Stereoelectronic Control of Bond Formation in *Escherichia coli* Tryptophan Synthase: Substrate Specificity and Enzymatic Synthesis of the Novel Amino Acid Dihydroisotryptophan†

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ABSTRACT: The reactions of the indole analogues indoline and aniline with the *Escherichia coli* tryptophan synthase α-aminoacrylate Schiff base intermediate have been characterized by UV–visible and ^{1}H NMR absorption spectroscopy and compared with the interactions of indole and the potent inhibitor benzimidazole. Indole, via the enamine functionality of the pyrrole ring, reacts with the α-aminoacrylate intermediate, forming a transient quinonoid species with λ_{max} 476 nm as the new C–C bond is synthesized. Conversion of this quinonoid to L-tryptophan is the rate-limiting step in catalysis [Lane, A., & Kirschner, K. (1981) *Eur. J. Biochem. 120*, 379–398]. Both aniline and indoline undergo rapid N–C bond formation with the α-aminoacrylate to form quinonoid intermediates; benzimidazole binds rapidly and tightly to the α-aminoacrylate but does not undergo covalent bond formation. The indoline and aniline quinonoids (λ_{max} 464 and 466 nm, respectively) are formed via nucleophilic attack on the electrophilic C-β of the α-aminoacrylate. The indoline quinonoid decays slowly, yielding a novel, new amino acid, dihydroisotryptophan. The aniline quinonoid is quasi-stable, and no new amino acid product was detected. We conclude that nucleophilic attack requires the precise alignment of bonding orbitals between nucleophile and the α-aminoacrylate intermediate. The constraints imposed by the geometry of the indole subsite force the aromatic rings of indoline, aniline, and benzimidazole to bind in the same plane as indole; thus nucleophilic attack occurs with the N-1 atoms of indoline and aniline. However, in the case of benzimidazole, this orientation places the reactive lone pair of electrons (the nonbonding pair on N-3) orthogonal to the direction necessary for nucleophilic attack. Since the π-system of the five-membered ring is too resonance stabilized to allow reaction to occur analogous to that of the indole enamine, benzimidazole is prohibited from reacting via stereoelectronic constraints.

In vivo, the bienzyme complex of tryptophan synthase (EC 4.2.1.20) from *Escherichia coli* catalyzes the synthesis of L-tryptophan from L-serine and 3-indolyl-D-glycerol 3′-phosphate (IGP) (Miles, 1979, 1986). The α subunits catalyze the conversion of IGP to indole and D-glyceraldehyde 3-phosphate. By use of the cofactor pyridoxal phosphate (PLP),[1] the β subunits catalyze the reaction of L-serine and indole to form L-tryptophan. Deamination of L-serine, yielding pyruvate and ammonia, occurs as a side reaction catalyzed by the β subunits in the absence of indole.

PLP is linked to the protein as a Schiff base (internal aldimine) to the ϵ-amino group of Lys 87. This internal aldimine reacts covalently with the neutral amino group of L-serine via a succession of chemical intermediates to form a quasi-stable species, the α-aminoacrylate intermediate (I) (see Scheme I) (Miles et al., 1982). The C-β of I is an electron-deficient carbon center and hence is subject to nucleophilic attack by the electron-rich C-3 carbon of indole (and the electron-rich centers of other nucleophiles). This C–C bond forming step yields a carbanionic or quinonoidal intermediate (II) (Davis & Metzler, 1972; Miles, 1986).

Pyridoxal phosphate derived intermediates have distinctive UV–visible characteristics which allow for easy detection. For example, tryptophan synthase bound quinonoidal intermediates (e.g., II), formed by the reaction of nucleophiles such as indole, indoline (III), or aniline (IV) (Dunn et al., 1987), are characterized by intense, long-wavelength absorption bands located in the 450–490-nm region and by fairly narrow spectral bandwidths.

The quinonoid abstracts a proton from an acidic residue to form the Schiff base of the new amino acid and PLP. Transamination of this Schiff base with Lys 87 releases the product and restores the internal aldimine. The rate-limiting step in the synthesis of tryptophan at pH 7.8 is the conversion of the quinonoid to product (Lane & Kirschner, 1981).

Substrate analogues have been used extensively in the study of enzyme mechanisms. Investigations of the reactions of tryptophan synthase ($\alpha_2\beta_2$) with indole and indole analogues indicate that $\alpha_2\beta_2$ exerts stereoelectronic control over bond formation. Previous work on the reactivity of various indole analogues (Wilcox, 1974; Tanaka et al., 1986) has focused on enzymatic synthesis of novel amino acids. Preliminary evidence indicating the synthesis of a new amino acid from indoline and L-serine using a cell culture of *Aerobacter aerogenes* (FO 3317) was reported by Kanamitsu and Kitajima (1975); however, neither the new amino acid nor the enzyme carrying out the synthesis was identified. In this paper, the reactions of indoline (2,3-dihydroindole) and aniline with the α-aminoacrylate of $\alpha_2\beta_2$ (I) are described. It will be shown that these

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; DIT, dihydroisotryptophan; BZI, benzimidazole; $\alpha_2\beta_2$, native form of tryptophan synthase; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide, reduced form; E(A-A), α-aminoacrylate; E(Q), enzyme-bound quinonoid; K_Q, association constant for quinonoid formation; K_p, equilibrium constant for enzymatic dihydroisotryptophan synthesis; 1PDNA, one-pulse decoupler on during acquisition pH*, pH meter reading in D_2O.

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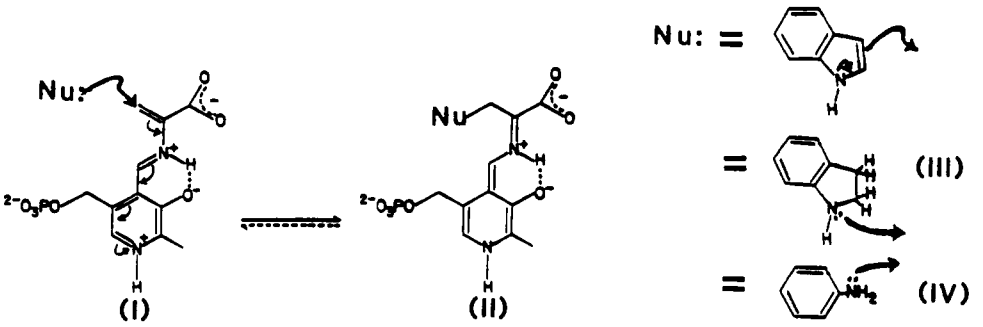
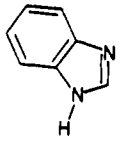
Scheme I



nucleophiles bind in the active site such that the anilino N occupies the same position normally occupied by the C-3 of the indole, and react with the C-β of the α-aminoacrylate via N–C bond formation. In contrast to indoline and aniline, benzimidazole (BZl) (V), a close electronic and structural analogue of indole, does not react but instead is a powerful competitive inhibitor (Heilmann, 1978; Lane & Kirschner, 1981; Dunn et al., 1987).



V

As will be shown, our investigation of the interaction of tryptophan synthase with these indole analogues provides new information concerning the relationship of substrate orientation to the stereoelectronic control of bond formation in this β-replacement reaction. A preliminary account for some of this work is presented in Dunn et al. (1987).

Materials and Methods

Materials. Indoline and aniline were purchased from Aldrich. Indoline was vacuum-distilled for further purification. L-Serine and NADH (Sigma) and the rabbit muscle lactate dehydrogenase (Boehringer Mannheim) employed in the coupled enzyme assay for pyruvate production were used without further purification. Tryptophan synthase was isolated and purified from *E. coli* W3110 trp R-Δ trp LD 102/FΔtrp ΔLD 102 according to a modification of the procedures of Adachi et al. (1974) as described in Drewe and Dunn (1985) and Tschopp and Kirschner (1980).

UV–Visible Spectral Measurements. Static absorption spectra were recorded on a HP-8450A spectrophotometer. Enzymatic reactions were carried out at 25 °C in 0.1 M potassium phosphate buffer, pH 7.8, with 1 mM EDTA. Indoline, aniline, and DIT are all photosensitive and, therefore, were protected from light. When the amount of DIT produced as a function of L-serine concentration was measured, indoline was kept at a concentration greater than L-serine to minimize pyruvate formation from serine deamination. The amount of pyruvate formed in the deamination side reaction was determined in a coupled enzyme assay by following the change in absorbance at 340 nm due to the LDH-catalyzed oxidation of NADH.

^{1}H FT NMR Spectral Measurements. NMR spectra were obtained by using a Nicolet NT 300 spectrometer. Kinetic runs were carried out at 25 ± 1 °C in degassed deuteriated sodium phosphate buffer, pH* 7.8. A tip angle of 33° was used in one-pulse experiments with a delay of 10 s. The internal reference was 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 1% v/v. Splitting patterns and the coupling of protons were established by single-frequency decoupling during acquisition (1PDNA).

Mass Spectral Measurements. The mass spectrum of DIT was determined with a VG-ZAD HF mass spectrometer with heat probe, T_p = 250 °C.

Synthesis and Purification of Dihydroisotryptophan. In this protocol, indoline and DIT were protected as much as possible from light. To carry out the enzymatic synthesis of DIT, 22 mM indoline, 12 mM L-serine, and 30 μM $\alpha_2\beta_2$ were incubated at 25 °C in 50 mM Tris-HCl, pH 8.0. The course of the reaction was followed by the increase in absorbance at 300 nm. When the reaction equilibrium had been established, the enzyme was precipitated with 6% v/v acetic acid and then pelleted with a brief centrifugation in a tabletop centrifuge. In a modification of the isolation protocol for 6-azidotryptophan (Miles & Phillips, 1985), 5 mL of the supernatant was applied to a column of Sephadex G-15 superfine (0.8 cm × 80 cm) and eluted with water. Fractions were collected every 2 mL with a flow rate of 2 mL/h. Excess serine eluted at about 30 mL. Because dextran gels adsorb aromatic substances (Janson, 1967), the elution of DIT (at 57 mL) and the indoline (at 70 mL) were retarded. The fractions containing DIT were pooled, lyophilized, and stored in a desiccator at −20 °C.

Results

UV–Visible Spectrum of the Enzyme-Bound Intermediate Formed in the Reaction of Indoline with E(A-A). The UV–visible spectrum of the internal aldimine of native $\alpha_2\beta_2$ (trace A of Figure 1), tryptophan synthase, shows an absorption maximum at 410 nm. L-Serine reacts rapidly and covalently with the internal aldimine, resulting first in the formation of a transient Schiff base (external aldimine, λ_{max} = 422 nm); then, after abstraction of the α-proton, a transient quinonoidal species is formed which undergoes β-elimination of the hydroxyl group, yielding a metastable intermediate, the α-aminoacrylate Schiff base (Drewe & Dunn, 1985) (structure I, trace B of Figure 1). Upon the addition of indole, the very rapid synthesis of a C–C bond between the C-3 of indole and C-β of the α-aminoacrylate occurs, and a transient quinonoidal intermediate with an absorption maximum at 476 nm is formed (structure II).

The reaction of the α-aminoacrylate with indoline, viz., Figure 1A, causes a rapid conversion of the UV–visible spectrum of the α-aminoacrylate to a new species with λ_{max} 464 nm. The relatively intense spectral band and narrow bandwidth of the 464-nm species (trace C) identifies this intermediate as a quinonoid. This quinonoid is quite stable over several hours. As will be shown, this quinonoid forms as a result of the nucleophilic attack of the N-1 nitrogen of indoline at the C-β carbon of the α-aminoacrylate. At high (8 mM) indoline concentrations, the amplitude of the 464-nm band saturates at an absorbance value that is about five times

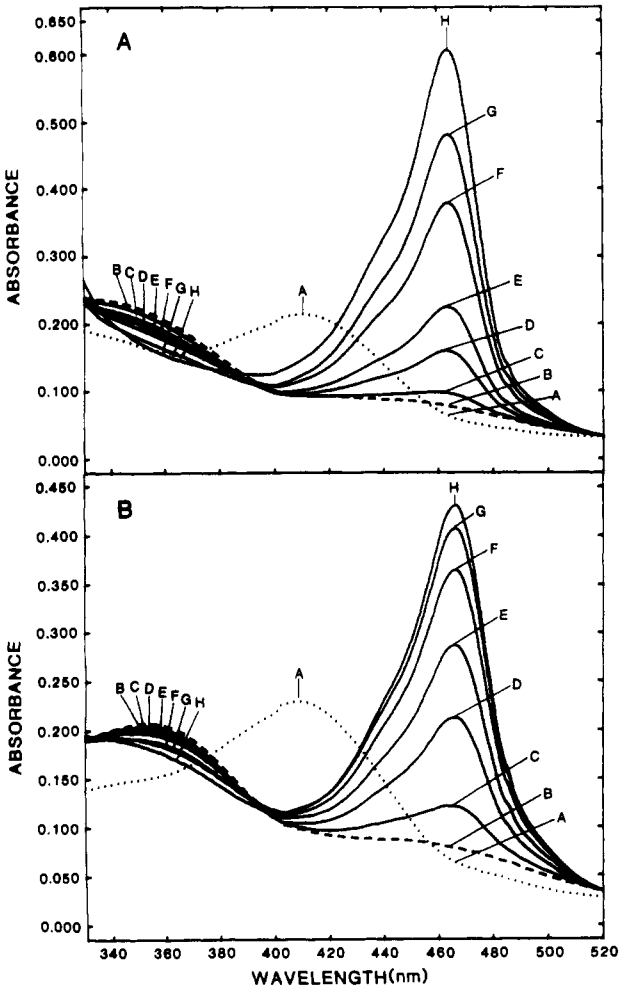


FIGURE 1: Panel A: UV–visible absorption spectra of the quinonoid formed in the reaction of indoline with the α-aminoacrylate intermediate. Trace A (dotted line): 13 μM $\alpha_2\beta_2$ in 0.1 M potassium phosphate, pH 7.8 at 22 °C (λ_{max} at 412 nm). Trace B (dashed line): $\alpha_2\beta_2$ and 40 mM L-serine. Spectra C–H (solid lines): $\alpha_2\beta_2$, 40 mM L-serine, and the addition of indoline in the following concentrations: 50 μM, 250 μM, 500 μM, 1.0 mM, 1.6 mM, and 3.3 mM. To determine K_Q, indoline was titrated into the α-aminoacrylate solution to give the following concentrations: 0.01, 0.05, 0.10, 0.25, 0.5, 1.0, 1.6, 3.3, 6.3, 9.6, and 12.8 mM. The absorption maximum of the quinonoidal intermediate is 464 nm. Panel B: UV–visible absorption spectra of the quinonoid formed in the reaction of aniline with the α-aminoacrylate intermediate. Trace A (dotted line): 15 μM $\alpha_2\beta_2$. Trace B (dashed line): 15 μM $\alpha_2\beta_2$ and 40 mM L-serine. Traces C–H (solid lines) were measured following the addition of aniline in the following concentrations: 0.71, 2.1, 4.1, 7.9, 14.6, and 31.7 mM. The absorption maximum of the quinonoidal intermediate is 466 nm.

greater than that of the 410-nm band for the internal aldimine.

Equilibrium Constant. The apparent equilibrium constant K_Q for the formation of the quinonoid from reaction of indoline with the α-aminoacrylate was determined from the titration of indoline (Figure 1) where

$$K_Q = [E(Q)]/\{[\text{indoline}][E(A\text{-}A)]\} \quad (1)$$

Assuming $\Delta ABS \propto \Delta[E(Q)]$ and ΔABS_{max} is the change in absorbance estimated from extrapolation to infinite indoline concentration, then the following relationship holds:

$$\Delta ABS = \Delta ABS_{max} \frac{[\text{indoline}]}{1/K_Q + [\text{indoline}]} \quad (2)$$

When the change in absorbance at 464 nm, corrected for dilution effects, is plotted as a function of free indoline concentration, computer analysis of the resulting hyperbolic curve gives a value for K_Q of 0.76 mM^{-1}.

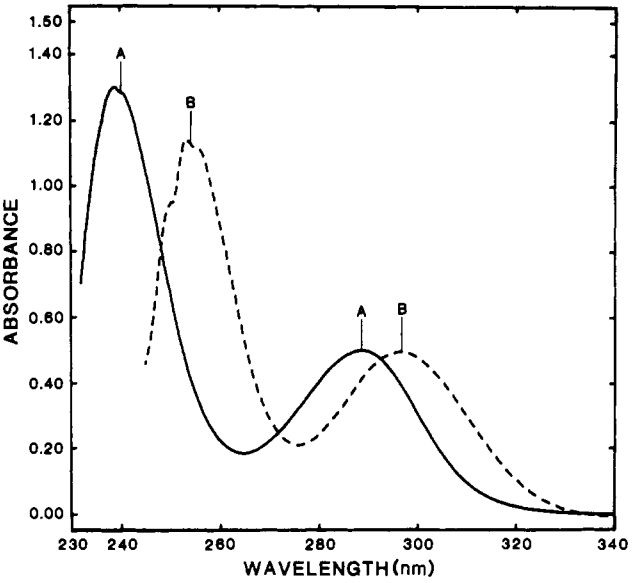


FIGURE 2: UV–visible absorption spectra of indoline and dihydroisotryptophan. Trace A (solid line with λ_{max} 239 and 289 nm) is 0.2 mM indoline at pH 7.8 in 0.1 M potassium phosphate buffer at 22 °C. Trace B (dashed line with λ_{max} 255 and 296 nm) is 0.2 mM of the new amino acid, dihydroisotryptophan, synthesized via the $\alpha_2\beta_2$-catalyzed reaction of indoline with L-serine (see text).

Indoline Turnover to DIT. When indoline is incubated with excess L-serine and catalytic amounts of enzyme, its absorption spectrum (λ_{max} 239 and 289 nm) slowly disappears and a new, red-shifted spectrum (λ_{max} 255 and 296 nm) appears (see Figure 2). This product has essentially the same extinction coefficients as indoline. Thus the chemical structure of the indoline ring portion of the new chromophore very likely is similar to that of indoline; clearly, a novel amino acid has been formed.

To quantitate the rate of β replacement of the L-serine β-hydroxyl with indoline, the absorption spectrum of the reaction mixture was measured at appropriate time intervals. A control mixture of reactants with buffer replacing the enzyme volume exhibited no spectral changes (provided the solution was not exposed to light). Although the rates of quinonoid formation are similar for indole and indoline [data not shown; see Dunn et al. (1987)], the initial steady state of β replacement with indoline is <1/100 the rate of tryptophan synthesis.

Mechanism of the Reaction of E(A-A) and Indoline. By analogy to indole, the proposed pathway of indoline reaction is

$$E(A\text{-}A) + \text{indoline} \underset{}{\overset{K_Q}{\rightleftharpoons}} E(Q) \underset{k_{-p}}{\overset{k_p}{\rightleftharpoons}} DIT + E \quad (3)$$

The formation of the indoline quinonoid has been shown to be reversible (Drewe & Dunn, unpublished results), but the release of dihydroisotryptophan (DIT) appears to be irreversible. When incubated with $\alpha_2\beta_2$, purified DIT at concentrations below 2 mM does not alter the UV–visible spectrum of the internal aldimine, perhaps due to an inability to bind. In addition, deamination of the aminoacrylate to pyruvate, i.e.

$$H_2O + E(A\text{-}A) \xrightarrow{k_s} \text{pyr} + NH_4 + E \quad (4)$$

is an irreversible side reaction that must be taken into account. As a result, the overall equilibrium constant could not be determined. Varying concentrations of L-serine were incubated with a fixed amount of indoline and enzyme. DIT production was followed by the increase in absorbance at 305 nm to

determine when equilibrium had been established. At the lower concentrations of L-serine, no pyruvate was detected, while at higher concentrations, small amounts of pyruvate were found at the longer incubation times. This finding implies that the rate of serine deamination is slowed in the presence of indoline and that little or no pyruvate is produced by the degradation of product. Thus, the following enzyme-catalyzed reaction can be written:

$$\text{indoline} + \text{L-serine} \xrightarrow{k_p} \text{DIT} \qquad (5)$$
$$\downarrow k_s$$
$$\text{pyr}$$

where $k_p > k_s$.

UV–Visible Spectrum of the Enzyme-Bound Intermediate Formed in the Reaction of Aniline with E(A-A). The spectrum resulting from the reaction of aniline with the α-aminoacrylate is shown in Figure 1B. With aniline as the nucleophile, the quinonoid formed (trace C) has an absorption maximum at 466 nm. The amplitude of this band at saturating concentration of aniline is about three times greater than the amplitude of the internal aldimine 412-nm band (trace A). Since the indoline quinonoidal band (λ_{max} 464 nm) has a greater amplitude than the aniline quinonoidal band (λ_{max} 466 nm), under high concentration (8 mM) of aniline, either the aniline quinonoid has a smaller extinction coefficient or a smaller fraction of the enzyme appears in the quinonoidal form with aniline as the nucleophile. Since the spectral bandwidths of the two quinonoids are similar, it is probable that the extinction coefficients are close in magnitude. Comparison of the spectra of these quinonoidal species (viz., trace C in Figure 1A and trace C in Figure 1B) shows more absorbance at 350 nm in the aniline system, indicating a lower proportion of aniline bound to the enzyme in the quinonoid form at equilibrium. When the α-aminoacrylate is titrated with either indoline or aniline, the set of spectra measured at quasi-equilibrium give fairly clean isosbestic points at 390 and 388 nm as absorbance decreases at 350 nm and increases at 464 or 466 nm.

Mechanism of the Reaction of E(A-A) with Aniline. When incubated with excess L-serine and catalytic amounts of $\alpha_2\beta_2$, the UV–visible spectrum of aniline, with absorption maxima at 231 and 279 nm, shows no change (data not shown). Either the enzymatic product of aniline and L-serine has a UV–visible spectrum indistinguishable from that of aniline alone or the rate of product release from the quinonoidal intermediate (and hence the amount formed) is below the limits of detection. Since the UV spectra of substituted anilines are different from that of aniline (e.g., *N*-methylaniline has absorption maxima at 245 and 293 nm; data not shown), the former does not appear probable. If the mechanism of the reaction of aniline with the α-aminoacrylate is analogous to that of indole, the following equation can be proposed:

$$\text{E(A-A)} + \text{aniline} \overset{K_Q}{\rightleftharpoons} \text{E(Q)} \overset{k_p}{\underset{k_{-p}}{\rightleftharpoons}} \text{E} + \text{product} \qquad (6)$$
$$\downarrow$$
$$\text{pyruvate}$$

If k_p is extremely slow and hence limits turnover (due to a high energy barrier) so that the second step goes undetected on the time scale of our experiments (days), then eventually, the quinonoid will decay away as the α-aminoacrylate is irreversibly degraded to pyruvate. As a result, little or no new amino acid accumulates.

^{1}H NMR. Except for the $\alpha_2\beta_2$-catalyzed exchange of the α-proton of serine for deuterium and the slow deamination side reaction (data not shown), there is no change in the ^{1}H NMR of aniline and L-serine in the presence of catalytic amounts of $\alpha_2\beta_2$ over a time period of 24 h. No hydrogen exchange with solvent deuterium was observed in the absence of enzyme. This result indicates that, under these experimental conditions, less than 2% of the aniline accumulates as new amino acid (the detection limits of the ^{1}H NMR experiments). As with indoline, any substitution at the amino group or on the ring should significantly change the chemical shifts of the peaks and/or the splitting pattern. When 10 mM substrate and 250 μM enzyme were incubated, no product was detectable 24 h later although the $\alpha_2\beta_2$ tested is active.

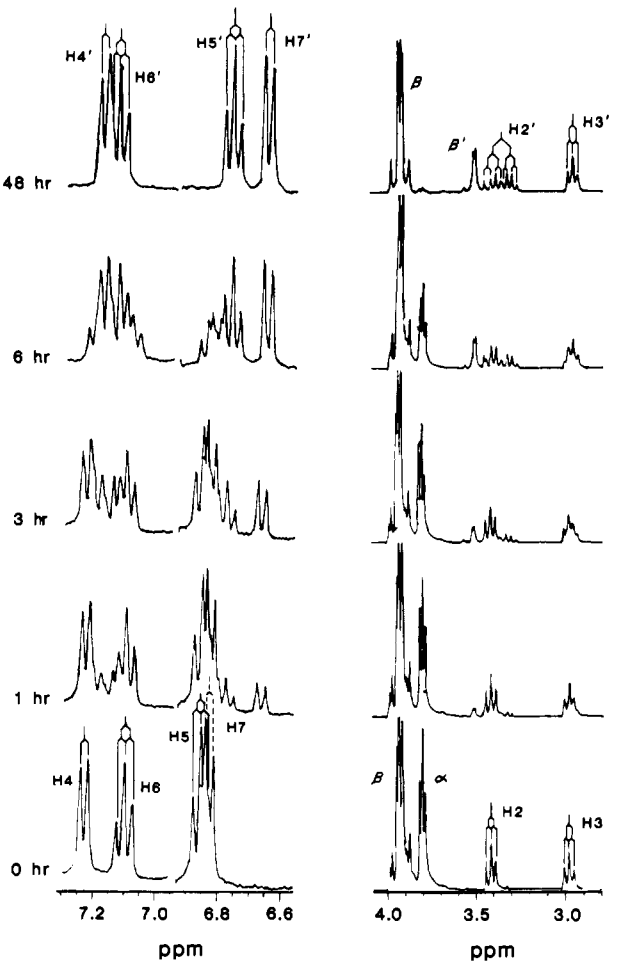


FIGURE 3: Time-resolved ^{1}H NMR spectra measured during the $\alpha_2\beta_2$-catalyzed conversion of L-serine and indoline to DIT. To initiate reaction, 6.0 mM L-serine, 1 mM indoline, and 25 μM $\alpha_2\beta_2$ were mixed in 0.1 M deuteriated sodium phosphate buffer, pH 8.2, at 25 °C. The α-proton of L-serine is enzymatically exchanged to deuterium, simplifying the signal of the β-protons to an AB quartet characteristic of a tightly coupled spin system. Splitting patterns depicted and the coupling of protons were determined by single-frequency off-resonance decoupling. The splitting patterns of the aromatic region over time indicate N–C bond formation and are not consistent with electrocyclic ring addition.

The time dependence of changes of the proton NMR spectra for indoline and L-serine in the presence of $\alpha_2\beta_2$ is shown in Figure 3. A control mixture of reactants without enzyme was also followed over time. The splitting patterns depicted were determined by 1PDNA experiments. These studies establish which protons are coupled to each other and the order of multiplicity. The number of protons was calculated by integration.

When indoline reacts with the α-aminoacrylate, there are two possibilities for a covalent reaction. The lone pair on the indoline nitrogen is capable of a nucleophilic attack (Sumpter

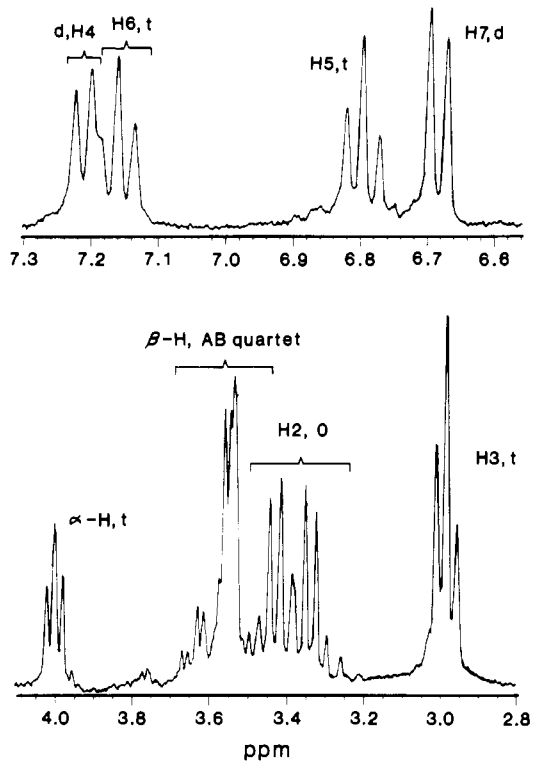


FIGURE 4: Proton assignments of the ^1H NMR spectrum of 2,3-dihydroiso-L-tryptophan in D_2O at 25 °C. The pH* was adjusted to 7.8 with NaOD. d = doublet, t = triplet, and o = octet.

& Miller, 1954) at the electron-deficient C-β of the aminoacrylate. Alternatively, if not sterically hindered, an enzyme-mediated electrocyclic addition on the aromatic ring of indoline could occur.

A meta addition is chemically unlikely (i.e., an addition at C-6 of structure III). For an ortho electrocyclic addition at C-7, the splitting patterns of the aromatic protons should change from two doublets and two triplets to two singlets and one doublet, whereas one singlet and two doublets are expected to be the result in the case of a para ring addition at C-5. Overlapping multiplets and the changes in chemical shift obscure coupling in the aromatic region of the spectra during the initial phases of the reaction. But, as depicted in Figure 3, the splitting patterns determined by 1PDNA remain the same. The upfield shift of the H-7 doublet and the downfield shift of the H-6 triplet are consistent with substitution at N-1 and the consequent effects on carbons positioned meta and ortho.

Since in these ^1H NMR experiments, $\alpha_2\beta_2$ catalyzes a relatively rapid exchange of the α-proton of L-serine in ^2H_2O, the α-protons of both L-serine and the product are enzymatically exchanged to deuterium. This exchange simplifies the signals of the β-protons to the AB quartets characteristic of tightly coupled nonequivalent spin systems (Figure 3). The aliphatic H-2 and H-3 protons of indoline give triplets located at 3.42 and 2.98 ppm, respectively. After completion of the reaction, the H-3 signal shows no change, whereas the peaks for H-2 are further split to a multiplet, probably an octet. The H-2 protons have become chemically nonequivalent, due to the now neighboring diastereotopic β-protons.

The ^1H NMR of the purified new amino acid (synthesized in H_2O) is shown in Figure 4. The α-proton of the product, dihydroisotryptophan (DIT), appears in this spectrum as a triplet at 4.0 ppm. The parent peak in the mass spectrum of DIT is 206.1053; the calculated mass for the structure is 206.1055.

Discussion

Dunathan's hypothesis (Dunathan & Voet, 1974) proposes that chemical transformations involving π-bond formation or π-bond reaction require prealignment of the reacting atoms so that orbital overlap is optimized during reaction. As for other PLP-requiring enzymes, it appears that tryptophan synthase exerts stereoelectronic control over bond formation.

Benzimidazole (BZl), similar in structure to indole except with a nitrogen at the 3-position (structure V), is a potent but reversible noncompetitive inhibitor of tryptophan synthase (Heilmann, 1978; Lane & Kirschner, 1981; Dunn et al., 1987). However, no detectable amounts of an amino acid product are synthesized from the incubation of benzimidazole with L-serine in the presence of $\alpha_2\beta_2$. The UV–visible spectrum of the $\alpha_2\beta_2$-bound α-aminoacrylate is only slightly perturbed by the binding of BZl. Consequently, *there is no evidence of covalent bond formation when BZl binds to the α-aminoacrylate*; certainly the interaction of BZl does not result in the formation of detectable amounts of quinonoidal species akin to structure II, nor are detectable amounts of new, covalent products produced (Heilmann, 1978; Lane & Kirschner, 1981; Dunn et al., 1987).

Indoline, aniline, and benzimidazole exhibit pK_a values of about 5, and all can behave as nucleophiles in organic reactions. Although indole is a planar heteroaromatic molecule, the ring nitrogen and adjacent double bond in the pyrrole ring convey enamine character to the system with significant delocalization of electron density from nitrogen to C-3. However, the pK_a of indole (with protonation at C-3) is much lower, ~ -3.5 (Hinman & Lang, 1964). With the exception of 3-substituted alkylindoles, electrophilic additions of electron-deficient olefins to indole and substituted indoles invariably occur at the 3-position (Sundberg, 1970). The resonance energy of indole has been calculated at 185.4 kJ mol^{-1} (Faour & Akasheh, 1985). In light of the pronounced chemical stability of benzimidazoles (Hofmann, 1953), the resonance energy of benzimidazole should be greater.[2] Benzimidazole undergoes acylation and alkylation at nitrogen as do indoline and aniline.

In nonenzymatic reactions, the imidazole nitrogen of BZl is a much better nucleophile than is the C-3 of indole. Therefore, the inability of BZl and the α-aminoacrylate to form a covalent bond must be due to some form of stereoelectronic control of covalent bond formation at the enzyme active site. According to current theories of chemical reactivity (such as Dunathan's hypothesis), for bond formation to occur between nucleophile and the β-carbon of the α-aminoacrylate, the bonding orbital of the nucleophile must overlap with the π-orbital of the β-carbon as the transition state is approached. It is reasonable to propose that the enzyme site has evolved to optimize this overlap by binding indole so that the reacting orbitals are aligned as depicted in Figure 5. This orientation should provide a low-energy pathway leading to the quinonoid, a highly planar structure. The lone pair of electrons on the secondary amine nitrogen of indoline (Sumpter & Miller, 1954), or on the primary amine nitrogen of aniline, is conjugated to some extent with the aromatic benzene ring. Full resonance interaction requires coplanarity of π-orbitals on ring carbons, and if the nitrogen is disubstituted, steric hindrance will reduce resonance interaction and make addition at N unfavorable. However, with one or fewer ortho substituents, there is little evidence to indicate N substitution causes a twist

[2] "...On the whole, nitrogen substitution has a stabilizing effect as compared with ... CH_2 group in five-membered rings..." (e.g., indazole > indole > indene) (Faour & Akasheh, 1985).

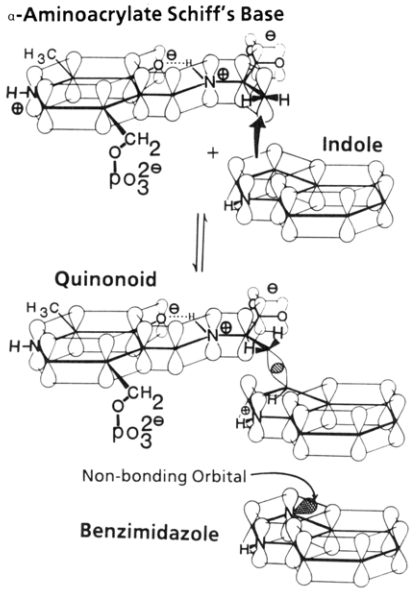
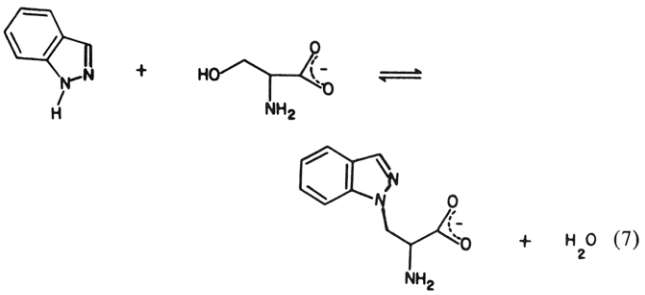


FIGURE 5: Stereoelectronic control of substrate specificity by tryptophan synthase. The pK_a's of benzimidazole (BZl), indoline, and aniline are all about 5.0. Electron density at the C-3 of indole renders this atom nucleophilic, while the lone pair on the nitrogens of indoline and aniline are nucleophilic. The "imine" nitrogen of BZl is a relatively good nucleophile and undergoes reaction with electrophilic carbon centers. Consequently, the lack of BZl reactivity must be due to the stereoelectronic requirements of the enzyme. As is shown with the π-orbital of the C-3 of indole, for bond formation in the transition state, the bonding orbital must overlap with the π-orbital of the β-carbon in the aminoacrylate intermediate. However, the analogous bond formation via the π-orbital of the corresponding nitrogen of BZl would result in a sp^3-hybridized center with a consequent loss of aromatic stability in the five-membered ring. In contrast to BZl, loss of resonance for indole does not result in a high activation energy for covalent bond formation because resonance stabilization of the indole 2,3 double bond is relatively low. The BZl nonbonding orbital does not react because the orientation is orthogonal to the π-orbital of the β-carbon.

away from a full, planar conjugation (Cowling & Johnstone, 1973). Consequently, the nitrogen lone pair may not have a perfect overlap with the reacting π-orbital of C-β but comes close enough for a reaction to occur.

If indole and BZl are constrained by the subsite to bind with identical orientations, as shown in Figure 5, then the bonding π-orbital of the BZl nitrogen is well placed for alignment with the π-system of the aminoacrylate, and the reaction would result in a transition from sp^2 hybridization to sp^3 at the reacting BZl nitrogen and at C-β of the α-aminoacrylate. However, reaction via this BZl orbital is energetically highly unfavorable because this bonding interaction would pull the nitrogen atom out of resonance with the delocalized planar aromatic system and would result in a corresponding loss in aromatic stability. Conversely, in the orientation shown in Figure 5, *the nonbonding lone pair of electrons on the BZl nitrogen cannot react because this orbital is pointed in a direction away from and nearly perpendicular to the π-orbitals of the α-aminoacrylate*. Indazole also reacts enzymatically with L-serine to form a novel amino acid (eq 7), with the new covalent bond between N-1 and C-β (Tanaka et al., 1986). Despite a resonance energy slightly greater than that of indole (215.2 kJ mol^{-1} vs 184.5 kJ mol^{-1}; Faour & Akasheh, 1985), the N-1 maintains sufficient sp^3 hybrid character for adequate orbital overlap.

Oddly enough, although aniline readily forms a quinonoidal intermediate, no detectable amount of new amino acid is released. Since a UV–visible spectral band, which is highly similar to that of the indoline quinonoid, is found (cf. Figure



1), a covalent bond is undoubtedly formed between aniline and C-β of the α-aminoacrylate intermediate. This result indicates that the energy barrier for conversion to product is so high that reaction does not proceed beyond formation of the quinonoid.

Apotryptophan synthase (i.e., enzyme from which all PLP has been removed) binds *N*-(phosphopyridoxyl)-L-serine tightly, while *N*-(phosphopyridoxyl)-L-tryptophan is bound only weakly. It has been proposed that the weak interaction of (phosphopyridoxal)-L-tryptophan is due to a different geometry at C-4′ compared to that of either the L-tryptophan quinonoid species or the bound *N*-(phosphopyridoxyl)-L-serine species (Tschopp & Kirschner, 1980). Stopped-flow kinetic studies investigating the catalytic mechanism of tryptophan synthase suggest that the rate of reprotonation of the α-carbanion of L-tryptophan is limited by rearrangements at the active site (Lane & Kirschner, 1983). The reprotonation of the L-tryptophan quinonoid (*si*) to give the external aldimine of L-tryptophan (*re*) probably requires an obligatory conformational change. In this proposal, a slow reorientation of the C-4′ atom from *si* to *re* is followed by a rapid proton transfer of the *re* face (Lane & Kirschner, 1983). This change in stereochemical orientation would require an obligatory enzyme conformational change. If the protein conformational change is not triggered, then the turnover of quinonoid to external aldimine could be highly unfavorable. According to this view of tryptophan synthase, substrate analogues of different structure will exhibit varying degrees of efficacy in mediating those tertiary structure changes essential to tryptophan synthase catalysis. Without the obligatory changes in protein structure, the corresponding transition states will be high in energy (perhaps too high) and turnover to product will be slow or cannot be completed.

Once DIT is released, there appears to be no enzymatic degradation to pyruvate, ammonia, and indoline (via the reverse reaction). The quinonoid formed in the reaction of aniline with the α-aminoacrylate does not yield detectable amounts of product. Aniline lacks the fused-ring system, whereas the indoline fused-ring system lacks the planarity of the indole chemical structure. Although BZl is a close structural analogue of indole, the highly resonance stabilized π-system of BZl prevents covalent bond formation with the α-aminoacrylate. These findings provide strong evidence indicating that resonance stabilization and stereoelectronic control of bond synthesis are critically important aspects of tryptophan synthase catalysis.

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Effect of Hydrostatic Pressure on the Mitochondrial ATP Synthase†

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ABSTRACT: The effects of hydrostatic pressure on three different preparations of mitochondrial H^+-ATPase were investigated by studies of the hydrolytic activity, of the spectral shift and quantum yield of the intrinsic protein fluorescence, and of filtration chromatography. Both membrane-bound and detergent-solubilized forms of the mitochondrial F_0-F_1 complex were reversibly inactivated in the pressure range of 600-1800 bar, whereas with soluble F_1-ATPase the inactivation was irreversible. Pressure inactivation of soluble F_1-ATPase was facilitated by decreasing the protein concentration, indicating that dissociation is an important factor. In the presence of 30% glycerol, soluble F_1-ATPase becomes inactivated by pressure in a reversible fashion, recovering the original activity. ATPase activity measured in an aqueous medium returns to the original values when incubated under high pressure in a glycerol-containing medium without substrate and is even enhanced when Mg-ATP is present. ATP hydrolysis returns to 80% of its original value in the case of the F_0-F_1 complex. Fluorescence studies under pressure revealed a red shift in the spectral distribution of the emission of tyrosine fluorescence of soluble F_1-ATPase. A decrease in the quantum yield of intrinsic fluorescence was also observed upon subjection to pressure. The fluorescence intensity decreased monotonically as a function of pressure when the sample was in an aqueous medium, whereas it presented a biphasic behavior in a 30% glycerol medium. Gel filtration studies demonstrated that the hydrodynamic properties of the F_1-ATPase are preserved if the enzyme is subjected to pressure in the presence of glycerol but they are modified when the same procedure is performed in an aqueous medium. It can be concluded that pressure dissociation of soluble F_1-ATPase is followed by reassociation to an inactive enzyme with altered hydrodynamic radius when the pressure is withdrawn. Protection against this irreversible effect by binding to the F_0 membrane component points directly to the importance of the coupling between membrane attachment and correct assembly of the F_1-ATPase.

Mitochondrial ATP synthase is the terminal enzyme in oxidative phosphorylation and catalyzes the synthesis of ATP with energy derived from electrochemical H^+ gradients. It is composed of a membrane sector, F_0, that permits the transport of protons generated by the respiratory chain to a soluble factor known as F_1 (Amzel & Pedersen, 1983; Senior & Wise, 1983). The F_1 moiety is composed of five different subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Amzel & Pedersen, 1983) and catalyzes ATP hydrolysis. Kinetic studies of the F_1-ATPase[1] have demonstrated that the three catalytic sites of the enzyme behave cooperatively (Gresser et al., 1982; Boyer et al., 1982; O'Neal & Boyer, 1984). These sites are ap-

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[1] Abbreviations: ATPase, adenosinetriphosphatase; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; SMP, submitochondrial particles.

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