Product Binding to the α-Carboxyl Subsite Results in a Conformational Change at the Active Site of *O*-Acetylserine Sulfhydrylase-A: Evidence from Fluorescence Spectroscopy†

G. David McClure, Jr.,‡,§ and Paul F. Cook*,‡,∥

Department of Biochemistry and Molecular Biology and Department of Microbiology and Immunology, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107

Received August 26, 1993; Revised Manuscript Received December 6, 1993®

ABSTRACT: The intrinsic fluorescence of the pyridoxal 5′-phosphate (PLP) enzyme *O*-acetylserine sulfhydrylase-A (OASS-A) was studied in order to gain insight into the structural basis for binding of substrates and products and for catalysis. Excitation of OASS-A with 298-nm light gives an emission spectrum with two maxima, 337 and 498 nm. OASS-A has two tryptophan residues, and the 337-nm maximum indicates that at least one of these is exposed somewhat to aqueous solvent. The 498-nm emission observed is due to fluorescence of the PLP Schiff base. Some of this long-wavelength fluorescence is likely due to direct excitation by incident radiation. However, the concomitant quenching of 340-nm emission and the enhancement of 498-nm emission observed upon reconstitution of apoenzyme with PLP support the conclusion that some of the long-wavelength emission is due to singlet–singlet transfer from at least one tryptophan residue to the PLP Schiff base. Enhancement of 498-nm fluorescence by either of the products, acetate or cysteine, of the enzymatic reaction without a quenching of 337-nm fluorescence is consistent with triplet–singlet transfer from one or both of the tryptophan residues to the PLP Schiff base. This would require a rigid environment for the tryptophan donor when the product is bound. However, a conformational change which affected principally the environment of the PLP Schiff base, resulting in a longer lifetime of its excited singlet state, would also increase the intensity of the 498-nm emission. Enhancement of OASS-A long-wavelength fluorescence by each product requires the unprotonated form of a different group on enzyme. Enhancement by acetate binding requires the unprotonated form of an enzyme group with a pK of 7 and is insensitive to substitution on the methyl group. L-Cysteine binding enhances 498-nm fluorescence when a group with a p*K* of 8 is unprotonated, and substitution at the thiol or the methylene bridge does not affect the enhancement elicited. Binding of L-cysteine to free enzyme (E) likely results in the formation of the external Schiff base accompanied by a conformational change giving fluorescence enhancement. The carboxylate moiety of acetate likely binds to the α-carboxylate subsite for amino acid reactants such as L-cysteine, resulting in a conformational change in the internal Schiff base and giving rise to the observed fluorescence enhancement. Data are interpreted in terms of the mechanism of OASS-A.

The structural basis for the specificity of the wide variety of enzymatic reactions that make use of the cofactor pyridoxal 5′-phosphate (PLP)[1] is an open question of both theoretical and practical importance [for an enlightening historical review of the most fully developed line of investigation into PLP-dependent enzyme reaction mechanisms, see Cooper and Meister (1989)]. Insight into the evolutionary relationship between enzyme domains to which substrates, products, and cofactor bind is enhanced when tertiary and quaternary structures of PLP-dependent enzymes are determined to high resolution (Kirsch *et al.*, 1984; Hyde *et al.*, 1988). Understanding the basis for rate enhancement by these versatile enzymes is incrased when their kinetic and acid–base chemical mechanisms are elucidated (Kiick & Cook, 1983; Houben *et al.*, 1989). Studies of the behavior in solution of PLP-dependent enzymes, either free or in the presence of substrates or products, can shed light on the link between the two roles of the cofactor in holoenzyme: imparting structure and increasing reaction rates.

The PLP-dependent enzyme *O*-acetyl-L-serine sulfhydrylase-A (EC 4.2.99.8; Becker *et al.*, 1969) from *Salmonella typhimurium* has recently been crystallized, and the three-dimensional structure of the enzyme, which makes acetate and L-cysteine from *O*-acetyl-L-serine and sulfide, is being determined (Rao *et al.*, 1993). Kinetic (Cook & Wedding, 1976, 1977; Tai *et al.*, 1993) and acid–base chemical (Nalabolu & Cook, 1993) mechanisms have been proposed for the β-replacement reaction catalyzed by OASS-A. The intrinsic fluorescence of the pyridoxal 5′-phosphate (PLP) enzyme *O*-acetylserine sulfhydrylase-A (OASS-A) was studied in order to gain insight into the structural basis for binding of substrates and products and for catalysis. This report is the first account of the effect of substrate or product binding on OASS-A structure and provides evidence consistent with enhanced nonradiative energy transfer from one or both tryptophan

† This work was supported by grants to P.F.C. from the National Institutes of Health (GM 36799) and the Robert A. Welch Foundation (B-1031). G.D.M. was a Robert A. Welch Foundation Graduate Fellow at the University of North Texas during the completion of this work.

‡ Department of Biochemistry and Molecular Biology.

§ Present Address: Mail Code 8859, Division of Allergy and Immunology, Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8859.

∥ Department of Microbiology and Immunology.

® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations: E, unliganded holoenzyme; OAS, *O*-acetyl-L-serine; OASS, *O*-acetylserine sulfhydrylase; PLP, pyridoxal 5′-phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/94/0433-1674$04.50/0

residues to the PLP internal aldimine and with a change in the conformation of the PLP Schiff base when either product is bound.

MATERIALS AND METHODS

Materials. OASS-A was purified from wild-type *S. typhimurium* LT-2 cells grown on Vogel-Bonner E medium (Vogel & Bonner, 1956) supplemented with reduced glutathione as the sulfur source (Kredich, 1971). The basis for all purifications was the scheme of Hara *et al.* (1990). Samples from pooled, concentrated final fractions were analyzed by SDS–PAGE and judged as being pure by inspection of Coomassie-stained gels and by examination of the UV–visible absorption spectrum. The ratio of the absorbance at 280 nm to the absorbance at 412 nm in native enzyme is in the range of 3.5 (Becker *et al.*, 1969) to 4.0 (Hara *et al.*, 1990). The extinction coefficient for OASS-A at 412 nm of 7600 M^{-1} cm^{-1} (Becker *et al.*, 1969) was used for determination of enzyme concentration.

For the spectroscopic procedures, all chemicals used were of the highest available grade.

Spectroscopic Methods. Spectra were taken of samples in Na^+ Hepes or K^+ phosphate, as indicated in Results. Spectra of samples prepared after extensive dialysis were customarily blanked against the final dialysis buffer.

Fluorescence spectra were taken on a Shimadzu RF5000U spectrofluorometer with a water-jacketed sample compartment for maintenance of temperature in the cuvettes at 25 °C. 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 spectra and excitation spectra. Spectra of blanks, i.e., of 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 in general chosen so as to minimize inner filter effects.

Ultraviolet and visible absorption spectra were taken at room temperature on a Hewlett-Packard 8452A diode array spectrophotometer. Samples were contained in self-masking quartz cuvettes able to hold volumes as great as 1.4 mL.

Circular dichroic spectra were taken at 25 °C 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.

Fluorescence Quenching. In experiments in which ionic quenchers of fluorescence were employed, ionic strength was held constant, and the method of Arrio-Dupont (1978) was followed. In this procedure, buffer and enzyme concentrations were identical in the cuvette, which contained a high concentration of NaCl, and in a test tube, which contained the same high concentration of CsCl or NaI. Aliquots from the tube were then added sequentially to the cuvette to achieve the indicated concentration of quencher. For preliminary experiments with enhancers of the long-wavelength emission of OASS-A, ionic strength was kept constnat by addition of concentrated NaCl into the cuvette. Once it was established that the long-wavelength emission enhancement did not depend on ionic strength, subsequent experiments were performed at a single concentration of buffer without added solutes other than the enhancer. Stock solutions of enhancers were titrated when necessary to a pH close to that at which the experiment would be performed. In experiments to determine the pH dependence of enhancement, solutions were buffered in 0.25 M potassium phosphate. The pH was read on a calibrated Radiometer PHM82 pH-meter immediately upon completion of the spectral scan.

Preparation of Apo-OASS-A. Apo-OASS-A was prepared by an adaptation of the method of Dowhan and Snell (1970) for the preparation of apo-D-serine dehydratase. Each is a modification of the overall scheme for the preparation of apophosphorylase *b* of Shaltiel *et al.* (1967). In this scheme, the protein is distorted by imidazole citrate, while the L-cysteine thiolate attacks C-4′ of the PLP internal aldimine. Deviations from the Dowhan and Snell procedure were (a) failure to purge solutions with inert gas; (b) mixture of the first substrate, OAS, at a concentration of 100 mM, with OASS-A immediately prior to the first dialysis. The rationale for b was to attempt to favor formation of the α-aminoacrylate intermediate in which the PLP is no longer bound to the enzyme as an internal aldimine. It was hoped that the PLP-α-aminoacrylate would diffuse away during dialysis before enzyme-catalyzed deacetylation (Cook *et al.*, 1992) and consequent reformation of the internal aldimine could occur.

Data Analysis. Determination of areas under fluorescence spectral peaks was performed on raw spectra by proprietary software of Shimadzu Scientific Instruments installed on the fluorometer. Determination of peak heights and bandwidths was performed by inspection of digitized data after smoothing the data by software internal to the fluorometer. The circular dichroic spectra were smoothed by a similar routine.

A blue shift in the wavelength of maximum fluorescence intensity for the longer-wavelength band ($\lambda_{max,1}$) was in general observed when the fluorescence of this band was enhanced. However, spectra showed an isoemissive point near 430 nm regardless of the blue shift. For this reason, rather than the ratio of peak heights, the area under the longer-wavelength curve (430–580 nm) and the area under the shorter-wavelength curve (310–430 nm) were used as estimators of the overall relative fluorescence of the two bands. Since numerator and denominator arise from the same spectral scan, both the ratio of peak heights and the ratio of band areas are relatively insensitive to long-term changes in lamp intensity and as such are more suitable for comparison of spectra than are raw data (such as untransformed relative fluorescence). For this reason, the fluorescence data presented below are not corrected for instrument response.

When it was necessary to determine binding constants for saturable processes of ligand binding to enzyme, an equation (eq 1) for hyperbolic saturation of a single binding site was fitted to observed fluorescence data. In eq 1, Y is the observed

$$Y = A(1 + X/K_{IN})/(1 + X/K_{ID}) \quad (1)$$

fluorescence, X is the concentration of ligand, A is the fluorescence in the absence of ligand, K_{ID} is the dissociation constant for ligand from the enzyme–ligand complex, and the product $A(K_{ID}/K_{IN})$ is the fluorescence at infinite ligand concentration.

To determine the pK for the process of enhancement of OASS-A fluorescence by acetate, the equation

$$-\log K_{enh} = \log C/(1 + H/K_A) \quad (2)$$

was fitted to the observed data. In eq 2, K_{enh} is the dissociation constant for acetate from the enzyme–acetate complex, H is the concentration of protons in solution, K_A is the dissociation constant for a proton from its complex with an ionizable group, and C is the pH-independent value of K_{enh}.

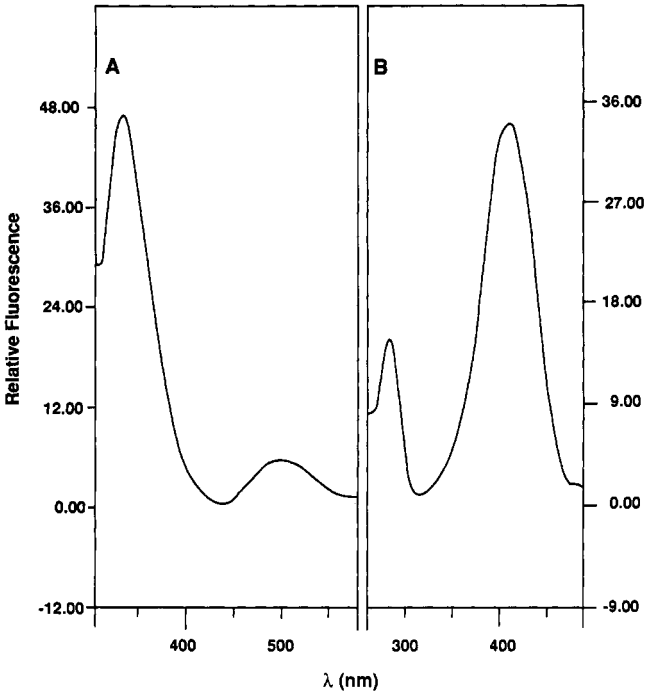

FIGURE 1: Fluorescence emission and excitation spectra for *Salmonella typhimurium* OASS-A. The protomer concentration of OASS-A was maintained at 2.5 μM in 100 mM NaHepes, pH 7. (A) Emission spectrum obtained with excitation at 298 nm. The ordinate represents relative fluorescence intensity, while the abscissa represents emission wavelength. (B) Excitation spectrum obtained by monitoring the emission at 500 nm. The ordinate represents relative fluorescence intensity, while the abscissa represents excitation wavelength.

The pK for the process of enhancement of enzyme fluorescence by L-cysteine was determined by fitting the equation

$$\log Y = \log ((Y_L + Y_H K/H)/(1 + K/H)) \quad (3)$$

to the data gathered. Here, Y is the reciprocal of the dissociation constant for L-cysteine from its complex with enzyme; Y_L is the local maximum of this reciprocal dissociation constant, at the low end of the pH range in question; Y_H is the local minimum of this same dissociation constant at the higher pH extreme; H is the concentration of protons in solution; and K is the dissociation constant for a proton from its complex with the ionizable group.

Equations 1–3 were fitted to the data by FORTRAN programs that execute the appropriate least-squares analysis (Cleland, 1979).

Values reported for the quenching constant, K_{SV}, are those obtained from linear regression analysis of the initial, linear portion of the data, if downward curvature was observed, or of the entire data set, if linear. Regression analysis of the quenching data was performed by a proprietary software package (Leatherbarrow, 1992).

RESULTS

Fluorescence Spectrum of OASS-A. The UV–visible absorption spectrum of OASS-A, with peaks near 280 and 412 nm, has been published (Becker *et al.*, 1969) and has recently been shown to retain these two major peaks over the pH range of 5.50–10.85 (Cook *et al.*, 1992). At pH 7.0 in 100 mM sodium Hepes, OASS-A from *S. typhimurium* shows two peaks in its fluorescence emission spectrum when excited at 298 nm (Figure 1A). The peak of the shorter-wavelength emission band ($\lambda_{max,1}$), at 337 nm, is typical of tryptophan residues in proteins. Tryptophan residues in aqueous environments emit maximally at 350 nm, while in nonpolar environments the emission maximum is near 325 nm (Burstein *et al.*, 1973). The peak of the longer-wavelength band ($\lambda_{max,2}$), at 498 nm, is typical of emission from Schiff bases of pyridoxal phosphate in a neutral, aqueous environment, where a 500-nm emission has been observed by other workers (Arrio-Dupont, 1970, 1971; Shaltiel & Cortijo, 1970), and of PLP-containing enzymes including tryptophan synthetase β_2 (Goldberg *et al.*, 1968; York, 1972) and D-serine dehydratase (Schnackerz, 1972; Federiuk & Shafer, 1983). The same long-wavelength band at 500 nm is also observed upon excitation at 412 nm. Either peak height or integrated area under the curve may be used as an estimator of the relative fluorescence of two bands in a spectrum. Under conditions in which λ_{max} does not change appreciably, peak height is a convenient estimator. The ratio of the height of the shorter-wavelength peak to the height of the longer-wavelength peak of OASS-A emission here is 8.8. This ratio is lower than that observed in the PLP-containing enzyme tryptophan synthetase in the absence of substrates (Goldberg *et al.*, 1968; Strambini *et al.*, 1992a,b), although tryptophan synthetase possesses only one tryptophan residue as opposed to the two of OASS-A. By comparison, then, the PLP Schiff base of OASS-A is highly fluorescent.

At pH 7.0 in 100 mM sodium Hepes, the excitation spectrum for the longer-wavelength emission of OASS-A measured at 500 nm shows two bands (Figure 1B). The peak of the shorter-wavelength excitation band is located at 284 nm, and the peak of the longer-wavelength excitation band is located at 413 nm. These peaks are very close to the peaks in the excitation spectrum for the 500-nm emission of PLP-valine observed by Arrio-Dupont (1970) and to those observed for D-serine dehydratase (275 and 400 nm; Ehrlich & Schnackerz, 1973; Federiuk & Shafer, 1983). When measured, the ratio of the peak heights tended to remain constant during the various experimental treatments to which OASS-A was subjected but was found to vary somewhat depending on the enzyme preparation and the buffer system employed.

OASS-A fluorescence, as evidenced by λ_{max} positions and the ratio of peak heights, was found to be independent of enzyme concentration in the range of 0.5–5 μM (protomer). In addition, $\lambda_{max,1}$, $\lambda_{max,2}$, and the ratio of peak heights were essentially independent of excitation wavelength between 295 and 298 nm.

Quenchers of Intrinsic Fluorescence as Probes of Tryptophan Environment. Even when the three-dimensional structure of an enzyme is known, crucial information about the environment of tryptophan residues in the protein as it exists in solution can be gained from studying the effects of quenchers of tryptophan fluorescence on the emission spectrum (Burstein *et al.*, 1973). Linear regression analysis of a Stern–Volmer plot of the quenching of the shorter-wavelength band of OASS-A fluorescence emission by acrylamide gave an estimate of $K_{SV} = 2.3 \pm 0.1$ M^{-1} (Figure 2). The Stern–Volmer plot for quenching by iodide was found to be nonlinear and showed downward curvature, possibly indicating quenching by the anion of more than one fluorescent species (Lehrer & Leavis, 1978; Eftink & Ghiron, 1981). An estimate of the quenching coefficient, $K_{SV} = 2.0 \pm 0.1$ M^{-1} was derived from linear regression analysis of the linear portion of this plot (Figure 2). The difference spectrum between that obtained with no iodide present and the spectrum in the presence of 160 mM iodide was constructed and found to possess a maximum intensity at 339.4 nm (data not shown). This value is close

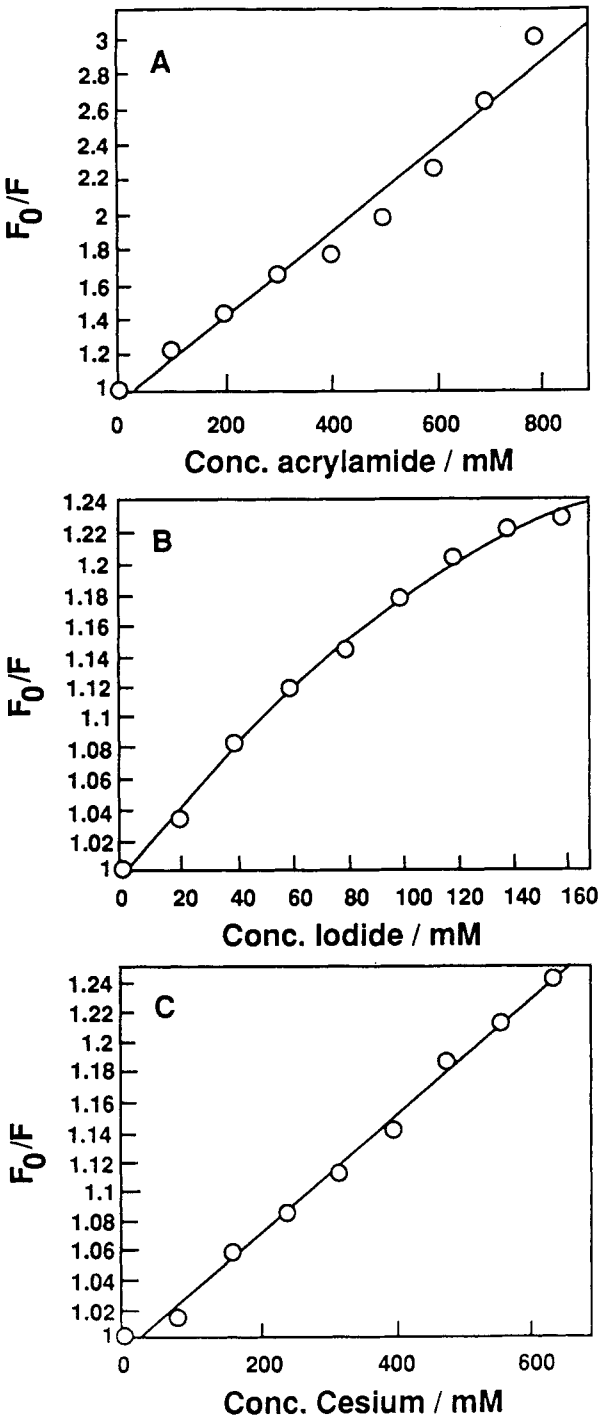

FIGURE 2: Stern–Volmer plots of quenching of *Salmonella typhimurium* OASS-A fluorescence. In each case the ordinate reflects the following quotient: (relative fluorescence in the absence of quencher)/(relative fluorescence at a given quencher concentration). The OASS-A protomer concentration is 2.5 μM, and data are collected in 100 mM NaHepes, pH 7. (A) Acrylamide; (B) iodide; (C) cesium.

to $\lambda_{max,I}$ for enzyme without added quencher. Given this proximity, it is possible that iodide quenches each of the two tryptophan residues (W51 and W162) of OASS-A, but with unequal efficiency. Quenching by cesium of the intrinsic fluorescence of OASS-A gave a linear Stern–Volmer plot from which an estimate of K_{SV} = 0.4 ± 0.1 M^{-1} was derived by linear regression analysis (Figure 2).

Apoenzyme Preparation as a Probe of Enzyme Structure. It has been informative for other investigators of PLP-dependent enzymes to prepare apoenzyme in order to either reconstitute holoenzyme (Dowhan & Snell, 1970) or to study the properties of isolated apoenzyme (Strambini *et al.*, 1992a). In order to probe further the relationship of tryptophan residues and the PLP Schiff base in OASS-A, apoenzyme was prepared from the OASS-A holoenzyme by using a modification of the method of Dowhan and Snell (1970). In order to adapt the method to OASS-A, it was necessary to add high concentrations of the first substrate OAS to the dialysis bag immediately prior to dialysis against the imidazole buffer. When excess OAS was not added, the spectrum of the enzyme after dialysis did not differ appreciably from that of native holoenzyme. This raises the question of the mechanism by which this procedure causes apoenzyme formation in the case of OASS-A. Although cysteine attacks C-4′ of PLP in the classic scheme of Shaltiel *et al.* (1967), this apparently does not occur in the case of OASS-A. Instead, formation of the α-aminoacrylate intermediate (Cook et al., 1992) appears to be sufficient for resolution of the PLP cofactor of OASS.[2] Since some precipitation did occur, it may be important both to keep DTT as a component of the dialysis buffer and also to purge the system with nitrogen.

Dowhan and Snell (1970) have reported that the apo-D-serine dehydratase they prepared had no detectable absorbance at 410 nm and that they could restore 99.9% of the enzymatic activity that was lost in the apoenzyme by incubation with equimolar or greater concentrations of PLP. The absorption spectrum of apo-OASS-A still has a slight positive value, but not a peak, at 412 nm (McClure, 1993), though this could be the result of light scattering rather than chromophore absorbance.

The fluorescence emission spectrum of apo-OASS-A shows a peak at 340 nm but none at 498 nm (Figure 3). The red-shift of the peak with respect to native holoenzyme reflects somewhat fuller exposure to aqueous solvent of the two tryptophan residues in apoenzyme. This could be the result of a gross conformational difference between apoenzyme and holoenzyme, of direct protection of tryptophan residues from solvent exposure by PLP in holoenzyme, or of the presence in solution of a fraction of enzyme that was fully denatured. The OASS data can be compared to those of Schnackerz (1972) for D-serine dehydratase, where both holoenzyme and apoenzyme have emission maxima at 335 nm.

To reconstitute holoenzyme, apo-OASS-A was incubated in the dark at room temperature with an excess of PLP such that the molar ratio of PLP to apoenzyme protomer was 2.25:1. The fluorescence emission spectrum showed a peak at 500 nm after 8-h incubation (Figure 3). Moreover, the fluorescence emission at 340 nm was remarkably quenched by incubation with PLP. The integrated area under the emission spectrum between 310 and 400 nm was decreased 40% by incubation of apoenzyme with PLP. The location of the tryptophan emission peak remained unchanged at 340 nm during this process. The quenching of the shorter-wavelength emission peak by PLP was confirmed in separate experiments in which the emission of apoenzyme at 340 nm consistently was found to be no more than one-half as great as that of an equal concentration of holoenzyme. Similar data have been obtained for D-serine dehydratase (Schnackerz, 1972). In such experiments, holoenzyme concentration was determined by A_{412} and apoenzyme concentration by A_{280} using published data on holoenzyme absorbance (Becker *et al.*, 1969; ϵ_{280} = 2170 M^{-1} cm^{-1}). Since PLP also absorbs at 280 nm, this procedure is biased toward overestimation of apoenzyme

[2] In agreement with this suggestion, a procedure has been developed for preparation of stable apo-OASS-A using OAS alone (K. D. Schnackerz and P. F. Cook, unpublished results).

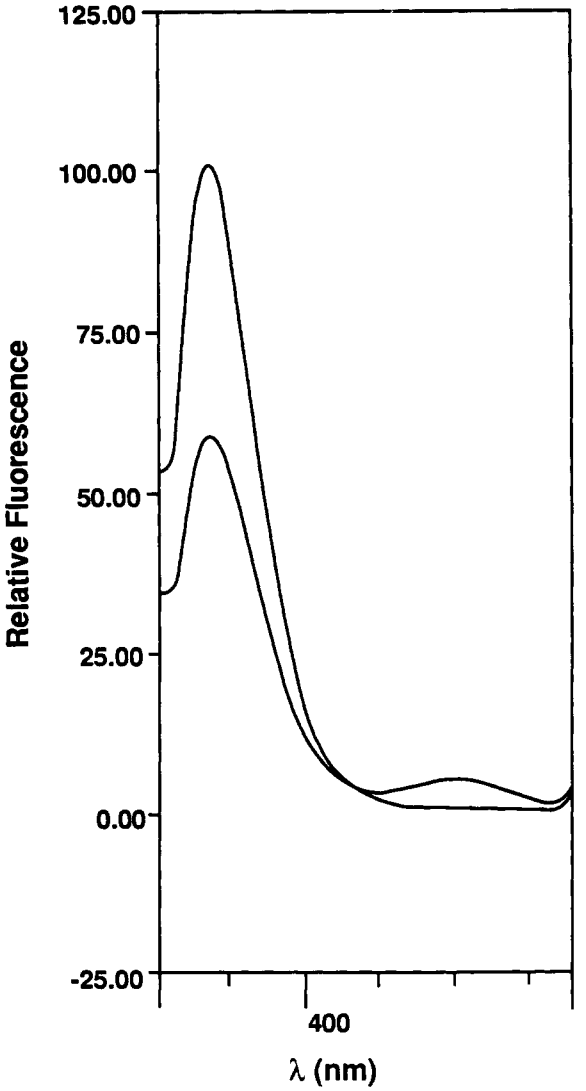

FIGURE 3: Fluorescence emission spectra of *Salmonella typhimurium* OASS-A in the absence and presence of PLP at a molar ratio of 2.25:1. The upper curve reflects the spectrum of apoenzyme prepared as in Materials and Methods. The lower curve represents the spectrum taken after 8-h incubation in the dark subsequent to mixing. The excitation wavelength is 295 nm. A protomer concentration of 4.4 μM was used in 100 mM potassium phosphate, pH 7. The ordinate is relative fluorescence, and the abscissa is emission wavelength.

concentration. Estimates of K_{SV} for cesium quenching of apoenzyme and holoenzyme tryptophan fluorescence were the same within experimental error.

Spectrum in the Presence of Substrates. The intensity of the longer-wavelength emission band is diminished considerably when OASS-A is incubated with an excess of the substrate OAS (data not shown). This diminution is a saturable process, as evidenced by titration of the fluorescence emission by OAS concentrations in the micromolar range. An estimate for $K_{d,OAS}$ of 0.44 ± 0.15 μM was obtained by fitting a hyperbolic equation (eq 1) to the data from such a titration. Since it is difficult to determine accurately the location of the diminished peak at higher OAS concentrations, integrated area under the curve was used as a measure of OAS binding. Underlying the proper application of eq 1 are the assumptions that OAS binding to OASS-A is a reversible process and that there is a one-to-one correspondence between OAS binding and the observed quenching of the fluorescence peak. L-Serine (400 mM, pH 7), an analog of OAS, was not found to give rise to a change in the OASS-A emission spectrum similar to that caused by OAS.

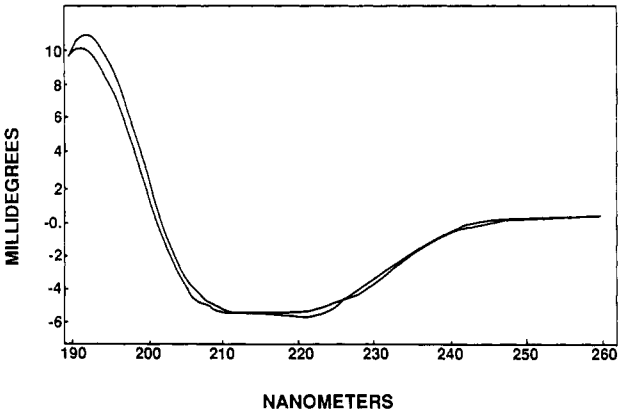

FIGURE 4: Circular dichroic spectra of *Salmonella typhimurium* OASS-A. The ordinate is θ, the relative rotation by the sample of the circularly polarized light, while the abscissa is the wavelength at which rotation was measured. The upper curve at shorter wavelengths is the spectrum of OASS-A in 10 mM potassium phosphate, pH 7, while the lower curve is for OASS-A in the presence of 1 mM OAS. Enzyme protomer concentration is 2.5 μM.

Addition of OAS to enzyme appears to proceed without major reorganization of the secondary structure of OASS-A, as evidenced by the overall similarity of the circular dichroic spectrum of the enzyme in the absence and presence of 1 mM OAS (Figure 4), a concentration independently shown to be saturating (Cook *et al.*, 1992). At neither 208 nor 225 nm was there a difference greater than 5% between the molar ellipticity of free OASS-A and OASS-A in the presence of 1 mM OAS.

Addition of an excess (e.g., 10 mM) of the second substrate sulfide to solutions containing OASS-A saturated with OAS (e.g., 4 mM) restored the full intensity of the longer-wavelength emission (data not shown). Volatilization of sulfide during the time required to take successive spectra precluded reliable estimation of the K_d for this process. However, sulfide alone, in the absence of OAS, appears not to affect the long-wavelength emission of OASS-A. Various analogs of sulfide, including azide, cyanide, thiosulfate, and 1,2,4-triazole, were similarly found not to affect the long-wavelength portion of the emission spectrum of OASS-A. However, unlike the physiological substrate, none of the sulfide analogs restored the long-wavelength emission when incubated with OASS-A and saturating concentrations of OAS.[3] It should be noted that the alternative second substrate, 5-thio-2-nitrobenzoate, absorbs too strongly in the relevant wavelength range (Ellman, 1959; Tai *et al.*, 1993) to permit its use as an analog in this case.

Spectrum in the Presence of Products. While a complex of the E form of OASS-A and L-cysteine was proposed some time ago on the basis of kinetic evidence (Cook & Wedding, 1976), until now there has been no direct spectroscopic evidence in support of this proposal. At pH 6.9, L-cysteine at 0.5 mM was not found to affect the fluorescence emission spectrum of OASS-A. However, at pH 10, 0.5 mM L-cysteine was found to enhance the longer-wavelength emission band while not significantly affecting the intensity of the shorter-wavelength band. The location of the peak of the shorter-wavelength band was unchanged, while the longer-wavelength band was blue-shifted and narrowed. Similar data have been

[3] Failure of sulfide analogs to restore the long-wavelength emission can be explained based on the ping-pong kinetic mechanism proposed for OASS-A (Tai *et al.*, 1993). The failure likely results from the relative rate of the second half-reaction with reactants other than sulfide being slower than the first half-reaction in which OAS is processed.

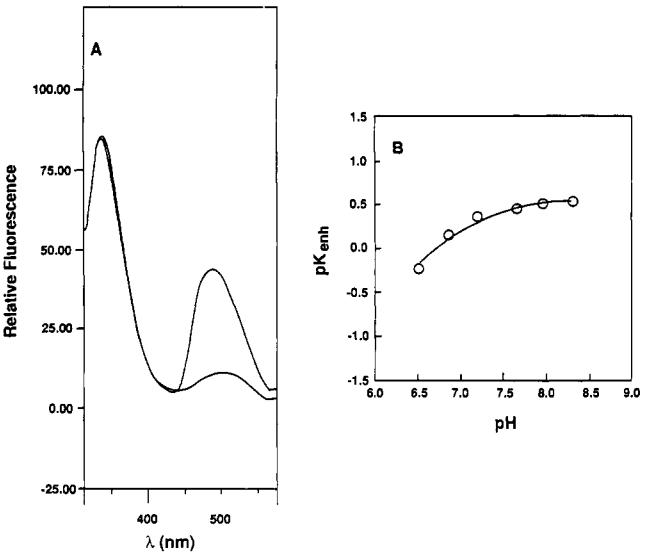

FIGURE 5: (A) Fluorescence emission spectra of *Salmonella typhimurium* OASS-A in the absence and presence of acetate. The upper curve at 500 nm reflects the spectrum of OASS-A in the presence of 1 M sodium acetate, while the lower curve represents OASS-A alone. The excitation wavelength is 298 nm. A protomer concentration of 2.5 μM was used in 0.25 M potassium phosphate, pH 6.87. The ordinate is relative fluorescence, and the abscissa is emission wavelength. (B) Dependence on pH of enhancement by acetate of OASS-A fluorescence. The abscissa is the negative common log of the dissociation constant for acetate from its complex with enzyme; the ordinate is the pH. Points are experimental values, while the line is the best fit of eq 2 to the experimental data.

obtained for the addition of L-serine to the β_2-subunit of tryptophan synthetase (Goldberg et al., 1968), and the addition of glycine or L-alanine to D-serine dehydratase (Ehrlich & Schnackerz, 1973; Federiuk & Shafer, 1983; Marceau et al., 1988).

The first product of the enzymatic reaction, acetate, was found to enhance the longer-wavelength emission band of OASS-A while not affecting the intensity of the shorter-wavelength band (Figure 5A). While the location of the peak of the shorter-wavelength band was virtually unchanged, the longer-wavelength band was narrowed and blue-shifted. (The fluorescence enhancement near 500 nm is obtained whether excitation is at 298 or 412 nm.) The addition of OAS to OASS-A and acetate was found to cause the longer-wavelength band to diminish considerably. Subsequent addition of excess sulfide to the mixture of OASS-A, acetate, and OAS restored the enhanced long-wavelength emission of OASS-A to that observed in the presence of acetate alone.

Since concentrations of acetate as high as hundreds of millimolar were required to elicit enhancement of the long-wavelength emission, there was some question whether this effect might be exerted by high ionic strength. Chloride at the same concentrations did not cause a like enhancement. However, at a concentration of 500 mM, each of the acetate analogs oxalate, propionate, chloroacetate, trichloroacetate, and trifluoroacetate also enhanced the long-wavelength emission.

pH Dependence of Fluorescence Enhancement by Enzymatic Reaction Products as a Probe of Enzyme Structure. Because of their sensitivity, even steady-state fluorescence techniques are well-suited to detect subtle changes in protein structure that occur upon ligand binding. Since enhancement of the longer-wavelength emission of OASS-A had been observed in the presence of either product of the enzymatic reaction, acetate or L-cysteine, and since the binding of L-cysteine appeared to be pH dependent, it was decided to make use of the observed enhancement to probe changes occurring in the vicinity of the enzyme's active site when the product is bound. Of particular interest to the elucidation of the acid–base chemical mechanism of OASS-A was the possibility of the presence of ionizable groups near the active site which must be in the proper protonation state in order for binding or catalysis to occur.

Potassium phosphate at 0.25 M was found to be an acceptable buffer for experiments with acetate and moderate concentrations of L-cysteine at pH values up to 10. At increasing values of pH, the longer-wavelength emission and then the shorter-wavelength emission both are quenched somewhat, resulting in a decline in each emission by pH 10. This finding is consistent with the observation by Arrio-Dupont (1970) of enhanced 500-nm fluorescence in PLP-valine at lower values of pH. It was also necessary to determine whether the potassium cation itself might affect the fluorescence properties of the enzyme. Cations used in common buffers can conceivably exert different specific effects, by binding to sites on enzyme, in addition to their colligative effects as solutes and their electrostatic effects as charged particles. The chloride salts of the monovalent cations lithium, sodium, potassium, cesium, and ammonium were tested to see if such salts all exerted the same effect on enzyme fluorescence in Hepes buffer. Each of the chloride salts behaved in a qualitatively similar way.

Enhancement of the longer-wavelength emission by acetate was found to be more facile at high pH. In order to characterize this process more fully, spectra were taken in the presence of various concentrations of acetate, over a range of several pH units. (As an example, the spectrum taken at pH 6.87 in the presence of 1 M sodium acetate is shown in Figure 5A.) Equation 2 was fitted to data obtained from titrations performed at each of these pH values. The dissociation constant was plotted as a function of pH (Figure 5B). A fit of eq 2 to these data indicated a pK for the enhancement of the longer-wavelength peak by acetate of 7.2 ± 0.1. Since this is far greater than the pK for acetate (4.56; Martell & Smith, 1977), this pK reveals a group on enzyme that must be unprotonated for enhancement to occur. An estimate of 0.27 ± 0.02 M was obtained for the pH-independent value of K_{enh}.

Likewise, enhancement of the longer-wavelength emission by L-cysteine was found to be much more facile at high pH. Spectra in the presence of various concentrations of L-cysteine were taken over a wide range of pH. (As an example, the spectrum taken at pH 10.34 in the presence of 0.5 M L-cysteine is shown in Figure 6A.) At each of these pH values, titrations were performed. Titration data were then fitted by eq 3, and the estimated dissociation constants were plotted as a function of pH (Figure 6B). A fit of eq 3 to the data gave a pK for the enhancement of the long-wavelength emission by L-cysteine of 8.0 ± 0.1. The local minimum and maximum values for the dissociation constant for L-cysteine from its fluorescent complex with enzyme were estimated as 0.81 ± 0.02 mM (at high pH) and 98 ± 4 mM (at low pH). The observed pK is close to that reported for the L-cysteine thiol (8.3; Grafius & Neilands, 1955), and it was thus possible that it must be ionized for optimum binding and enhancement of fluorescence. However, at pH 9.2 and 10.8, the estimated dissociation constant for L-cystine from its complex with enzyme is of the same order as that for L-cysteine (data not shown). These data suggest that the product's thiol group is not important to binding and indicate that the observed pK of 8.0 does not belong to L-cysteine. In addition, at netural pH, the cysteine

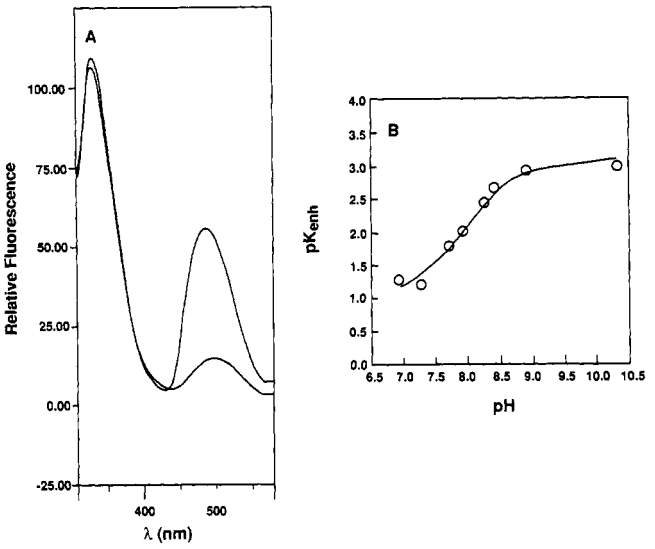

FIGURE 6: (A) Fluorescence emission spectra of *Salmonella typhimurium* OASS-A in the absence and presence of L-cysteine. The upper curve at 500 nm reflects the spectrum of OASS-A in the presence of 500 μM L-cysteine, while the lower curve represents OASS-A alone. The excitation wavelength is 298 nm. A protomer concentration of 2.5 μM was used in 0.25 M potassium phosphate, pH 10.34. The ordinate is relative fluorescence, and the abscissa is emission wavelength. (B) Dependence on pH of enhancement by L-cysteine of OASS-A fluorescence. The abscissa is the negative common log of the dissociation constant of L-cysteine from its complex with enzyme. This reciprocal dissociation constant is denoted by Y in eq 3; the ordinate is the pH. Points are values from experiments, while the line represents the best fit of eq 3 to the data.

analog L-penicillamine was found to enhance the longer-wavelength OASS-A fluorescence.

DISCUSSION

Interpretation of the OASS-A Fluorescence Spectrum. The fluorescence emission spectrum of OASS-A is consistent with expectation for an enzyme containing at least one tryptophan residue and one PLP Schiff base. Upon excitation in the 280–298-nm range (emission being independent of the wavelength of excitation if a single fluorophore is emitting; Arrio-Dupont, 1978), tryptophan residues in proteins will emit in the range of 324–350 nm (Burstein *et al.*, 1973). The blue extreme (324 nm) indicates protection of the side chain from the aqueous solvent in a hydrophobic environment; the red extreme (close to 350 nm) indicates full exposure of the residue to aqueous solvent. The emission maximum of OASS-A at 337 nm falls between these two extremes. Since the deduced primary structure of the enzyme contains two tryptophans (Byrne *et al.*, 1988), there are three plausible interpretations of the 337-nm peak. The first is that each of the two residues is exposed to solvent to a similar, meaningful extent, so that each residue is neither highly exposed to solvent nor completely buried in the hydrophobic interior of the protein. Another interpretation is that the 337-nm peak is a convolution or average of two peaks, one from the emission of a relatively buried tryptophan and the other from a tryptophan more fully exposed to solvent. It is difficult to distinguish between these possibilities. Since OASS-A is present in solution as a catalytically active dimer, there is also the formal possibility that the 337-nm peak is an average of quite different contributions from each protomer. Recent evidence consistent with half-sites reactivity (unpublished work of J. W. Simmons III in this laboratory), in which only one protomer at a time is catalytically active, does not allow neglect of this last interpretation.

The longer wavelength emission peak, at 487 nm in native enzyme, is consistent with what has been seen in other systems for emission of PLP Schiff bases in aqueous environments at neutral pH (Arrio-Dupont, 1970). The excitation spectrum for the 500-nm emission is also consistent with the expected excitation spectrum of a PLP Schiff base emitting at that wavelength. Electronic transitions involving only the pyridine ring (π to π^*) would absorb radiation in the 280-nm range (the B-band), while those involving the extended conjugation of the PLP Schiff base (also π to π^*) would absorb in the 410–415-nm range (the K-band; Silverstein *et al.*, 1981). The re-emission of absorbed radiation in the neighborhood of 500 nm reflects both the aqueous nature of the solvent in which the Schiff base is found (Shaltiel & Cortijo, 1970) and the equilibrium of tautomers of the PLP Schiff base present at neutral pH (Arrio-Dupont, 1970).

Shaltiel and co-workers (Shaltiel & Cortijo, 1970; Cortijo *et al.*, 1971, 1976) recorded fluorescence spectra of phosphorylase *b* in aqueous buffer at several values of pH and of the Schiff base of PLP with *n*-hexylamine in a series of nonaqueous solvents. On the basis of their observation that the maximum emission of PLP Schiff bases tended to occur at longer wavelengths, toward 535 nm, in less polar solvents and at shorter wavelengths in aqueous solutions, Shaltiel and co-workers concluded that the environment of the Schiff base of PLP with lysine in phosphorylase *b* was hydrophobic in nature (Shaltiel & Cortijo, 1970; Cortijo *et al.*, 1971, 1976). The findings of Shaltiel and co-workers can be applied to interpret the fluorescence emission spectrum of OASS-A. Since the longer-wavelength emission maximum of OASS-A is near 500 nm, it is reasonable to conclude that the PLP Schiff base in OASS-A is exposed to aqueous solvent. The model compound studies of Cortijo would suggest that a blue-shift in the long-wavelength fluorescence of an aqueous solution of OASS-A diluted into dioxane (McClure, 1993) reflects a fuller exposure of the PLP Schiff base to water molecules in the solvent mixture. However, the full structural basis for this spectral change, similar to that induced by acetate or L-cysteine, merits further investigation.

There does not appear to be a specific effect on OASS-A fluorescence of the monovalent cations most frequently used as counterions in common buffers. Instead, an increase in ionic strength is in general accompanied by a relative quenching of the longer-wavelength peak and an enhancement of the shorter-wavelength peak (McClure, 1993). Even the cesium cation can be seen to exert this effect, as was observed also in the case of aspartate aminotransferase (Arrio-Dupont, 1978). Fluorescence emission also appears reasonably independent of the buffers employed in the studies presented here. Even at the extremes of pH required for fuller characterization of OASS-A, both the short-wavelength and the long-wavelength emission peaks were observed, and their behavior was thus able to be studied.

The linearity of the Stern–Volmer plot for quenching of the 337-nm emission by acrylamide indicates that acrylamide quenches a population of fluorophores more or less uniformly accessible to solvent.[4] The K_{SV} of 2.3 M^{-1} falls into the low end of the normal range of values for a Stern–Volmer constant for acrylamide. This could mean that the fluorescence lifetimes of the fluorophores quenched are rather short or

[4] A fit of the data using a parabolic function is not as well conditioned as a linear fit correcting for the number of parameters.

that acrylamide collides with them at a relatively low rate. Although from first principles and the evidence at hand it is impossible to distinguish between these alternatives, empirical evidence in other proteins favors a low rate constant for collision (Eftink & Ghiron, 1976). That is, the fluorophores in OASS-A that are quenched by acrylamide are not particularly accessible to solvent. Consistent with these data are those obtained with iodide. Although iodide quenches a heterogeneous population of fluorophores, as inferred from the curvature in the Stern–Volmer plot, the rather low value, 2.0 M^{-1}, of K_{SV} again indicates fluorophores not particularly highly exposed to solvent. In addition, the maximum of the difference spectrum (spectrum in absence of iodide minus that observed in iodide's presence) indicates that the environment of the tryptophan residues quenched by iodide is not much different from that of the tryptophans giving rise to the fluorescence emission in native enzyme. Finally, the linearity of the Stern–Volmer plot for cesium quenching of the 337-nm emission indicates quenching of fluorophores uniformly exposed to solvent, but the very low value, 0.4 M^{-1}, of K_{SV} suggests that quenching is very inefficient.

The results of reconstitution and of experiments in which apoenzyme and holoenzyme were compared directly are consistent with a model in which a substantial fraction (at least 40%) of the tryptophan emission evident in apoenzyme is quenched in holoenzyme. This quenching is concomitant with the appearance of the 498-nm peak and is consistent with a model in which energy transfer occurs between at least one tryptophan residue and the PLP Schiff base (see below). The virtual identity of the values for K_{SV} for Cs^+ quenching obtained with holoenzyme and apoenzyme favors a model in which access of this quencher to the tryptophan residues is the same regardless of the presence of PLP bound as Schiff base.

pH Dependence of Fluorescence Enhancement by Products as a Structural and Mechanistic Probe. Enhancement of long-wavelength fluorescence in OASS-A by either product proceeds without concomitant quenching of the short-wavelength emission. If singlet–singlet transfer caused the enhancement, one would expect a decrease in the shorter-wavelength emission. One would also observe a change in shorter-wavelength emission if a shift in tautomeric equilibrium occurred.

Arrio-Dupont (1970) synthesized Schiff bases of PLP with valine and recorded their fluorescence spectra at various values of pH. Emission maxima of 430 and 500 nm were observed at values of pH between 6 and 11. Arrio-Dupont attributed the 430-nm emission to a PLP tautomer that absorbs maximally at 330 nm, and the 500-nm emission was attributed to a PLP tautomer that absorbs maximally at 280 and 410 nm. The excitation spectrum for the 500-nm emission of OASS-A in neutral, aqueous buffer has maxima near 284 and 413 nm. These data are consistent with the presence in substrate-free OASS-A of a PLP Schiff base. Since the enzymatic reaction products affect the 498-nm emission but not the 430-nm emission, their binding appears to affect the fluorescence of only one of the forms of the PLP Schiff base that might be present at neutral pH. Therefore, a shift in tautomeric equilibrium could not account for all the spectral changes reported here.

The findings of Metzler and co-workers (1980) bear directly upon the interpretation of the OASS-A fluorescence spectra. Whereas Arrio-Dupont examined the fluorescence spectra of model Schiff bases, Metzler and co-workers examined the absorption spectra of several Schiff bases under a variety of conditions. Their finding that is most relevant to interpretation of the OASS-A spectra is that PLP Schiff bases absorb to some degree at 298 nm, no matter the pH, and hence no matter the predominant tautomeric form of PLP Schiff base present. Therefore, the observation that the 430-nm emission of OASS-A is insensitive to product binding and that the 500-nm emission is quite sensitive strengthens the following conclusion: binding of product to OASS-A affects the fluorescence of only one tautomer of PLP Schiff base. This means that product binding does not affect the equilibrium between tautomers of PLP that might be present in OASS-A.

Strambini and co-workers (Strambini *et al.*, 1992a,b) have recently investigated the phosphorescence and fluorescence of tryptophan synthetase multimeric apoenzyme, holoenzyme, and reduced holoenzyme. The Strambini group found long-wavelength phosphorescence and fluorescence in holoenzyme. They attributed a delayed 520-nm fluorescence to triplet–singlet energy transfer from an excited tryptophan residue to the PLP Schiff base in holoenzyme. Singlet–singlet transfer from tryptophan to the PLP Schiff base was also postulated as a mechanism to account for certain observations. The 500-nm region was the portion of the fluorescence spectrum that was most affected by the binding of ligands to the enzyme. Given the degree of homology in primary structure (42%; G. D. McClure, Jr., unpublished observation) between tryptophan synthetase β-subunit and OASS-A and the fact that each enzyme can catalyze a β-replacement reaction, it is tempting to conclude that similar phosphorescence and fluorescence phenomena might well be observed in OASS-A. A more conservative interpretation is that the Strambini findings show that 500-nm fluorescence emission in a PLP-dependent enzyme can be directly affected by ligand binding without other portions of the emission spectrum changing greatly.

If, as stated above, product binding does not affect the equilibrium between tautomers of PLP, enhancement of the 498-nm emission by ligand binding may be due to an increase in the efficiency of nonradiative energy transfer from a donor to the PLP Schiff base acceptor. Possibilities for nonradiative transfer include singlet–singlet transfer, singlet–triplet transfer, triplet–singlet transfer, triplet–triplet transfer, and electron-exchange transfer (Turro, 1978).

When acetate or L-cysteine binds to OASS-A, the emission in the vicinity of 338 nm is virtually unaffected. Therefore it would appear that the decay of the excited singlet state of tryptophan residues is not affected by the binding of either of these ligands. However, since emission in the 498-nm region is enhanced, decay of the excited singlet state of the PLP Schiff base is being affected. If a nonradiative process of energy transfer is occurring upon product binding in which tryptophan is the donor and the PLP Schiff base is the acceptor, it must therefore involve the triplet state of tryptophan and the singlet state of the PLP Schiff base. The delayed fluorescence observed by Strambini and co-workers (Strambini *et al.*, 1992a,b) in tryptophan synthetase was attributed to triplet–singlet energy transfer from an excited tryptophan residue to the PLP Schiff base in the holoenzyme. The Strambini group claimed that this was the first report of triplet–singlet energy transfer for a protein–cofactor pair. Their more recent finding of tryptophan phosphorescence in *Escherichia coli* aspartate aminotransferase suggests that this mechanism may be obtained more generally in PLP-dependent enzymes (Cioni *et al.*, 1992).

Another possibility for the mechanism of enhancement is electron-exchange transfer (Turro, 1978). Electron-exchange transfer requires the donor and the acceptor to be physically close, within a few van der Waals radii. If r is the distance

between donor and acceptor, the efficiency of electron-exchange transfer is proportional to e^{-r}. Triplet–singlet transfer also requires the donor and the acceptor to be physically close, but in this case the efficiency of transfer is proportional to r^{-6} (Turro, 1976).

It is not possible to conclude solely on the basis of the work presented here whether or not the orientation between either tryptophan residue and PLP changes once ligand is bound. However, a correlate of the enhancement of acceptor fluorescence would be an increase in the lifetime of its excited state. The hypothesis of triplet–singlet transfer could be tested if the lifetimes of the 337- and 498-nm emissions could be measured directly in the presence or absence of ligand. If the lifetime of the 337-nm emission remained unchanged but that of the 498-nm emission were considerably lengthened, then the hypothesis would be supported. Lifetime experiments are planned to test these hypotheses.

It is interesting to note that findings of enhanced fluorescence of D-serine dehydratase upon glycine binding were interpreted by Federiuk and Shafer (1983) to indicate a conformational change that occurs upon transimination to form the external (amino acid) Schiff base. In the case of the cysteine-enhanced fluorescence of OASS-A, the explanation put forth by these authors likely also applies. That is, formation of the external Schiff base with cysteine is accompanied by a conformational change that results in a change in the orientation of the PLP, thereby increasing either the efficiency of energy transfer or the lifetime of the excited state of the PLP Schiff base. In this regard, the external Schiff base with L-cysteine can be prepared by the addition of L-cysteine to OASS-A without further reaction (unpublished work of J. W. Simmons, III, in this laboratory). It will be of interest to determine whether the excited-state lifetime of the external Schiff base has been affected compared to that of the internal Schiff base.

Effect of Substrate Binding on Intrinsic Fluorescence. There is little change in the emission at 337 nm upon the binding of the substrate OAS. The 498-nm emission is much more sensitive to the presence of this substrate. Since the 498-nm emission in holoenzyme appears to be the reuslt of excitation of the PLP Schiff base with the active site lysine, the disappearance of this emission upon addition of OAS to the enzyme is expected. The OASS reaction 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 & Wedding, 1976; Tai *et al.*, 1993). Concomitant with the conversion of the PLP Schiff base to the α-aminoacryalte Schiff base, changes in the absorption spectrum of OASS-A are observed (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 absorption band of the PLP Schiff base which emits maximally at 498 nm is no longer present when OAS is added to OASS-A. The addition of sulfide to the α-aminoacrylate Schiff base then results in a regeneration of the PLP Schiff base of free enzyme and thus restores the 498-nm emission.

Structural Requirements for Enhancement of Long-Wavelength Fluorescence and Implications with Regard to Enzyme Mechanism and Structure. Data obtained at neutral pH are consistent with the existence of a complex between OASS-A and the product, acetate. A methyl group as such is not required for product binding, since trifluoroacetate, an isostere of acetate, elicits the same enhancement of intrinsic fluorescence. Therefore, an explanation of product binding based primarily on hydrophobic interactions finds no support. Instead, it appears that substitution of even three chlorines for the methyl hydrogens does not abolish the ability of a product analog to bind, as evidenced by the enhancement evoked by trichloroacetate. Hence, there does not appear to be much if any steric hindrance to the binding of the portion of the molecule opposite the carboxylic acid moiety. Finally, since propionate also seems to be accommodated by the putative acetate-binding site, a three-carbon chain does not preclude binding. [This is reminiscent of the finding of Cook and Wedding (1976) that propionate can reverse the changes in the absorption spectrum caused by OAS binding.] Therefore, it is reasonable to hypothesize that orientation of the carboxylate group into a binding site on enzyme may be a determinant of acetate binding.

Both L-cysteine and L-cystine similarly enhance long-wavelength fluorescence at pH above 10. Hence, one may conclude that the product's thiol group is not the sole determinant of binding of L-cysteine to enzyme. The enhancement of 498-nm fluorescence by L-penicillamine at neutral pH indicates that derivatization of the methylene bridge does not preclude binding. Therefore the carboxylate, the amino, or both of these groups on L-cysteine are likely involved in its binding to the enzyme.

The functional group common to both products is a carboxylate. Federiuk and Shafer (1983) have suggested that the carboxylate of an enzyme-bound amino acid alters a charge–charge interaction between groups on enzyme, thereby facilitating a conformational transition. It appears likely that this explanation also holds for OASS-A. In the case of cysteine binding, formation of the external Schiff base may be accompanied by or preceded by binding of the α-carboxyl group, breaking a salt bridge on enzyme, and facilitating a conformational change. Acetate cannot form a Schiff base, but can bind to the α-carboxyl amino acid subsite. However, acetate is also a product of the reaction and, as a product, binds to a site distinct from the α-carboxyl subsite (Cook *et al.*, 1992; Nalabolu *et al.*, 1993). The affinity of acetate for its site as a product (Nalabolu *et al.*, 1993) and the affinity of acetate for the enzyme found in these studies are distinctly different. As a product inhibitor, acetate binds with a pH-independent dissociation constant of about 30 mM (Nalabolu *et al.*, 1993), an order of magnitude lower than the pH-independent value of 270 mM found in these studies. Thus, these studies document the binding of acetate to a second site with weaker affinity. The second site is likely the α-carboxyl subsite. Once bound, acetate likely disrupts a salt bridge, allowing the internal Schiff base to assume a conformation similar to that of the external Schiff base. The latter results in fluorescence enhancement. This is to our knowledge the first reported example of cofactor Schiff base fluorescence enhancement by a non-amino acid reactant for a PLP-dependent enzyme.

In the proposed acid–base chemical mechanism for OASS-A, two enzyme groups that must be unprotonated are observed in the V/K for OAS. These data indicate the requirement for these groups to be unprotonated on free enzyme for binding and catalysis. One of the groups, with a pK of 7, is thought to polarize the substrate's carbonyl carbon to make acetate a better leaving group (Nalabolu & Cook, 1993). On the basis of data presented here, we conclude that acetate binds more avidly to the enzyme when this enzyme group with pK 7 is unprotonated. Deprotonation of the gruop with a pK of 7 may result in an opening up of the active site, facilitating the binding of acetate to the α-carboxyl subsite.

There is no group with a pK of 8 observed in the V/K for OAS representing free enzyme. However, as shown in eq 4, the addition of L-cysteine to enzyme can result in the formation of an external Schiff base, with the lysine that formed the internal Schiff base protonated. The latter has a pK of 8.2 (Cook *et al.*, 1992) and, when deprotonated, would enhance the binding of cysteine according to eq 4.

$$\text{E} + \text{L-cysteine} \rightleftharpoons \underset{(\text{lysine-}NH_3^+)}{\text{cysteine Schiff base}} \rightleftharpoons \underset{(\text{lysine-}NH_2)}{\text{cysteine Schiff base}} \quad (4)$$

In agreement with this proposal, an external Schiff base between the pyridoxal form of the enzyme and cysteine has been identified via spectrophotometric titration. In addition, optimum binding of cysteine occurs at high pH once a group with a pK of 8 has been titrated (unpublished work of J. W. Simmons, III, in this laboratory).

There are two tryptophan residues, at positions 51 and 162, in the deduced primary structure of OASS-A (Byrne *et al.*, 1988). Tryptophan residue 162 in OASS-A can be seen to be strictly conserved when the amino acid sequences of OASS from plant (Saito *et al.*, 1992; Romer *et al.*, 1992; Rolland *et al.*, 1993) and bacterial (Byrne *et al.*, 1988; Sirko *et al.*, 1990) sources are aligned (G. D. McClure, unpublished observations). The fluorescence data presented above are consistent with the presence near the enzyme's active site of a tryptophan residue, the orientation or position of which, relative to PLP, is sensitive to the presence of substrates and products. We speculate that tryptophan 162 is likely close to the active site and gives rise to much of the fluorescence signal enhanced by each product's presence there. Fluorescence spectra of OASS-A mutants that lack W51 but still possess W162 still show enhanced 498-nm emission in the presence of acetate (unpublished observations of J. Davis in this laboratory).

REFERENCES

Arrio-Dupont, M. (1970) *Photochem. Photobiol. 12*, 297.

Arrio-Dupont, M. (1971) *Biochem. Biophys. Res. Commun. 44*, 653.

Arrio-Dupont, M. (1978) *Eur. J. Biochem. 91*, 369.

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

Burstein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263.

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

Cioni, P., Onuffer, J. J., & Strambini, G. B. (1992) *Eur. J. Biochem. 209*, 759.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104.

Cook, P. F., & Wedding, R. T. (1976) *J. Biol. Chem. 251*, 2023.

Cook, P. F., & Wedding, R. T. (1977) *J. Biol. Chem. 252*, 3459.

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

Cooper, A. J. L., & Meister, A. (1989) *Biochimie 71*, 387.

Cortijo, M., Steinberg, I. Z., & Shaltiel, S. (1971) *J. Biol. Chem. 246*, 933.

Cortijo, M., Llor, J., Jimenez, J. S., & Garcia-Blanco, F. (1976) *Eur. J. Biochem. 65*, 521.

Dowhan, W., Jr., & Snell, E. E. (1970) *J. Biol. Chem. 245*, 4618.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199.

Ehrlich, J. H., & Schnackerz, K. D. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 1183.

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

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

Goldberg, M. E., York, S., & Stryer, L. (1968) *Biochemistry 7*, 3662.

Grafius, M., & Neilands, J. B. (1955) *J. Am. Chem. Soc. 77*, 3389.

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

Houben, K. F., Kadima, W., Roy, M., & Dunn, M. F. (1989) *Biochemistry 28*, 4140.

Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857.

Kiick, D. M., & Cook, P. F. (1983) *Biochemistry 22*, 375.

Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., & Christen, P. (1984) *J. Mol. Biol. 174*, 497.

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

Leatherbarrow, R. J. (1992) *Grafit Version 3.0. Erithacus Software*, Staines, U.K.

Lehrer, S. S., & Leavis, P. C. (1978) *Methods Enzymol. 49*, 222.

Marceau, M., Lewis, S. D., & Shafer, J. A. (1988) *J. Biol. Chem. 263*, 16934.

Martell, A. E., & Smith, R. M. (1977) *Critical Stability Constants*, Vol. 3, Plenum, New York.

McClure, G. D., Jr. (1993) Ph.D. Dissertation, University of North Texas.

Metzler, C. M., Cahill, A., & Metzler, D. E. (1980) *J. Am. Chem. Soc. 102*, 6075.

Rao, G. S. J., Mottonen, J., Goldsmith, E., & Cook, P. F. (1993) *J. Mol. Biol. 231*, 1130.

Rolland, N., Droux, M., Lebrun, M., & Douce, R. (1993) *Arch. Biochem. Biophys. 300*, 213.

Romer, S., d'Harlingue, A., Camara, B., Schantz, R., & Kuntz, M. (1992) *J. Biol. Chem. 267*, 17966.

Saito, K., Miura, N., Yamazaki, M., Hirano, H., & Murakoshi, I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8078.

Schnackerz, K. D. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1565.

Shaltiel, S., & Cortijo, M. (1970) *Biochem. Biophys. Res. Commun. 41*, 594.

Shaltiel, S., Hedrick, J. L., & Fischer, E. H. (1967) *Methods Enzymol. 11*, 675.

Silverstein, R. M., Bassler, G. C., & Morrill, T. C. (1981) *Spectrometric Identification of Organic Compounds*, John Wiley, New York.

Sirko, A., Hryniewicz, M., Hulanicka, D., & Bock, A. (1990) *J. Bacteriol. 172*, 3351.

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

Strambini, G. B., Cioni, P., Peracchi, A., & Mozzarelli, A. (1992b) *Biochemistry 31*, 7535.

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

Turro, N. J. (1978) *Modern Molecular Photochemistry*, Benjamin/Cummings, Menlo Park.

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

York, S. S. (1972) *Biochemistry 11*, 2733.