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Identification and Spectral Characterization of the External Aldimine of the *O*-Acetylserine Sulfhydrylase Reaction†

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ABSTRACT: The *O*-acetylserine sulfhydrylase (OASS) reaction has been studied using a number of spectral probes including UV−visible, fluorescence, circular dichroism, and ^{31}P NMR spectroscopy. The addition of L-cysteine, L-alanine, and glycine to OASS results in a shift in λ_{max} of 412 nm for the internal Schiff base to 418 nm resulting from the formation of the external Schiff base. The addition of L-serine or *O*-methyl-D,L-serine gives decreases of the absorbance of unliganded enzyme at 412 nm of about 50% and 20%, respectively, concomitant with an increase in the absorbance at 320 nm and a shift in the λ_{max} of the remaining visible absorbance to 418 nm. The spectral shifts observed in the presence of L-serine are suggestive of establishing an equilibrium between different forms of external Schiff base. The concentration dependence of the changes at 440 (L-cysteine) and 320 nm (L-serine) provides an estimate of the dissociation constant for the external aldimine. The pH dependence of the dissociation constant suggests the α-amine of the amino acid must be unprotonated for nucleophilic attack at C4′ of PLP, and an enzyme side chain must be unprotonated to hydrogen-bond the thiol or hydroxyl side chain of the amino acid. When L-cysteine is the amino acid, the thiol side chain must be protonated to hydrogen-bond to the unprotonated enzyme side chain. The ^{31}P NMR chemical shift is increased from 5.2 ppm for unliganded enzyme to 5.3 ppm in the presence of L-cysteine, signaling a tighter interaction at the 5′-phosphate upon formation of the external Schiff base. The ^{31}P NMR chemical shift is decreased to 4.4 ppm in the presence of L-serine, suggesting a looser binding of the 5′-phosphate compared to free enzyme or the external Schiff base with L-cysteine. However, the line width in the latter case is very broad at about 50 Hz, suggesting the presence of more than one species. The spectrum in the presence of L-serine gives a change in the chemical shift to higher field as the temperature decreases. Addition of L-cysteine or L-serine significantly changes the far-UV CD spectrum, likely reflecting the closing of the active site. In addition, an induced dichroism of the PLP cofactor is observed in the visible region of the CD spectrum. Free enzyme gives a positive Cotton effect at 412 nm, while in the presence of L-cysteine or glycine the positive Cotton effect shifts to 418 nm with the same intensity as that observed for free enzyme. The visible CD in the presence of L-serine exhibits positive Cotton effects at 320 and 418 nm, while formation of the α-aminoacrylate intermediate results in a band with a negative Cotton effect centered at 470 nm.

The synthesis of L-cysteine in *Salmonella typhimurium* proceeds via a two-step enzymatic pathway (Kredich & Tomkins, 1966). In the first step, catalyzed by serine transacetylase, the β-hydroxyl group of L-serine is activated for elimination by acetylation using acetyl CoA as the acetyl donor. The *O*-acetyl-L-serine (OAS)[1] intermediate then undergoes a β-substitution reaction catalyzed by the PLP-dependent *O*-acetylserine sulfhydrylase (EC 4.2.99.8) in which acetate is eliminated and replaced by sulfide to give L-cysteine.

A number of PLP enzymes, including OASS, catalyze the nucleophilic displacement of a substituent at the β-position of amino acids. A general mechanism for the PLP-dependent β-replacement reaction is given in Scheme 1 (Miles, 1986). In Scheme 1, a number of the intermediates expected to occur in a PLP-dependent β-elimination reaction are pictured in terms of their structure and spectral properties. Enzymes are isolated with PLP bound in Schiff base linkage to the ε-amino group of an enzymic lysine. The internal aldimine will absorb in the visible region at 400−430 nm if the imine nitrogen is protonated, allowing the formation of an intramolecular hydrogen bond and bringing the π system of the imine into conjugation with the pyridine ring. Two of the predominant resonance forms of the protonated internal

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

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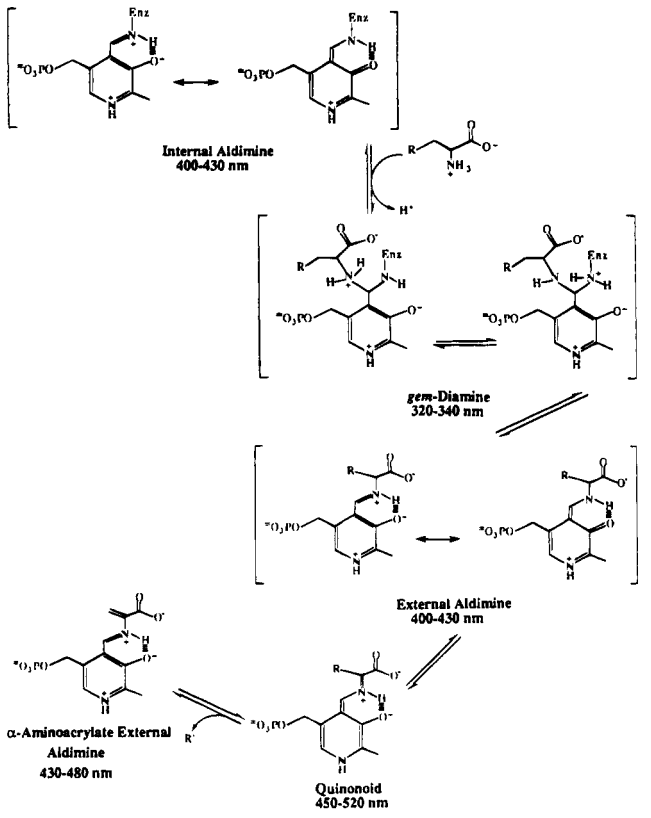
Scheme 1



aldimine are shown. The imine nitrogen may also either be unprotonated or the proton may reside on the phenolic oxygen of PLP, giving absorbance at the lower wavelength of 310−330 nm for the internal aldimine. Addition of the amino acid substrate leads to the formation of a *geminal*-diamine intermediate via nucleophilic attack of the α-amino group of the substrate at C4′ of the PLP imine. Intramolecular proton transfer from the incoming α-amino group to the departing ϵ-amino group must precede collapse of the *gem*-diamine to the external aldimine between PLP and the amino acid substrate. The external aldimine has properties that are similar to the internal aldimine. Abstraction of the α-proton of the external aldimine and delocalization of electrons into the pyridine ring gives a quinonoid intermediate, which absorbs at a wavelength higher than the external aldimine. Formation of the quinonoid intermediate may not be necessary for all reactions depending on the nature of the leaving group in the β-elimination reaction. Elimination of the β-substituent occurs next with the formation of α-aminoacrylate in external aldimine linkage with PLP. The α-aminoacrylate external aldimine has two λ_{max} values in the 320−330 and 450−470 nm regions of the spectrum (Schnackerz et al., 1979). Once elimination of the β-substituent has taken place, another nucleophile attacks at the β-position of α-aminoacrylate to give the new amino acid by reversal of the steps in Scheme 1.

As seen from the above description of the PLP-dependent β-replacement reaction, familiarity with the spectral properties of PLP and its reactions with amines and amino acids is basic to an understanding of the function of the coenzyme. *O*-Acetylserine sulfhydrylase has a prominent absorption band at 412 nm, indicating that the Schiff base between an enzyme lysine and PLP is protonated (Cook et al., 1992); these authors have also shown that the imine remains protonated over the pH range 5−11. Addition of OAS to OASS causes a decrease in the absorbance at 412 nm with a concomitant increase in the absorbance at 330 and 470 nm (Cook & Wedding, 1976; Cook et al., 1992). The model studies of Schnackerz et al. (1979), and the known ping pong nature of the reaction (Cook & Wedding, 1976; Tai et al., 1993) indicate that the species formed upon the addition of OAS is a protonated Schiff base between PLP and α-aminoacrylate formed by elimination of acetate from OAS.

In this study, amino acid substrates and analogs are used in an attempt to stop the OASS reaction at specific points along the reaction pathway. Addition of L-cysteine, L-alanine, or glycine to OASS results in formation of the external Schiff base, while L-serine and *O*-methyl-D,L-serine gives an equilibrium mixture of species that likely represents different forms of the external Schiff base. The above-mentioned intermediates are characterized with respect to their spectral properties using UV−visible, fluorescence, circular dichroism, and ^{31}P NMR spectroscopy, and the equilibria for formation of the intermediates are determined as a function of pH.

MATERIALS AND METHODS

Chemicals. Mops, Taps, Ches, and Caps were from Sigma, while Hepes and Mes were from Research Organics. The analogs L-alanine, D-alanine, L-cysteine, L-serine, D-serine, and *O*-acetyl-L-serine were obtained from Sigma, while glycine and *O*-methyl-D,L-serine were obtained from United States Biochemical. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

Enzyme. *O*-Acetylserine sulfhydrylase-A was prepared from wild-type *Salmonella typhimurium* LT-2 using the method of Hara et al. (1990) adapted to the HPLC (Tai et al., 1993).

UV−Visible Spectral Measurements. All spectra were collected at 25 °C in reaction cuvettes 1 cm in path length and 1 mL in volume. Absorption spectra were measured utilizing a Hewlett Packard 8452A diode array spectrophotometer. All measurements in these studies (unless otherwise indicated) 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; Caps, 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 spectral measurements.

Generally, spectra were measured as a function of pH from 6 to 10. A typical assay consisted of 30 μM OASS-A in the appropriate buffer (see above), 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. In all cases, correction was made for absorbance of the amino acid, particularly below 340 nm. When changes in the baseline absorbance was observed, spectra were corrected using the difference in absorbance at 600 nm.

^{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. Generally, 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 values 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. The standard deviation of the chemical shift values is on the order of 1% (Cook et al., 1992). An estimate of the error on the line width is obtained by using the upper and lower bounds of the baseline compared to the average value used to calculate the line widths. Using this method, a maximum error of 10−20% is estimated, but is likely ≤10%.

Fluorescence Spectroscopy. Fluorescence spectra were taken on a Shimadzu RF 5000U spectrofluorometer equipped with a water-jacketed sample compartment to maintain the temperature in the cuvette at 25 °C. In all cases, excitation was at 298 nm, and the emission monochromator was scanned from 300 to 600 nm. Excitation and emission slit widths were 5 nm. Spectra of blanks, i.e., of samples containing all components except OASS, were taken immediately prior to measurements of samples containing protein. Blank spectra were subtracted from spectra of samples containing enzyme. Ligand and substrate concentrations were chosen to minimize inner filter effects.

Circular Dichroism Spectroscopy. Circular dichroic spectra were recorded using an AVIV 62 DS spectropolarimeter and quartz cuvettes with 0.2 cm path length. The temperature of the cell compartment was maintained constant at 25 °C with a RC-6 Lauda circulating bath. All far-UV spectra were scanned from 260 to 190 nm with a protein concentration of 100 μg/mL at intervals of 1 nm with 1.5 nm slit width and a 3-s dwell time. All visible spectra were scanned from 550 to 300 nm with a protein concentration of 3.2 mg/mL at intervals of 1 nm with 1.5 nm slit width and a 3-s dwell time. The buffer used for far-UV spectra was 20 mM KH_2PO_4, pH 7, while that for visible spectra was 100 mM Hepes, pH 7. Each spectrum reflects an average of three scans. The sample spectra were corrected for the appropriate buffer blanks. The digital data for the corrected far-UV spectra were converted to mean residue ellipticity according to

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

where $[\theta]$ is the mean residue ellipticity (in deg cm^{-2} $dmol^{-1}$), $[\theta]_{obs}$ 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 l is the path length (in centimeters). These molar ellipticity values were used to obtain estimates of secondary structure using the computer program PROSEC (PROtein SECondary structure) supplied by the instrument manufacturer. The program is based on a binary search algorithm and uses the reference spectra of Chang et al. (1978) and estimates α-helix, β-sheet, β-turn, and coil directly from the instrument data. Values for the secondary structural components obtained from PROSEC were verified using a second program BLEND, which combines pure secondary structural components to produce a synthetic spectrum using multiple regression analysis and the same reference spectra used in PROSEC. Spectra thus recorded were superimposable with the experimental spectra in all cases.

Data Processing. Data were fitted using the FORTRAN programs developed by Cleland (1979). The dissociation constant, K_d, for L-serine or *O*-methyl-D,L-serine was determined using eq 2. A is the value of ΔA_{320} at zero X, where X is amino acid concentration and K_{ID} is the dissociation constant. The expression $A(K_{ID}/K_{IN})$ is the value of ΔA_{320} at infinite X, and K_{IN} is a term that causes ΔA_{320} to plateau at a finite value.

$$\Delta A_{320} = A[1 + X/K_{IN}]/[1 + X/K_{ID}] \quad (2)$$

Fractional changes used to determine the dissociation constants for alanine, glycine, or L-cysteine were fitted using eq 3, where Y is the fractional change at any concentration of A, A is the analog concentration, and K_d is the dissociation constant for the analog.

$$Y = A/(K_d + A) \quad (3)$$

The pK values obtained from the pH dependence of the K_d values for L-serine and L-cysteine were from fits using eqs 4 and 5. The value of C is the pH-independent value of $1/K_d$, H is the hydrogen ion concentration, while K_1, K_2, and K_3 are the acid dissociation constants for enzyme or amino acid functional groups.

$$\log (1/K_d) = \log [C/(1 + H/K_1 + H^2/K_1K_2)] \quad (4)$$

$$\log (1/K_d) = \log [C/(1 + H/K_1 + H^2/K_1K_2 + K_3/H)] \quad (5)$$

RESULTS

Reactant and Analog Studies. Absorbance spectra for the reaction of L-cysteine with OASS-A at pH 9.0 are shown in Figure 1A. There is no increase in absorbance at 470 or 320 nm, indicating that no significant elimination of SH to form the α-aminoacrylate intermediate has taken place, and this was confirmed by enzyme assay for sulfide formation (Cook et al., 1992; Cook & Wedding, 1976). Addition of L-cysteine does give a noticeable shift in the visible absorbance maximum from 412 to 418 nm. The observed shift in λ_{max} represents the formation of an external Schiff base with PLP. The fractional changes in absorbance were used to calculate the K_d for external Schiff base formation. Experiments were then repeated as a function of pH. A plot of log $1/K_d$ versus pH (Figure 1B) has limiting slopes of +2 and −1 and yields an average pK value of 7.4 ± 0.2 for the low pH range and a pK of 9.6 ± 0.4 at high pH. The pH-independent value of the L-cysteine dissociation constant is 120 ± 20 μM.

At high pH, the reaction of L-serine with OASS-A results in a decrease in the absorbance at 412 nm with a concomitant increase in absorbance at 320 nm and a shift in the λ_{max} of the remaining absorbance to 418 nm. In Figure 2A, spectrum 1 is measured with 30 μM OASS-A alone, while spectrum 2 reflects 30 μM OASS-A in the presence of 128 mM L-serine. The presence of two new absorption bands, 320 and 418 nm, suggests the establishment of an equilibrium between species. The shift in λ_{max} for the remaining visible absorbance is consistent with the external Schiff base being one of the species present in the equilibrium mixture. There is no observable increase in the absorbance at 470 nm, indicating that, as for L-cysteine, there is a lack of formation

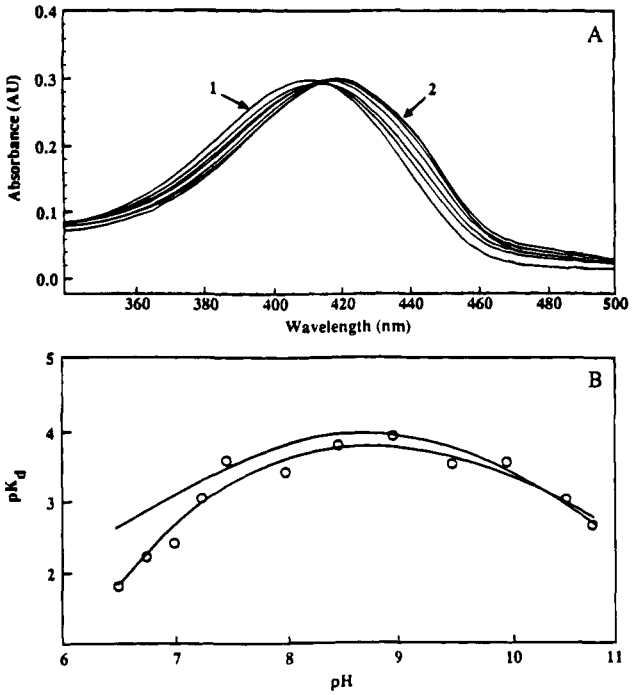


FIGURE 1: (A) Visible absorbance spectrum of *O*-acetylserine sulfhydrylase in the absence and presence of increasing concentrations of L-cysteine. The spectra were obtained at pH 9, 100 mM Ches. The spectrum marked 1 is for free enzyme, while that marked 2 is in the presence of 5 mM L-cysteine. (B) Plot of pK_d obtained from the treatment shown against pH. The points are experimental while the curve is theoretical for a fit using eq 5. The second curve depicts a limiting slope of 1 on the acid side of the profile.

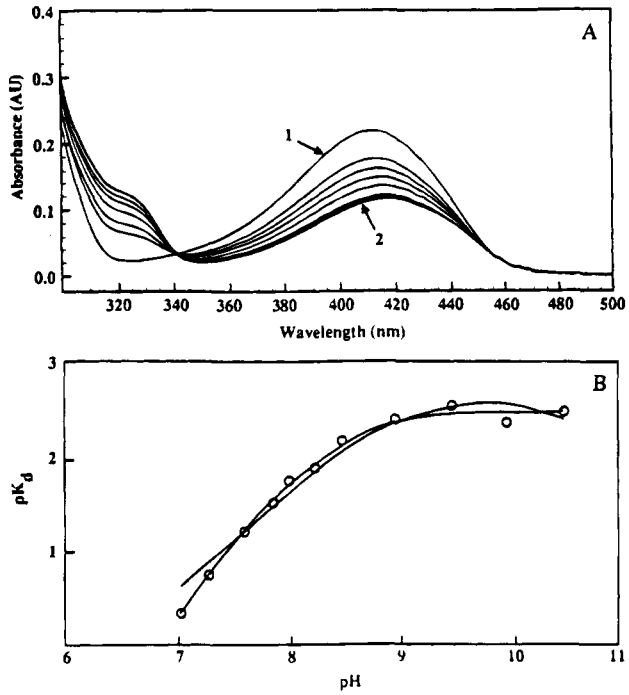


FIGURE 2: (A) Visible absorbance spectrum of OASS in the absence and presence of increasing concentrations of L-serine. The spectra were obtained at pH 10.5, 100 mM Caps. The spectra marked 1 and 2 represent 0 and 128 mM L-serine, respectively. (B) Plot of pK_d obtained from the treatment shown against pH. The points are experimental while the curve is theoretical for a fit using eq 4. The second curve depicts a limiting slope of 1 on the acid side of the profile.

of the α-aminoacrylate intermediate resulting from the elimination of the β-hydroxyl. Fractional changes were used to calculate the K_d, and experiments were then repeated as a function of pH. A plot of log $1/K_d$ versus pH (Figure 2B) has a limiting slope of +2 and yields pK values of 7.6 ± 0.2 and 8.4 ± 0.2. The pH-independent value of the L-serine dissociation constant is 4.2 ± 0.1 mM.

Table 1: Dissociation Constants of External Schiff Base Obtained at pH 9.5

amino acid	K_d (mM)
Obtained at 418 nm	
L-cysteine	0.12 ± 0.02
L-alanine	8 ± 1
D-alanine	160 ± 20
glycine	10 ± 2
Obtained at 320 nm	
L-serine	4.2 ± 0.1
O-methyl-D,L-serine	4.4 ± 0.9

The absorbance spectra of the reaction of OASS-A with L-alanine and glycine at pH 9.5 give spectral changes identical to those observed with L-cysteine (data not shown). The K_d values for dissociation of the external Schiff base obtained for these analogs are 8 ± 1 and 10 ± 2 mM, respectively. D-Alanine also forms an external Schiff base with OASS, but with much less affinity. The K_d for D-alanine is 160 ± 20 mM at pH 9.5 (data not shown). *O*-Methyl-D,L-serine behaves in a manner similar to L-serine, giving a decrease at 412 nm by about 20% concomitant with an increase in the absorbance at 320 nm and a shift in the λ_{max} of the remaining absorbance to 418 nm; the K_d estimated from the 320-nm species at pH 9.5 is 4.4 ± 0.9 mM. The K_d values for all of the amino acid analogs are summarized in Table 1.

^{31}P NMR Spectroscopy. Native OASS-A shows a pH independent ^{31}P NMR signal of the cofactor phosphate group at 5.2 ppm with a line width of 20.5 Hz (Cook et al., 1992). In the presence of 10 mM L-cysteine at pH 9.0, the ^{31}P NMR signal is shifted further downfield to 5.3 ppm, with a line width of 32.5 Hz. In the presence of 36.4 mM L-serine, the ^{31}P NMR signal is shifted upfield to 4.4 ppm (90%) with a small portion (10%) still present at 5.2 ppm, likely representing native OASS (Figure 3). The line width at 20 °C is substantially increased from the value of 20.5 Hz for free enzyme to a value of 50.5 Hz. Addition of OAS does not change the position or line width of the ^{31}P NMR signal of the enzyme (Cook et al., 1992).

To determine whether the broad resonance centered at 4.4 ppm is a result of an equilibrium between two species, the temperature dependence of the ^{31}P NMR spectrum in the presence of L-serine was measured as a function of temperature (Figure 4). The ^{31}P NMR resonance decreases from 4.4 ppm at 20 °C to 4.2 and 3.6 ppm at 15 and 4 °C, respectively. The other resonance, attributed to free enzyme, decreases from 5.2 to 5.0 to 4.8 ppm over the same temperature range. The visible absorbance spectrum of OASS in the presence of 50 mM L-serine at 4 and 15 °C are unchanged from those measured at 20 °C (data not shown).

Fluorescence Spectroscopy. The emission spectrum of native OASS-A exciting at 298 nm exhibits two maxima at 337 and 500 nm, respectively (Figure 5). Addition of L-cysteine causes an enhancement in the intensity of the long wavelength band accompanied by a blue shift in the λ_{max} from 500 to 490 nm with no significant changes in the 337-nm band (McClure & Cook, 1994). The addition of L-serine gives an identical blue shift in the λ_{max} of the long wavelength band, but gives slightly less than half the enhancement found with L-cysteine (Figure 5).

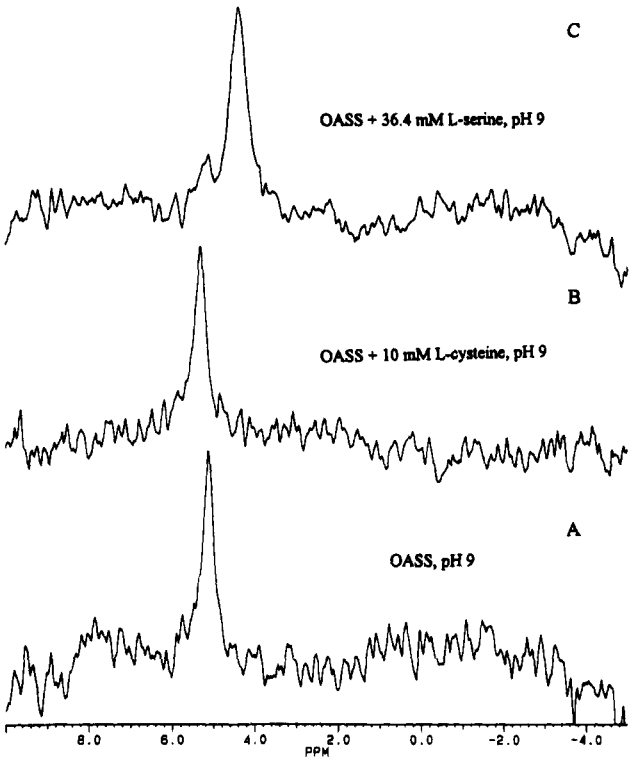


FIGURE 3: ^{31}P NMR spectra of OASS in the absence (A) and presence of 10 mM L-cysteine (B) and 36.4 mM L-serine (C). Spectra were collected at pH 9, 100 mM Ches, as discussed in Materials and Methods.

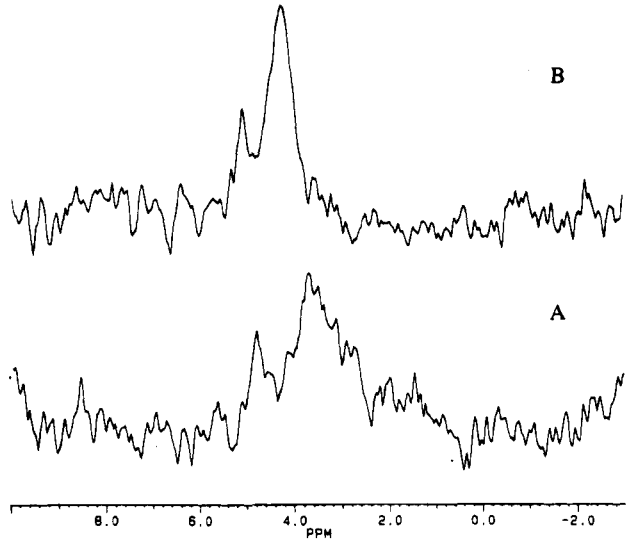


FIGURE 4: ^{31}P NMR spectra of OASS in the presence of 36.4 mM L-serine at pH 9, 100 mM Ches; temperature was maintained at 4 °C (A) and 15.5 °C (B).

Circular Dichroism Spectroscopy. The far-UV CD spectrum for OASS-A exhibits a broad trough from 225 to 205 nm with no significant change in the presence of OAS (McClure & Cook, 1994). However, significant differences in the far-UV CD spectrum is observed upon addition of either L-serine or L-cysteine (Figure 6A). Secondary structural components associated with the spectra recorded in Figure 6A have been determined. The enzyme alone has α-helix, β-structure, and remaining structure of 29%, 24%, and 47%, respectively, while addition of OAS gives values of 27%, 24%, and 49%, respectively. Thus, no difference is observed in the enzyme structure whether it is present as free enzyme or α-aminoacrylate intermediate. These data

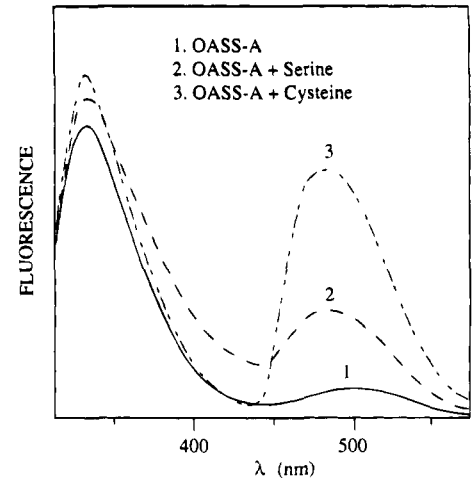


FIGURE 5: Fluorescence emission spectra of OASS in the absence and presence of 150 mM L-serine and 15 mM L-cysteine at pH 9, 100 mM Ches. Excitation was at 298 nm.

are in agreement with the qualitative interpretation of McClure and Cook (1994), who obtained similar data. In the presence of L-cysteine, the above components are 33%, 28%, and 39%, respectively, while in the presence of L-serine values of 39%, 23%, and 38%, respectively, are obtained. The structure of OASS is similar whether the L-cysteine or L-serine external Schiff base has been prepared. There is clearly a difference, however, between the structure of enzyme plus or minus OAS and the structure of the enzyme when it exists as the external Schiff base. The difference is manifest as an increase in the α-helical content with a concomitant decrease in remaining structure.

Induced CD spectra of the PLP cofactor were obtained in the absence and presence of OAS, L-cysteine, and L-serine (Figure 6B). The spectrum for unliganded enzyme exhibits a positive Cotton effect centered on the visible absorption band of the cofactor, 412 nm, as has been found for many PLP enzymes including the β_2 subunit of tryptophan synthase (Balk et al., 1981) and D-serine dehydratase (Marceau et al., 1988), which catalyze reactions very similar to that catalyzed by OASS-A. The addition of L-cysteine or glycine gives a shift in the λ_{max} to a slightly higher wavelength, but with the same relative ellipticity as free enzyme. The presence of L-serine gives a similar slight shift to higher wavelength, but with only 50% ellipticity relative to enzyme alone. In contrast, OAS causes a change in sign of the Cotton effect now centered around the visible absorption band of the α-aminoacrylate intermediate, 470 nm. The effect of OAS is a direct result of formation of the intermediate, as shown by the change of Cotton effect from positive to negative, measured as a function of the OAS concentration (Figure 6C). The amount of OAS required for the titration (~100 μM) is consistent with the amount of OASS present in the titration (~116 μM).

DISCUSSION

L-Cysteine Forms the External Schiff Base upon Binding to OASS. Reaction of L-cysteine with OASS-A at pH 9 gives a slight shift in the λ_{max} from 412 to 418 nm. No increase in the absorbance at 470 nm is observed, indicating that the α-aminoacrylate intermediate is not formed, i.e., the second half-reaction catalyzed by OASS-A is for all intents and purposes irreversible. The lack of appearance of absorbance at 470 nm agrees with the kinetic data of Cook and Wedding

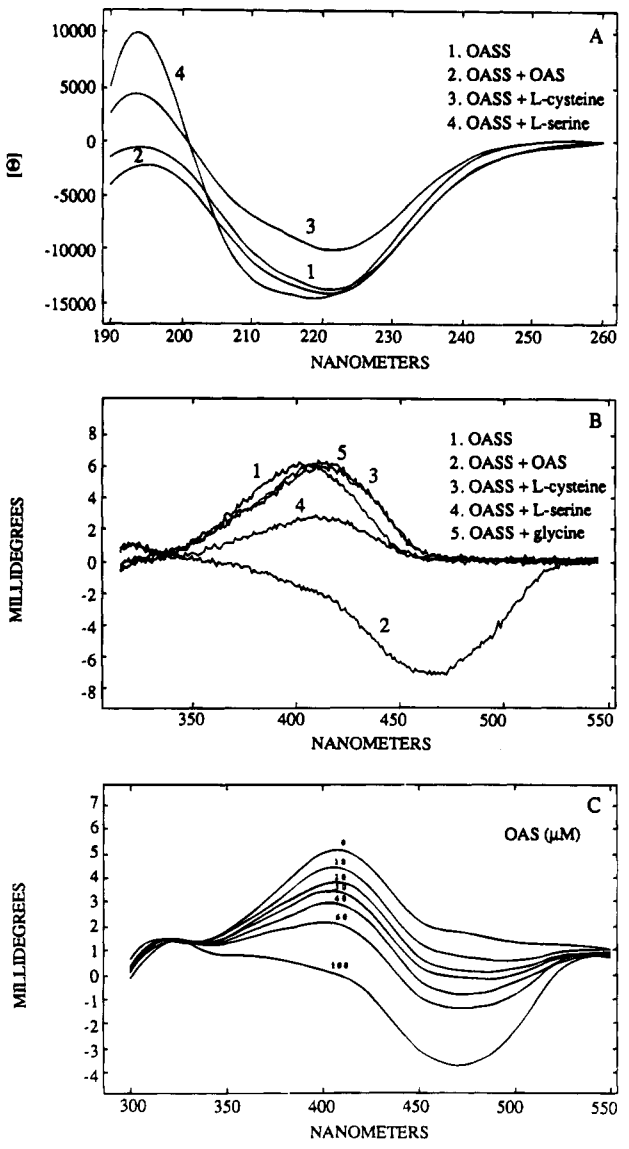


FIGURE 6: Circular dichroic spectra of OASS. (A) Far-UV spectra obtained with OASS at 10 μg/mL, at pH 7, 100 mM potassium phosphate for enzyme alone (1); enzyme plus 1 mM OAS (2); at pH 9, 20 mM borate buffer for enzyme plus 0.2 mM L-cysteine (3), and 1 mM L-serine (4). (B) Visible CD spectra obtained with OASS at 3.2 mg/mL, at pH 7, 100 mM potassium phosphate for enzyme alone (1), enzyme plus 1 mM OAS (2), at pH 9, 20 mM borate buffer for enzyme plus 5 mM L-cysteine (3), 15 mM L-serine (4), and 100 mM glycine (5). (C) Titration of the change in the induced CD upon addition of OAS from 0 to 100 μM. Conditions are as in panel B, with the exception that the OASS concentration is 4 mg/mL.

(1976) and Tai et al. (1993), who failed to demonstrate activity with L-cysteine. However, the L-cysteine/sulfide half-reaction does exhibit isotope exchange of ^{35}S into L-cysteine (Cook & Wedding, 1976). Thus, it appears that although reversible, the second half of the reaction catalyzed by OASS has an equilibrium constant far toward L-cysteine. Spectral data obtained with OASS and L-cysteine are consistent with the formation of an external Schiff base with L-cysteine, as shown in the first two steps of Scheme 1. The ϵ_{418} of 7840 M^{-1} cm^{-1} for the L-cysteine external Schiff base is slightly higher than the ϵ_{412} of 7600 M^{-1} cm^{-1} for the internal Schiff base (Becker et al., 1969). Identical results were obtained with L-alanine and glycine at pH 9.5 (data not shown), with estimated ϵ_{418} of 7840 and 8160 M^{-1} cm^{-1}, respectively. Interestingly, no quinonoid intermediate was detected with any of the analogs tested. Studies of tryptophan synthase from *Escherichia coli* have shown that the formation of tryptophan from L-serine and indole glycerol phosphate is a complex multistep process involving several kinetically and spectroscopically detectable intermediates.[2] These studies, utilizing rapid-scanning UV−visible spectroscopy in combination with rapid mixing, stopped-flow equilibrium studies have assigned a spectral absorbance maximum of 420 nm to the external aldimine of L-serine with tryptophan synthase (Miles & McPhie, 1974; Lane & Kirschner, 1983; Drewe & Dunn, 1985). A similar external Schiff base with a λ_{max} of 420 nm has also been detected with glutamate decarboxylate using stopped-flow methods (Karube & Matsushima, 1977; Morozov et al., 1982). The external Schiff base of serine hydroxymethyltransferase with glycine was found to have a λ_{max} of 425 nm (Schirch, 1982).

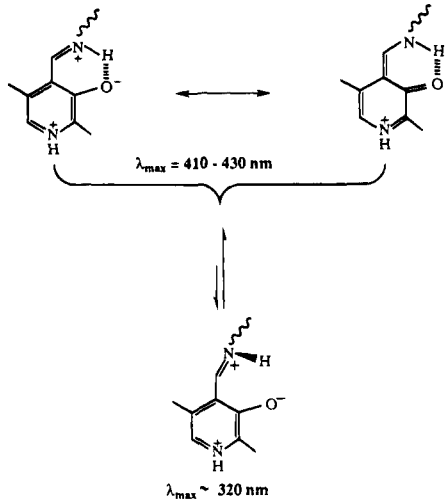
Formation of the external Schiff base with L-cysteine at pH 9 is accompanied by a slight downfield shift of the ^{31}P signal of the cofactor 5′-phosphate to 5.3 ppm from 5.2 ppm for enzyme alone at any pH (Cook et al., 1992). In addition, there is an increase in the line width from 20.5 Hz for enzyme alone to 32.5 Hz for the external Schiff base. The value of 5.2 ppm observed for free OASS is the highest one reported for the free PLP form of an enzyme. The high value of the chemical shift along with the observed line width of 20.5 Hz was interpreted by Cook et al. (1992) to indicate that the cofactor was rigidly bound to enzyme by strong salt bridge-(s). The higher value of the chemical shift measured in the presence of L-cysteine suggests an even stronger salt bridge to the bound cofactor. The increase in line width suggests the presence of more than one species in equilibrium (probably different tautomeric forms of the external Schiff base) with an average chemical shift of 5.3 ppm.[3] Thus, a change in the orientation of the cofactor must be realized upon formation of the external Schiff base. In agreement with the suggested change in orientation of the cofactor are the data of McClure and Cook (1994). These authors demonstrated that excitation of OASS at 298 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. The addition of L-cysteine caused a significant enhancement of the 500-nm fluorescence emission band, interpreted as indicating a conformational change at the active site resulting in either a change in the orientation of the PLP with respect to the tryptophan or an increase in the lifetime of the Schiff base excited state.

Finally, the far-UV CD of OASS changes significantly when the external Schiff base is compared to free enzyme or the α-aminoacrylate intermediate. These data likely signal the closing of the active site to a catalytic conformation. In agreement with these data, a mutant OASS in which the Schiff base lysine (K42) has been replaced with an alanine is isolated as an external Schiff base between PLP and a

[2] It is interesting to note that L-cysteine and L-serine are substrates for tryptophan synthase but apparently not for OASS. These data likely reflect differences in the geometry of the external Schiff bases with L-cysteine, L-serine, and α-aminoacrylate and interactions with the hydroxyl and thiol side chains.

[3] It is known that the PLP cofactor is rigidly bound to phosphorylase (Feldmann & Hull, 1977). Using the line width of the ^{31}P signal in phosphorylase (55−60 Hz), the molecular weight of phosphorylase (200 000), and the line width of the ^{31}P signal of OASS (20.5 Hz), a molecular weight of 68 000−74 500 is calculated, in excellent agreement with the MW of 68 900 (Byrne et al., 1988).

Scheme 2



free amino acid. The K42A-OASS external Schiff base is resistant to reduction by sodium borohyride unless 3−5 M guanidinium is present (unpublished work of V. Rege in this laboratory).

The visible CD of the OASS-bound PLP also changes upon addition of L-cysteine. The induced or extrinsic Cotton effect reflects the interaction of the symmetric cofactor with the asymmetric environment provided by the protein. The effect results from the $\pi \rightarrow \pi^*$ transitions of the protonated Schiff base chromophore (Miles & Moriguchi, 1977). The λ_{max} of the maximum ellipticity is red-shifted from 412 nm in free enzyme to 418 nm in the presence of L-cysteine, mimicking what is observed in the visible spectrum. The sign of the induced Cotton effect is positive for the external Schiff base as it is for the internal Schiff base, suggesting that the cofactor is bound in a manner in which the protein still interacts with the same face of the cofactor.

L-Serine Gives a Mixture of Species upon Binding to OASS. Reaction of L-serine with OASS-A at pH 9 gives a decrease in the absorbance at 412 nm by about 60%, concomitant with an increase in absorbance at 320 nm and a shift in the λ_{max} of the remaining absorbance to 418 nm. A single tight isosbestic point at about 340 nm is observed for the above changes, suggesting the interconversion of two species, one with a major absorption band at 412 nm and the second with absorption bands at 320 and 418 nm. Based on the changes observed with L-cysteine, the 418-nm band likely indicates formation of the external Schiff base with L-serine. The 320-nm band would thus result from a different tautomeric form of the external Schiff base or an equilibrium mixture of protonated and unprotonated forms of the external Schiff base. However, since the changes in the presence of saturating L-serine are pH independent, the former is indicated. As reviewed by Johnson and Metzler (1970), the tautomeric forms shown in Scheme 2 have been observed in both model and enzyme systems. The structure on the bottom of Scheme 2 reflects a slight rotation around the C4−C4′ bond such that the π-system of the imine is no longer in the plane of the pyridine ring and the proton on the imine nitrogen projects out of the plane. (The opposite rotation with the proton projecting behind the pyridine ring is also possible.) The broad line width of the ^{31}P signal would also suggest two species in equilibrium. If equilibration between two species is slow on the NMR time scale, two distinct resonances will be observed for the individual species. If the equilibration is rapid, a single sharp resonance is found, while if the equilibration is intermediate, a broad signal is observed positioned at the average of the two resonances (Nageswara Rao, 1989). For intermediate equilibration, a decrease in temperature should resolve the individual resonances. In the case of the reaction of OASS with L-serine at pH 9, an upfield chemical shift to 4.4 ppm from 5.2 ppm for enzyme alone (Cook et al., 1992) is observed with a large increase in the line width from 20.5 Hz for enzyme alone to 50.5 Hz. As a result, the equilibration between the two species must be in the intermediate to fast range. A decrease in the temperature from 20 to 4 °C, however, did not resolve the putative equilibrium mixture.

As suggested above, the binding of L-serine to OASS gives an apparent equilibrium mixture between an external Schiff base protonated on the Schiff base nitrogen and hydrogen-bonded to the phenolic O3′ anion and either the unprotonated form of the Schiff base or the protonated form in which the proton resides on O3′ (Kallen et al., 1985). Since the ϵ_{412} for both the internal Schiff base and the external Schiff base with L-cysteine are pH independent, it is the latter that is most likely, i.e., the O3′-protonated Schiff base might be favored as a result of weakening an interaction with O3′, e.g., a hydrogen bond. Knowing that the chemical shift for the N-protonated external Schiff base is 5.3 ppm and given an average value of 4.4 ppm in the presence of L-serine, a value of 3.5 ppm is calculated for the O3′-protonated Schiff base, considerably upfield from the value of 5.3 ppm. The low value of the chemical shift suggests a significant loosening in the binding of the cofactor at the 5′-phosphate with O3′ protonated, consistent with a change in interaction between enzyme and cofactor. Considering the chemical shifts of the individual species in equilibrium and the line width, one can obtain an estimate of the rate of exchange between the two species according to (Gadian, 1982):

$$\Delta\nu = \pi(\nu_A - \nu_B)^2/2k \qquad (6)$$

In eq 6, $\Delta\nu$ is the line width (50.5 Hz), $\nu_A - \nu_B$ is the difference in frequency between the two species in equilibrium (218.7 Hz), and k is the first-order rate constant for exchange, estimated to be 1500 s^{-1}.

As has been shown for the addition of L-cysteine, the addition of L-serine gives an enhancement of the long wavelength fluorescence band upon excitation at 298 nm. However, the amount of enhancement is 45% that observed with L-cysteine, approximately consistent with the amount of N-protonated external Schiff base present based on the UV−visible titration of OASS with L-serine (Figure 2). Thus, at least that portion of the enzyme that exists as N-protonated (and perhaps also O3′-protonated) external Schiff base in the presence of L-serine undergoes a conformational change similar to that elicited by L-cysteine (vide supra). Energy transfer is not observed to that fraction of the enzyme that exists as the O3′-protonated external Schiff base, since it does not fluoresce at 500 nm.

The changes in the far-UV CD of OASS in the presence of L-serine are pronounced despite the fact that serine is not saturating (saturating serine, >40 mM, produces too high a dynode voltage). These data suggest a rearrangement in protein structure similar to that discussed above for L-cysteine, i.e., reflecting a closing of the enzyme active site. Changes in the induced CD of the OASS-bound PLP upon addition of L-serine are similar to those observed with

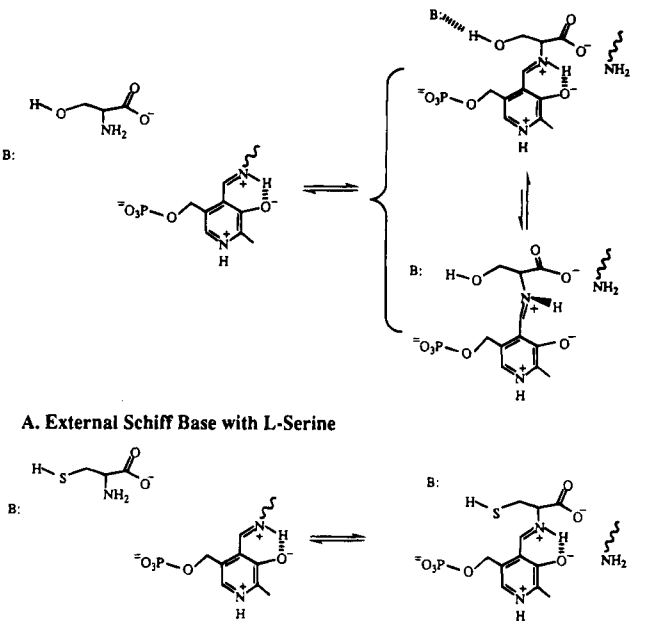
L-cysteine but attenuated as observed for the fluorescence spectra. The λ_{max} of the maximum ellipticity is red-shifted from 412 nm in free enzyme to 418 nm in the presence of L-serine, and the relative ellipticity of the external Schiff base is reduced to about 60% compared to enzyme alone, again mimicking what is observed in the visible spectrum. In addition, there is the appearance of a second cofactor band near 320 nm attributed to the O3′-protonated external Schiff base. The Cotton effects for both the N-protonated and O3′-protonated external Schiff bases are positive as they are for the internal Schiff base, suggesting that if the cofactor is bound differently in the internal and external Schiff bases, only subtle differences in binding occur.

Characterization of the α-Aminoacrylate Intermediate. Addition of OAS to OASS results in a decrease in the absorbance at 412 nm with concomitant increases at 330 and 470 nm, indicative of formation of the α-aminoacrylate intermediate (Kredich et al., 1969; Cook & Wedding, 1976; Cook et al., 1992). In the case of tryptophan synthase, the α-aminoacrylate intermediate is obtained with the $\alpha_2\beta_2$ complex in the presence of α-glycerol 3-phosphate and L-serine (Miles et al., 1987). The ^{31}P NMR spectrum of the OASS α-aminoacrylate intermediate is identical to that of free enzyme (Cook et al., 1992), suggesting that the rigidity of binding of the 5′-phosphate is identical to that in free enzyme. There is no long wavelength band in the fluorescence spectrum upon excitation at 298 nm (McClure & Cook, 1994) as a result of elimination of the 412-nm band of the protonated internal Schiff base that acts as the fluorescence energy transfer acceptor. The far-UV CD of the intermediate is very similar to that of the free enzyme, indicating no gross conformational alterations (McClure & Cook, 1994). However, the induced CD of the cofactor is dramatically altered. Although the λ_{max} for the induced CD of the cofactor is centered at 470 nm, the λ_{max} found in the UV−visible absorption spectrum has the same relative ellipticity as does free enzyme, the Cotton effect is negative, likely reflecting a difference in chromophore. Interestingly, although the α-aminoacrylate intermediate of tryptophan synthase absorbs maximally at 330 nm, the induced CD of the cofactor also exhibits a negative Cotton effect centered at 330 nm (Kayastha et al., 1987).

pH Dependence of Dissociation Constants for Amino Acid Analogs. The equilibrium constant for dissociation of the external Schiff base to give free enzyme and L-serine is pH dependent. The pH-independent dissociation constant is 4 mM and increases as the pH decreases giving two p*K*'s of 7.6 and 8.4. The p*K* of 8.4 likely reflects the unprotonated α-amine of L-serine, which has a p*K* of 9.0 (Martell & Smith, 1974)[4] to facilitate nucleophilic attack on C4′ of the internal Schiff base, and the pK of 7.6 reflects an enzyme side chain that must be unprotonated for the formation of the external Schiff base with L-serine (Scheme 3). A possible role of this group will be discussed below.

The equilibrium constant for dissociation of the external aldimine to give free enzyme and L-cysteine is also pH dependent. The dissociation constant is about 120 μM at pH 9 and increases as the pH is increased or decreased, giving two p*K*'s with an average of 7.4 at low pH and a p*K* of 9.5 at high pH. The difference between the data obtained with L-serine and L-cysteine is the presence of an additional functional group in the pH profile that must be protonated for formation of the external Schiff base. The most logical possibility for assignment of the p*K*'s is based on the mechanism proposed with L-serine. Thus, the α-mine of L-cysteine with an observed p*K* of 9.6 (10.3; Martell & Smith, 1974)[4] will be unprotonated, and one of the two groups with a p*K* of 7.4 most likely represents the enzyme residue that must be unprotonated to form the external Schiff base with L-cysteine. The second group with a p*K* of 7.4 then most likely reflects the thiol group of L-cysteine (8.2; Martell & Smith, 1974), which should be protonated either to form a hydrogen-bond to the enzyme residue or to maintain a specific charge in the active site, Scheme 3. Thus, the unprotonated α-amine of L-cysteine and the protonated thiol group of L-cysteine are in reverse protonation states. That is although the p*K* for the α-amine of L-cysteine is observed on the basic side and the p*K* for the cysteine thiol is observed on the acid side of the profile, the former must be unprotonated and the latter protonated (Cleland, 1977). The assignment of the unprotonated α-amine of L-serine and L-cysteine is consistent with the proposed chemical mechanism of the OASS-A reaction (Tai et al., 1995).

Scheme 3



A. External Schiff Base with L-Serine

B. External Schiff Base with L-Cysteine

The enzyme group that must be unprotonated for formation of the external Schiff bases with L-serine or L-cysteine is also observed in the *V*/*K* pH profile for β-chloroalanine (Tai et al., 1995) but must be protonated. The opposite protonation state for the enzyme group in the reaction with β-chloroalanine (first half-reaction) and with cysteine (second half-reaction) suggests a role for the enzyme group in the mechanism of OASS. A reasonable explanation would require the protonated enzyme group to hydrogen-bond the carbonyl of the acetyl side chain. There is no need for general base catalysis in the OASS reaction; acetate is a very good leaving group and needs only be held out of the plane formed by C2 and C3 of the amino acid external Schiff base for elimination. The protonated enzyme group could cer-

[4] The value of 9 reported by Martell and Smith (1974) was obtained at 0.1 ionic strength. Abase titration of L-serine gave us a value of about 8.9, for all intents identical to the reported value. Although the value of 8.4 is not identical to that of 8.9, it is similar when the errors on both determinations have been taken into account. This is also true for the value of 9.6 obtained for L-cysteine and the reported value of 10.3, once the relatively large error of 0.4 on the former.

tainly fulfill the role of holding the acetate out of plane. It is thus also possible that the unprotonated enzyme group accepts a hydrogen bond from the hydroxyl group of L-serine. It is not known whether the enzyme group could either accept a hydrogen bond or simply be required to be unprotonated for formation of the external Schiff base of L-cysteine.

Formation of the external Schiff base with cysteine leads to a significant enhancement of the 500-nm emission band that results from energy transfer of a tryptophan near the active site to the protonated Schiff base (McClure & Cook, 1994). Titrations of the fluorescence enhancement of the 500-nm band as a function of the L-cysteine concentration and pH provide an estimate of 800 μM for the pH-independent K_d value of the cysteine. The K_d value of 120 μM reported from absorbance measurements is almost an order of magnitude smaller. In addition, only a single group that must be unprotonated with a pK of 8 is observed in the fluorescence titration studies. McClure and Cook suggested that the observed pK of 8 reflects deprotonation of the lysine that originally formed the internal Schiff base with PLP (Lys42) and is displaced by L-cysteine in the transaldimination reaction. Lys42 has been assigned a pK of about 8.2 based on the first-order rate for disappearance of the α-aminoacrylate intermediate (Cook et al., 1992). The pK of 8.2 reflects a group that must be unprotonated for a deacetylase activity of OASS-A, proposed to result from displacement of the α-aminoacrylate intermediate with Lys42 to regenerate free enzyme. The requirements for formation of the external Schiff base and the fluorescence enhancement are clearly different, regardless of whether the assignment of the pK 8 observed in the fluorescence studies to Lys42 is correct or whether it should be attributed to some other enzyme residue that must be unprotonated for fluorescence enhancement. (The thiol of cysteine was ruled out as a result of similar fluorescence enhancement by cystine and cysteine.)

Comparison of K_d Values. The dissociation constants for the external Schiff base using a number of amino acid analogs were measured at pH 9.5, allowing a comparison of the data obtained for all analogs (Table 1). The K_d for the external Schiff base with L-cysteine is 120 μM, more than order of magnitude more favorable than the formation of Schiff base with L-serine.[5] The larger thiol group of cysteine binds with much higher affinity than the smaller, more hydrophilic hydroxyl group of serine. The latter may be the reason L-serine gives more than a single species of the external Schiff base. Replacement of the serine hydroxyl with a proton (L-alanine), replacement of the hydroxymethyl with a proton (glycine), or modification of the hydroxyl (*O*-methyl-D,L-serine) makes almost no difference to the value of the dissociation constant, indicating that little of the binding strength of serine comes from the side chain interaction. With L-serine and *O*-methyl-D,L-serine, two forms of the external Schiff base are obtained, suggesting that the methyl group of the latter interacting with the enzyme provides a similar configuration to that of serine. The K_d for D-alanine is 160 mM compared to 8 mM for L-alanine, indicating that OASS forms the external Schiff base stereospecifically with L-amino acids.

[5] The actual value of the dissociation constant for L-cysteine will be closer to 1 μM since the thiol and α-amine are in their incorrect protonation state for optimum binding. The difference between the two pK values is about 2 pH units, indicating that the fraction of cysteine in the correct protonation state for formation of the external Schiff base is about 1%.

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