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Biophysical Chemistry 61 (1996) 9–22

Biophysical Chemistry

Time-resolved fluorescence of tryptophan synthase

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Received 4 July 1995; revised 28 February 1996; accepted 28 February 1996

Abstract

Time-resolved and steady-state fluorescence of the tryptophan synthase $\alpha_2\beta_2$ complex and of the β_2 dimer from *Salmonella typhimurium* were measured to characterize the conformational properties of the β subunit in the presence and in the absence of the α subunit when the catalytic species internal aldimine, external aldimine and α-aminoacrylate Schiff bases were selectively accumulated within the β active site.

The fluorescence decay of the coenzyme pyridoxal 5′-phosphate, bound via a Schiff base in the β subunit of the $\alpha_2\beta_2$ complex (internal aldimine species), is accounted for by two lifetimes (2.9 and 0.9 ns) of almost equal fractional intensity that are slightly affected by pH. Accordingly, both the absorption and emission spectra were found to be pH independent. The emission properties of the internal aldimine in the β_2 dimer are pH dependent, suggesting that the α-subunit binding alters the microenvironment of the β-subunit active site. This conclusion is also supported by the emission of the single tryptophanyl residue of the enzyme (Trp-177β).

In the reaction of L-serine with the $\alpha_2\beta_2$ complex, the predominant catalytic intermediate is the external aldimine ($\lambda_{max} = 422$ nm) at pH 10, and the α-aminoacrylate ($\lambda_{max} = 350$ nm) at pH 7. The external aldimine exhibits a high fluorescence intensity at 500 nm that decays with a single lifetime of 6.2 ns in the $\alpha_2\beta_2$ complex, at pH 10, and at a similar value in the β_2 dimer. The emission properties of the external aldimine with respect to the internal aldimine, and the small effects induced by α-subunit binding indicate a shielding of the coenzyme and a stabilization of its excited state. In contrast, the short fluorescence lifetime (0.4 ns) and the weak fluorescence emission of the α-aminoacrylate Schiff base indicate an increase of non-radiative processes possibly due to a more tight coupling of this intermediate with the protein matrix with respect to the external aldimine.

Whereas the internal aldimine is distributed in two tautomeric forms, both the external aldimine and the α-aminoacrylate are present in single conformational states with distinct structural and/or dynamic properties that may modulate regulatory intersubunit signals.

Keywords: Protein conformational states; Pyridoxal 5′-phosphate-dependent enzyme; Frequency domain fluorescence; Pyridoxal 5′-phosphate and tryptophan fluorescence decays

Abbreviations: PLP, pyridoxal 5′-phosphate; GP, DL-glycerol-3-phosphate; Bicine, N,N-bis(2-hydroxyethyl)glycine; Mops, 3-(N-morpholino)-propane sulphonic acid; MBP buffer, 50 mM Mops, 50 Bicine, 50 mM Proline, 1 mM EDTA

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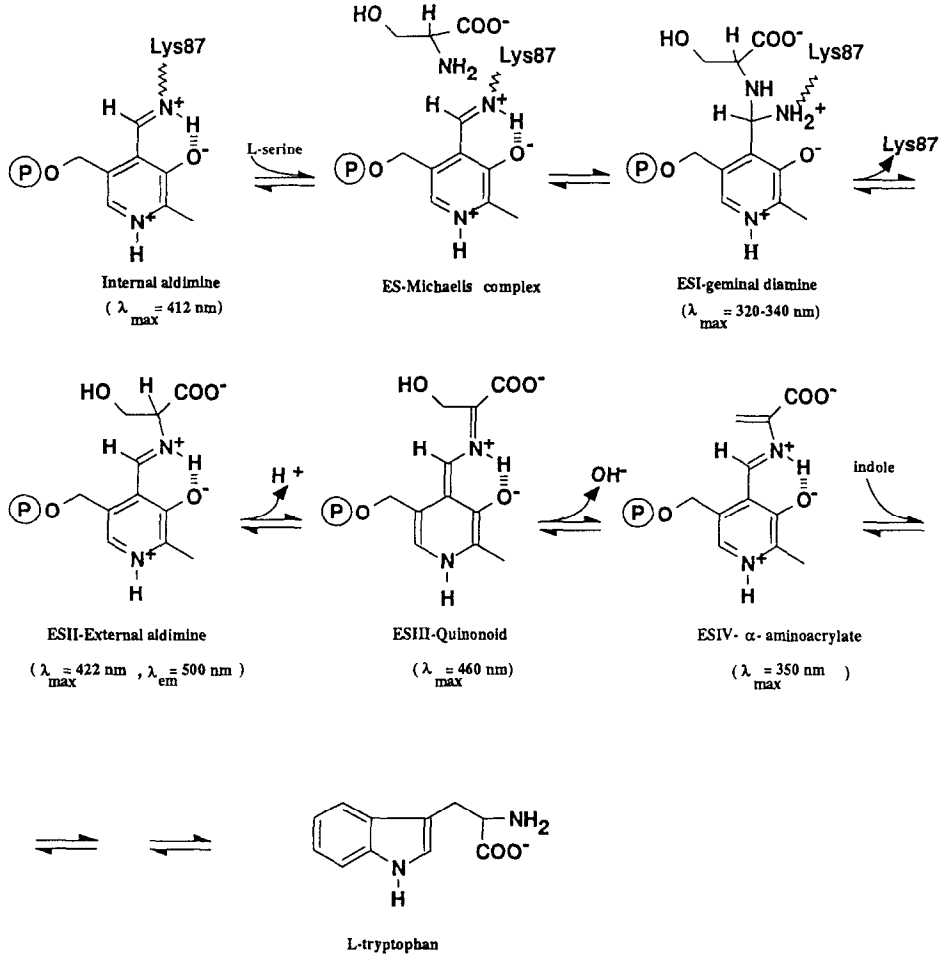
PII S0301-4622(96)00020-8

1. Introduction

A ligand-dependent equilibrium between "open" and "closed" states of a protein has been postulated, and in a few cases crystallographically demonstrated, to explain their functional properties [1–5], including the PLP-dependent enzyme aspartate aminotransferase [6–8]. "Open" and "closed" states are associated with low and high catalytic activity, respectively, since domain closure may bring catalytic group in close contact with the substrate and expel water molecules from the active site [9]. "Open" and "closed" states have also been postulated in the bifunctional tryptophan synthase $\alpha_2\beta_2$ complex to serve both a catalytic role by selectively stabilizing distinct catalytic intermediates, and a regulatory role by triggering different intersubunit signals [10–13].Tryptophan synthase from *Salmonella typhimurium* (E.C. 4.2.1.20) is a bienzyme complex composed of two α subunits and a tightly associated β_2 dimer with one PLP bound per β subunit [14–16]. The enzyme catalyzes the final step in the biosynthesis of L-tryptophan according to the following (αβ) reaction:

indole − 3 − glycerol phosphate + *L* − serine
→ *L* − tryptophan + glyceraldehyde − 3
− phosphate + H_2O



Scheme 1. Mechanism of the reaction catalyzed by the β-subunit of the tryptophan synthase $\alpha_2\beta_2$ complex. Absorption and emission properties are reported below each species [15,16].

which results from the sum of two half-reactions (α and β reactions respectively) catalyzed by the individual subunits:

indole − 3 − glycerol phosphate

⇌ indole + *D* − glyceraldehyde − 3 − phosphate

indole + *L* − serine → *L* − tryptophan and H_2O

The β-reaction proceeds through a series of PLP-enzyme species, characterized by distinct spectral properties (Scheme 1). In the absence of indole, the predominant catalytic intermediates are the external aldimine (Scheme 1, ESII) and the β-elimination product, the α-aminoacrylate Schiff base (Scheme 1, ESIV). The equilibrium between the external aldimine and the α-aminoacrylate depends on pH, α-subunit ligands, temperature and monovalent cations [17–20]. At constant temperature and ion concentration, low pH or α-subunit ligands favor the α-aminoacrylate species, whereas high pH favors the external aldimine [19]. Thus, by changing the experimental conditions it is possible to selectively accumulate either the external aldimine or the α-aminoacrylate Schiff base.

The catalytic activities of the α- and the β-active site are reciprocally modulated by β- and α-subunit ligands [10–13,21–30]. The external aldimine is unable to trigger signals to the α subunit, whereas the α-aminoacrylate causes an activation of the α reaction. It has been proposed that changes in the structure of the catalytic intermediates are accompanied by changes in the conformation of the β subunit. In particular, the external aldimine and the α-aminoacrylate might stabilize an "open" and a "closed" conformation of the β subunit, respectively [10–13]. Whereas the relevance of ligand binding in the allosteric regulation of the activity of α and β subunits is fairly well established, both the existence of "open" and "closed" states of the β subunit and the coupling between the conformation of the β-active site and intersubunit signals have not yet been proved. In fact, the three-dimensional structure of the enzyme from *S. typhimurium* (Fig. 1), determined in the presence and in the absence of the α-subunit ligand indole propanol phosphate, show local conformational changes at the α-active site and no effects at the β-active site [31]. X-ray crystallographic studies of β-subunit catalytic intermediates

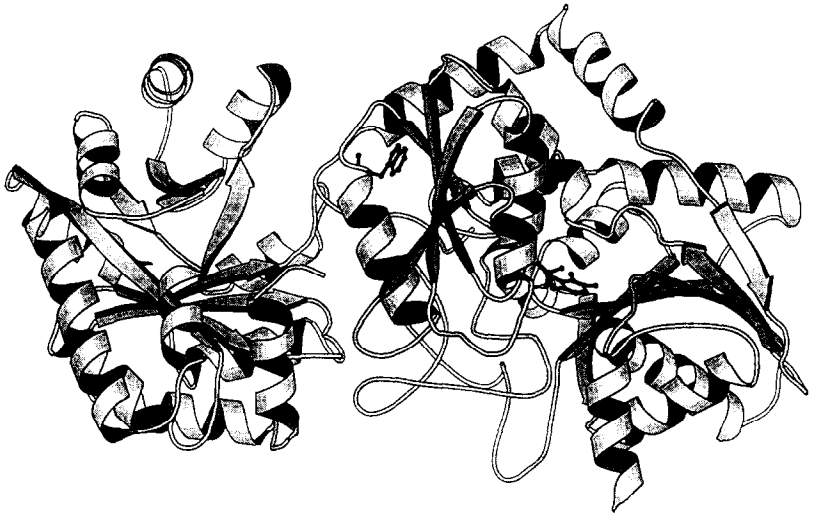


Fig. 1. Ribbon diagram of the α-β subunit pair of the tryptophan synthase $\alpha_2\beta_2$ complex. The complex has a linear αββα quaternary structure. Both pyridoxal 5′-phosphate and Trp-177β are displayed as balls and sticks. The β-subunit is shown on the right with the coenzyme at the center and Trp-177β on the top of a helix near the subunit interface. The coordinates are obtained from the file 1WSY.PDB [31], and the figure was produced using the program MOLSCRIPT [76]

are not available, although the crystalline enzyme is catalytically competent [32] and most of the catalytic intermediates can be accumulated in the crystal [17]. Moreover, the phosphorescence properties of the unique Trp-177β, located near the subunit interface in the N-domain of the β-subunit (Fig. 1), at about 23 Å from the coenzyme, demonstrate that the formation of the external aldimine does not affect the viscosity of the microenvironment near the probe and the mobility of the α-active site, whereas the binding of the α-subunit ligand GP alters the α-active site conformation and significantly decreases the flexibility of the β-subunit [33,34].

Fluorescence lifetime measurements of intrinsic probes of the $\alpha_2\beta_2$ complex, such as the unique tryptophanyl residue 177β and the coenzyme, are well suited for the conformational characterization of molecular species in the excited state [35–38]. The fluorescence decay of tryptophanyl residues have been widely studied [36], while the fluorescence decay of PLP has been investigated only in a few PLP-dependent enzymes including 4-aminobutyrate aminotransferase [39], glycogen phosphorylase b [40], aromatic L-aminoacid decarboxylase [41], and in PLP-labeled proteins [42,43]. In the case of tryptophan synthase, fluorescence lifetime decays were measured to investigate the accessibility of the β-active site in the β_2 dimer and in the $\alpha_2\beta_2$ complex from *Escherichia coli* [44].

In the present work, we exploited steady-state and time-resolved fluorescence of both PLP and Trp-177β to gain insight on the conformational properties of the β-subunit when the catalytic PLP species internal aldimine, external aldimine and α-aminoacrylate Schiff bases were present in the active site either in the absence or in the presence of the α-subunit.

Since structural and functional information has recently been obtained on mutated and wild-type forms of the enzyme from *S. typhimurium* [16], we investigated the protein from this source.

2. Materials and methods

2.1. Materials

The tryptophan synthase $\alpha_2\beta_2$ complex from *S. typhimurium* was purified and assayed according to Ahmed and co-workers [32,46,45]. The β_2-dimer was prepared by thermal denaturation of the α-subunits, as described by Miles et al. [47]. Protein concentration was determined spectrophotometrically on the basis of the extinction coefficients at 278 nm for the β_2 dimer and for the $\alpha_2\beta_2$ complex [47]. L-Serine, GP and bicine were purchased from Sigma; Mops and proline were purchased from Fluka; EDTA was purchased from Merck. All reagents were of the best available quality and were used without further purification.

2.2. Buffers

pH measurements were carried out with a pHM 83 Radiometer, equipped with a U402-M3 Ingold microelectrode, calibrated with three standard buffers at 20°C. Experiments were carried out at 10°C in a three-buffers system (MBP buffer) containing 50 mM Mops, 50 mM bicine, 50 mM proline and 1 mM EDTA. The pH was raised with sodium hydroxide up to 11.2, and the buffer was then back-titrated with HCl to the desired pH value. By this procedure both the ionic strength and sodium ion concentration were kept nearly constant at pH values between 6.5 and 10.5.

2.3. Luminescence spectra

For spectroscopic measurements, a solution containing the enzyme (10–30 mg ml^{-1}), 50 mM bicine, 1 mM EDTA, pH 7.8, was directly diluted into the buffer. The pH of the final solution was determined in the cuvette after spectral recordings. Static fluorescence (emission and excitation) spectra were collected using a Perkin-Elmer LS 50 B luminescence spectrophotometer. The fluorescence of the solvent was automatically subtracted. Emission spectra are not corrected for the photomultiplier response. The cuvette holders of the fluorometer were thermostatted at 10 ± 0.5°C.

2.4. Fluorescence decay measurements

The fluorescence decays were studied using the phase-modulation technique [48,49]. The sample is excited with light of modulated intensity. The phase shift and the demodulation of the emitted light are

determined as a function of the frequency of the modulation. Phase (τ_p) and modulation lifetimes (τ_m) are calculated according to the equations:

$$\tan P_m = \omega\tau_p$$

$$M_m = \left[1 + (\omega\tau_m)^2\right]^{-1/2}$$

where P_m is the measured phase shift, M_m is the measured relative modulation ratio, and ω is the angular modulation frequency. The phase and modulation lifetimes values are determined by assuming either a sum of exponential decays or a continuous lifetime distribution. The measured values are compared with the calculated values of phase and modu-

Table 1
Fluorescence decay parameters for PLP in the β_2 dimer and in the $\alpha_2\beta_2$ complex, in the absence and in the presence of L-serine, at different pH values, 10°C (excitation at 410 nm for the native enzyme and at 420 nm for L-serine-enzyme derivatives)

Parameter	pH						
	7	7.6	7.8	8.5	9.5	10	10.5
β_2 dimer							
τ_1	4.42 ± 0.27 [a]					1.50 ± 0.06 [a]	
τ_2	1.15 ± 0.04						
f_1	0.32 ± 0.02					1	
f_2	0.68 ± 0.02						
χ^2	6.5					12.2	
$\alpha_2\beta_2$ complex							
τ_1	2.88 ± 0.06		2.32 ± 0.05			2.28 ± 0.07	
τ_2	0.92 ± 0.02		0.76 ± 0.02			0.83 ± 0.03	
f_1	0.54 ± 0.01		0.61 ± 0.01			0.60 ± 0.02	
f_2	0.46 ± 0.01		0.39 ± 0.01			0.40 ± 0.02	
χ^2	1.7		1.6			2.3	
β_2 dimer + L-serine							
τ_1	5.54 ± 0.17 [a]					6.38 ± 0.19 [a]	
f_1	1					1	
χ^2	6					7.1	
$\alpha_2\beta_2$ complex + L-serine							
τ_1	5.33 ± 0.10 [a]	5.74 ± 0.10 [a]	5.75 ± 0.10 [a]	5.75 ± 0.10	6.00 ± 0.10	6.18 ± 0.08	6.26 ± 0.10
τ_2	0.37 ± 0.18	0.26 ± 0.18 [a]	– [b]	– [b]	– [b]	– [b]	– [b]
f_1	0.92 ± 0.01	0.92 ± 0.01	0.99 ± 0.01	0.97 ± 0.01	0.98 ± 0.01	0.98 ± 0.01	0.97 ± 0.01
f_2	0.08 ± 0.01	0.08 ± 0.01	– [b]	– [b]	– [b]	– [b]	– [b]
χ^2	5.1	5.3	5.3	2.8	3.0	2.5	3.0
$\alpha_2\beta_2$ complex + L-serine + GP							
τ_1	5.36 ± 0.12		5.75 ± 0.24			5.75 ± 0.23	
τ_2	0.33 ± 0.04		0.55 ± 0.14			0.64 ± 0.11	
f_1	0.87 ± 0.01		0.89 ± 0.02			0.92 ± 0.01	
f_2	0.13 ± 0.01		0.11 ± 0.02			0.08 ± 0.01	
χ^2	3.4		10.8			10.0	

Fluorescence lifetimes τ_1 and τ_2 are expressed in ns and fractional intensities are f_1 and f_2, respectively. Emissions were collected at $\lambda > 470$ nm using the Oriel 52095 filter. GP concentration was 50 mM. In the case of measurements on a single sample, high χ^2 values are generally observed when the emission signal was weak. Measurements carried out with an enzyme concentration between 0.41 and 1.2 mg ml^{-1} gave identical lifetimes and fractional intensities. Measurements carried out after 5 h of incubation of the enzyme with L-serine at 10°C gave identical lifetimes and fractional intensities. The binding of GP to the native $\alpha_2\beta_2$ complex does not produce any observable change in lifetimes and fractional intensities.

[a] Average values of 2–3 measurements performed on different samples. In this case high χ^2 values also reflect the variability between different samples.

[b] A contribution with a lifetime of the order of the instrumental resolution (0.05 ns) was found to be necessary to adequately fit the data.

lation for an intensity decay using the following equations:

$$P_c = \tan^{-1}[S(\omega)/G(\omega)]$$

$$M_c^2 = S(\omega)^2 + G(\omega)^2$$

$S(\omega)$ and $G(\omega)$ have different expressions depending on the model assumed for the fit. For a sum of exponentials, the functions $S(\omega)$ and $G(\omega)$ are given by:

$$S(\omega) = \sum_i [f_i \omega \tau_i / (1 + \omega^2 \tau_i^2)]$$

$$G(w) = \sum_i [f_i / (1 + \omega^2 \tau_i^2)]$$

$$\sum f_i = 1$$

where the index i depends on the number of exponentials used for the fit, τ_i is the lifetime of the ith component, and f_i is the fractional intensity of the ith component. The value of the reduced χ^2, used to judge the "goodness" of the fit, is defined as:

$$\chi^2 = \sum \left\{ [(P_c - P_m)/\sigma_p]^2 + [(M_c - M_m)/\sigma_m]^2 \right\} / (2n - F - 1)$$

where n is the number of modulation frequencies, F is the number of free parameters and σ_p and σ_m are the standard deviations of each phase and modulation measurement, respectively.

Time-resolved fluorescence measurements were carried out on a multifrequency cross-correlation phase and modulation fluorometer GREG 200 (ISS Inc., Champaign, IL) operating with a digital filter for the selection of the suitable cross-correlation frequency and equipped with a 300 W xenon arc lamp [50]. The temperature of the samples was maintained at 10 ± 0.5°C using a circulating water bath and a jacketed cell holder. In order to reduce the color effect in the system [51], solutions of *p*-terphenyl ($\tau = 1.05$ ns) and *p*-bis[2-(5-phenyloxazoyl)]benzene (POPOP, $\tau = 1.35$ ns) in absolute ethanol were employed as references for excitation at $\lambda = 295$ nm and at 340–350 nm, respectively. Glycogen scatter was used as the reference for excitation in the 410–420 nm range. The wavelength of the excitation light was selected by a Jobin–Yvon monochromator with 16 nm bandwidth. Oriel cutoff filters and the Corning 7-54 interference filter were used to collect the light for different emissions (see Tables 1–3). Evaluation of the buffer solution indicated that background fluorescence and/or scattered light contributed less than one percent to the measured emissions. However, for tryptophan excitation at 295 nm the scattered light contribution was higher (approximately 10%), due to the proximity of the excitation band to the cutoff wavelength of the filters that were employed.

For each sample, the phase-shift and modulation at 10–15 frequencies in the 10–200 MHz range were measured. In a few cases, the fall of phase and modulation values for 150 MHz has limited the

Table 2
Fluorescence decay parameters for PLP in the $\alpha_2\beta_2$ complex in the absence and in the presence of L-serine, at different pH values (excitation at 340 nm for the native enzyme and at 350 nm for L-serine-enzyme derivatives)

Parameter	pH		
	7	7.6	10
$\alpha_2\beta_2$ complex			
τ_1	3.33 ± 0.20		2.45 ± 0.10
τ_2	0.89 ± 0.03		0.73 ± 0.03
f_1	0.44 ± 0.02		0.58 ± 0.02
f_2	0.56 ± 0.02		0.42 ± 0.02
χ^2	6.0		4.2
$\alpha_2\beta_2$ complex + L-serine			
τ_1	5.30 ± 0.20 [a]	5.61 ± 0.08	
τ_2	0.32 ± 0.03	0.47 ± 0.02	
f_1	0.76 ± 0.02	0.76 ± 0.01	
f_2	0.24 ± 0.02	0.24 ± 0.01	
χ^2	5.7	1.9	
$\alpha_2\beta_2$ complex + L-serine + GP			
τ_1	5.72 ± 0.45		
τ_2	0.42 ± 0.04		
f_1	0.39 ± 0.03		
f_2	0.61 ± 0.03		
χ^2	13.3		

Fluorescence lifetimes τ_1 and τ_2 are expressed in ns and the fractional intensities are f_1 and f_2, respectively. Emissions for the $\alpha_2\beta_2$ complex were collected at $\lambda > 470$ nm using a Oriel 52053 filter, and for the L-serine-$\alpha_2\beta_2$ complex at $\lambda > 380$ nm using the Oriel 52095 filter. GP concentration was 50 mM. High χ^2 values are observed in measurements in which the signal was weak and in the case of measurements on different samples, thus also reflecting sample variability.

[a] Average values of 2 measurements on different samples.

Table 3
Fluorescence decay parameters for Trp-177 in the β_2 dimer and in the $\alpha_2\beta_2$ complex in the absence and in the presence of L-serine at pH 7 and 10 (excitation at 295 nm)

Parameter	pH	
	7	10
β_2 dimer		
τ_1	1.78 ± 0.18	1.60 ± 0.08
τ_2	0.14 ± 0.04	0.32 ± 0.03
f_1	0.45 ± 0.03	0.50 ± 0.02
f_2	0.55 ± 0.03	0.50 ± 0.02
χ^2	9.5	1.5
$\alpha_2\beta_2$ complex		
τ_1	2.17 ± 0.23	1.96 ± 0.26
τ_2	0.11 ± 0.03	0.09 ± 0.04
f_1	0.38 ± 0.02	0.37 ± 0.03
f_2	0.62 ± 0.02	0.63 ± 0.03
χ^2	8.5	13
β_2 dimer + L-serine		
τ_1	1.83 ± 0.11	1.69 ± 0.10
τ_2	0.22 ± 0.03	0.28 ± 0.02
f_1	0.50 ± 0.02	0.40 ± 0.02
f_2	0.50 ± 0.02	0.60 ± 0.02
χ^2	3	1.1
$\alpha_2\beta_2$ complex + L-serine		
τ_1	2.78 ± 0.30	2.02 ± 0.22
τ_2	0.19 ± 0.03	0.17 ± 0.03
f_1	0.33 ± 0.02	0.34 ± 0.02
f_2	0.67 ± 0.02	0.66 ± 0.02
χ^2	6.0	7.0
$\alpha_2\beta_2$ complex + L-serine + GP		
τ_1	2.74 ± 0.36 [a]	1.72 ± 0.12
τ_2	0.13 ± 0.03	0.19 ± 0.02
f_1	0.33 ± 0.02	0.40 ± 0.02
f_2	0.67 ± 0.02	0.60 ± 0.02
χ^2	11.5	2.4

Fluorescence lifetimes τ_1 and τ_2 are expressed in ns and the fractional intensities are f_1 and f_2, respectively. Emission filters were Oriel 51255 and Corning 7-54 that allow to collect light in the wavelength range 300–400. Variability of χ^2 is due to the low fluorescence signal.

[a] The binding of GP to the native $\alpha_2\beta_2$ complex does not produce any observable change in lifetimes and fractional intensities.

possibility to collect data at higher frequency values and, consequently, shorter components of lifetime with low fractional intensity were not well represented. For each frequency the instrument acquired a minimum of four to a maximum of eight measurements of phase and modulation with a number of integration cycles up to 80 for each measurement in order to obtain a constant, frequency-independent standard deviation of 0.2° for σ_p and 0.004 for σ_m. The data analysis was performed with a software (ISS Inc.), that uses a non-linear least-squares method based on the Marquardt algorithm. The best fit of the experimental data was evaluated from the plot of residuals, the autocorrelation functions and the reduced χ^2 values. In the cases of low-intensity signals and large noise in the phase and modulation measurements the experimental standard deviations are different from the pre-defined standard deviations and the χ^2 values might be different from one even for a well-fitting model. In this case, to evaluate the goodness of the fit between the experimental data and the assumed model, the distribution around zero of the deviations between the simulated and the calculated values was considered. The error in the determination of lifetimes and fractional intensities is calculated by the error correlation matrix. Experimental data were fitted using a model that assumes a sum of discrete lifetimes. Data were also fitted with a model that assumes a continuous distribution of lifetimes [52–55]. The statistical analysis, used to judge the "goodness" of the fitting of the data to a model, and physicochemical information reporting a discrete distribution of components for the coenzyme [33,56,57] have allowed to prefer the former model for the fluorescence decay of PLP derivatives.

The decay of single tryptophan-containing proteins is rarely monoexponential [36]. The multiple components can arise from different local conformations of the protein around the probe, and consequently, different orientations of the indole ring. The biexponential fluorescence decay of free tryptophan in solution has also been explained assuming a model in which two rotamers of the indole ring are differentially quenched and, therefore, exhibit very distinct lifetimes [58,59]. Since phosphorescence decays indicate the existence of two conformational states of the tryptophanyl residue [33,34], the discrete model was preferred over the continuous distribution model in the analysis of Trp-177β fluorescence decay, although the calculated χ^2 values assuming the discrete lifetimes model were slightly higher than those for a model that assumes a Lorentzian distribution of lifetimes and a discrete component with $\tau \approx 0$ (which

includes all the scattered light, but possibly also some short-lived fluorescence).

3. Results

3.1. Emission and excitation spectra of the tryptophan synthase $\alpha_2\beta_2$ complex and β_2 dimer in the absence and in the presence of L-serine

Absorption spectra (300–550 nm) of the internal aldimine Schiff base in the β_2 dimer and in the $\alpha_2\beta_2$ complex from *S. typhimurium* are characterized by a major band centered at 412 nm with a shoulder around 350 nm [60]. The relative intensities of these bands are not affected by pH[1], as previously observed for the *E. coli* enzyme [61]. After reaction of the $\alpha_2\beta_2$ complex with L-serine, a complex spectrum appears with absorption bands at 422 nm and at 350 nm. The red band has been assigned to the external aldimine whereas the blue band has been assigned to the α-aminoacrylate [15,16,60,62,63]. The relative intensity of these bands depends on pH [18,19]. A pH increase causes a rise of the 422 nm band and a concomitant decrease of the 350 nm band. This effect results from a pH-induced change in the equilibrium between the external aldimine and the α-aminoacrylate and it is not due to tetramer dissociation [17–19]. In contrast, in the case of L-serine-β_2 dimer derivatives, the external aldimine is the only species that accumulates over the pH range 6–10 [19].

When the β_2 dimer is excited at 410 nm, the emission spectrum consists of a band peaked at 500 nm that originates from the ketoenamine tautomer of the internal Schiff base [33,64]. The emission band is about 25% more intense at pH 10 than at pH 7.0. For the L-serine-β_2 derivative, a strong emission centered at 500 nm is observed upon excitation at 420 nm (data not shown), in agreement with several studies describing the fluorescence properties of the external aldimine Schiff base of the *E. coli* enzyme

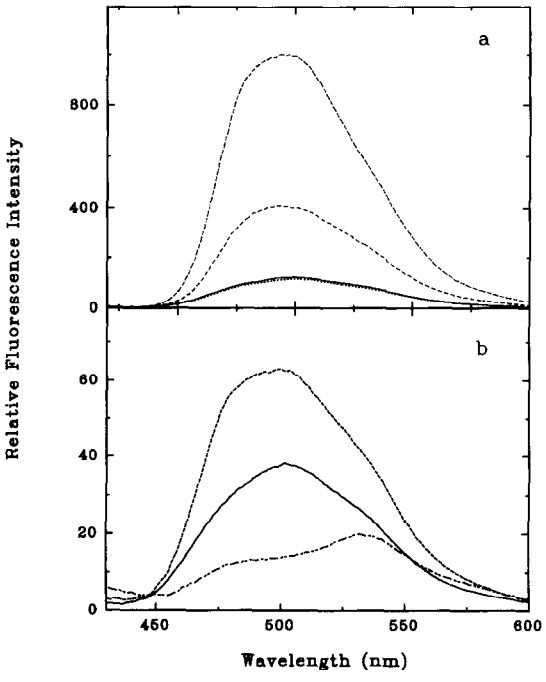


Fig. 2. Emission spectra of the $\alpha_2\beta_2$ complex in the absence and in the presence of L-serine. Emission spectra were recorded for a solution containing 10 μM enzyme, MBP buffer, at 10°C. Excitation and emission slits were 2.5 and 2.5 mm, respectively. (a) Upon excitation at 410 nm, in the absence of L-serine at pH 7 (————) and 10 (- - -) and, upon excitation at 420 nm, in the presence of 100 mM L-serine at pH 7 (– – –) and 10 (— – —). (b) Upon excitation at 350 nm, in the absence of L-serine (————), in the presence of 100 mM L-serine (- - -) and in the presence of 100 mM L-serine and 50 mM GP (— – —), at pH 7.

[44,57,60,62,65]. The broad emission band is blue-shifted by about 7 nm with respect to the emission of the internal aldimine, with a Stokes shift reduced from 88 to 71 nm and is eight times more intense [44]. The intensity of the emission band increases on pH, as previously observed in the β_2 dimer from *E. coli* [60]. This increase, not accompanied by a pH-dependent accumulation of the external aldimine [19], has been attributed to the interaction of the chromophore with an ionized residue [60]. The excitation spectra of PLP emission in the native β_2 dimer (emissions at 475, 495 or 535 nm, data not shown) agree with the absorption data.

The fluorescence spectrum (excitation at 410 nm) of the internal aldimine of the $\alpha_2\beta_2$ complex (Fig. 2a) exhibit an emission centered at 500 nm, that, at variance with the internal aldimine of the β_2 dimer,

[1] Since pH changes are exploited to accumulate either the external aldimine or the α-aminoacrylate species in the reaction of L-serine with the $\alpha_2\beta_2$ complex, we have also investigated the effect of pH on the spectroscopic properties of the native enzyme.

is pH-independent. The L-serine-$\alpha_2\beta_2$ derivatives exhibit the typical blue-shifted emission band of the external aldimine (Fig. 2a). The intensity of this emission increases with pH due to the accumulation of the external aldimine [19] and, at pH 10, it is eight times higher than that of the internal aldimine. The emission spectrum was also recorded upon excitation of the $\alpha_2\beta_2$ complex at 350 nm ($\lambda_{em} \geq 420$ nm) either at pH 7 (Fig. 2b) and 10 (data not shown). The intensity is three times less than that observed upon excitation at 410 nm. Furthermore, the emission spectrum of the external aldimine, collected upon excitation at 350 nm, exhibits a small shoulder on the red side of the broad 493 nm band (Fig. 2b), that is not present in the spectrum recorded with excitation at 420 nm (Fig. 2a). When the emission spectrum is recorded for the L-serine-$\alpha_2\beta_2$ derivatives in the presence of saturating concentrations of GP (excitation at 350 nm) the shoulder becomes a peak centered at 570 nm (Fig. 2b). This red emission is likely to be attributed to the α-aminoacrylate Schiff base that accumulates under these experimental conditions [19].

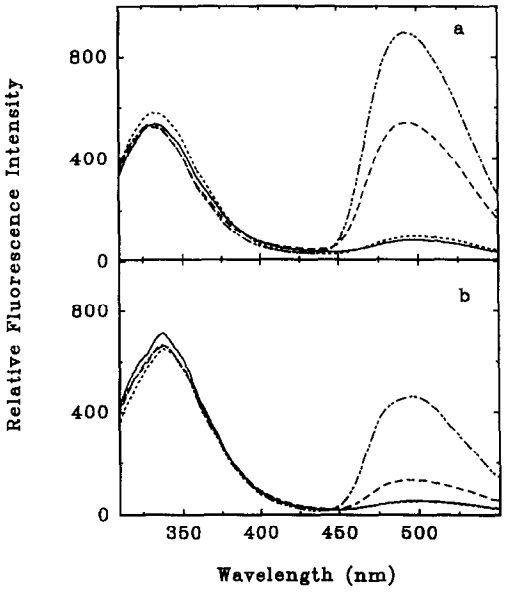


Fig. 3. Emission spectra of the β_2 dimer and the $\alpha_2\beta_2$ complex in the absence and in the presence of L-serine, at pH 7 and 10, recorded with excitation at 295 nm. Emission spectra were recorded for a solution containing 10 μM enzyme, MBP buffer, at pH 7 (————) and 10 (- - -) in the absence of L-serine, and at pH 7 (– – –) and 10 (— — —) in the presence of 100 mML-serine, at 10°C. The excitation wavelength was 295 nm. a: excitation and emission slits, 2.5 and 5 mm; b: 3 and 2.5 mm, respectively.

The excitation spectra of PLP bound to $\alpha_2\beta_2$ complex (emission at wavelengths higher than 450 nm, data not shown) agree with the corresponding absorption spectra.

The emission spectra, collected with excitation at 295 nm for the β_2 dimer (Fig. 3a) and for the $\alpha_2\beta_2$ complex (Fig. 3b), are due essentially to the emission of the unique tryptophanyl residue Trp-177β, although the coenzyme is also exhibiting some absorption at this wavelength [66]. The fluorescence spectra exhibit a band at 334–337 nm that is pH-independent and is slightly blue shifted in the presence of L-serine. The emission spectra reveal also a second band in the 500 nm-region [33], which increases and becomes the most relevant spectral feature after reaction with L-serine. This band has been observed in Schiff bases of PLP [67–69] and in other PLP-dependent enzymes [70,71]. In the latter cases the 500 nm band has been attributed to the emission of the coenzyme following energy transfer from a Trp residue [33,39]. In the presence of L-serine, the 500 nm band increases as a function of pH, due to the accumulation of the external aldimine.

3.2. Fluorescence decay of PLP bound to the β_2 dimer and to the $\alpha_2\beta_2$ complex in the absence and in the presence of L-serine

Fluorescence multifrequency phase and modulation data of PLP for the $\alpha_2\beta_2$ complex either in the absence or in the presence of L-serine are shown in Fig. 4. In the case of the internal aldimine, two distinct components of comparable fractional intensity are present (Tables 1 and 2 and Fig. 4a). The long-lived component shows a lifetime of 2.88 ns, at pH 7.0, that slightly decreases as pH increases, while the fast component shows a lifetime that is close to 1 ns and is nearly pH independent. In the presence of L-serine, at pH 7.0, two components were required to fit the data with lifetimes of 5.33 ns ($f = 0.92$) and 0.37 ns (Fig. 4b). By increasing pH values, i.e. by stabilizing the external aldimine, the fast component becomes negligible, while the long-lived component exhibits an increase of its amplitude. In contrast, when the fluorescence decays were measured in the presence of both L-serine and GP, an allosteric

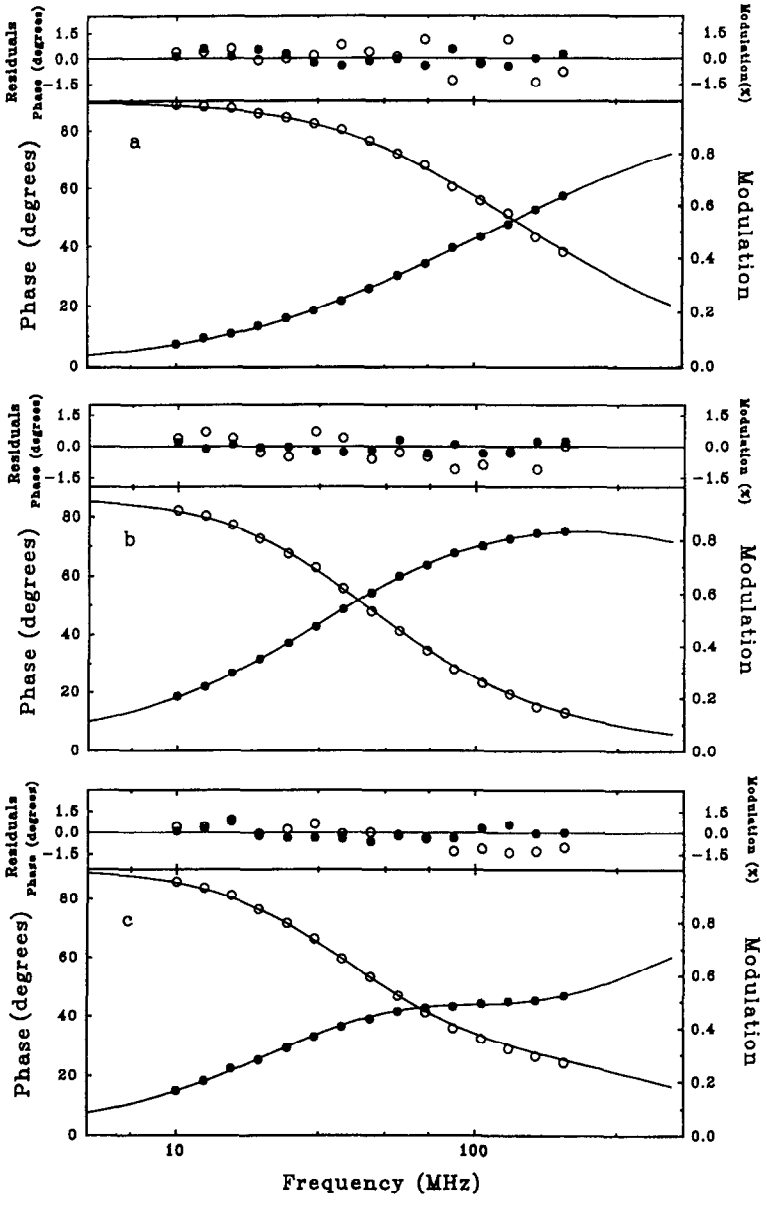


Fig. 4. Multifrequency phase and modulation data for the $\alpha_2\beta_2$ complex in the absence and in the presence of L-serine. The fluorescence decay of the PLP-enzyme complex was monitored for a solution containing a, 0.65 mg ml^{-1} $\alpha_2\beta_2$ complex; b and c, 0.52 mg ml^{-1} $\alpha_2\beta_2$ complex, 100 mM L-serine, MBP buffer, pH 7, at 10°C. Samples were excited at 410 (a), 420 nm (b) and 350 nm (c). Emission was measured using a Oriel 52095 filter. The multifrequency phase (○) and modulation data (●) were collected between 10 and 200 MHz. Solid lines through data points are the least squares best fit to two exponential decays with $\tau_1 = 2.88 \pm 0.06$ ns, $\tau_2 = 0.92 \pm 0.02$ ns, $f_1 = 0.54 \pm 0.01$, $f_2 = 0.46 \pm 0.01$ and $\chi^2 = 1.7$ (a), $\tau_1 = 5.30 \pm 0.04$ ns, $\tau_2 = 0.37 \pm 0.04$ ns, $f_1 = 0.92 \pm 0.01$, $f_2 = 0.08 \pm 0.01$ and $\chi^2 = 1.0$ (b), and $\tau_1 = 5.38 \pm 0.08$ ns, $\tau_2 = 0.33 \pm 0.02$ ns, $f_1 = 0.77 \pm 0.01$, $f_2 = 0.23 \pm 0.01$ and $\chi^2 = 2.0$ (c). The deviation in phase (○) and modulation (●) between measured and calculated values is shown in the upper part of each panel. Systematic standard deviations of modulation higher than the imposed level (0.004), even when collecting the maximal number of experimental data (see Section 2), are due either to low modulation values (0.2) (panel b) or to very low signal (panel c), at high modulation frequency.

effector that significantly favors the accumulation of the α-aminoacrylate [18,19], the contribution of the fast component increases, and becomes detectable even at pH values higher than 9 (Table 1).

Fluorescence decay of the coenzyme for the $\alpha_2\beta_2$ complex and for the L-serine-enzyme complexes were also recorded upon excitation at 340 nm and at 350 nm, respectively. The fluorescence lifetime parameters are reported in Table 2. The fluorescence decay of the internal aldimine is similar to that observed upon excitation at 410 nm. In the L-serine-$\alpha_2\beta_2$ complex at pH 7.0, the emission decay, upon excitation at 350 nm, is accounted by two components with the same lifetimes determined upon excitation at 420 nm, but with a larger contribution of the short component. Remarkably, when GP is added to the reaction mixture, at pH 7, the component with the short lifetime becomes prevalent (Table 2).

The fluorescence decay of the internal aldimine in the β_2 dimer is best fitted by two components with lifetimes of 1.15 ($f = 0.68$) and 4.42 ns, at pH 7.0, and a single component with a lifetime of 1.5 ns, at pH 10 (Table 1). The decay of the external aldimine in the β_2 dimer is mono-exponential both at pH 7.0 and 10 with a lifetime of 5.54 ns at pH 7.0 that increased to 6.38 ns at pH 10. The 5.54 ns lifetime is identical to that determined for the *E. coli* β_2 dimer at pH 7.6, 25°C, using the single photon counting technique [44].

3.3. Fluorescence decay of Trp-177β for the β_2 dimer and for the $\alpha_2\beta_2$ complex in the absence and in the presence of L-serine

The excitation of Trp-177β was performed at 295 nm, in order to minimize the contribution of tyrosine emission. In Table 3, we report the parameters for the decay of Trp-177β in the β_2 and in the $\alpha_2\beta_2$ complex, in the absence and in the presence of L-serine, at pH 7 and 10. Since the filters used to collect the emitted fluorescence in the 300–400 nm region allow passage of about 10% transmittance at 300 nm, a high contribution of excitation light was detected and overlapped with the emission. The low signal intensity and the presence of such scattered light complicate the analysis of the fluorescence decay of Trp-177β and do not allowed to evaluate the presence of components with very short lifetimes.

4. Discussion

Tryptophan synthase is a model enzyme for the investigation of the coupling between protein conformation, catalysis and allosteric regulation since distinct regulatory signals are associated to different catalytic intermediates [10–13], possibly via the formation of "open" and "closed" conformational states of the β-subunit. In previous works, by exploiting the extreme sensitivity of phosphorescence decay to changes of local protein viscosity, the dynamic properties of the apo- and holo-enzyme at the α- and β-active sites and near Trp-177β were determined [33,34]. Here, we have characterized the conformational properties of the internal aldimine, the external aldimine and the α-aminoacrylate Schiff bases.

4.1. Conformational properties of the internal aldimine

The presence of two absorption bands in the 300–500 nm region is a feature shared by almost all PLP dependent enzymes. In the case of tryptophan synthase, the 412 nm band is attributed to the ketoenamine tautomer of the internal Schiff base [56], while the nature of the shoulder in the 330–340 nm region remains puzzling. A possible attribution is that the blue absorbing species is the enolimine form of the internal Schiff base. This PLP tautomer is favored in apolar environments since it bears no charge. The two bands might therefore reflect an equilibrium between different conformations of the active site, one more and one less polar [56,57]. Our data show that absorption spectra and emission intensity of the $\alpha_2\beta_2$ complex are virtually pH-independent in the range 7–10. Moreover, we detected two fluorescence lifetimes with values and fractional intensities that are only slightly pH-dependent. These results indicate that the internal aldimine in the $\alpha_2\beta_2$ complex is distributed in two tautomeric forms of the coenzyme that are linked by a pH-independent equilibrium. The existence of a structural heterogeneity of the internal aldimine was also evidenced by Trp-177β phosphorescence decay [33]. Since fluorescence lifetimes, fractional intensities and absorption and emission spectra are pH-dependent in the β_2

dimer, these findings suggest that the interaction of the α-subunit with the β-subunit has significantly altered the conformational equilibrium present in the isolated β-subunits by favoring a particularly stable set of conformational states of the β-subunit. The increase of lifetimes in the fluorescence decay (Table 3) and in phosphorescence decay [72] of Trp-177β in the $\alpha_2\beta_2$ complex with respect to the β_2 dimer indicates a reduced flexibility of the β-subunit in the presence of the α subunit.

Finally, the emission spectra of Trp-177β in the β_2 dimer and in the $\alpha_2\beta_2$ complex, centered at 334–337 nm, are blue shifted with respect to Trp in aqueous solution ($\lambda = 350$ nm) and red shifted with respect to Trp in non polar environments ($\lambda = 325$ nm), indicating a partially shielded position of Trp in the hydrophobic interior of the protein. This conclusion is also supported by the increased values of lifetimes of Trp-177β with respect to free Trp in aqueous solution.

4.2. Fluorescence properties of the external aldimine and the α-aminoacrylate Schiff bases

The formation of the external aldimine in the β_2 dimer and in the $\alpha_2\beta_2$ complex increases the fluorescence intensity in comparison with the fluorescence of the internal Schiff base. A similar behaviour has been observed in the reaction of D-serine dehydratase with glycine [73] and *O*-acetylserine sulfhydrylase-A with cysteine [71]. These findings were interpreted as indication of a conformational change associated with the formation of the external aldimine. The reduced Stokes shift, the increased fluorescence intensity and the lengthening of the lifetimes of PLP as a function of pH are in agreement with such interpretation. These findings and the blue shift in the emission suggest that the conformational change that accompanies L-serine binding leads to a shielding of the PLP [42], and, consequently, to a stabilization of the excited states, as previously demonstrated by static fluorescence quenching measurements [44]. The enhanced fluorescence and similar excited state lifetimes for the external aldimine in the β_2 dimer and in the $\alpha_2\beta_2$ complex indicate that the conformation of this intermediate is not significantly affected by the presence of the α-subunit.

The fluorescence decay of the L-serine-$\alpha_2\beta_2$ complex (excitation at 420 nm) at pH higher than 7.8, where the external aldimine is the predominant species, is monoexponential, and become biexponential at lower pH values, where the L-serine enzyme derivatives are distributed between the external aldimine and the α-aminoacrylate. The slow component exhibits lifetimes that are characteristic of the external aldimine, and fractional intensities that increase as pH increases. The fast component exhibits a fractional contribution that is low at pH 7, but, in the fluorescence decays upon excitation at 350 nm, is significantly higher and becomes predominant when the concentration of the α-aminoacrylate is increased by the presence of GP. These findings allow to attribute the fast component of the decay to the fluorescence of the α-aminoacrylate. The fluorescence of the α-aminoacrylate is weak and can clearly be detected under experimental conditions that lead to its predominant accumulation, i.e. low pH and the presence of GP, and with excitation at its absorption peak ($\lambda = 350$ nm). The emission spectrum is characterized by a very high Stokes shift and a low quantum yield with respect to the emission of the external aldimine. This latter finding explains the small fractional intensity of the fast component in the fluorescence decay monitored with excitation at 420 nm and the relatively higher contribution of the fast component for the fluorescence decay with excitation at 350 nm. The reduced fluorescence intensity and the low value of the lifetime suggest that the emission properties of the α-aminoacrylate are characterized by highly efficient non-radiative transitions, possibly due to a tight coupling with the protein matrix and/or different orientation or distance between the coenzyme and nearby residues. A change of PLP orientation in the catalytic transformation of the external aldimine to the α-aminoacrylate seems to be excluded on the basis of polarized absorption measurements on single crystals of the $\alpha_2\beta_2$ complex. The calculated polarization ratio [2] of the internal aldimine, the external aldimine and the α-aminoacrylate Schiff base is fairly constant [17,74].

[2] Single crystal polarized absorption spectra of tryptophan synthase are obtained with light linearly polarized parallel to two orthogonal extinction directions ([17],[75]). The polarization ratio is defined as the ratio of the absorption intensity at each wavelength for the two orthogonal directions.

Since this parameter is very sensitive to changes in the orientation of the plane of the pyridine ring where the dipole electronic transition moment lays, this finding suggests that PLP orientation is independent of the nature of the Schiff base that is bound to PLP.

Remarkably, whereas the internal aldimine is present in two tautomeric forms, both the external aldimine and the α-aminoacrylate appear to be present as single conformations with rather distinct emission properties, that might reflect not only differences in their excited states but also in structural and/or dynamic properties of the β-active site. These changes seem to be large enough to modify the network of interactions between α-and β-active sites and to result in either an "open" or a "closed" state of the enzyme [10–13].

Since the emission properties of Trp-177β are very similar when either the external aldimine or the α-aminoacrylate are present (Table 3), this finding suggests that Trp-177β is localized in an environment not affected by the chemical modifications that take place at the β- active site.

The three dimensional structure of the $\alpha_2\beta_2$ complex [31] (Fig. 1) indicates that the wall of the β-active site more close to the subunit interface is formed by aminoacid residues characterized by temperature factors higher than residues forming the other walls of the active site. When the external aldimine is formed in the β-active site, water molecules leave the β-active site and conformational changes might occur. Removal of both the β-hydroxyl group and the α-proton from the external aldimine to form the α-aminoacrylate or the change in the L-serine α-carbon from a sp^3 to sp^2 hybridization may induce distinct conformational changes that are communicated via the mobile wall to the interface and, then, to the α-active site. These hypothetical series of events can be tested either by solving the three-dimensional structure of both the external aldimine and the α-aminoacrylate Schiff bases or by carrying out molecular dynamics simulation of these intermediates. Both approaches are in progress.

Acknowledgements

We are grateful to G.B. Strambini for making available to us luminescence measurements prior to publication, and to E.W. Miles for providing us with the *E. coli* strain containing the plasmid encoding the *S. typhimurium* tryptophan synthase genes. A.P. and A.M. are indebted to G.L. Rossi for the encouragement in pursuing this work. This work was supported by funds from the Italian Ministry of Universities and Research in Science and Technology, the National Institute of Physics of Matter and the National Research Council Target Project on Biotechnology and Bioinstrumentation

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