission spectra of the C42 mutant enzymes. Fluorescence spectra of C42S-OASS (A) and C42A-OASS (B) were measured at 25 °C in the absence (1) and presence (2) of OAS (50 mM C42S; 80 mM C42A). Excitation was at 298 nm, and the ordinate is relative fluorescence.

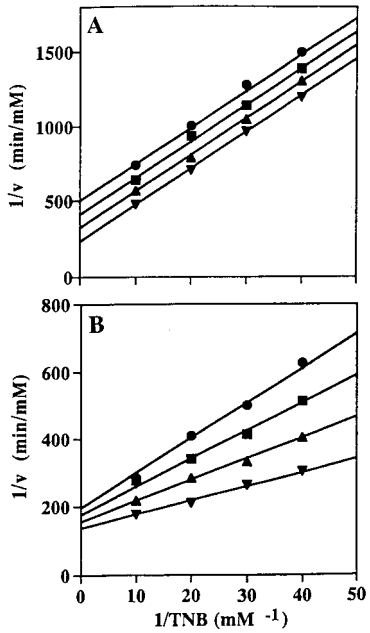


FIGURE 2: Initial velocity pattern for C42S-OASS. Data were obtained at 25 °C by varying the concentration of TNB as indicated at each of the following OAS concentrations: pH 5.5, 2 mM (●), 2.5 mM (■), 3.33 mM (▲), and 5 mM (▼); pH 7, 5 mM (●), 6.67 mM (■), 10 mM (▲), and 20 mM (▼). The following pH values were used: (A) pH 5.5, 100 mM Mes; (B) pH 7, 100 mM Hepes. The points are experimental, while lines are theoretical from a fit of eq 3 (B) or eq 2 (A) to the data.

Table 1: Kinetic Parameters for Wild-Type and C42 Mutant OASS-A

parameter	wild type[a]	C42S[a]	C42A[b]
		OAS/TNB	
K_{OAS} (mM)	15 ± 3	3.4 ± 0.5	0.4 ± 0.1
K_{TNB} (mM)	0.6 ± 0.1	0.018 ± 0.002	0.9 ± 0.2
V/E_t (s^{-1})	0.56 ± 0.08	0.032 ± 0.001	0.018 ± 0.004
$V/(K_{OAS}E_t)$ (M^{-1} s^{-1})	37 ± 5	9 ± 1	43 ± 3
$V/(K_{TNB}E_t)$ (M^{-1} s^{-1})	950 ± 55	1900 ± 200	20.9 ± 0.3
		OAS/Sulfide	
$V/(K_{OAS}E_t)$ (M^{-1} s^{-1})	(1.4 ± 0.7) × 10^5	(1.4 ± 0.1) × 10^3	
$V/(K_{S^{2-}}E_t)$ (M^{-1} s^{-1})	(2.4 ± 1.8) × 10^7	(1.44 ± 0.07) × 10^5	

[a] Parameters were obtained at pH 7, 100 mM Hepes. [b] Parameters were obtained at pH 7, 100 mM Mes.

wavelength band (λ_{max} 500 nm) with the appearance of a new band at 532 nm with a F_{342}/F_{532} of about 16 for C42S and about 10 for C42A mutant proteins (Figure 1).

The far-UV CD spectra of the two mutant enzymes and that of native OASS (*16*) are superimposable over the wavelength range 200−250 nm, indicating that no gross structural changes have occurred as a result of the C42S and C42A mutations (data not shown). The induced CD spectra of the C42S(or C42A)-OASS alone and in the presence of amino acids are also essentially identical to those observed for the wild-type enzyme (*13*), indicating that the orientation of the bound cofactor has not changed in the mutant proteins compared to WT OASS.

The ^{31}P NMR spectra of native C42S-OASS in the presence or absence of OAS exhibit a single pH-independent resonance at 5.2 ppm, over the pH range 6.1−8 (data not shown), identical to those of the wild-type enzyme (*3*); an identical ^{31}P signal is observed at pH 6.6 for C42A-OASS. In the presence of L-cysteine, the C42S and C42A mutants give chemical shifts at 5.4 and 5.3 ppm, respectively, compared to a value of 5.3 ppm observed for the WT OASS (*13*). The presence of 100 mM L-serine gives a chemical shift at 4.2 ppm for the C42S mutant enzyme, identical to that observed for the WT OASS (*13*).

Initial Velocity Studies. The initial velocity pattern obtained for C42S-OASS depends on the pH at which it is measured. At pH values from 5.5, a series of parallel lines were observed (Figure 2A), indicating a ping-pong kinetic mechanism. At pH values of 6 and above, the initial velocity pattern intersects to the left of the ordinate (Figure 2B), suggestive of a sequential kinetic mechanism. The initial velocity pattern for C42A-OASS, measured at pH 6.5, exhibits a series of parallel lines indicating a ping-pong kinetic mechanism, which is similar to WT OASS. Estimated kinetic parameters are provided in Table 1.

pH Dependence of Kinetic Parameters. The pH dependence of V/K_{OAS} appears to be qualitatively similar to that of WT OASS (data not shown) but is difficult to measure because of the high K_{OAS} and ϵ_{412} for TNB. The V/K_{TNB} is pH-dependent and very similar to the profile observed for the wild-type enzyme (Figure 3), giving a pK value of about 6.5. There is some scatter in the data as a result of the high substrate K_m values and an inability to achieve saturation, but the pH-dependent trend in the data is consistent with the data obtained for the wild-type enzyme. The pH-independent value of $V/(K_{TNB}E_t)$ is 3000 ± 400 M^{-1} s^{-1}.

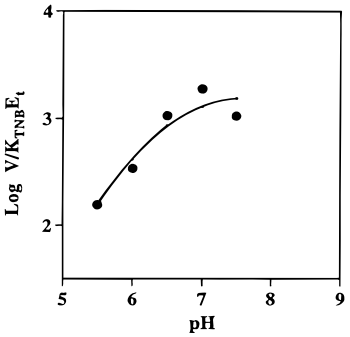


FIGURE 3: pH dependence of V/K_{TNB} for C42S-OASS. V/K_{TNB} was obtained at 25 °C by varying the concentration of TNB with OAS fixed at 50 mM, at each of the pH values indicated. The points are experimental, while the line is theoretical from a fit of eq 5 to the data.

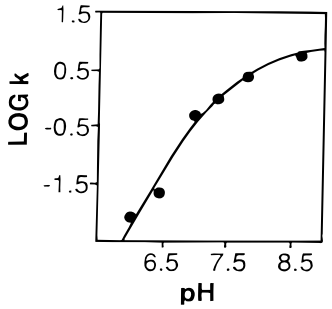


FIGURE 4: pH dependence of the first-order rate constant for the OAS:acetate lyase activity of C42S-OASS. Activity was obtained with 10.8 μM enzyme and 12 μM OAS at 25 °C. The points are experimental, while the curve is theoretical based on a fit using eq 5.

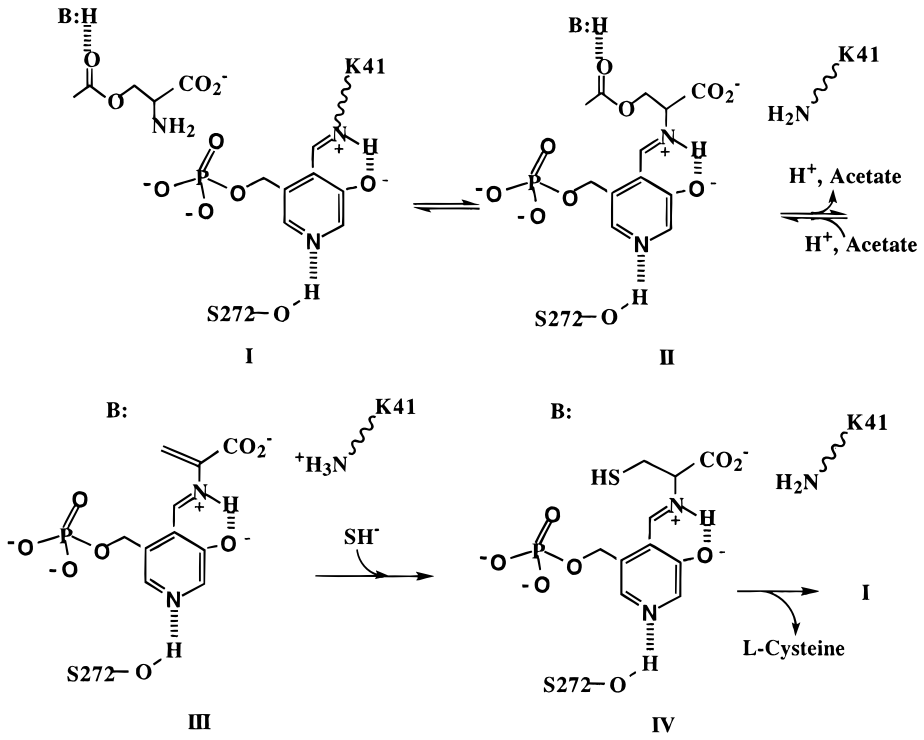
Deuterium Isotope Effects. The primary deuterium isotope effect on V/K_{OAS} was obtained for both C42S and C42A mutant enzymes using OAS-2-*d*, stereospecifically deuteriated on the α-carbon (*7*). Since C42S-OASS adheres to a ping-pong kinetic mechanism below pH 5.5, a $^D(V/K_{OAS})$ of 1.55 ± 0.05 was measured at pH 5.5. A $^D(V/K_{OAS})$ of 1.4 ± 0.3 was also observed at pH 6.5 for C42A-OASS.

*O-Acetyl-*L*-serine:Acetic Acid Lyase Activity*. The pH dependence of the first-order rate constant for the C42S-OASS-catalyzed OAS:acetate lyase activity is shown in Figure 4. The plot exhibits a limiting slope of 1 at low pH, giving an estimated pK of 8.2, and a pH-independent value of about 5 s^{-1} for the first-order rate constant. The OAS:acetate lyase activity of C42S-OASS was estimated to be about 3 × 10^{-4} s^{-1} at pH 7.5.

DISCUSSION

Spectral Properties. The UV−visible, fluorescence, far-UV and visible CD, and ^{31}P NMR spectra of the C42S mutant enzyme in the absence and presence of amino acid analogues are nearly identical to those of the wild-type enzyme (*3*, *13*, *16*). The only differences noted are concerned with the extinction coefficient of the α-aminoacrylate intermediate, 11 000 M^{-1} cm^{-1}, compared to a value of 9800 M^{-1} cm^{-1} for the native enzyme (*3*). In addition, the fluorescence spectrum in the presence of OAS gives a low-intensity 532 nm band which has not been reported previously. However, a recent extension of previous work indicates the presence

Scheme 1: Proposed Chemical Mechanism for OASS-A



of a 532 nm band for native enzyme in the presence of OAS (*18*). Thus, changing C42 to a serine has not affected the structure of the protein or the bound cofactor significantly nor has it affected the ability of the enzyme to produce the α-aminoacrylate intermediate or the external Schiff bases of L-serine or L-cysteine.

Replacement of the side-chain thiol of C42 with a proton (C42A) does not significantly change the gross structure of the enzyme, as shown by all of the spectral properties of the free enzyme. There is, however, a slight decrease in the fluorescence quantum yield of the 500 nm band and a slight decrease in the molar ellipticity at 412 nm, suggesting that the cofactor orientation has been altered slightly. Changes do not extend to the 5′-phosphate binding site, since the ^{31}P NMR chemical shift is identical to that of native enzyme. Similar slight quantitative changes are observed in the values of λ_{max}, extinction coefficient, fluorescence quantum yield, and molar ellipticity of the L-serine and L-cysteine external Schiff bases, consistent with a slight change in the orientation of the cofactor. The only significant change is in the extinction coefficient of the α-aminoacrylate intermediate, and this will be discussed further below.

Activity Studies. Although only slight changes are observed in almost all of the spectral properties of the C42S and C42A mutant proteins, the kinetic properties differ significantly from those of the WT enzyme. In the case of the C42S mutant enzyme, the pH dependence of kinetic parameters and the OAS:acetate lyase activity yield, within error, identical p*K* values. However, initial velocity patterns are intersecting at pH values higher than 5.5, and the absolute values of the kinetic parameters differ compared to the WT OASS. The pH-independent value of the OAS:acetate lyase reaction is 0.07 s^{-1} for the WT enzyme (*3*). An increase in the pH-independent value of the OAS:acetate lyase activity of more than 50-fold (5 s^{-1}) is observed for the C42S mutant enzyme, while a decrease of more than 200-fold (∼0.0003 s^{-1}) is found for the C42A mutant enzyme.

Interpretation of the V and V/K pH Profiles for C42S-OASS. On the basis of the pH dependence of kinetic parameters (*10*), pre-steady-state studies (*19*), spectral properties (*3*, *13*), and the crystal structure (*20*), the mechanism shown in Scheme 1 has been proposed. At the beginning of the first half-reaction, an enzyme side chain must be protonated and may interact with the OAS side chain as shown or play a structural role (**I** in Scheme 1). (The same group is observed in the second half-reaction but in the opposite protonation state.) The substrate OAS binds as the monoanion, i.e., with its α-amino unprotonated to attack the Schiff base carbon and generate the external Schiff base (**II** in Scheme 1). A second group is required protonated to start the second half-reaction and must be unprotonated to catalyze the OAS:acetate lyase reaction (**III** in Scheme 1). The latter was attributed to the ϵ-amino group of K41, and this was proven using site-directed mutagenesis, since the K41A mutant will give the external Schiff base, but not the α-aminoacrylate intermediate, unless small primary amines are added (*6*). Attack by sulfide gives the cysteine external Schiff base (**IV** in Scheme 1), followed by displacement of cysteine and rebinding of OAS to give **I** in Scheme 1.

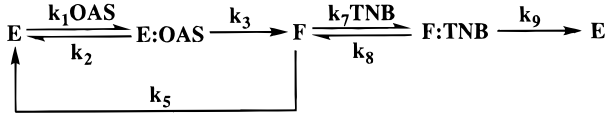
The V_{max} profile represents the p*K* values of groups on the enzyme in the rate-limiting half-reaction(s) when reactants are saturating. In the case of C42S-OASS, the V_{max} pH profile is pH-independent from 5.5 to 7.5 and thus provides little information.

With TNB as the nucleophilic substrate the V/K_{OAS} and V/K_{TNB} pH profiles are similar to those of WT OASS, indicating that mutation of C42 to S or A does not significantly affect the p*K* values of active site groups involved in catalysis and/or binding. In agreement with this suggestion, the p*K* obtained from the pH dependence of the

OAS:acetate lyase activity, which reflects the protonation state of K41 once the α-aminoacrylate intermediate has been formed, is also unchanged compared to the wild-type enzyme.

Interpretation of the Intersecting Initial Velocity Pattern for C42S-OASS. The initial velocity pattern for C42S-OASS depends on the pH of the measurement. The initial velocity pattern intersects to the left of the ordinate above pH 7, and intersects on the ordinate between 6 and 7, while at pH 5.5 the pattern is parallel, consistent with the proposed ping-pong kinetic mechanism. The intersecting initial velocity pattern is the result of a rapid OAS:acetate lyase activity for the C42S-OASS relative to the net rate constant for the second half-reaction at limiting concentrations of TNB.

Scheme 2



As described by Cook et al. (*21*), the initial velocity pattern obtained in the absence of added inhibitors will intersect to the left of the ordinate, as a result of an unstable F, mimicking a sequential kinetic mechanism. Consider the mechanism in Scheme 2, where the intermediate F is converted to E at a rate within the time scale of the steady-state assay. Expressions for the kinetic parameters (V/E_t), $(V/(K_aE_t))'$, $V/(K_bE_t))'$, and $V/(K_{ia}K_bE_t)$ are given in eqs 6−11. (The prime is used to distinguish the rate constants obtained in the mechanism derived from Scheme 1 from a normal ping-pong kinetic mechanism.)

$$(V/E_t)' = k_3k_9/(k_3 + k_9) \quad (6)$$

$$(V/(K_aE_t))' = k_1k_3/(k_2 + k_3) \quad (7)$$

$$(V/(K_bE_t))' = k_3k_7k_9/(k_8 + k_9)(k_3 + k_5) \quad (8)$$

$$V/(K_{ia}K_bE_t) = k_1k_3k_7k_9/k_5(k_8 + k_9)(k_2 + k_3) \quad (9)$$

$$(K_a)' = K_{ia}(1 + k_3/k_2)/(1 + k_3/k_9) \quad (10)$$

$$(K_b)' = K_{ib}[(1 + k_9/k_8)/(1 + k_3/k_9)](k_3 + k_5)/k_9 \quad (11)$$

The rate constant k_5 for C42S-OASS decreases below a pK of 8.2. At pH 5.5, k_5 is no longer significant with respect to the rate of the second half-reaction. At pH values between 5.5 and 6.5, k_9 likely limits the overall reaction; i.e., $k_9 \ll k_3$. As a result, K_a' approaches zero.

The product of the $(V/K)'$ values divided by $V/(K_{ia}K_bE_t)$ effectively subtracts out the rate of the normal ping-pong reaction giving the maximum rate of the OAS:acetate lyase reaction.

$$V_{\text{OAS:acetate lyase}}/E_t = k_3k_5/(k_3 + k_5) \quad (12)$$

The rate constant k_5, which reflects the OAS:acetate lyase activity, has a pH-independent value of 5 s^{-1}, which would give a value of 0.3 s^{-1} at pH 7. Data would thus suggest that the maximum rate of the OAS:acetate lyase reaction, obtained at limiting TNB concentration, is limited by the rate of breakdown of F (the α-aminoacrylate intermediate) to pyruvate and ammonia, that is, k_5. In agreement with the suggested limitation by k_5, k_3 is estimated at about 1−5 s^{-1} on the basis of the similarity in the values of $V/(K_{OAS}E_t)$ for WT and C42S enzymes (Table 1) and rapid-scanning stopped-flow studies of WT and cofactor-substituted enzymes (*7*, *21*). The latter allows a determination of the rate-limiting step in the overall reaction of OAS and TNB to give acetate and *S*-CNP-L-cysteine, given by eq 6, $k_3k_9/(k_3 + k_9)$, and estimated as 0.03 s^{-1} (Table 1). Since k_3 is ∼1 s^{-1}, k_9 must be 0.03 s^{-1}, the limiting step for the overall reaction, which includes nucleophilic attack by TNB on the α-aminoacrylate intermediate to form the *S*-CNP-cysteine external Schiff base and release of the product amino acid (Scheme 1).

Identification of the rate-limiting step must await further study. On the basis of the above considerations, the V/K for the second half-reaction given by eq 7 above reduces to k_9/K_{iTNB}, where K_{iTNB} is k_8/k_7. Thus, the OAS:acetate lyase activity will be important even at the highest concentrations used in these studies ($5K_{TNB}$), which gives a value of 0.2 s^{-1} compared to the effective value of 0.3 s^{-1} for the OAS:acetate lyase rate.

The above mechanism predicts that V/K_{OAS} for C42S, which has an increased OAS:acetate lyase activity, should be identical to that obtained for the wild-type OASS-A, while the V/K_{TNB} should be decreased (*21*). However, a glance at Table 1 shows the opposite effects, i.e., a 4-fold decreased V/K_{OAS} and a 2-fold increased V/K_{TNB}. Thus, effects other than an increase in the rate of the OAS:acetate lyase activity must result from the C42S mutation. It is likely that the affinity of the C42S mutant enzyme for OAS and TNB has increased compared to that of the wild-type OASS-A. An indication of this is obtained from a comparison of the K_m values for TNB. With the alternative nucleophilic substrate, TNB, a second-order rate constant 10^4-fold lower than that measured for the natural substrate, sulfide, is obtained (Table 1) (*5*). The K_{TNB} value is almost certainly a dissociation constant,[2] and the value of K_{TNB} is decreased (33 ± 6)-fold for the C42S mutant, compared to the wild-type enzyme. Equal decreases in V and V/K_{TNB} are observed for the C42A mutant protein, suggesting a decrease in the rate of the limiting second half-reaction, conversion of the α-aminoacrylate intermediate and TNB to free enzyme and *S*-CNP-L-cysteine.

With sulfide as the nucleophilic substrate, both V/K values are decreased 2 orders of magnitude for C42S, compared to the wild-type enzyme (Table 1). It is not possible to be certain since a value for V could not be obtained for C42S, but data suggest a decrease in the rate of interconversion of binary reactant/product complexes for both half-reactions.

Deuterium Isotope Effects. The primary deuterium isotope effects on V/K_{OAS} are 1.55 and 1.4 for C42S and C42A mutant enzymes, respectively. At pH 5.5, α-proton abstraction is rate-determining for the WT enzyme (*7*). Assuming α-proton abstraction remains rate-determining for the mutant enzymes, the transition state for the concerted α,β-elimination of acetic acid from the OAS external Schiff base has become either earlier or later. Determination of transition state effects in these mutant protins must await further study.

[2] The same cannot be stated for K_{OAS}, since OAS is a "sticky" substrate (*7*).

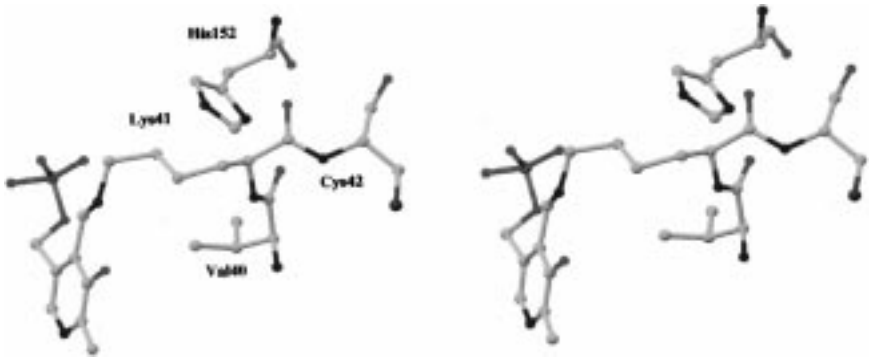


FIGURE 5: Stereoview of the active site of OASS-A viewing the PLP edge-on. The entry to the active site is toward the *re* face of the cofactor to the left of the figure. Ser272 is hydrogen-bonded to N1 of the pyridinium of PLP. Note the thiol of cysteine 42 behind the active site.

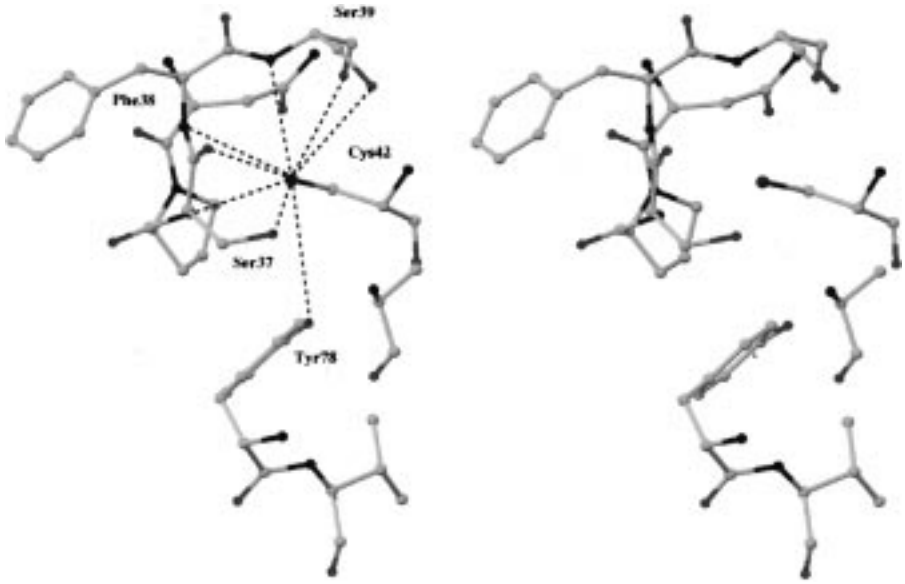


FIGURE 6: Stereoview showing the number of hydrophilic groups around the thiol of cysteine 42 that could serve a potential hydrogen-bonding role. Distances between the sulfur and the hydrophilic groups pictured are provided in Table 3.

Table 2: Active Site Sequence Alignment of OASS-A and -B

species	OASS	active site sequence	ref
Salmonella typhimurium	-A	^{39}SVK**C**RIGA	2
Escherichia coli	-A	^{39}SVK**C**RIGA	2
Triticum aestivum	-A	^{48}SVK**D**RIGY	22
Spinacia oleracea	-A	^{47}SVK**D**RIGF	23
Spinacia oleracea L.	-A	^{106}*SVK**D**RIGY	24
Haemophilus influenzae	-A	^{40}SVK**C**RIGA	25
Arabidopsis thaliana	-A	^{44}SVK**D**RIGF	26
Flavobacterium sp.	-A	^{40}SIK**D**RIAL	27
Capsicum annuum	-A	^{106}SVK**D**RIGF[a]	28
Bacillus subtilis	-A	^{43}SVK**D**RIGL	29
Mycobacterium tuberculosis	-A	^{42}SVK**D**RIGV	30
Zea mays	-A	^{47}SVK**D**RIGY	31
Salmonella typhimurium	-B	^{39}SVK**D**RAAL	32
Escherichia coli	-B	^{39}SVK**D**RAAL	33
Campylobacter jejuni	-B	^{38}SIK**D**RAAF	34
Ralstonia eutropha	-B	^{41}SVK**D**RPAR	35

[a] Precursor sequence.

Structural Information. Cysteine 42 is the sole cysteine in the primary sequence of OASS-A (*2*). It is located immediately C-terminal to the Schiff base lysine. Comparison of the sequence around the Schiff base lysine of OASS-A and -B from different species indicates that either cysteine or aspartate is allowed immediately C-terminal to the active site lysine (Table 2).

Table 3: Distance (Å) between the S of C42 and Neighboring Hydrophilic Functional Groups

γ-O of Ser37	4.18
backbone carbonyl of Ser37	2.79
backbone NH of Ser37	2.95
backbone NH of Phe38	3.55
γ-O of Ser39	4.50
backbone carbonyl of Ser39	3.34
backbone NH of Ser39	3.78
p-hydroxyl of Tyr78	4.85

The three-dimensional structure of OASS-A has recently been solved (*20*). Cysteine 42 is located behind the cofactor, pointing away from the active site, toward the interior of the protein structure (Figure 5). The cysteine is within hydrogen-bonding distance to a number of hydrogen bond donor and acceptor functional groups (Figure 6, Table 3). Table 3 details the close proximity of six potential hydrogen bond donors, hydroxyls of serine and tyrosine and backbone NHs, as well as two carbonyl oxygens, providing a relatively hydrophobic environment. The hydrophilic environment would tend to polarize the soft thiol (or thiolate), allowing a closer approach of the larger sulfur to the hydrogen bond donors. [On the basis of the hydrogen-bonding potential of the carboxylate side chain found in most species (Table 2), data suggest that cysteine (the only allowable replacement)

in the A-isozymes of *S. typhimurium* and *Escherichia coli* may be present as a thiolate, rather than a thiol.]

Conversion of C42 to a serine would result in a localized movement of the serine hydroxyl (compared to the cysteine thiol) to allow better hydrogen-bonding interactions with hydrogen bond donors (for example, hydroxyls and NHs of S37 and S39). The closer interactions result in a repositioning of the main chain and side chains around and including K41, the Schiff base lysine. A subtle movement of the ϵ-amino group of K41 could be thought of as improving the geometry for nucleophilic displacement of α-aminoacrylate from PLP, leading to an overall decreased stability of the intermediate. It is equally likely that conversion of C42 to alanine results in movement of the methyl (compared to the thiomethyl of cysteine or hydroxymethyl of serine) away from hydrophilic groups, hindering the OAS:acetate lyase activity. Data demonstrate how single amino acid substitutions may cause only subtle changes in protein structure but large changes in the efficiency of the enzyme in catalyzing its reaction.

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