Tryptophan Luminescence as a Probe of Enzyme Conformation along the *O*-Acetylserine Sulfhydrylase Reaction Pathway†

Giovanni B. Strambini,‡ Patrizia Cioni,‡ and Paul F. Cook*,§

Consiglio Nazionale della Ricerche—Istituto di Biofisica, Via S. Lorenzo, 26-56127 Pisa, Italy, and Department of Biochemistry and Molecular Biology, University of North Texas Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107-2699

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ABSTRACT: *O*-Acetylserine sulfhydrylase A (OASS-A) is a pyridoxal 5′-phosphate- (PLP-) dependent enzyme that catalyzes the last step in the synthesis of L-cysteine, the β-replacement of acetate in *O*-acetyl-L-serine (OAS) by sulfide. The phosphorescence properties of the two tryptophans of wild-type OASS-A, W51 and W162, and of W162 in the W51Y mutant protein have been characterized over the temperature range 170−273 K. In glasses at 170 K, the apoenzyme exhibits a phosphorescence spectrum which is the superposition of two spectra with well-resolved 0,0 vibronic bands centered at 405 and 410 nm, the blue λ_{max} suggesting that one of the two Trp residues in OASS-A is in a polar pocket, while the other is in a relatively hydrophobic pocket. The presence of PLP in the OASS-A holoenzyme reduces the intrinsic fluorescence by 40−45%, but the spectrum is unaltered except for the appearance of the internal Schiff base ketoenamine fluorescence band centered at 484 nm. The phosphorescence is strongly quenched by PLP, with about 70% reduction in intensity and lifetime. Further, the phosphorescence spectrum of the holoprotein exhibits a single and narrow 0,0 vibronic band centered at 405 nm and a broad band in the 450−550-nm range resulting from delayed fluorescence of the ketoenamine tautomer of the internal Schiff base, sensitized by triplet−singlet energy transfer from tryptophan to the ketoenamine tautomer of PLP. Comparison with data obtained for the W51Y mutant strongly suggests that the 405-nm phosphorescence band derives from W162, and that W51 in the wild type is entirely quenched either by singlet or triplet energy transfer to PLP or by some local group in the protein. From the rate of energy transfer, the separation between W162 and PLP is estimated to be about 25 Å. Substrates other than OAS affect only the intensity of the coenzyme fluorescence band (484 nm) and the intensity of delayed fluorescence relative to that of phosphorescence, effects that are attributable to changes in fluorescence quantum yield of the ketoenamine chromophore. Addition of OAS, on the other hand, leads to a splitting of the 0,0 vibronic band in the phosphorescence spectrum of W162, yielding poorly resolved peaks at 406 and 408.5 nm, indicating thereby a change in the environment of the tryptophan residue and therefore in the conformation of the macromolecule as the internal Schiff base is converted to the α-aminoacrylate Schiff base. In buffer at 273 K, both the fluorescence and phosphorescence spectra relax to longer wavelengths and the phosphorescence lifetime is reduced to a few milliseconds, all indications that W162 is in a flexible region of the macromolecule, probably in close proximity to the aqueous interface. The phosphorescence lifetime in fluid medium reveals conformational heterogeneity in OASS-A and unveils important structure modulating effects of cofactor, substrates, and pH. Binding of PLP to the apoprotein increases the rigidity of the polypeptide in the region of W162 (in agreement with the greater thermal stability of the holoprotein), while OAS and L-serine have an opposite effect. Increasing the pH from 6.5 to 9 results in a 1.7-fold increase in τ_{av} and a change in the relative amplitudes of the two lifetime components. Since the phosphorescence originates from a single tryptophan residue, the two τ components reflect distinct conformations of the subunit. In this case the conformational equilibrium (slow on the phosphorescence time scale) is governed by one or more groups in the protein with a p*K* around 8.

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 intermediate then undergoes a β-substitution reaction, catalyzed by the pyridoxal 5′-phosphate- (PLP-) dependent *O*-acetylserine sulfhydrylase, in which acetate is eliminated and replaced by sulfide to give L-cysteine.

The kinetic mechanism for OASS-A is ping-pong with competitive inhibition by both substrates, indicative of E−sulfide and F−OAS dead-end complexes (Cook & Wedding, 1976; Tai et al., 1993). Rapid-scanning stopped-flow experiments indicate a rate-determining first half-reaction, with formation of the α-aminoacrylate intermediate

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‡ CNR—Istituto di Biofisica.

§ University of North Texas Science Center at Fort Worth.

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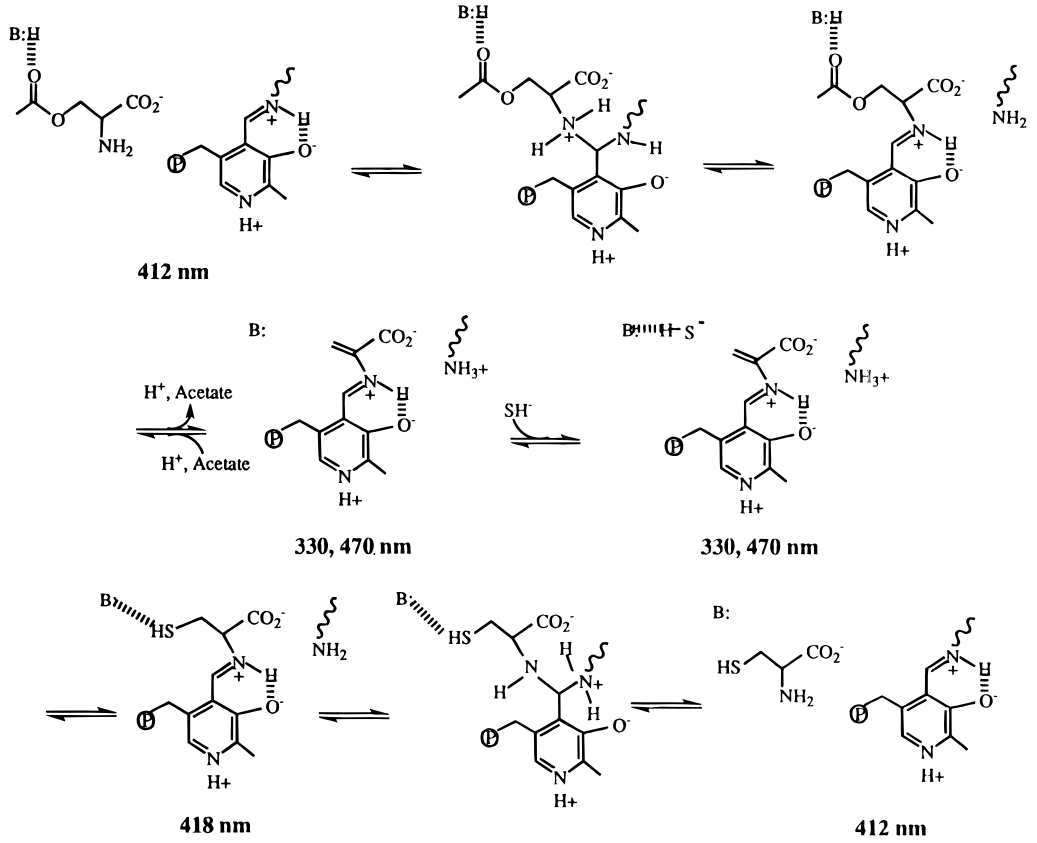
Scheme 1



one of the slow steps within the first half-reaction (Woehl et al., 1996).

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. An absorption maximum at 412 nm is observed for unliganded OASS, due to the formation of a protonated Schiff base between an active-site lysine and the active-site PLP (Cook & Wedding, 1976; Cook et al., 1992). Addition of OAS to the native enzyme results in the disappearance of absorbance at 412 nm and the appearance of new absorption maxima at 330 and 470 nm, indicative of the formation of a protonated Schiff base between PLP and α-aminoacrylate upon the β-elimination of acetate from OAS (Cook & Wedding, 1976; Schnackerz et al., 1979; Cook et al., 1992). The addition of L-cysteine to OASS results in the formation of the external Schiff base with a λ_{max} of 418 nm (Schnackerz et al., 1995). The addition of L-serine results in establishing an equilibrium between two different tautomeric forms of an external Schiff base absorbing at 320 and 418 nm (Schnackerz et al., 1995). No change is observed in the far-UV CD upon addition of OAS, but a significant change is seen upon addition of either cysteine or serine, thought to suggest a closing of the active site (Schnackerz et al., 1995). The ^{31}P NMR chemical shift is increased from 5.2 to 5.3 ppm, signaling a tighter interaction at the 5′-phosphate upon formation of the cysteine external Schiff base, while no change is observed upon addition of OAS. In the case of serine, the ^{31}P NMR signal is decreased to 4.4 ppm, suggesting a loosening of the binding at the 5′-phosphate. The line width is indicative of an intermediate to rapid exchange between two species, presumably the two Schiff base tautomers.

The fluorescence emission spectrum of OASS-A with excitation at 298 nm gives a ketoenamine band at 500 nm in addition to tryptophan fluorescence at 337 nm (McClure & Cook, 1994). The red fluorescence band is enhanced in the presence of cysteine as a result of a conformation change that occurs upon formation of the external Schiff base (McClure & Cook, 1994). The external Schiff base with serine also exhibits an enhancement of the long-wavelength fluorescence, but not to the same extent as with cysteine, rather by an amount consistent with the amount of ketoenamine tautomer present (Schnackerz et al., 1995). There are two tryptophans in the primary sequence of OASS-A, at positions 51 and 162 (Byrne et al., 1988).

The pH dependence of the ultraviolet−visible absorption spectrum in the absence and presence of OAS (Cook et al., 1992) and the pH dependence of kinetic parameters using natural and alternative reactants have also been measured (Tai et al., 1995) and a chemical mechanism of OASS-A has been proposed (Scheme 1). *O*-Acetyl-L-serine binds to the enzyme with an active-site internal Schiff base with its α-amine unprotonated to form the protonated external Schiff base with the side-chain acetyl carbonyl hydrogen-bonded to a protonated enzyme group, which aids in the β-elimination of acetate. The enzyme lysine that was in Schiff base linkage with the active-site PLP deprotonates the α-carbon in the β-elimination reaction and a proton is likely released with the acetate product. Sulfide likely binds as HS^- to undergo nucleophilic attack on the α-aminoacrylate intermediate, followed by protonation of the α-carbon by the enzyme lysine. The HS^- may be hydrogen-bonded to the enzyme group that assists in the β-elimination of acetate.

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 the positive Cotton effect shifts to 418 nm with a slightly decreased intensity compared to 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 induced CD data provide information on conformational changes localized to the active site in the vicinity of the PLP cofactor.

The present study aims at investigating the conformational state of OASS-A and the changes induced by pH, cofactor, and substrate complexation, by means of intrinsic phosphorescence of its two Trp residues, W51 and W162. Spectral energies are sensitive to the polarity of the intermediate chromophore's environment, while the excited-state lifetime in rigid glassy medium can report on the rate of quenching interactions such as energy transfer to the ketoenamine tautomer of the PLP Schiff base (Strambini et al., 1992a), from which Trp−PLP distance relationships can be derived. Further, the room-temperature phosphorescence lifetime provides an exquisitely sensitive probe of the local fluidity of the protein matrix (Strambini & Gonelli, 1985, 1995), a parameter that has proven useful for several enzymes in uncovering the subtle changes in polypeptide conformation brought about by the binding of substrates and allosteric effectors (Cioni & Strambini, 1989; Strambini & Gonelli, 1990; Strambini et al., 1992b), changes that otherwise remained undetected with conventional spectroscopic techniques. The characterization of the individual emission properties of W51 and W162 was aided by the construction of a single Trp mutant, W51Y-OASS-A. The results demonstrate that, in holo-OASS-A, W51 is silent so that the intrinsic phosphorescence is due exclusively to W162. The latter is involved in energy transfer to the ketoenamine tautomer, and spectral shifts along with significant changes in the room-temperature phosphorescence τ, attest to important structural modulating effects of pH and substrate complexation.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli* NM522 (*hsds5 s(lac−pro)* [*F′ pro*$^+$ *laclqZsM15*]) and *S. typhimurium* LB500 (*metA metE551 trpD2 leu hsdLT hsdA hsdB* and m^+ for all three modification systems) were obtained from ATCC. The *S. typhimurium* strain DW378 (*trpC109 cysK1772 cysM1770*) and plasmid pRSM40 were obtained from Dr. N. M. Kredich at Duke Medical School.

Enzymes. Wild-type *O*-acetylserine sulfhydrylase A from *S. typhimurium* LT-2 or the W51Y mutant was purified by the method of Hara et al. (1990) as modified by Tai et al. (1993). The enzyme preparation used in this study was >95% pure on the basis of SDS−PAGE. The enzyme had a specific activity of 800 units/mg with sulfide as the second substrate and assayed using a sulfide ion-selective electrode (Hara et al., 1990). The mutant enzyme had a specific activity $^1/_{10}$ that of the wild-type OASS-A using 5-thio-2-nitrobenzoate as the nucleophilic substrate (Tai et al., 1993). The procedure for preparing the W51Y mutant will be described in a manuscript characterizing the mutant enzyme.

The apoenzymes of OASS-A and the W51Y mutant were prepared according to Schnackerz and Cook (1995). Generally, enzyme was dialyzed for 24 h against 100 mM Mes, pH 6.5, with 10 mM OAS and 5 M guanidinium chloride.

Chemicals. *O*-Acetyl-L-serine and Na_2S were obtained from Sigma. All other reagents and chemicals were obtained from commercially available sources and were of the highest quality available.

Luminescence Studies. A conventional homemade instrument was employed for all fluorescence and phosphorescence measurements in glasses and in fluid solutions (Strambini, 1983). Continuous excitation for fluorescence and phosphorescence spectra was provided by a xenon lamp and the excitation wavelength was selected by a 0.25-m grating monochromator with a 10-nm bandpass. The emitted light was collected through another 0.25-m grating monochromator (Jobin-Yvon, H25) with 3-nm bandwidth and detected by an EMI9635QB photomultiplier.

Phosphorescence and delayed fluorescence decays in glasses were obtained after steady-state excitation by the xenon lamp. In fluid solutions, the phosphorescence lifetimes were obtained following pulsed excitation by a frequency-doubled flash-pumped dye laser with a pulse duration of 1 μs and a typical energy/pulse of 1−10 mJ. The decay of tryptophan phosphorescence was detected by an apparatus with high sensitivity and a time resolution of 10 μs (Strambini & Gonnelli, 1995). All phosphorescence decays were analyzed in terms of a sum of exponential components by a nonlinear least-squares fitting algorithm (Global Unlimited, LFD, University of Illinois).

To obtain reproducible phosphorescence data in solution, it is of paramount importance to thoroughly remove all dissolved oxygen. The procedure followed to obtain satisfactory deoxygenation has been described in a previous report (Strambini & Gonnelli, 1990).

RESULTS

Luminescence Spectral Properties of OASS-A at 170 K in a Glycerol/Buffer Glass

Spectral Properties and Emission Intensities. Upon excitation at 295 nm, the apoenzyme in a glycerol/buffer glass exhibits a partly structured fluorescence spectrum (Figure 1) with a λ_{max} of 326 nm (shoulder at 315 nm), which is blue-shifted 8−10 nm relative to the spectrum of buffer at room temperature. Molecular relaxations around the excited chromophore are typically characterized by a chromophoric red shift, and thus the Trp environments in OASS are likely fairly mobile in fluid solutions. The corresponding phosphorescence spectrum (Figure 1) is the superposition of two spectra with well-resolved 0,0 vibronic bands centered at 405 and 410 nm. Tryptophan residues located in hydrophobic pockets generally have a $\lambda_{0,0}$ of 411−412 nm, while those exposed to aqueous solvent have a $\lambda_{0,0}$ of 407−408 nm. The 405-nm component of the OASS phosphorescence spectrum is blue-shifted relative to those in the hydrophobic and aqueous environments, implying that one of the two Trp residues in OASS-A is in a relatively polar pocket. Raising the pH from 6.5 to 9.5 shifts the 410-nm band about 1 nm to the blue, suggesting that the environment of the putative Trp residue is affected by the pH change.

The presence of PLP in the OASS-A holoprotein reduces the intrinsic Trp fluorescence by 40−45%, but the spectrum is unaltered (Figure 1) except for the appearance of the internal Schiff base ketoenamine fluorescence band centered at 484 nm (Scheme 1). With excitation at 330 nm, outside

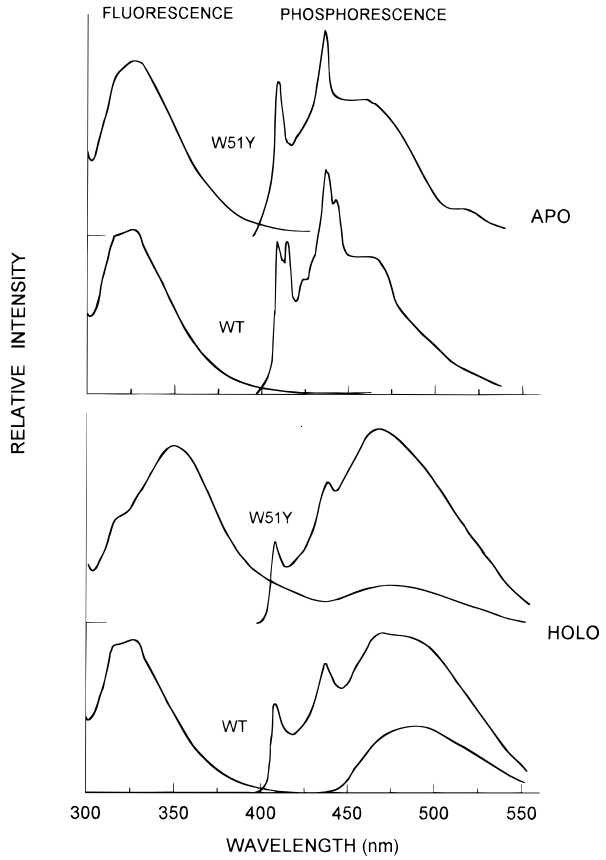


FIGURE 1: Tryptophan fluorescence and phosphorescence spectra of apo- and holo-OASS-A wild type and W51Y mutant. Spectra were measured in phosphate buffer pH 6.5/glycerol (50/50 w/w) at 170 K. The excitation wavelength was 295 nm. Protein concentrations were typically 4 μM. Spectra were not corrected for instrumental response and the intensity scale is different for each spectrum.

the Trp absorption range, the coenzyme fluorescence consists of two bands, a major one centered at 484 nm (ketoenamine tautomer) and a weaker band centered at 362 nm (spectrum not shown). The latter is characteristic of the enolimine tautomer that, according to the absorption spectrum, is barely detectable in this protein. The Trp phosphorescence spectrum of holo-OASS-A (Figure 1) exhibits a single and narrow 0,0 vibronic band centered at 405 nm, as opposed to the apoprotein spectrum which exhibits two bands at 405 and 410 nm. Further, at wavelengths greater than 440 nm there is a long-lived coenzyme emission that alters the phosphorescence spectrum by superimposing on it a broad band in the range of 440−550 nm. Following spectral decomposition, the latter emission is seen to be identical to the prompt coenzyme fluorescence, that is, the 484-nm band. The lack of phosphorescence upon selective excitation of the PLP moiety in the holoenzyme at 330 nm, and the decay characteristics of the observed fluorescence emission (essentially identical to that of Trp phosphorescence), confirm that the 484-nm band observed in the phosphorescence spectrum results from delayed fluorescence of the ketoenamine tautomer of the internal Schiff base, sensitized by energy transfer from the phosphorescence state of Trp. The latter phenomenon has been observed previously for tryptophan synthase (Strambini et al., 1992a) and aspartate aminotransferase (Cioni et al., 1992).

The efficiency of triplet−singlet energy transfer, Φ_{ET}, can be obtained directly from the reduction in phosphorescence intensity of the holoprotein relative to the apoprotein (P_{rel}, Table 1). An accurate measure of Φ_{ET} is provided by comparing the intensity ratio (phosphorescence at 435 nm)/(fluorescence at 326 nm), since the ratio also accounts for any variation in intersystem crossing quantum yield due to quenching processes that might occur at the precursor fluorescence state. The estimated value of Φ_{ET} is 0.70 ± 0.02, suggesting that in the holoprotein about 70%, relative to apoprotein, of the phosphorescence is quenched by energy transfer to the ketoenamine tautomer of PLP.

Effects of Reactants. The luminescence of apo- and holo-OASS-A was also examined in the presence of OAS (1 mM, pH 6.5), L-serine (100 mM, pH 9.5), potassium acetate (1 M, pH 9.5) and L-cysteine (4 mM, pH 9.5). With the exception of OAS, the reactants and analogs have practically no influence on either the spectral features of Trp emission (both apo- and holoenzyme) or the magnitude of Φ_{ET} (Table 1). A substrate effect is instead evident on the intensities of the coenzyme fluorescence band (484 nm), i.e., F_{484}/F_{326}, as well as on the intensity of delayed fluorescence (*DF*) relative to that of phosphorescence (*P*). Because Φ_{ET} is essentially constant in the various complexes, the substantial changes in the *DF/P* ratio are attributable to changes in fluorescence quantum yield of the ketoenamine chromophore, a change that in good part accounts also for the substrate- or pH-induced enhancement of the red coenzyme band (F_{484}/F_{326}) in the prompt fluorescence spectrum.

The complex with OAS results in the formation of the α-aminoacrylate Schiff base, a very weakly luminescent chromophore that absorbs at longer wavelengths ($\lambda_{max} = 470$ nm). As a consequence, the coenzyme band at 484 nm is lacking almost entirely in both prompt and delayed fluorescence. More interesting, OAS leads to a splitting of the 0,0 vibronic band in the phosphorescence spectrum yielding poorly resolved peaks at 406 and 408.5 nm (not shown). The emergence of spectral heterogeneity together with wavelength shifts clearly imply a change in the environment of the Trp residues and therefore in the conformation of the macromolecule as the internal Schiff base is converted to the α-aminoacrylate intermediate. No OAS effect was observed on the apoprotein.

Phosphorescence Decay Kinetics. In rigid glasses the phosphorescence lifetime, τ, of Trp, both free in solution or in proteins, is typically around 6 s. Smaller values of τ imply specific interactions, such as energy or electron transfer with a group in the immediate vicinity. At pH 6.5, the phosphorescence intensity of the apoprotein decays as an exponential function with a τ of 6.1 ± 0.1 s, independent of the emission wavelength. By contrast, the decay of the holoprotein measured at 405 nm, a wavelength selective to Trp phosphorescence, is more rapid and highly nonexponential (Figure 2). When fitted in terms of two discrete components, lifetimes of about 5 and 1 s are obtained, with the long-lived component having an amplitude not exceeding 10% (Table 2). The decay of delayed fluorescence was measured at 510 nm where the contribution of Trp phosphorescence to the overall emission is only ∼25%. After the phosphorescence component is subtracted out, the remainder of the intensity decays with a lifetime of about 1 s, in agreement with that of the precursor phosphorescent state.

The lifetime reduction from apo- to holoprotein affords a direct measure of the rate of triplet−singlet energy transfer, $k_{TS} = 1/\tau_{holo} - 1/\tau_{apo}$, and therefore of Φ_{ET} ($\Phi_{ET} = 1 -$

Table 1: Fluorescence and Phosphorescence Parameters of OASS-A and Its Reactant Complexes in a Glass at 170 K

sample	pH	fluorescence	phosphorescence			Φ_{ET}	
		(F_{484}/F_{326})	$\lambda_{0,0}$ (nm)	P_{rel}	DF_{460}/P_{405}	$(1 - P_{rel})$	$[1 - (\tau/\tau_{apo})]$
apo-OASS	6.5		405, 410	1.00			
holo-OASS	6.5	0.37	405	0.30	0.91	0.70	0.61
holo-OASS + OAS	6.5	0.02	406, 408.5	0.28	0.05	0.72	0.38
apo-OASS	9.5		405, 409	1.00			
holo-OASS	9.5	0.50	405	0.33	1.66	0.67	0.61
holo-OASS + serine	9.5	0.47	405	0.32	1.32	0.68	0.60
holo-OASS + acetate	9.5	0.65	405	0.38	2.33	0.62	0.64
holo-OASS + cysteine	9.5	0.44	405	0.29	1.48	0.71	0.61

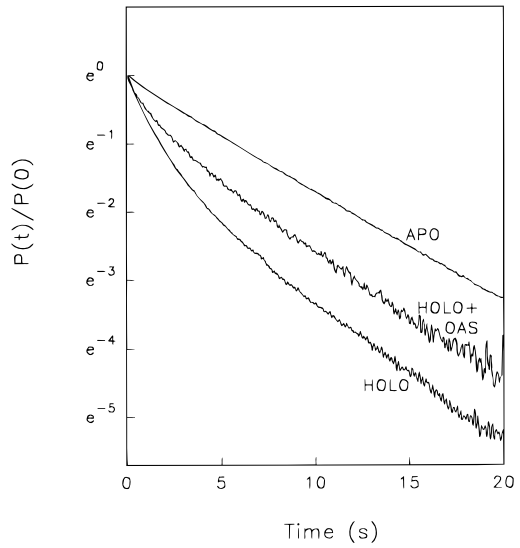


FIGURE 2: Decay of tryptophan phosphorescence intensity (λ_{em} = 405 nm) of apo- and holo-OASS-A and of holo-OASS-A plus 1 mM OAS. The time course of Trp phosphorescence was recorded upon steady-state excitation at 295 nm. Experimental conditions are as in the legend to Figure 1.

Table 2: Phosphorescence Decay Kinetics (λ_{em}, 405 nm) of OASS-A and Its Reactant Complexes in a Glass at 170 K

sample	pH	τ_1 (s)	τ_2 (s)	α_1	τ_{av}[a]	τ_{av}/τ_{apo}
apo-OASS	6.5	6.1			6.1	1.00
holo-OASS	6.5	4.9	1.0	0.10	2.4	0.39
holo-OASS + OAS	6.5	5.2	1.0	0.27	3.8	0.62
apo-OASS	9.5	6.2			6.2	1.00
holo-OASS	9.5	5.9	1.4	0.07	2.4	0.39
holo-OASS + serine	9.5	5.7	1.4	0.09	2.5	0.40
holo-OASS + acetate	9.5	5.1	1.3	0.07	2.2	0.36
holo-OASS + cysteine	9.5	5.1	1.3	0.09	2.4	0.39

[a] $\tau_{av} = f_1\tau_1 + f_2\tau_2$, where f_1 and f_2 are the fractional steady-state intensities with lifetimes τ_1 and τ_2, respectively. Note that $f_i = \alpha_i\tau_i/\omega\alpha_i\tau_i$.

$[\tau_{av}(holo)/\tau(apo)]$, where $\tau_{av} = \sum f_i\tau_i$, and f_i is the fractional steady-state intensity with lifetime τ_i). The observation of two phosphorescence lifetimes for the holoprotein implies that there are two transfer rates with magnitudes of 0.83 and 0.04 s^{-1}. Table 1 shows a good agreement between intensity-determined and τ-determined Φ_{ET}, an indication that these two rates of transfer account adequately for the coenzyme-induced decrease in phosphorescence intensity. From the relative amplitudes of the decaying components (Table 2), it can be seen that the very small or negligible rate of transfer affects only 10% of the zero-time phosphorescing population. There are three possible origins of the transfer heterogeneity. (1) Only one of the two Trp residues has favorable geometry (distance/orientation) for efficient transfer to the coenzyme. (2) Essentially only one Trp residue is phosphorescent in the holoprotein (405-nm band) and the vanishingly small rate of transfer represents 10% of subunits with either unbound PLP or with the Schiff base present in tautomeric forms other than the red-absorbing ketoenamine, the only one that satisfies the requirement of emission/absorption spectral overlap. (3) Conformational heterogeneity of the macromolecule exists, with about 10% of the subunits having an unfavorable geometric requirement for energy transfer. The weak coenzyme fluorescence band at 360 nm provides evidence for tautomers other than the ketoenamine and therefore the second possibility appears to be a more plausible explanation for the transfer heterogeneity.

Effects of Reactant Binding on the Decay Kinetics. Among the reactants tested, only OAS significantly affects the phosphorescence decay. The main difference in the presence of OAS (that is, formation of the α-aminoacrylate intermediate) compared to unliganded enzyme or the complexes with L-cysteine, L-serine, and acetate is a 3-fold increase in the amplitude (α_1) of the component decaying with a lifetime of 5 s (Table 2), very similar to the lifetime expected for Trp in a rigid glass. A further peculiarity of the α-aminoacrylate intermediate is that, relative to the apoprotein, the reduction in lifetime does not fully account for the actual decrease in phosphorescence intensity. Indeed the Φ_{ET} predicted from intensity measurements is 1.9 times larger than that estimated from the reduction in lifetime (Table 1). The discrepancy in the two estimates can be reconciled if a fraction of Trp residues, estimated theoretically around 0.34, transfers their excitation energy to the coenzyme more rapidly than 1 s^{-1}, resulting in total quenching of their emission. From the above considerations, it follows that in the α-aminoacrylate intermediate, only roughly 66% of the Trp residues (or subunits) phosphoresce, 18% with a τ of about 5 s similar to the apoprotein and the remainding 47% with a τ of about 1 s. The increase from 10% to 18% of the 5-s component (nontransferring Trp residues) is in keeping with the greater proportion of enolimine tautomer present in the α-aminoacrylate intermediate relative to the free enzyme as documented by an enhancement of the 360-nm fluorescence band. Finally, although the phosphorescence of the α-aminoacrylate intermediate is spectrally heterogeneous, it is difficult to establish whether the two broad 0,0 vibronic bands are associated to components with a particular lifetime.

Low-Temperature Luminescence of a Mutant OASS-A with Tryptophan-51 Replaced by a Tyrosine

The replacement of Trp-51 with Tyr eliminates one of the two Trp residues in the protein, with the remaining being at position 162 (Byrne et al., 1988). The luminescence

Table 3: Fluorescence and Phosphorescence Characteristics of OASS-A W51Y Mutant and Its Reactant Complexes in a Glass at 170 K

sample	pH	fluorescence (F_{360}/F_{480})	phosphorescence $\lambda_{0,0}$ (nm)	P_{rel}	DF_{460}/P_{405}	Φ_{ET} $(1 - P_{rel})$	$[1 - (\tau/\tau_{apo})]$
apo-OASS	6.5		405.5	1.00			
holo-OASS	6.5	4.3	406	0.30	1.37	0.70	0.66
holo-OASS + OAS	6.5	7.5	407, 413	0.29	0.45	0.71	0.43
apo-OASS	9.5		405.5	1.00			
holo-OASS	9.5	2.5	405.5	0.37	1.79	0.63	0.57
holo-OASS + serine	9.5		405.5	0.35	1.40	0.65	0.62
holo-OASS + acetate	9.5	3.6	405.5	0.36	1.86	0.64	0.61
holo-OASS + cysteine	9.5	2.6	406	0.36	1.32	0.64	0.62

Table 4: Phosphorescence Decay Kinetics (λ_{em}, 405 nm) of OASS-A W51Y and Its Reactant Complexes in a Glass at 170 K

sample	pH	τ_1 (s)	τ_2 (s)	α_1	τ_{av}	τ_{av}/τ_{apo}
apo-OASS	6.5	6.1		1.00	6.1	1.00
holo-OASS	6.5	4.6	1.4	0.08	2.1	0.34
holo-OASS + OAS	6.5	5.3	1.4	0.24	3.5	0.57
apo-OASS	9.5	6.2		1.00	6.2	1.00
holo-OASS	9.5	5.3	1.5	0.11	2.6	0.43
holo-OASS + serine	9.5	5.2	1.5	0.07	2.3	0.38
holo-OASS + acetate	9.5	5.0	1.4	0.10	2.4	0.39
holo-OASS + cyateine	9.5	5.3	1.4	0.08	2.3	0.38

characteristics of the mutant protein (W51Y) are summarized in Figure 1 and in Tables 3 and 4. In apo-W51Y, Trp emission differs from that of wild-type protein only in terms of the phosphorescence spectrum, which is now homogeneous with a single narrow 0,0 vibronic band centered at 405.5 nm. Since it is practically the same wavelength as one of the two 0,0 vibronic bands of the wild-type enzyme, W162 is assigned to the 405-nm band, while the 410-nm band is assigned to W51. The 405.5-nm band is insensitive to pH over the range 6.5−9.5, identical to the native enzyme.

Holo-W51Y fluorescence (Figure 1) differs from that of the wild-type enzyme in that there is a large coenzyme emission with a maximum around 350−360 nm, in the region of the fluorescence of the enolimine tautomer of the internal Schiff base. Hence, relative to wild-type enzyme there is a shift in the tautomeric equilibrium in favor of the enolimine tautomer of the internal Schiff base, as is also evident in the absorption spectrum. Because of the strong coenzyme fluorescence emission, Trp fluorescence in the mutant appears merely as a shoulder at 315 nm.

The most salient feature of the phosphorescence of the holo-W51Y-OASS-A mutant is its striking similarity to that of wild-type enzyme, despite the lack of W51. The phosphorescence spectrum is practically identical. Likewise, the phosphorescence to fluorescence intensity ratio is reduced to about 0.35 that of the apoprotein and the loss in intensity is compensated by the delayed fluorescence of the coenzyme. Even more significant, the phosphorescence decay kinetics of the mutant protein are as heterogeneous as those of the wild-type enzyme with similar preexponential amplitudes and lifetimes (Table 4). Since there is now a single Trp in each subunit of the W51Y mutant enzyme, the difference in rates of energy transfer that are responsible for lifetime heterogeneity must reflect a difference among the subunits, with about 10% either unable to transfer their phosphorescence to the coenzyme or transfer very weakly.

Binding of reactants alters the relative intensity of the two coenzyme fluorescence bands (360 and 480 nm) as well as the quantum yield of delayed fluorescence (*DF/P* ratio) from the ketoenamine tautomer (Table 3). As for the wild-type

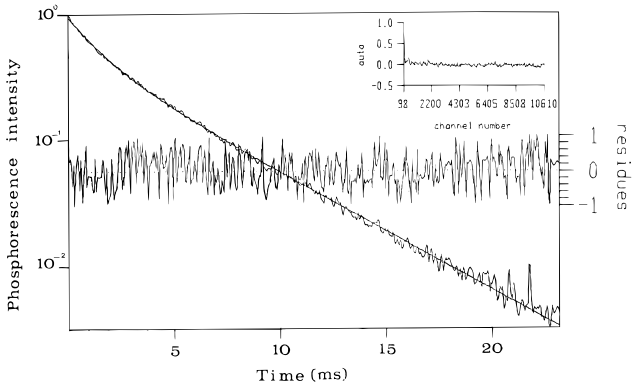


FIGURE 3: Example of phosphorescence decay of holo-OASS-A in 0.1 M phosphate buffer, pH 8, at 273 K upon pulsed excitation at 295 nm. The protein concentration is 4 μM. Fitting the data to a biexponential rate law yields a value of 1.1 for χ^2.

enzyme, formation of the α-aminoacrylate intermediate upon addition of OAS results in a resolution of the phosphorescence spectrum into two broad 0,0 vibronic bands peaked at 407 and 413 nm. Such spectral heterogeneity provides unequivocal proof of conformational heterogeneity of the polypeptide in the region of W162. Again, for the α-aminoacrylate intermediate, Φ_{ET} predicted from intensity measurements is larger than that estimated from the reduction in lifetime, and from the difference it can be estimated that in roughly 25% of subunits the phosphorescence is quenched by energy transfer at a rate greater than 1 s^{-1}.

Tryptophan Phosphorescence of Wild-Type and Mutant Enzymes in Fluid Solution

Raising the temperature above the glass transition of the glycerol/water solvent mixture ($T_g \sim 190$ K) dramatically decreases the viscosity of the medium, and the protein gradually acquires its natural flexibility. At 215 K the phosphorescence spectrum of the holoprotein has relaxed to longer wavelength, $\lambda_{0,0} \sim 408$ nm, and both steady-state intensity and lifetime have decreased by over a factor of 20. At 273 K, in buffer, the phosphorescence intensity is roughly 10^{-3} times that in a glass, as is the decrease in lifetime. Spectral relaxation is a consequence of rapid reorientation of the solvent shell about the excited chromophore, while the magnitude of τ reflects the rigidity of its environment. An early (low-temperature) spectral relaxation and a large drop in τ emphasize the high mobility of the Trp site(s), a flexibility typical of regions close to the protein surface.

An example of the phosphorescence decay of native holo-OASS-A in buffer, at 273 K, is shown in Figure 3. The decay is distinctly nonexponential, and according to the distribution of residues and value of χ^2, the intensity is adequately fitted in terms of two discrete lifetime compo-

Table 5: Phosphorescence Lifetime (τ) and Amplitudes (α) Derived from a Biexponential Fitting of the Phosphorescence Decay of OASS-A and Some of Its Reactant Complexes in Phosphate Buffer at 0 °C

sample	pH	[reactant] (M)	τ_1 (ms)	τ_2 (ms)	α_1	τ_{av}	χ^2
Wild Type							
apo-OASS	6.5		2.4	0.42	0.69	1.79	1.1
holo-OASS	6.5		4.8	1.91	0.22	2.55	1.0
holo-OASS + OAS	6.5	10^{-3}	1.7	0.40	0.52	1.08	0.9
apo-OASS	9.0		2.2	0.52	0.59	1.51	1.2
holo-OASS	9.0		4.9	2.01	0.81	4.35	1.0
holo-OASS + serine	9.0	10^{-2}	3.7	1.73	0.54	2.81	1.3
holo-OASS + cysteine	9.0	10^{-4}	<0.01				
holo-OASS + acetate	9.0	1	2.0	0.72	0.83	1.78	1.1
W51Y Mutant							
apo-OASS	6.5		1.5	0.33	0.13	0.48	1.4
holo-OASS	6.5		1.0	0.30	0.31	0.52	1.2
holo-OASS + OAS	6.5		0.25	0.08	0.31	0.13	1.2
apo-OASS	9.0		1.1	0.30	0.13	0.40	1.0
holo-OASS	9.0		1.4	0.20	0.33	0.60	1.1
holo-OASS + serine	9.0	10^{-2}	0.25	0.08	0.40	0.15	1.2

nents. At pH 6.5, the derived lifetimes are 4.8 and 1.9 ms, the short-lived component having an amplitude of 0.8. It should be pointed out that the values of τ are larger than 0.5−1.0 ms, typical of solvent-exposed Trp residues in proteins (Gonelli & Strambini, 1995), and confirm that the triplet probe, although located in a flexible site of the macromolecule, is shielded from contact with the solvent. Should the emission originate from only one of the two Trp residues of OASS-A, as some evidence seems to indicate, the heterogeneity of τ would establish a heterogeneity in the dynamic structure of the subunits, at least in the region of the probe.

The average phosphorescence lifetime was found to be affected by the removal of the coenzyme, by pH, and by complex formation with reactants. The parameters that describe the phosphorescence decay kinetics of OASS-A in buffer at 273 K, under various experimental conditions, are summarized in Table 5. Coenzyme removal causes a substantial reduction in τ_{av} and indicates that the apoprotein is more flexible, a finding that is also consistent with the lower thermal stability of the apoenzyme. More interesting is the effect of pH. Increasing the pH from 6.5 to 9 results in a 1.7-fold increase in τ_{av}, but without a significant change in the magnitude of the individual lifetime components. The relative amplitude (α) of the two components, however, does change significantly. Figure 4 shows the pH dependence of τ_{av} and clearly indicates that even at intermediate pH values, changes in τ_{av} are well accounted for by changes in amplitude. It should be noted that shifts in α alone are difficult to explain in terms of emission from both the Trp residues of OASS-A. One must invoke either selective quenching of one chromophore or a pH modulation of fluorescence energy migration between the two chromophores that would be responsible for effectively changing the distribution of the triplet-state population among the two residues. The first process requires a reduction in phosphorescence intensity, while changes in energy transfer are often associated with changes in the polarization of the emission. Since neither the intensity nor the fluorescence polarization is markedly affected by the pH change, it would seem more plausible that most of the fluorescence and phosphorescence emission originates from a single Trp residue and that the

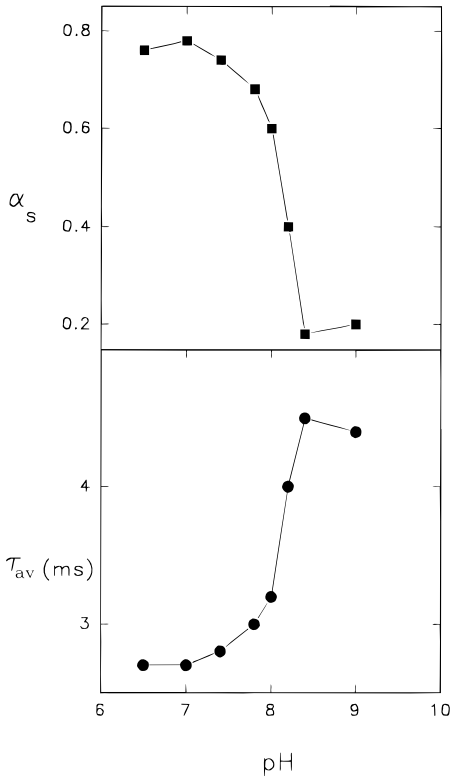


FIGURE 4: pH dependence of the average phosphorescence lifetime, where $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$, for holo-OASS-A in 0.1 M phosphate buffer at 273 K. The top panel represents the pH dependence of the preexponential amplitude of the short-lived (2 ms) component. The protein concentration is typically 4 μM.

two τ components reflect distinct conformations of the subunit. In this case the conformational equilibrium (slow on the phosphorescence time scale) is governed by one or more groups in the protein with a pK around 8.

The presence of reactants and analogs, acetate, L-serine, L-cysteine, and OAS, invariably leads to a decrease in τ_{av}. In the case of L-cysteine the effect is dramatic and τ decreases to below 10 μs, the detection limit of the apparatus. Since the cysteine effect occurs even at concentrations that give only partial saturation (~50 μM) and affect both apo- and holo-OASS-A (a stable complex is likely not even formed in the former case), the reduction of τ must be due to a specific, diffusion-mediated, quenching reaction of L-cysteine. Since it is known that sulfhydryl compounds are effective quenchers of Trp phosphorescence and that the interaction occurs at relatively close range, diminishing drastically when the edge to edge quencher−chromophore separation exceeds 2−3 Å, the chromophore in OASS-A must be very close to the aqueous interface. Because roughly the same decrease in τ occurs with apo- and holoprotein, a quenching effect (probably by trace impurities) could also be responsible for the decrease of τ_{av} in the presence of 1 M acetate. Clear evidence of conformational changes is provided instead for the L-serine Schiff base and the α-aminoacrylate intermediate. While the ligand amino acids do not affect the phosphorescence decay of the apoprotein, the τ of the two components decreases and the two amplitudes become very similar in the holoprotein.

The mutant protein, in spite of a single Trp per subunit, also exhibits a nonexponential decay, confirming conformational heterogeneity of the subunits in the millisecond time scale. Both apo- and holo-W51Y possess shorter phospho-

rescence lifetimes relative to wild type, and since none of the lifetime components are similar to those of the wild-type enzyme, the mutant has an increased flexibility of the region surrounding W162. In addition, W51Y lacks the alkaline transition observed for wild-type enzyme, i.e., the increase in τ_{av} above pH 8. Also, the extent of the lifetime response to L-serine and OAS binding differ. The two amino acids induce a larger decrease in τ_{av} in the mutant compared to wild type. Finally, similar to wild-type OASS-A, L-cysteine completely quenches the phosphorescence of apo- and holo-W51Y. Since L-cysteine is too large a molecule to migrate through the protein matrix, this finding once again indicates that W162 is close to the aqueous interface.

DISCUSSION

Assignment of Tryptophan Phosphorescence to Individual Tryptophan Residues. A reasonable model for the phosphorescence data assigns the 405-nm band in the phosphorescence spectrum of apo- and holo-OASS-A to the emission from W162, while the 410-nm band of apo-OASS-A is assigned to W51. In the holoprotein the phosphorescence represented by a single band at 405 nm originates entirely from W162, while W51 is practically silent, quenched at the fluorescence level either statically, by a reaction with a local group, or through fluorescence energy transfer to either PLP (accounting for the fluorescence quenching from apo- to holoenzyme) or to W162.

Also in agreement with the assignment of W162 as the Trp responsible for energy transfer to the ketoenamine tautomer of the internal Schiff base are data from sequence alignment obtained for OASS enzymes in a number of different species from bacteria to plants (V. Rege, unpublished results). Of the two Trp residues in OASS-A from *S. typhimurium*, only W162 is conserved in all species. In addition, also based on sequence alignment, W162 corresponds to the same position as the sole Trp present in the β-subunit of tryptophan synthase, an enzyme that has phosphorescence properties very similar to those of OASS-A (Strambini et al., 1992a).

There are a number of lines of evidence that support the above assignments. The apo-W51Y-OASS-A has only the 405-nm band, which is then assigned to W162. In addition, the phosphorescence spectrum, decay kinetics, and Φ_{ET} of wild-type holoenzyme (which contains both W162 and W51), alone or in complex with reactants, are strikingly similar to the parameters obtained for the holo-W51Y enzyme (which contains only W162), alone or in complex with reactants. Thus, data strongly suggest that in both wild-type and mutant enzymes, the phosphorescence is due to one and the same Trp residue, W162. The alkaline transition in τ_{av} and lifetime amplitudes, α_s, are also consistent with emission from a single chromophore per subunit and would be difficult to explain otherwise. Finally, the room-temperature phosphorescence (RTP) of the wild-type and mutant enzymes is equally quenched by L-cysteine, suggesting that in both cases the RTP emission comes from a residue (or residues) in a similar environment.

Environment around W162. The λ_{max} of the 0,0 vibronic band for W162 phosphorescence is 405 nm, indicative of a polar site but not one that is solvent-exposed ($\lambda_{0,0}$ is 408 nm for a solvent-exposed residue). In addition, $\tau_{RTP} > \tau_{H_2O}$ is also consistent with the residue being somewhat shielded from solvent. The site must be quite flexible, as suggested by fluorescence and phosphorescence spectral relaxation as the temperature is increased and the small value of τ_{RTP} (Gonelli & Strambini, 1995). Accessibility to quenching by L-cysteine, a quencher relegated to the aqueous phase with an interaction range of 2−3 Å, implies that W162 is near the protein surface. In agreement with these data, McClure and Cook (1994) suggested on the basis of static fluorescence studies that at least one of the two Trp residues in OASS-A is not buried in the hydrophobic interior and is somewhat exposed to solvent.

Table 6: Triplet−Singlet, Tryptophan−Coenzyme Energy Transfer Parameters and Estimated Donor/Acceptor Separations

	J^a ($cm^3\ M^{-1}$)	R_0^b (Å)	Φ_{ET}	$R(\Phi_{ET})^c$ (Å)	k_T (s^{-1})	$R(k_T)^d$ (Å)
OASS-A	1.32×10^{-14}	29	0.70	24	0.83	22
OASS-A + OAS	2.90×10^{-14}	34	0.72	28	0.83	26

[a] $J = \int_0^\infty P_d(\lambda)\epsilon_a(\lambda)\lambda^4\ d\lambda / \int_0^\infty P_d(\lambda)\ d\lambda$, where ϵ_a is the molar absorption coefficient of the acceptor and P_d is the donor phosphorescence intensity. [b] $R_0^6 = (8.8 \times 10^{-25})\kappa^2 n^{-4}\phi J$ in centimeters. $\phi = k_p\tau_d = 0.45$ is the phosphorescence quantum yield of Trp on the basis of a radiative transition probability $k_p = 0.09\ s^{-1}$ (Galley & Stryer, 1969). The orientation factor κ^2 is taken as 0.476, the value for random static donor−acceptor orientations. n is the refractive index of the medium and is assumed to be 1.4. [c] $R(\Phi_{ET})$ is given by $\Phi_{ET} = R_0^6/(R_0^6 + R^6)$. [d] $R(k_T)$ is given by $k_T = (1/\tau_{apo})(R_0/R)^6$.

Extent of Triplet−Singlet Phosphorescence Energy Transfer and Estimate of the Distance between W162 and PLP. The reduction in phosphorescence intensity and lifetime as PLP binds to the apoprotein and the appearance of delayed fluorescence from the ketoenamine tautomer of PLP imply that phosphorescence quenching of W162 is a result of triplet−singlet Förster-type resonance energy transfer, a process reported previously for other PLP enzymes (Cioni et al., 1992; Strambini et al., 1992a). Förster theory describes the dependence of the transfer efficiency (or rate) on the distance (R) between the donor−acceptor chromophores, their mutual orientation (κ^2), and the spectral overlap (J) between donor emission and acceptor absorption. Estimates of the separation between W162 and PLP, together with the critical transfer distance, R_0, calculated assuming a random, static orientation between donor and acceptor are given in Table 6. A value of R equal to about 25 Å is estimated from both transfer efficiencies and rates. It should be stressed, however, that unless the coenzyme and/or W162 have substantial rotational mobility, a narrow distribution of donor−acceptor orientations is anticipated, and the assumption of random distribution can lead to large errors. Since the estimate of R is similar for energy transfer to the ketoenamine tautomer of PLP and to the α-aminoacrylate intermediate, chromophores with different transition moments and presumably different orientations, the approximations involved in the estimation of κ^2 are likely reasonable. Similar values have been estimated for the donor−acceptor distance with W177 and PLP in tryptophan synthase (Strambini et al., 1992a), the closest relative to OASS-A based on sequence alignment, and the distance is within error equal to that obtained from the three-dimensional structure (Hyde et al., 1988).

Evidence of Conformational Changes in OASS-A. Several lines of evidence from phosphorescence spectral and decay data point to changes in conformation that are induced by

pH and complexation with PLP (apoenzyme), OAS, and L-serine (holoenzyme) of the native enzyme and the W51Y mutant. At low temperature, the OAS-induced red shift and splitting of the 405-nm band into two (centered at 406 and 408.5 nm for holo-OASS-A and at 407 and 413 nm for the holo-W51Y-OASS-A mutant) are indicative of a change in polarity of the W162 environment. In fluid solutions, a most sensitive conformational parameter is the phosphorescence lifetime, since changes in τ imply changes in the dynamical structure of the protein in the region of W162. On the basis of τ data, the release of PLP from the holoenzyme or the binding of OAS or L-serine to it is accompanied by an enhanced flexibility at the site of W162 in both wild-type and W51Y mutant enzymes.

There have also been indicators of conformational heterogeneity in previous work, particularly based on the formation of intermediates along the reaction pathway resulting from the addition of reactant and reactant analogs to OASS-A. Addition of OAS has, for a number of years, been known to generate the α-aminoacrylate intermediate (Becker et al., 1969; Cook & Wedding, 1976; Cook et al., 1992), and that two forms with λ_{max} values of 330 and 470 nm, respectively, of the intermediate are in equilibrium, consistent with two different tautomers. More recently, using static fluorescence measurements, addition of L-cysteine (or acetate) was shown to enhance the long-wavelength fluorescence of the ketoenamine tautomer of the Schiff base (McClure & Cook, 1994). A similar phenomenon has been observed for the β-subunit of tryptophan synthase (Federiuk & Shafer, 1983). Further, the concentration dependence of the enhancement of the long-wavelength fluorescence of the internal Schiff base (acetate) and external Schiff base (L-cysteine) is pH-dependent, giving a p*K* of about 7 (acetate) to 8 (L-cysteine), similar to the pH dependence observed for the alkaline transition noted in the present studies (Figure 4). A follow-up of the fluorescence studies using far-UV CD, PLP-induced CD, and ^{31}P NMR supports a change in conformation upon formation of the external Schiff base (Schnackerz et al., 1995). Similarly, addition of L-serine results in the formation of the external Schiff base with dramatic changes in the far-UV CD and ^{31}P NMR but also clearly shows a mixture of two tautomers in the UV−visible absorbance spectrum (Schnackerz et al., 1995). Finally, a site-directed mutant in which the Schiff base lysine is replaced by an alanine is isolated stably as the external Schiff base with an amino acid from solution (V. Rege, unpublished work). The resultant mutant external Schiff base is resistant to reduction by borohydride unless 3−5 M guanidinium chloride is present, taken as evidence that the active site may be closed upon formation of the external Schiff base. Consistent with this interpretation, the far-UV CD spectrum changes significantly only in the presence of L-serine and L-cysteine (both generate the external Schiff base) but not OAS (generates the α-aminoacrylate intermediate) (McClure & Cook, 1994; Schnackerz et al., 1995).

Evidence of Heterogeneity in the Subunit Conformation. Some of the above evidence is also consistent with a heterogeneity in the conformation of the individual subunits in the OASS-A dimer. Data include the finding of at least two distinct rates of triplet−singlet energy transfer in the α-aminoacrylate intermediate (OASS-A plus OAS) and heterogeneity in the phosphorescence spectral parameters in the same complex, especially compelling evidence for the W51Y mutant enzyme which has only a single Trp. Also consistent is the heterogeneous room-temperature phosphorescence decay in both wild-type and mutant enzymes and its modulation by pH and substrates. Interestingly, apparent negative cooperativity has been observed for the OASS-A reaction at high concentrations of the nucleophilic substrate analog 5-thio-2-nitrobenzoate (Tai et al., 1993).

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