Conformational Changes and Subunit Communication in Tryptophan Synthase: Effect of Substrates and Substrate Analogs†

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ABSTRACT: The transmission of regulatory signals between the α- and β-subunits of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* has been investigated by monitoring the luminescence properties of the enzyme in the presence and in the absence of the α-subunit ligand DL-α-glycerol 3-phosphate, the α- and β-subunit substrate indole, and the β-subunit substrate analog L-histidine. The β-subunit contains as intrinsic probes Trp-177 and pyridoxal 5′-phosphate, whereas the α-subunit has been mutagenized by replacing Ala-129 with a Trp residue. In contrast to the inertness of L-histidine, DL-α-glycerol 3-phosphate was found (i) to alter the phosphorescence spectrum of Trp-129, (ii) to shift the fluorescence thermal quenching profile of both Trp-177 and coenzyme to higher temperature, (iii) to slow down the triplet decay kinetics of Trp-177 in fluid solution, and (iv) to affect the equilibrium between different conformations of the enzyme. These findings provide direct evidence that DL-α-glycerol 3-phosphate binding affects the structure of the α-subunit and, in the presence of coenzyme, induces a conformational change in the β-subunit that leads to a considerably more rigid structure. As opposed to DL-α-glycerol 3-phosphate, the shortening of the phosphorescence lifetime upon indole binding suggests that this substrate increases structural fluctuations in the β-subunit. Implications for the mechanism of the allosteric regulation between α- and β-subunits are discussed.

Allosteric enzymes play a crucial role in the regulation of biological systems, since they allow a fine-tuning of metabolite concentrations in response to cell requirements. These enzymes are multimeric proteins in which the allosteric control is achieved by intersubunit communication.

Tryptophan synthase provides a very interesting example of allosteric protein. The enzyme is an $\alpha_2\beta_2$ complex in which each type of subunit catalyzes a distinct reaction:

α-subunit

indole-3-glycerol phosphate ⇌ indole + D-glyceraldehyde 3-phosphate

β-subunit

indole + L-serine → L-tryptophan + H_2O

The $\alpha_2\beta_2$ complex catalyzes the overall reaction with a 100-fold increase in efficiency (Yanofsky & Crawford, 1972; Miles, 1979, 1991a). Binding of L-serine to the β-active site decreases the dissociation constants for substrate and substrate analogs of the α-subunit (Creighton, 1970; Lane & Kirschner, 1983a; Kawasaki et al., 1987). Similarly, the presence of α-subunit ligands strengthens the binding of β-subunit substrates and substrate and intermediate analogs, and causes a redistribution of covalent intermediates formed in the β-active site (Lane & Kirschner, 1981, 1983a; Miles, 1980; Mozzarelli et al., 1989, 1991; Houben & Dunn, 1990). The reciprocal influence of α- and β-subunit ligands is also revealed by their effect on α- and β-reaction kinetics (Lane & Kirschner, 1981, 1983b, 1991; Houben & Dunn, 1990; Dunn et al., 1990, 1991; Kirschner et al., 1991; Anderson et al., 1991; Kayastha et al., 1991).

The three-dimensional structure of the $\alpha_2\beta_2$ complex from *Salmonella typhimurium* either in the presence or in the absence of indole-3-propanol phosphate indicates local conformational changes at the α-active site but no changes in the β-active site (Hyde et al., 1988; Hyde & Miles, 1990). However, it has been reported that indole-3-propanol phosphate bound to the α-site perturbs the spectrum of the pyridoxal 5′-phosphate bound at the β-active site (Kirschner et al., 1975). Since the α- and β-active sites are 25 Å apart, this interaction can only be indirect and transmitted through the protein matrix. The nature of such an interaction can be either electrostatic or structural via conformational changes that involve intersubunit contacts. The latter hypothesis has been usually the favorite (Miles, 1991a). An X-ray investigation of L-serine–$\alpha_2\beta_2$ complexes in the presence and in the absence of α-subunit ligands is not yet available nor is there structural or spectroscopic evidence on the effect of β-subunit ligands on the α-active site. Since the conformational changes associated with the allosteric regulation might be very small and escape X-ray analysis or conventional spectroscopic techniques, their detection requires the application of techniques very sensitive to subtle conformational and environmental changes.

In the preceding paper (Strambini et al., 1992), it was demonstrated that the sole tryptophan residue (Trp-177) of the β-subunit is highly luminescent and interacts with the coenzyme through energy transfer from both the fluorescent and phosphorescent states. Furthermore, its phosphorescence emission is long-lived even at ambient temperature, and the decay kinetics, which are very sensitive to the flexibility of the surrounding polypeptide chain, provide unequivocal evidence

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for both a conformational heterogeneity and a structuring role of bound coenzyme.

In this work, we exploit fluorescence and phosphorescence quantum yields and triplet decay kinetics as monitors of conformational changes in the β-subunit that may result from binding of DL-α-glycerol 3-phosphate (GP)[1] and indole in the α-subunit and L-His in the β-subunit. Moreover, by taking advantage of a mutagenized $\alpha_2\beta_2$ complex in which Ala-129 in the α-subunit is replaced by Trp, we seek to probe structural changes specific of the α-subunit. The results provide clear evidence that GP binding is responsible for a conformational change in both subunits. The α-subunit ligand affects the equilibrium between different conformers of the macromolecule and, in the presence of coenzyme, significantly increases the rigidity of the β-subunit.

MATERIALS AND METHODS

Holo, reduced holo, and apo forms of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* were prepared and utilized as described in the preceding paper (Strambini et al., 1992). A mutant form of the $\alpha_2\beta_2$ complex in which the α-subunit alanine-129 was replaced by tryptophan was a generous gift of Drs. E. W. Miles and S. Nagata. Oligonucleotide-directed mutagenesis was used to change the *trp*A alanine-129 codon to the tryptophan codon by the methods of Nagata et al. (1989) (Nagata and Miles, unpublished results). The mutagenetic oligomer employed was a 21-mer complementary to nucleotides 376–398 in the mutant *trp*A gene with the sequence 5′-CGCGACATC-C*C*A*GACCAGCAC-3′. The complementary codon is underlined; the asterisks follow bases that differ in the complementary wild-type codon. The specific activities of this mutant $\alpha_2\beta_2$ complex were 5% and 60% of those of the wild-type $\alpha_2\beta_2$ complex in the α- and β-reaction, respectively (Nagata and Miles, unpublished results).

Sample preparation, luminescence studies, and statistical analyses of kinetic data were carried out as described in the preceding paper (Strambini et al., 1992). Binary, ternary, and quaternary complexes with DL-α-glycerol 3-phosphate (GP) (Sigma), L-histidine (His) (Sigma), and indole (In) (Sigma) were formed by incubating the enzyme with a ligand concentration of 40 mM, 200 mM, and 100 μM, respectively. While for GP and His these concentrations correspond to a very high saturation of their respective binding sites both in Bicine buffer and in PG/Bicine buffer (Mozzarelli et al., unpublished results), that of indole corresponds to about 10% saturation (Weischet & Kirschner, 1976). Larger concentrations soon became prohibitive in terms of inner filter effects. Since the binding constants for indole and GP to apo and reduced forms of the bienzyme complex are not known, we have assumed that they do not significantly differ from those reported for the holoenzyme. The results presented in this work justify this assumption.

RESULTS

Ligand Effects on Coenzyme Fluorescence. Pyridoxal 5′-phosphate bound in the β-active site through a Schiff's base with Lys-87 exists as an equilibrium of tautomeric species which exhibit distinct absorption and fluorescence spectra. The red-absorbing ketoenamine tautomer fluoresces with maximum wavelength around 480–500 nm (red band), whereas the enolimine fluoresces around 360–380 nm (blue band). The relative intensity of the two bands is therefore a function of the tautomer equilibrium and of the respective quantum yields.

Table I: Relative Coenzyme Fluorescence Intensities of the $\alpha_2\beta_2$ Complex in the Presence and in the Absence of α- and β-Ligands[a]

	F (blue band)		F (red band)		F_b/F_r	
sample	160 K	273 K	160 K	273 K	160 K	273 K
holo-$\alpha_2\beta_2$	13.5	7.0	5.8	17.0	2.32	0.96
+GP	20.1	8.1	7.4	30.0	2.72	0.73
+His	13.6	6.1	5.9	18.3	2.31	0.77
+GP + His	21.3	7.1	6.2	25.8	3.49	0.95
+GP + In	13.6	9.7	7.0	27.1	1.94	0.70
+His + In	14.3	7.8	6.7	22.0	2.13	0.76
+GP + His + In	23.8	6.5	7.5	24.0	3.17	0.86
reduced holo-$\alpha_2\beta_2$	100	19.9				
+GP	83.6	38.4				
+In	94.5	16.4				

[a] The fluorescence intensity (λ_{ex} = 330 nm) was measured at the maximum wavelength of the blue (λ = 365 nm) and red (λ = 495 nm) bands in PG/Bicine buffer, at 160 and 273 K. The values of fluorescence for the blue and red bands in different species, at 160 K, are given as the percent of the fluorescence intensity of the blue band of the reduced holo-$\alpha_2\beta_2$ complex. The values at 273 K are given as the percent of the fluorescence of the same species at 160 K. The standard error is typically 7–8% at 160 K and 1.5% at 273 K.

The effects of GP, L-His, and indole on the coenzyme fluorescence (λ_{ex} = 330 nm) of holo- and reduced holo-$\alpha_2\beta_2$ complex were investigated at various temperatures, and the results of measurements at 160 and 273 K are reported in Table I. In the various binary and ternary complexes examined, the wavelength of the band peak is practically constant. At 160 K, the fluorescence intensity of the reduced cofactor is only slightly affected by GP binding. On the contrary, the intensity ratio, F_{365}/F_{495}, of the Schiff's base form is significantly larger in almost all complexes that contain GP, with the exception of the ternary complex with indole (Table I). The change might reflect either an enhancement of the blue band yield or a shift in the equilibrium between different tautomers. At ambient temperature, the absorption spectrum of the holoenzyme shows a modest increment ($\approx$5%) of the absorbance around 320–340 nm upon addition of GP (data not shown) that indicates a small shift in the equilibrium toward non-ketoenamine tautomers. The extent of this shift is not sufficient to justify the large increase of F_{365}/F_{490} at low temperature. In contrast to GP, His alone or together with indole has no detectable influence on the coenzyme fluorescence at low temperature.

Another consequence of GP binding to the α-subunit is a smaller thermal quenching of the fluorescence. Inspection of Table I shows that for the reduced coenzyme the residual intensity at ambient temperature is 2 times larger in the presence than in the absence of GP. For the Schiff's base PLP–enzyme form, protection against thermal quenching is most pronounced for the red band. The residual intensity of this band at 273 K is typically around 26–30% of the value at 160 K for GP complexes, 17–18% for other complexes or unliganded protein, and less than 2% for the PLP Schiff's base complex with L-valine (Strambini et al., 1992). The effect of GP on the Schiff's base F_{365}/F_{490} ratio is almost canceled at ambient temperature by the differential thermal behavior of the two bands. Thus, under the usual conditions of fluorescence measurements, only small effects of GP will be apparent on the shape of the spectrum. Finally, the enhancement by over 14-fold of the red band intensity between free and bound coenzymes suggests a convenient method for

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; PG, propylene glycol; GP, DL-α-glycerol 3-phosphate; In, indole.

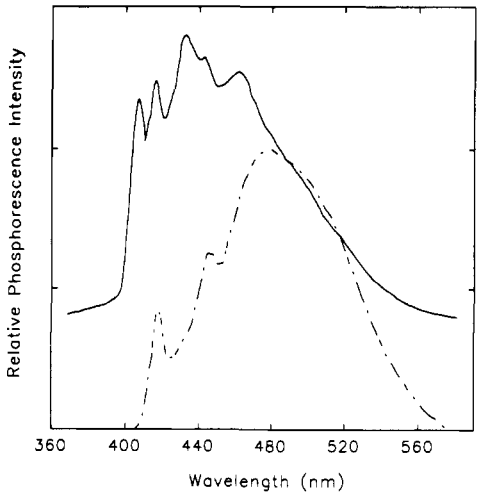

FIGURE 1: Phosphorescence spectra of the wild type and the α-subunit mutant Ala[129]-Trp tryptophan synthase. The $\alpha_2\beta_2$ complex in PG/Bicine buffer (50/50, v/v) was excited at 298 nm. The phosphorescence spectra were recorded for the wild type (– · –) and for the mutant (—) at 195 K. The spectra are not corrected for instrumental response.

determining binding equilibria for the coenzyme in the presence of GP.

Since several variables can influence the observed fluorescence emission, a rationalization of GP effects in terms of equilibria and fluorescence yields can only be approximate. While the blue band enhancement at low temperature could be consistent with a large temperature- and/or ligand-induced shift of the tautomer equilibrium, the generalized increase in the average fluorescence yield at ambient temperature with both reduced and Schiff's base forms implies less quenching in GP complexes. Since excited-state proton-transfer reactions, which are the main route of deactivation of the fluorescence state, are strongly favored in fluid media (Kallen et al., 1985), the lower quenching in these complexes is probably associated with a more rigid structure at the active site of the β-subunit.

Ligand Effects on Trp Luminescence Spectra at Low Temperature. At 195 K, the addition of GP and/or His to the apo- and holoenzyme does not alter the spectral energies of excited singlet and triplet states of Trp-177. Since this parameter depends on specific interactions with the chromophore environment (Galley, 1976), such constancy suggests that complex formation with α- and β-subunit ligands does not involve major structural rearrangements in the N-domain of the β-protomer.

Structural effects on the α-subunit were probed by analyzing the spectral properties of Trp placed at position 129 of the α-subunit. This amino acid is localized at the α-active site and participates in the formation of a hydrophobic pocket that binds the indole moiety of the indole-3-glycerol phosphate (Figure 4) (Hyde et al., 1988; Lim et al., 1991).

The fluorescence intensity of the mutant enzyme is about twice as large as that of the wild type, and the spectrum is very similar. Its phosphorescence spectrum, however, is clearly heterogeneous with two distinct 0–0 vibronic bands, separated by 10 nm (Figure 1). The bands are centered at 408.5 and 418.5 nm, the latter wavelength coinciding with the band maximum of the wild type. This finding and the thermal quenching profile (following section) assign the 408.5 nm band to Trp-129 and also confirm that the mutation has not perturbed the environment of Trp-177.

Binding of GP or GP plus His to the mutant causes a 2-nm red shift of the 408.5-nm band. His by itself has no effect. The spectral red shift elicited by GP binding attests to a change

Table II: Trp Fluorescence (λ_{em} = 325 nm) and Phosphorescence (λ_{em} = 420 nm) Intensities for Various Complexes of Tryptophan Synthase in a PG/Bicine Buffer Glass (195 K) and in Bicine Buffer at 293 K[a]

sample	$F/F_{(apo)}$ 195 K	$F/F_{(apo)}$ 293 K	$PF_{(apo)}/P_{(apo)}F$ 195 K
apo-$\alpha_2\beta_2$ + GP	0.96	0.99	1.01
apo-$\alpha_2\beta_2$ + His	1.15	1.03	0.98
reduced holo-$\alpha_2\beta_2$	0.25	0.46	0.96
+GP	0.23	0.58	1.65
holo-$\alpha_2\beta_2$	0.28	0.50	0.22
+GP	0.30	0.54	0.29
+His	0.32	0.50	0.24
+GP + His	0.29	0.56	0.31

[a] The standard error is typically 7–8% for low-temperature fluorescence intensities and 1–2% for P/F ratios and high-temperature fluorescence intensities.

in the immediate environment of the indole ring that could be attained either by direct interaction with the ligand or by a ligand-induced structural rearrangement of the polypeptide chain. Since the Cα carbon of Ala-129 is at least 13 Å apart from the presumed GP binding pocket, a conformational change of the α-subunit appears to be involved.

Ligand Effects on the Quenching of Trp Luminescence by the Coenzyme. The presence of the coenzyme, either in a reduced state or in Schiff's base form, in the β-active site of tryptophan synthase decreases by roughly a factor of 2 the fluorescence quantum yield of Trp-177 (Strambini et al., 1992). The extent of quenching in binary and ternary complexes with GP and His in PG/buffer at 195 K and in buffer at 293 K is given in Table II. A clear, although modest, reduction of quenching was observed only at high temperature for GP complexes. In binary and also ternary complexes with His, GP reduces the fluorescence quenching by about 6% with the Schiff's base and by 12% with the reduced coenzyme. There is no effect of His and GP on the apoenzyme.

The increments in the fluorescence quantum yield may be understood in terms either of a greater intrinsic quantum yield, ϕ_{F_0}, or of less efficient energy transfer to the coenzyme caused by a less favorable distance/orientation between indole and pyridoxal rings. The observation that ϕ_{F_0} in the apoprotein is 0.45 ± 0.05 of its cold temperature value [see Table I of Strambini et al. (1992)] and that GP enhancements are concurrent with the drop in ϕ_{F_0}(240–300 K) supports the first hypothesis. Hence, the greater fluorescence yields of Trp and coenzyme in GP complexes appear to be caused by a shift of the thermal quenching profile to higher temperatures. This is consistent with a less flexible structure of the β-subunit.

Quenching of phosphorescence, as embodied in a decrease in the P/F intensity ratio relative to the apoprotein, involves triplet–singlet energy transfer to the ketoenamine tautomer and is therefore limited to the Schiff's base enzyme. P/F ratios at 195 K (Table II) point out that once again only GP affects the efficiency of energy transfer with a 7–9% reduction. Since the triplet lifetime and, therefore, transfer rates are practically identical among complexes, the result (barring other causes, see below) would point to a reduction in the ketoenamine fraction.

Control experiments with apo- and reduced holoprotein, which have similar P/F ratios, demonstrated an unexpected (because the triplet lifetime is constant) 65% increment of the ratio for the complex of GP with the reduced form. Since there is no proportional lengthening of the triplet lifetime nor a heavy-atom effect to justify the greater phosphorescence

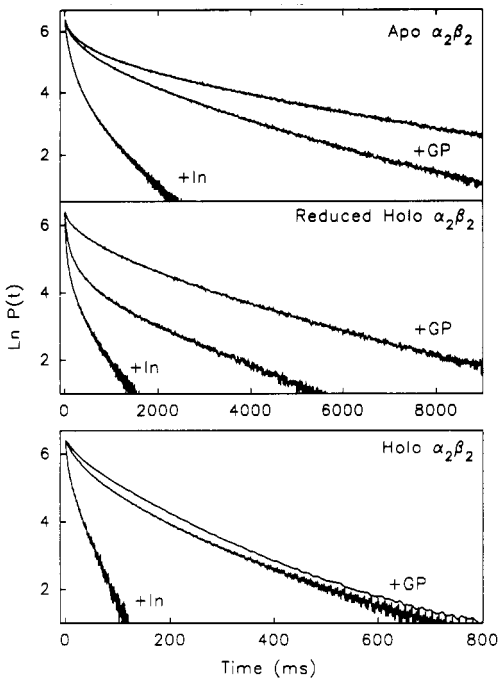

FIGURE 2: Influence of indole and GP on the Trp phosphorescence decay of apo-, holo-, and reduced holotryptophan synthase. GP (40 mM) or indole (100 μM) was added to an enzyme solution containing PG/Bicine buffer (50/50, v/v). The time courses of Trp phosphorescence decay for apo-, reduced holo-, and holo-$\alpha_2\beta_2$ complex were recorded at 250 K.

intensity (the intersystem crossing yield with the holoprotein is that found with Trp), we conclude that the phosphorescence of Trp-177 is partly sensitized at the triplet level. The only plausible route is triplet–triplet energy transfer from nearby Tyr^- (Longworth, 1971). If confirmed, this would indicate that binding of GP lowers the p*K* of some of the four Tyr residues that are within energy-transfer distance (12–15 Å) of Trp-177.

Effects of GP, His, and Indole on the Phosphorescence Decay Kinetics. In glass matrices, indole added to the wild type and Trp-129 present in the mutant form exhibit blue-shifted vibronic bands (406 and 408.5–410.5 nm, respectively) with respect to Trp-177 (418.5 nm) that allow for separate lifetime determinations. At these low temperatures, their lifetimes are typically 5.5–6 s. At warmer temperatures (240 K), their phosphorescence is selectively quenched over that of Trp-177, and their lifetimes are less than 1 ms, the detection limit of the apparatus. The implication is that both position 129 in the α-subunit and indole binding "sites" either in the channel (Hyde et al., 1988) or in the α- and β-active sites are regions of the macromolecule characterized by considerable flexibility. Of course, indole molecules within the channel that are sufficiently close to the pyridoxal ring for energy transfer will be silent.

Above 240 K, the emission is due solely to Trp-177, and all decay kinetics in fluid solutions pertain exclusively to it. Other results obtained with the mutant will not be mentioned since in fluid solutions they are practically indistinguishable from those of the wild type.

In fluid solutions, phosphorescence decays are always heterogeneous, and complex formation with α-subunit ligands (GP or indole) has a remarkable influence on both amplitudes and decay rates. A comparison of phosphorescence decays at 250 K is shown in Figure 2. Indole drastically shortens the triplet lifetime, and its effects are quite general with all three form of the enzyme (apo-, holo-, and reduced holoenzyme).

Table III: Triplet Lifetimes (τ) and Preexponential Terms (α) Derived from a Biexponential Fitting of the Phosphorescence Decay of Several Complexes of Tryptophan Synthase in Bicine Buffer at Two Selected Temperatures

sample	τ_S (ms)	τ_L (ms)	α_S	α_L
	273 K			
apo-$\alpha_2\beta_2$	17.3	55.5	0.45	0.55
apo-$\alpha_2\beta_2$ + GP	10.1	53.1	0.86	0.14
apo-$\alpha_2\beta_2$ + His	17.2	38.0	0.63	0.37
apo-$\alpha_2\beta_2$ + In	5.0	7.5	0.1	0.9
reduced holo-$\alpha_2\beta_2$	29.5	79.4	0.56	0.44
reduced holo-$\alpha_2\beta_2$ + GP	26.7	178.0	0.75	0.25
reduced holo-$\alpha_2\beta_2$ + In	5.9	22.0	0.68	0.32
holo-$\alpha_2\beta_2$	28.2	48.5	0.46	0.54
holo-$\alpha_2\beta_2$ + GP	33.2	65.8	0.30	0.70
holo-$\alpha_2\beta_2$ + His	27.8	48.8	0.44	0.56
holo-$\alpha_2\beta_2$ + GP + His	35.2	64.5	0.33	0.67
holo-$\alpha_2\beta_2$ + GP + In	14.0	30.5	0.71	0.29
holo-$\alpha_2\beta_2$ + His + In	3.6	13.5	0.79	0.21
holo-$\alpha_2\beta_2$ + His + GP + In	13.3	31.5	0.64	0.36
	293 K			
apo-$\alpha_2\beta_2$	4.7	16.8	0.71	0.29
apo-$\alpha_2\beta_2$ + GP	4.0	19.2	0.88	0.12
apo-$\alpha_2\beta_2$ + His	4.1	13.2	0.87	0.13
apo-$\alpha_2\beta_2$ + In	2.1	4.0	0.30	0.11[a]
reduced holo-$\alpha_2\beta_2$	11.6	25.2	0.58	0.42
reduced holo-$\alpha_2\beta_2$ + GP	10.0	49.7	0.85	0.15
reduced holo-$\alpha_2\beta_2$ + In	3.6	11.4	0.41	0.13[a]
holo-$\alpha_2\beta_2$	14.6	23.6	0.66	0.34
holo-$\alpha_2\beta_2$ + GP	13.3	29.6	0.21	0.79
holo-$\alpha_2\beta_2$ + His	13.9	22.5	0.69	0.31
holo-$\alpha_2\beta_2$ + GP + His	14.4	28.7	0.30	0.70
holo-$\alpha_2\beta_2$ + GP + In	4.1	13.6	0.61	0.23[a]
holo-$\alpha_2\beta_2$ + His + In	3.6	7.5	0.37	0.21[a]
holo-$\alpha_2\beta_2$ + His + GP + In	5.2	14.7	0.43	0.17[a]

[a] Under these conditions, $\alpha_S + \alpha_L < 1$ since part of phosphorescence intensity decays at times shorter than the detection limit of the apparatus.

GP is more selective. When GP is present, the decay of the apoprotein is slightly more rapid, whereas the opposite is true for the holoprotein. The lengthening of the triplet lifetime is particularly dramatic with reduced holo-$\alpha_2\beta_2$, and, due to the absence of energy transfer, it allows direct monitoring of its intrinsic value. Once again, the presence of His has no effect. At 250 K, the fitting of the decay curves in terms of two exponential components gives, as with unliganded proteins (Strambini et al., 1992), a $\chi^2 > 2$, an indication that a two-conformer model is inadequate to describe the data.

The main features that characterize the phosphorescence decays of complexes in PG/buffer at 250 K are present also in buffer at ambient temperature, except that, at higher temperature, a two-component model gives better fitting statistics. The parameters α_i and τ_i, derived from a biexponential analysis of decays in buffer at 273 and 293 K, correspond to the amplitude and the lifetime of the short- and long-lived component, respectively, and are collected in Table III. Ligand effects in terms of the average lifetime, $\tau_{av} = \alpha_S\tau_S + \alpha_L\tau_L$, and the amplitude of preexponential factors are shown in Figure 3.

In the apoprotein, τ_{av} is lowered by both His and GP. A large part of the effect is due to an increased amplitude of the short-lived component (α_S). The observed effect of His is somewhat surprising, since His is thought to bind to the enzyme through a Schiff's base with pyridoxal 5′-phosphate. A possible explanation is that His can penetrate in the empty β-active site and indirectly interacts with Trp-177.

A constant feature of GP binding to the holoenzyme, whether in binary or ternary complexes with His, is a considerable increase in τ_L. The lengthening effect on τ_{av} is more or less evident according to the corresponding change

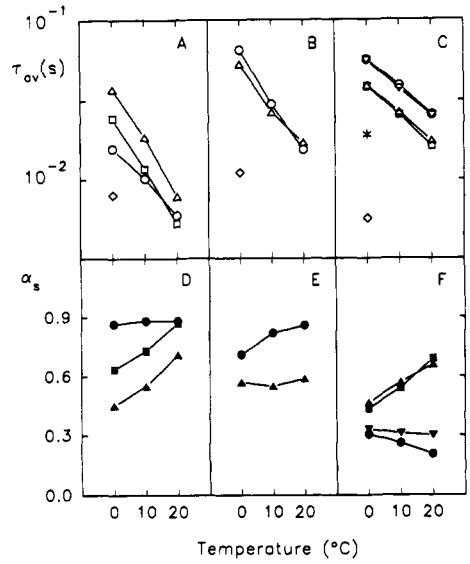

FIGURE 3: Temperature and ligand effects on the average phosphorescence lifetime, τ_{av}, and the preexponential term, α_S. The $\tau_{av} = \alpha_S\tau_S + \alpha_L\tau_L$ (open symbols) and the preexponential term α_S (filled symbols) are reported for the apo (panels A and D), reduced holo (panels B and E), and holo (panels C and F) forms of tryptophan synthase in the absence (△, ▲) and in the presence of GP (○, ●), His (□, ■), indole (◇), GP and His (▽, ▼), and GP and indole (★).

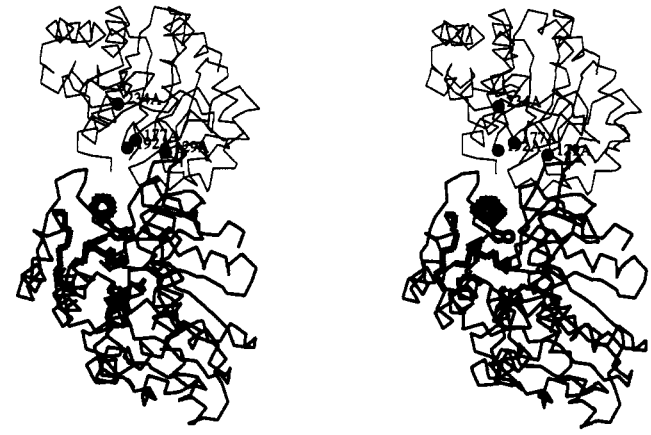

FIGURE 4: Stereoview of tryptophan synthase $\alpha\beta$ dimer. The α-subunit is drawn with a light line and the β-subunit with a medium line. Only the amino acid α-carbons are shown except for Lys-87 with bound PLP and Trp-177. These groups, the α-helix that contains Trp-177, and the adjacent three strands that are part of the walls of the β-active site are drawn with heavy lines. The view is almost parallel to the axis of the helix. Small filled circles indicate amino acid residues of the α-subunit, 129A, 177A, 192A, and 234A. The coordinates are obtained from the Brookhaven Protein Data Bank (file 1WSY.PDB; Hyde et al., 1988).

in amplitudes and τ_S. Thus, the 30–40% and 100% increase in τ_L with holo- and reduced holo-$\alpha_2\beta_2$ appears as an increase in τ_{av} of 45% and 8%, respectively. GP binding to apo and reduced forms or to the Schiff's base enzyme respectively increases and decreases the value of α_S. Furthermore, the amplitudes in GP complexes show a very slight temperature dependence when compared to His complexes or the unliganded protein.

In conclusion, the triplet lifetime of Trp-177 reveals unequivocal structural changes upon GP binding to the α-subunit. The nature of such changes is profoundly different in the presence or in the absence of coenzyme. The correlation between τ and local viscosity (Strambini & Gonnelli, 1985) demonstrates that in the holoprotein complex formation results in a considerably greater rigidity of the N-domain in contrast with the greater flexibility of the apoprotein. GP also shifts the equilibrium between the two major conformations of the macromolecule that are inferred from the biexponential decay law. The direction of the shift is opposite for the two states of oxidation of the coenzyme.

The shortening of the triplet lifetime by indole is universal and dramatic. At 20 °C, a good fraction of the phosphorescence intensity is so short-lived that is no longer detectable. Because at a concentration of 100 μM indole saturation of holo-$\alpha_2\beta_2$ is only around 10% (Weischet & Kirschner, 1976), reduction of the intrinsic lifetime of the entire protein sample could be due to either rapid binding and release or an external quenching action by excess free substrate. To test the importance of phosphorescence quenching by free indole in solution (diffusion-limited bimolecular process), phosphorescence decays were obtained with equimolar quantities of Trp and *N*-acetyltryptophanamide (NATA), two indole analogues that should have similar accessibility to the protein surface but be unable to penetrate the indole channel connecting α- and β-active sites. The results indicate that these anologues have a limited effect on the decay rate of holo-$\alpha_2\beta_2$, reducing τ_{av} by 5–7% (data not shown). While this finding minimizes any external quenching activity of indole, nothing can be said of quenching pathways involving the channel. Due to this ambiguity, decay rates of indole complexes cannot be related in a simple way to the flexibility of the polypeptide chain near Trp-177. Nevertheless, it is significant that a structure-tightening ligand like GP is able to restore, at least in part (Figure 3), slow decay kinetics (data obtained with holo-$\alpha_2\beta_2$ only). Since GP partially blocks indole access to the channel (Dunn et al., 1990), we must conclude that its presence either opposes the structure-loosening effects associated with indole complex formation or restricts indole mobility within the channel, thereby cutting down on its quenching efficiency.

DISCUSSION

The α-subunit ligands indole-3-propanol phosphate and DL-α-glycerol 3-phosphate have been extensively utilized to elicit and to characterize the allosteric regulation between α- and β-subunits of tryptophan synthase. Indole-3-propanol phosphate binds at the center of the α-subunit near residues Glu-49 and Asp-60 (Hyde et al., 1988), that are involved in indole-3-glycerol phosphate cleavage (Yutani et al., 1987; Miles et al., 1988; Nagata et al., 1989). GP should bind in the specific α-subsite for the glycerol phosphate moiety of the natural substrate near residues 211–213 and 234–235 (Figure 4). X-ray crystallographic studies do not indicate any positively charged residues that can interact with these ligands.

Whereas both GP and indole-3-propanol phosphate have no effects on the functional properties of isolated β_2-subunits, in the $\alpha_2\beta_2$ complex α-subunit ligands act on the β-reaction by increasing the rate of some catalytic steps and by decreasing the rates of some others (Miles, 1979, 1991a). As a consequence of these effects, the equilibrium distribution of catalytic intermediates is dramatically modified in the presence of α-subunit ligands. These results have been explained on the basis of either a conformational change induced by α-subunit ligands at the β-active site (Kirschner et al., 1975; Lane & Kirshner, 1981, 1983a,b; Kawasaki et al., 1987) or the selective stabilization of a preexisting protein conformation (Houben & Dunn, 1990).

A wealth of luminescence data both from the chromophores intrinsic to the β-protomer (Trp-177 and coenzyme) and from that introduced into the α-subunit (Trp-129) concurs in demonstrating that GP complexation induces unequivocal con-

formational changes in both α- and β-subunits. A structural rearrangement of the α-subunit is inferred from a red shift in the phosphorescence spectrum of Trp-129, in agreement with circular dichroism measurements on the interaction between isolated α-subunits and α-subunit ligands (Heyn & Weischet, 1975). On the other hand, the effects on the spectroscopic properties of β-subunit chromophores are manifold, but the common basis of most of these changes is represented by a considerable tightening of the β-polypeptide chain both in the active-site region and in the N-domain. A more rigid environment near Trp-177 can be deduced from the smaller thermal quenching of its fluorescence and from the slower triplet decay kinetics. The latter provides an estimated increase in the effective viscosity of about a factor of 2 (Strambini & Gonnelli, 1985). Likewise, a tighter, less flexible site for the coenzyme is inferred from the smaller thermal quenching of its fluorescence relative to unliganded holo-$\alpha_2\beta_2$ and the complex with His.

Multiexponential phosphorescence decays in fluid solutions emphasize the conformational heterogeneity of the bienzyme complex. According to this parameter, GP changes the equilibrium between the two main conformers in the native holoenzyme (identified by their distinct triplet τ) in favor of the more compact form, increases the structural rigidity of both conformations, and reduces the temperature dependence of the equilibrium constant. It is singular that the structure-tightening effect of GP is strictly associated with the presence of bound coenzyme. In fact, in the apoprotein, its structural influence is opposite, resulting in a slight increase in the flexibility of the two conformers and a shift of the equilibrium in favor of the less rigid form.

L-His reacts with the enzyme, leading to a mixture of several catalytic intermediates possibly associated with distinct protein conformations (Houben & Dunn, 1990). The multiexponential phosphorescence decay observed in the L-His–enzyme complexes is unlikely to be related to this intermediate distribution since a similar heterogeneous phosphorescence decay has been found in the unliganded species.

Indole, one of the substrates for the β-reaction, is produced at the α-active site and channeled to the β-active site through the protein matrix (Hyde et al., 1988; Dunn et al., 1990). Unlike GP, its association with the apo- and holoenzyme drastically increases the triplet decay rate, an effect that in the holo-$\alpha_2\beta_2$ form is in large part reversed by the simultaneous binding of GP. We cannot discriminate how much of the effect is due to an increased flexibility of the local structure as opposed to a direct quenching interaction between ground-state indole in the channel and excited Trp-177. The latter process has a steep dependence on the separation between centers. According to the crystallographic coordinates of the holo-$\alpha_2\beta_2$ complex (Hyde et al., 1988), the distance of closest approach between the indole ring of the aromatic amino acid and the indole substrate is about 5 Å from the channel and 9 Å from the protein–solvent interface. The observation that indole analogues in solution are inefficient quenchers suggests that this limitation should apply also to the substrate in the channel and that indole effects are likely to represent increases in the flexibility of the N-domain. A local loosening of the protein structure upon indole binding to the α-subunit has been previously suggested on the basis of calorimetric and thermal denaturation studies (Wiesinger & Hinz, 1984). It is tempting to speculate that since the N-domain constitutes one side of the channel the increased structural fluctuations monitored in the presence of indole may result from local, rapid changes in the channel cross section that, according to the crystallographic coordinates, are necessary for indole migration. Only experiments with indole derivatives that bind to the α-subunit but are unable to enter the channel could discriminate between this hypothesis and one in which the conformational change originates subsequent to indole binding to α- or β-sites. One such compound is indole-3-propanol phosphate, an analog of the natural substrate 3-indole-D-glycerol 3′-phosphate. This inhibitor is an allosteric effector (Kirschner et al., 1975; Lane & Kirschner, 1981, 1983b), and because the propanol phosphate moiety might mimic the function of GP, interpretation of luminescence data in terms of a specific indole effect would be unjustified.

The influence of the natural substrate L-serine on subunit conformation cannot be investigated by luminescence measurements since pyruvate, formed in significant amounts by L-serine deamination at the relatively high concentration of enzyme required by these experiments, absorbs and interferes with the spectral properties of the intrinsic chromophores. We have therefore used the substrate analog L-histidine (Dunn et al., 1987; Houben et al., 1989; Houben & Dunn, 1990). L-His binding has been suggested to affect the K_{diss} of GP to α-subunits, and, similarly, GP binding causes a 4-fold decrease of the K_{diss} of L-His to the β-subunit and a redistribution of L-His–enzyme derivatives (Houben & Dunn, 1990). The effect of GP on L-His binding is much lower than that observed on the natural substrate L-tryptophan (Houben & Dunn, 1990). We have found that, in contrast to GP and indole, L-His has no apparent influence on the structure of the holoenzyme. The first simple explanation would be that L-His does not bind to tryptophan synthase from *Salmonella typhimurium*, in contrast to that found for the enzyme from *Escherichia coli* (Dunn et al., 1987; Houben et al., 1989; Houben & Dunn, 1990). Titration measurements at room temperature allow us to determine a K_{diss} of about 60 mM, only 3 times higher than that reported for the enzyme from *E. coli* (Houben & Dunn, 1990). We have to point out that the reaction of L-His with the enzyme from *S. typhimurium* leads, however, to an intermediate distribution in which the quinonoid species observed in the same reaction with the enzyme from *E. coli* (Houben & Dunn, 1990) is absent (Mozzarelli et al., unpublished results), suggesting that the enzyme from the two sources behaves differently in the reaction with L-His.

Propylene glycol, present in the buffer at cold temperatures, resembles glycerol phosphate and might interfere with L-His binding or with the effects elicited by L-His. We have found that propylene glycol exhibits small effects on phosphorescence lifetimes and amplitudes. Moreover, it slightly modifies absorption spectra of L-His–enzyme complexes and does not appear to compete with the GP binding site, but does decrease the concentration of the quinonoid formed in the reaction of L-His in the presence of GP (Mozzarelli et al., unpublished results). However, the absence of any detectable influence of L-His on the luminescence properties of both α- and β-subunits cannot be ascribed to propylene glycol, since the same pattern of effects, induced by GP, indole, and L-His at cold temperature in PG/Bicine buffer solutions, is conserved at ambient temperature in Bicine solutions. We conclude that the conformational changes triggered by L-His on α- and β-subunits are small and might escape detection. In support of this conclusion, there is the finding that L-His is unable to form α-aminoacrylate (Dunn et al., 1987; Houben et al., 1989; Houben & Dunn, 1990), the catalytic intermediate recently indicated as critical in the cross-activation between α- and β-active sites (Dunn et al., 1991; Anderson et al., 1991).

A structural parameter sensitive to the distance/orientation between the indole side chain of Trp-177 and the pyridoxal ring is the efficiency of energy transfer (Lakowicz, 1983; Strambini et al., 1992). The constancy of Trp fluorescence and phosphorescence yields in the various complexes of the native enzyme suggests that the structural changes observed in phosphorescence do not involve different distance/orientation (tilts) of the pyridoxal moiety relative to Trp-177. Coenzyme reorientation has been suggested to take place in the catalytic step between the external aldimine and α-aminoacrylate on the basis of circular dichroism measurements and stereochemical studies (Lane & Kirschner, 1983b; Miles et al., 1982). Since the reaction of L-His with the $\alpha_2\beta_2$ complex leads to the predominant formation of the external aldimine, our present results can only confirm that the PLP ring does not tilt in going from the internal to the external aldimine both in the presence and in the absence of an allosteric effector.

Finally, a bonding network for signal transmission between the two active sites can be tentatively identified on the basis of the following evidence: (i) The phosphate moiety of IPP induces a movement of residues 234–235 of the α-subunit by more than 1 Å (Figure 4) (Hyde et al., 1988). (ii) GP has no effect on the β-reaction when Arg-179 in the α-subunit is replaced by leucine (Kawasaki et al., 1987). (iii) Trypsin cleaves the α-subunit at Arg-188, leaving an $\alpha_2\beta_2$ complex catalytically active, but insensitive to the GP allosteric effect (Miles, 1991b). (iv) Circular dichroism measurements, carried out on isolated α-subunits, have evidenced the occurrence of conformational changes upon indole-3-propanol phosphate binding (Heyn & Weischet, 1975). (v) The polypeptide chain of the α-subunit is disordered between residues 177 and 192 (Hyde et al., 1988), suggesting that this loop is particularly flexible or can assume different positions. A similar loop, present in two other enzymes of tryptophan pathway, is supposed to be involved in substrate binding and substrate-induced conformational changes (Wilmanns et al., 1991). The distance between the GP binding site and Leu-177 of the α-subunit ranges from 6 to 12 Å, and the distance between Leu-177 and Trp-177 in the β-subunit is about 15 Å. (vi) GP seems to affect the pK_a of an ionizable residue in the N-domain of the β-subunits near Trp-177. By inspection of the three-dimensional structure, this residue might be one of the three tyrosines (181, 186, and 197) about 3.5 Å away from Trp-177.

The allosteric change triggered by GP binding to the α-active site might involve a conformational rearrangement transmitted via Arg-179 to the flexible loop of the α-subunit, that, in turn, interacts with the helix that ends with Trp-177 of the β-subunit. A movement of this helix acts on three strands that form part of the β-active site (Figure 4), causing a small but functionally significant change in the active-site geometry. The net result is an increase in the compactness and, possibly, of the hydrophobicity of the β-active site. Furthermore, since both the strands and the helix of the β-subunit involved in the transmission of information between the subunits are walls of the channel, GP could decrease the rate of indole transfer from the α- to β-active site not only by blocking the inlet of the tunnel (Dunn et al., 1990) but also by decreasing the outlet space at the β-active site. The decrease in the rate of L-tryptophan release induced by α-subunit (Lane & Kirschner, 1981) ligands might also be explained by the increase in rigidity and apolarity of the β-active site.

A recent proposal by Miles and co-workers (Ahmed et al., 1991) suggests that the β-active site exists in two conformations. One, called "open", is proper of the β_2-subunit and of several site-directed mutagenized forms of the $\alpha_2\beta_2$ complex. The other, called "closed", pertains only to the native $\alpha_2\beta_2$ complex. On the basis of our results, we can extend such a model, suggesting that, at room temperature, the "closed" conformation is indeed the sum of at least two conformations characterized by distinct dynamic properties (Strambini et al., 1992). α-Subunit ligands change the conformational equilibria toward an even more "closed" averaged state.

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