Proton Transfer and Carbon−Carbon Bond Cleavage in the Elimination of Indole Catalyzed by *Escherichia coli* Tryptophan Indole-Lyase

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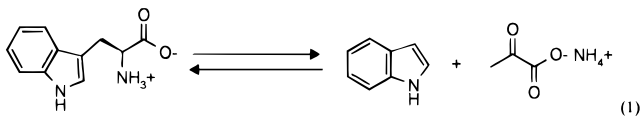
Received May 17, 1999

Abstract: Tryptophan indole-lyase from *Escherichia coli* catalyzes the reversible cleavage of L-tryptophan to indole and ammonium pyruvate. This reaction is mechanistically interesting since it involves the elimination of an aromatic carbon leaving group. We have been studying the mechanism of tryptophan indole-lyase using rapid-scanning stopped-flow spectrophotometry. Recently, we demonstrated that the rate constant for α-aminoacrylate intermediate formation from α-^{2}H-L-tryptophan exhibits an isotope effect of 3.0 (Sloan, M. J.; Phillips, R. S. *Biochemistry* **1996**, *35*, 16165−16173). We have confirmed this previous result ($^Dk = 2.99 \pm 0.30$) and we have now found that β,β-di-^{2}H-L-tryptophan also exhibits a secondary isotope effect ($^Dk = 1.17 \pm 0.03$) on the elimination reaction. Furthermore, α,β,β-tri-^{2}H-L-tryptophan exhibits a multiple isotope effect ($^Dk = 4.42 \pm 0.67$) on the elimination of indole. In addition, there is a significant solvent isotope effect ($^Dk = 1.79 \pm 0.11$) on indole elimination in D_2O. This solvent isotope effect combines with the effect of α-deuterium, since elimination of α-^{2}H-L-tryptophan in D_2O exhibits $^Dk = 4.30 \pm 0.16$. In addition, the rate constant for indole elimination shows a linear Eyring plot between 5 and 35 °C. In the direction of tryptophan synthesis, the reaction of the α-aminoacrylate intermediate with indole to form a quinonoid intermediate also exhibits a kinetic isotope effect for 3-^{2}H-indole, with $^Dk = 1.88 \pm 0.19$. In contrast to our expectations, the results suggest that the proton transfer and carbon−carbon bond cleavage in the elimination reaction are very nearly simultaneous and that the indolenine structure is a transient intermediate which occupies a very shallow well on the reaction coordinate, or a transition state, in the reaction of Trpase.

Introduction

Tryptophan indole-lyase (Trpase, EC 4.1.99.1) catalyses the reversible hydrolytic cleavage of L-tryptophan to form indole and ammonium pyruvate (eq 1). This enzyme is found in a



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number of enteric bacteria including *Escherichia coli*,[1] *Proteus vulgaris*,[2] and *Haemophilus influenzae*.[3] The production of indole during the bacterial putrefaction of protein was first reported by Bopp in 1849.[4] The source of the indole in protein remained obscure until the synthesis and structure elucidation of tryptophan by Hopkins and Cole in 1903.[5] In addition to the elimination reaction shown in eq 1, Trpase can catalyze the elimination in vitro of a wide range of amino acids with leaving groups attached to the β-carbon, including L-serine,[6] *O*-alkyl-L-serines,[6] *O*-acyl-L-serines,[7] *S*-alkyl-L-cysteines,[6] *S*-(*o*-nitrophenyl)-L-cysteine,[8] β-chloroalanine,[6] and 2,3-diaminopropionate.[6] All of these nonphysiological substrates possess chemically reasonable leaving groups which contain relatively electronegative elements. However, the physiological reaction of Trpase is mechanistically intriguing, as it requires the elimination of a formally unactivated aromatic carbon leaving group. Hence, there must be additional catalysis to activate the indole ring to make it a suitable leaving group under physiological conditions. Studies performed with α-^{2}H-L-tryptophan have demonstrated that there is intramolecular transfer of the α-proton to C-3 of indole in the reaction.[9,10] Previously, we demonstrated that analogues of the indolenine tautomer of L-tryptophan,[11] 2,3-dihydro-L-tryptophan and oxindolyl-L-alanine (Chart 1), are potent competitive inhibitors of Trpase and tryptophan synthase.[12,13] Furthermore, the inhibition of Trpase is stereospecific

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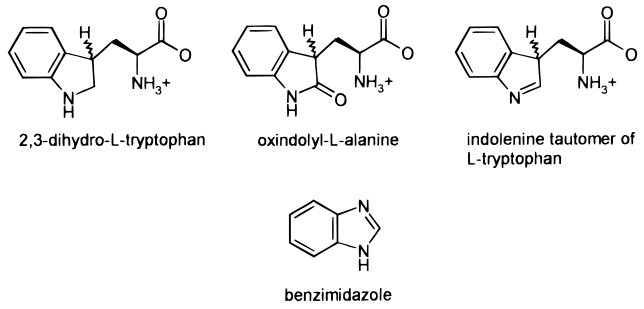
10.1021/ja991647q CCC: $19.00
Published on Web 01/29/2000

Chart 1

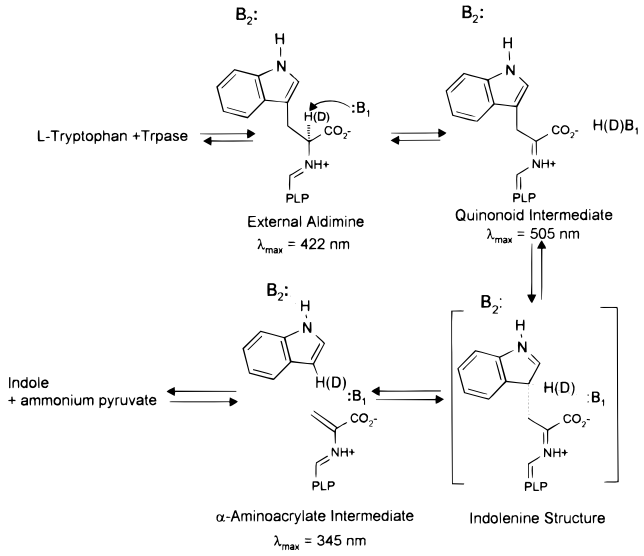


for (3*R*)-2,3-dihydro-L-tryptophan.[13] We interpreted these results as evidence for an indolenine intermediate in the reactions of both Trpase and tryptophan synthase. In the present work, we examined the effects of isotopic substitution of tryptophan and solvent on the pre-steady-state kinetics of the indole elimination reaction of Trpase to obtain evidence for an indolenine intermediate. In contrast to our expectations, the results suggest that the proton transfer and carbon−carbon bond cleavage in the elimination reaction are very nearly simultaneous and that the indolenine structure is a transient intermediate which occupies a very shallow well on the reaction coordinate, or a transition state, in the reaction of Trpase.

Results

α-Deuterium Isotope Effect on Indole Elimination. In previous pre-steady-state kinetic studies on the reaction of Trpase with L-tryptophan using rapid-scanning stopped-flow spectrophotometry, we found that inclusion of benzimidazole in reaction mixtures results in the accumulation of a previously undetected reaction intermediate, with λ_{max} at about 345 nm, that was assigned to an α-aminoacrylate structure[10,14] (Scheme 1). Benzimidazole is an uncompetitive inhibitor (K_i = 0.81 mM[14]) since it is a product analogue of indole that binds only to the enzyme intermediate after indole release, and hence, including it makes indole release quasi-irreversible. Furthermore,

Scheme 1



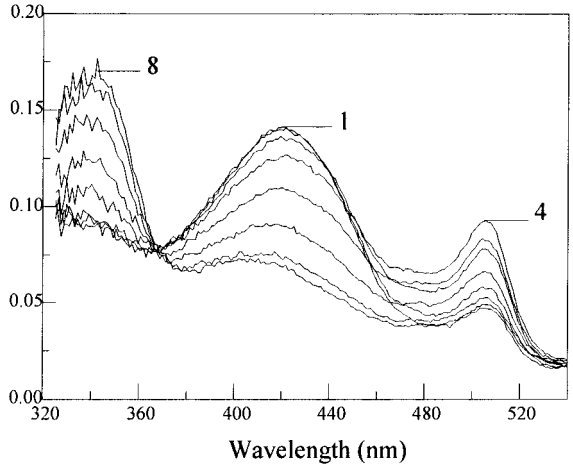


Figure 1. Rapid-scanning stopped-flow spectra of the reaction of *E. coli* Trpase with 10 mM L-tryptophan and α-^{2}H-L-tryptophan in the presence of 5 mM benzimidazole. The scan times are the following: 1, 0.001 s; 2, 0.003 s; 3, 0.005 s; 4, 0.010 s; 5, 0.020 s; 6, 0.040 s; 7, 0.080 s; 8, 0.160 s.

the α-aminoacrylate complex with benzimidazole releases pyruvate at a considerably slower steady-state rate. The 345 nm intermediate forms concomitant with decay of the external aldimine (λ_{max} = 422 nm) and quinonoid (λ_{max} = 505 nm) intermediates, with an isosbestic point at 364 nm and clean first-order kinetics (Figure 1). In contrast, in the absence of benzimidazole, only an increase in the absorbance of the 422 and 505 nm peaks is observed when Trpase is mixed with L-tryptophan.[10,14] The observed rate constant for formation of the 345 nm intermediate at 25 °C is 32 ± 2 s^{-1}, and this rate constant is not significantly affected by the concentration of either L-tryptophan or benzimidazole, although the amplitude is increased with [benzimidazole].[14] The same rate constant can be obtained either by fitting the absorbance increase at 345 nm (Figure 2A), the absorbance decrease at 422 nm (Figure 2B), or the absorbance decrease in the second phase at 505 nm (Figure 2C). The absorbance changes at 345 nm show more noise than those at 422 or 505 nm due to the lower output of the Xe lamp in the near-UV region and to the reduced efficiency of the visible gratings in the stopped-flow instrument below 350 nm (see Figure 1). However, the noise level of the data obtained at 345 nm does not significantly affect the results of fitting. For this study, rate constants were obtained from single exponential fitting of the first 200 ms of the reaction at either 345 or 422 nm, as fitting of the 505 nm time courses can be problematic due to the rapid rise−fall behavior (Figure 2C). These data at either 345 or 422 nm can be fit to a single exponential process with very high precision. At longer times, the 345 nm absorbance peak slowly decreases (Figure 2A), concomitant with an increase in absorbance at 422 nm (Figure 2B) and 505 nm (Figure 2C), due to the slowly increasing [indole] which competes with benzimidazole for the α-aminoacrylate intermediate.

Previously, we demonstrated that there is a significant primary isotope effect of about 3 on the formation of the 345 nm intermediate when the reaction is performed with α-^{2}H-L-tryptophan.[10] In the present work, we redetermined and confirmed the α-deuterium isotope effect of 2.99 ± 0.30 on the formation of the 345 nm intermediate from α-^{2}H-L-tryptophan (Figure 2A and Table 1). The amplitude of the absorbance changes at 345, 422, and 505 nm are also decreased for the reaction of α-^{2}H-L-tryptophan due to the previously determined primary isotope effect of 3.6 [16] on formation of the quinonoid

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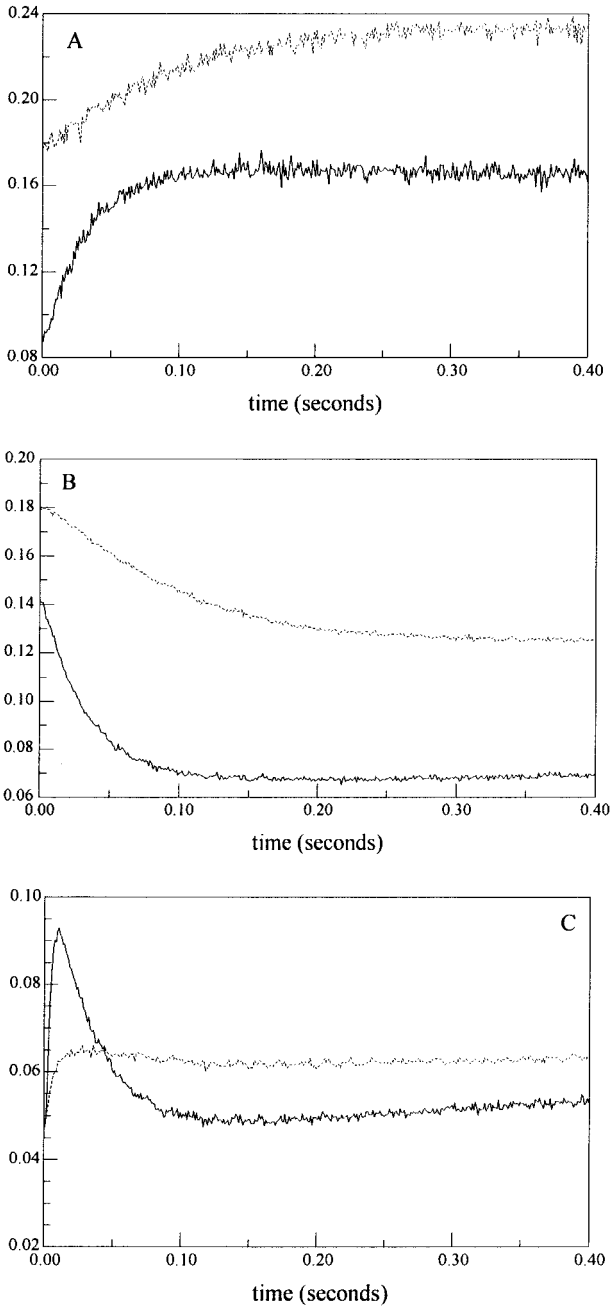

Figure 2. (A) Time courses at 345 nm for the reaction of *E. coli* Trpase with L-tryptophan and α-^{2}H-L-tryptophan in the presence of benzimidazole: solid line, L-tryptophan; dashed line, α-^{2}H-L-tryptophan. (B) Time courses at 422 nm for the reaction of *E. coli* Trpase with L-tryptophan and α-^{2}H-L-tryptophan in the presence of benzimidazole: solid line, L-tryptophan; dashed line, α-^{2}H-L-tryptophan. (C) Time courses at 505 nm for the reaction of *E. coli* Trpase with L-tryptophan and α-^{2}H-L-tryptophan in the presence of benzimidazole: solid line, L-tryptophan; dashed line, α-^{2}H-L-tryptophan.

Table 1. Kinetic Isotope Effects on Elimination of Indole from L-tryptophan

reaction	^Dk
α-^{2}H-L-tryptophan in H_2O	2.99 ± 0.30
α,β,β-^{2}H-L-tryptophan in H_2O	4.42 ± 0.67
β,β-^{2}H-L-tryptophan in H_2O	1.17 ± 0.03
L-tryptophan in D_2O	1.79 ± 0.11
α-^{2}H-L-tryptophan in D_2O	4.30 ± 0.16

intermediate (Figure 2C). We also examined the reaction of β,β-di-^{2}H-L-tryptophan (47% β,β-dideuterated and 38% β-mono-deuterated), and we found that there is a significant secondary isotope effect of 1.17 ± 0.03 on the formation of the 345 nm intermediate (Table 1). α,β,β-Tri-^{2}H-L-tryptophan (55% α,β,β-trideuterated and 25% α,β dideuterated) was prepared, and we found that the isotope effects on the reaction are approximately multiplicative, resulting in the observed isotope effect of 4.42 ± 0.67 (Table 1). In addition, we examined the effect of D_2O on the formation of the 345 nm intermediate. There is a significant solvent isotope effect of 1.79 ± 0.11 on the formation of the 345 nm intermediate (Table 1), indicating that one or more protons involved in the transition state are partially exchangeable with solvent. The solvent isotope effect is also seen in the reaction of α-^{2}H-L-tryptophan, where the observed isotope effect is increased from 3 to 4.30 ± 0.16 (Table 1). In addition, the temperature dependence of the rate constant of formation of the 345 nm intermediate was examined, and it exhibits a linear Eyring plot from 5 to 35 °C (Figure 3), consistent with only a single process contributing to the observed rate constant. The Eyring activation parameters for the elimination obtained from Figure 2 are $\Delta H^{\ddagger} = 17.2$ kcal/mol and $\Delta S^{\ddagger} = 6.4$ cal/(mol deg).

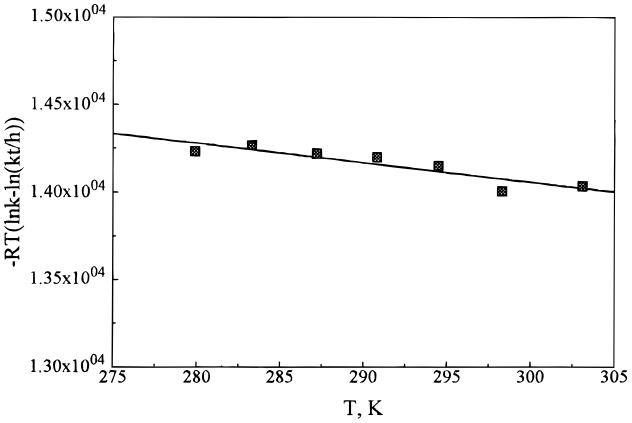

Figure 3. Eyring plot of the temperature dependence of the rate constant of formation of the 345 nm intermediate.

Isotope Effects on Indole Addition to the α-Aminoacrylate Intermediate. Addition of L-serine or ammonium pyruvate together with benzimidazole to Trpase results in a steady-state spectrum with a peak at 345 nm, very similar to that of the transient complex seen in the rapid-scanning reactions.[14] When this preformed α-aminoacrylate intermediate is mixed in the stopped-flow instrument with indole or 5-fluoroindole, a very rapid reaction is observed that forms a transient quinonoid intermediate, with λ_{max} at 505 nm. The rate constant for the formation of the 505 nm peak is dependent on the indole concentration (Figures 4 and 5). At [indole] > 0.6 mM, the rate constant becomes so fast that the 505 nm peak is completely formed within the dead time of the stopped-flow instrument. A plot of the apparent rate constant for the quinonoid intermediate formation against [indole] at concentrations below 0.6 mM is linear, indicating that this is a second-order process reacting under pseudo-first-order conditions (Figure 5, circles). When 3-^{2}H-indole is used, the rate constant for formation of the quinonoid intermediate is decreased significantly (Figure 5, squares). The slopes of the lines fitted by linear regression analysis to the data in Figure 5 give the apparent second-order rate constants for the reaction of indole with the α-aminoacrylate intermediate. These are (1.33 ± 0.11) × 10^6 M^{-1} s^{-1} for indole and (0.70 ± 0.04) × 10^6 M^{-1} s^{-1} for 3-^{2}H-indole. Thus, there is a significant isotope effect of 1.88 ± 0.19 on the formation of the quinonoid intermediate from 3-^{2}H-indole. This second-

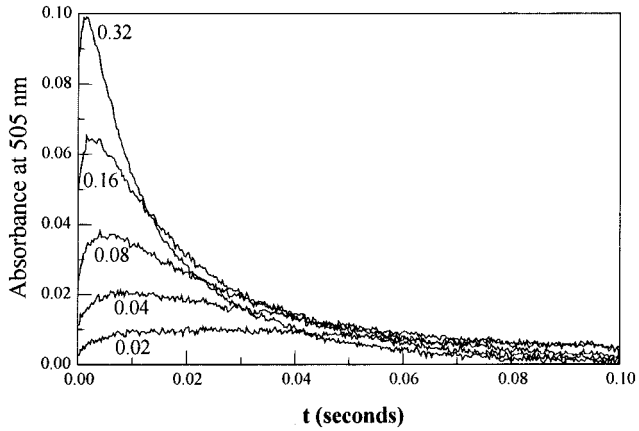

Figure 4. Time courses for the reaction of *E. coli* Trpase with varying [indole] in the presence of 0.5 M L-serine and 10 mM benzimidazole at 505 nm. The [indole] are 0.02, 0.04, 0.08, 0.16, and 0.32 mM.

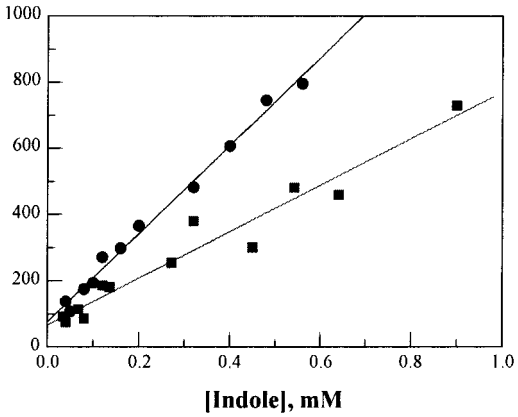

Figure 5. Concentration dependence of the apparent rate constant for formation of the 505 nm peak from indole (filled circles) and 3-^{2}H-indole (filled squares). The lines are the result of linear regression analysis of the data, with the parameters given in the text.

order rate constant is also in good agreement with the k_{cat}/K_m value for indole from steady-state kinetic measurements of 4×10^6 M^{-1} s^{-1}. The intercepts of the plots in Figure 5 are 78 s^{-1} for indole and 68 s^{-1} for 3-^{2}H-indole. For the reaction of 5-fluoroindole, the corresponding rate constants from the slopes and intercepts of a plot similar to that in Figure 5 (data not shown) are $(6.9 \pm 0.1) \times 10^5$ M^{-1} s^{-1} and 38 ± 2 s^{-1}. This is also in good agreement with the k_{cat}/K_m value of 4×10^5 M^{-1} s^{-1} for 5-fluoroindole in steady-state kinetics.

Discussion

The formation of indole during the bacterial putrefaction of protein was first described by Bopp 150 years ago.[4] The catabolism of L-tryptophan to indole and ammonium pyruvate by the action of Trpase, an enzyme produced by enteric bacteria, is now known to be the source of this indole. From a mechanistic standpoint, the rapid elimination of a carbon leaving group is intriguing, since it implies that the enzyme has to activate the indole ring for elimination to take place. Davis and Metzler proposed in an insightful review published in 1972[11] that the indolenine tautomer of tryptophan was an intermediate in the reaction mechanism of both Trpase and tryptophan synthase, which catalyzes the synthesis of L-tryptophan from L-serine and indole. Later, we demonstrated that structural analogues of the proposed indolenine tautomer, 2,3-dihydro-L-tryptophan and oxindolyl-L-alanine (Chart 1), are potent competitive inhibitors of Trpase and tryptophan synthase, and we concluded that this was in agreement with the intermediacy of the indolenine tautomer of tryptophan in the reaction mechanisms of both enzymes.[12] Furthermore, the inhibition of Trpase is stereospecific for (3*R*)-2,3-dihydro-L-tryptophan; hence, we suggested that the (3*R*)-indolenine diastereomer of L-tryptophan is the reaction intermediate.[13] In contrast, (3*S*)-2,3-dihydro-L-tryptophan was found to be a potent inhibitor of tryptophan synthase.[13] Thus, we proposed that the reaction of indole is a stepwise process in the mechanism of both Trpase and tryptophan synthase.[13] In the case of Trpase, we proposed a mechanism involving proton transfer to the indole ring to form a discrete indolenine intermediate, followed by the carbon−carbon bond cleavage. In the present work, we performed pre-steady-state kinetic isotope effect studies in order to obtain kinetic evidence for the existence of the proposed indolenine intermediate.

The minimum kinetic mechanism for the Trpase reaction is given in eq 2, where E_{al} is the L-tryptophan external aldimine,

$$\text{L-Trp} + \text{Trpase} \overset{K_1}{\rightleftharpoons} E_{al} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_q \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_{aa} + \text{In} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{Trpase} + NH_4^+ + CH_3COCO_2^- \quad (2)$$

E_q is the quinonoid intermediate, and E_{aa} is the aminoacrylate intermediate. In previous work using single-wavelength and rapid-scanning stopped-flow methods, we studied the reaction of L-tryptophan with the enzyme at 25 °C.[10,14,15,16] The rate constant for formation of the external aldimine of L-tryptophan, E_{al}, is too fast to measure even by stopped-flow techniques, but from the concentration dependence of the formation of the quinonoid intermediate, E_q, we estimated the value of K_1 to be 11.6 mM. The deprotonation of the α-carbon of the L-tryptophan external aldimine is fast, with an extrapolated value of k_2 of about 760 s^{-1}, and the reprotonation rate constant, k_{-2}, is about 60 s^{-1}.[10,14,15,16] As we found previously[10,14] and in this work, the formation of the α-aminoacrylate intermediate, with λ_{max} at 345 nm, occurs with an apparent rate constant, k_3, of 32 s^{-1}. The observed relaxation may be expected to be a combination of rate constants involving this step and the previous steps. However, it appears that the α-deprotonation step does not significantly contribute to the observed rate constant for formation of the 345 nm intermediate, since there is no significant concentration dependence of k_{obs} on [L-tryptophan], whereas the rate constant for formation of the 505 nm quinonoid intermediate shows a marked dependence on [L-tryptophan].[10,14,15,16] The clean isosbestic point between the 422 and 505 nm absorbance peaks and the 345 nm intermediate (Figure 1) requires that the external aldimine [E_{al}] and quinonoid [E_q] intermediates are at equilibrium during the formation of the 345 nm intermediate. In the presence of benzimidazole, k_{-3} is essentially zero in the pre-steady state since the reaction is quasi-irreversible in the first turnover. Furthermore, the observed rate constant for formation of the 345 nm intermediate is identical, within experimental error, to the rate constant, k_3, for indole elimination obtained by fitting rapid chemical quench data to the proposed mechanism in eq 2 with FITSIM.[15] Hence, the formation of the 345 nm absorbance peak in the presence of benzimidazole provides a convenient direct spectroscopic measurement of the first-order rate constant for indole elimination from the quinonoid intermediate, without significant complications due to kinetic complexity. Thus, the isotope effects that we observe on this apparent rate constant are primarily those on k_3, the step of indole elimination from the quinonoid intermediate of L-tryptophan.

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For the mechanism given in eq 2, k_{cat}/K_m for L-tryptophan obtained in steady-state kinetic studies is given by eq 3, since

$$k_{cat}/K_m = k_2k_3/(K_1(k_{-2} + k_3)) \quad (3)$$

indole release is the first irreversible step under initial rate conditions. Applying the values for the kinetic parameters obtained in this and previous pre-steady-state studies to eq 3 gives a predicted k_{cat}/K_m for L-tryptophan of 3.5×10^4 M^{-1} s^{-1}, in excellent agreement with steady-state kinetic measurements of 3×10^4 M^{-1} s^{-1}.[17] Furthermore, in previous steady-state kinetic isotope effect experiments, we observed a primary isotope effect on k_{cat}/K_m of 2.8 for α-^{2}H-L-tryptophan.[17] At that time, we made a resonable assumption that the only isotope sensitive step in the reaction of α-^{2}H-L-tryptophan was α-deprotonation. However, it is now clear from the present data that α-deuteration affects both α-deprotonation (k_2) and indole elimination (k_3), and thus the observed deuterium isotope effect on k_{cat}/K_m comes from both k_2 and k_3.

The observed isotope effect of α-^{2}H-L-tryptophan on k_3 was initally surprising. This isotope effect could arise either from direct transfer with internal return of the α-proton to the indole leaving group or indirectly through a hydrogen-bonding network. The intramolecular transfer of a deuterium atom from α-^{2}H-L-tryptophan to C_3 of indole was reported by Vederas et al. in 1978,[9] based on NMR analysis of indole isolated from reaction mixtures of α-^{2}H-L-tryptophan. Depending on the reaction conditions, the retention of deuterium in the indole product derived from α-^{2}H-L-tryptophan was only 7.9% in H_2O but 100% in D_2O. Vederas et al. concluded that the isotope transfer was intramolecular by examining the product of a mixture of α-deuterated and ring-deuterated tryptophans.[9] Our kinetic studies are in agreement with this result, as the rate constant for indole elimination shows a significant isotope effect of about 3 with α-^{2}H-L-tryptophan. An increase in the observed isotope effect on the elimination to 4.30 is observed when the reaction of α-^{2}H-L-tryptophan is performed in D_2O (Table 1), which suggests that there is some partial exchange of the α-proton prior to transfer to the indole ring even in a single turnover. The value of 4.3 must be the maximum isotope effect on indole formation, since Vederas et al. found that the indole product contains 100% deuterium at C_3 under these conditions.[9] The solvent and α-substrate deuterium isotope effects on indole elimination are not strictly multiplicative, indicating that they are not independent. This would be the case if partial exchange of a single proton were taking place. A solvent isotope effect of 1.79 is seen on elimination of indole when the reaction of L-tryptophan is performed in D_2O (Table 1). This result is in agreement with, but somewhat smaller than, the solvent isotope effect of 2.5 ± 0.4 that we observed previously on the second phase of formation of the quinonoid intermediate.[16] This difference likely reflects the experimental difficulty in accurately fitting the intermediate phase of a three exponential process. The present experiments isolate the elimination step as a single exponential process, giving much greater precision in the fitting. These solvent isotope effects on the reaction of Trpase are considerably smaller than the solvent isotope effect of 6.9 ± 2.3 determined by Kiick from steady-state kinetic measurements.[18] However, Kiick determined that multiple waters were involved and concluded that the solvent effect was due to changes in solvation of the enzyme. It is possible that a solvent sensitive conformational change takes place after indole release and prior to or concomitant with release of the pyruvate product.

The reaction of β,β-^{2}H-L-tryptophan shows a normal secondary isotope effect on α-aminoacrylate intermediate formation, as expected for the $C_\beta-C_3$ bond cleavage, which takes place with an sp^3 to sp^2 hybridization change at C_β. The secondary isotope effect for β,β-dideuterio-L-tryptophan, calculated by comparison of the reactions of normal and β,β-dideuteriotryptophan, is 1.17. Since the substrate used contained about 65% deuterium on the β-position, the calculated maximum secondary isotope effect is 1.28. This is considerably less than the secondary isotope effect of 1.81 on the formation of the α-aminoacrylate intermediate from β,β-di-^{2}H-*O*-acetyl-L-serine by *O*-acetylserine sulfhydrylase.[19] The reaction of α,β,β-^{2}H-L-tryptophan also shows a significantly larger isotope effect than α-^{2}H-L-tryptophan, demonstrating that the primary and secondary deuterium isotope effects on the $C_\beta-C_3$ bond cleavage are multiplicative. This is consistent with the proton transfer to C_3 of indole and the $C_\beta-C_3$ bond breakage taking place simultaneously rather than stepwise. In contrast, for the expected stepwise mechanism, with proton transfer to the *ipso* carbon of the indole to form a discrete indolenine intermediate preceding $C_\beta-C_3$ bond cleavage, the observed primary isotope effect would be expected to decrease for the multiply labeled substrate. The temperature dependence of the rate constant for α-aminoacrylate intermediate formation also shows a linear Eyring plot (Figure 3), which is consistent with a single step in the indole elimination. This conclusion is also consistent with studies of the reaction of 6-(difluoromethyl)tryptophan with Trpase by Woolridge and Rokita, which showed no evidence for fluoride elimination and hence argued against deprotonation of the indole NH in the elimination mechanism.[20]

The reaction of indole with the preformed α-aminoacrylate intermediate, prepared by addition to the enzyme of benzimidazole together with L-serine, forms a transient quinonoid intermediate with λ_{max} at 505 nm (Figure 4), resulting from the Michael-type addition of indole at C_3 to the β-carbon of the α-aminoacrylate complex. The decay of this transient intermediate is biphasic, since it has two possible modes of reaction, either elimination of the indole or reprotonation to tryptophan. For the reaction of indole with the α-aminoacrylate intermediate, E_{aa}, the apparent rate constant for formation of the quinonoid intermediate, E_q, should be given by eq 4. From the results

$$1/\tau = k_{-3}[\mathrm{In}] + (k_{-2} + k_3) \quad (4)$$

shown in Figure 5, which show second-order kinetics under pseudo-first-order conditions, we can conclude that there is no kinetically significant noncovalent complex of indole with the α-aminoacrylate intermediate, E_{aa}. The value of k_{-3} of 1.33×10^6 M^{-1} s^{-1} comes from the slope in Figure 5. The intercept of the plot in Figure 5 should give the sum of the rate constants of the two ways in which the quinonoid intermediate can break down, by indole elimination, k_3, and α-protonation, k_{-2}. This intercept value is 76.8 ± 12.3 s^{-1}, in reasonable agreement with the predicted value of 92 s^{-1}, calculated from 32 s^{-1} for k_3 obtained in the forward direction with benzimidazole and 60 s^{-1} for k_{-2} obtained in the reaction of L-tryptophan.[10,14,15,16] For the reaction of 3-^{2}H-indole, the rate constant for quinonoid intermediate formation, taken from the slope of the line in Figure 4, is 6.4×10^5 M^{-1} s^{-1}. The isotope effect observed with 3-^{2}H-indole is thus 1.88, which is consistent with a primary isotope effect of the C_3-indole hydrogen on the formation of the quinonoid intermediate. A secondary isotope effect might well

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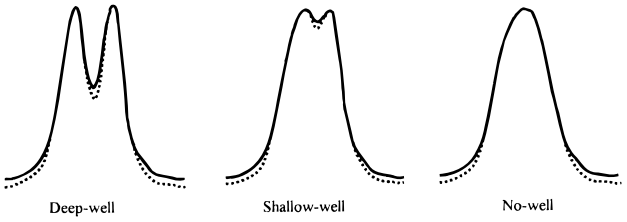
be expected on the addition of 3-^{2}H-indole to form an indolenine intermediate. However, such a secondary isotope effect should be no larger than about 1.2 and, furthermore, should be inverse, since the hybridization change on C_3 in the Michael addition reaction is from sp^2 to sp^3. The intercept value for the reaction of 3-^{2}H-indole, 68 s^{-1}, is in good agreement with the predicted value of 70 s^{-1} (10 s^{-1} for k_3 and 60 s^{-1} for k_{-2}). Hence, in the direction of tryptophan synthesis, the C_3-H bond appears to be broken concomitant with the formation of the C_3-C_β bond. These results are in contrast to those reported for tryptophan synthase, where no isotope effect is observed in the reaction of 3-^{2}H-indole with the α-aminoacrylate intermediate.[21] Hence, Lane and Kirschner concluded that the mechanism of indole reaction for tryptophan synthase is stepwise, with an indolenine intermediate.[21]

Thus, the mechanism which is consistent with these results is given in Scheme 1. External aldimine formation is complete within the dead time of the stopped-flow instrument, and subsequent α-deprotonation of the Schiff's base by a catalytic base, B_1, occurs rapidly to give a quinonoid intermediate with $\lambda_{max} = 505$ nm. A second base, B_2, which interacts with the indole NH, was identified by steady-state pH dependence studies.[17] Elimination of indole from the quinonoid intermediate then takes place at 32 s^{-1}, producing an α-aminoacrylate intermediate with $\lambda_{max} = 345$ nm. This intermediate could be the α-aminoacrylate Schiff's base, or it could also be the *gem*-diamine adduct with Lys270, as we proposed previously.[14] If the elimination of indole catalyzed by Trpase occurs by a stepwise mechanism, as we had anticipated, then the reaction coordinate between the quinonoid intermediate and the α-aminoacrylate intermediate should exhibit two maxima and a relatively deep well for the indolenine intermediate (Chart 2, left). The deuterated substrate, intermediate, and product are slightly lower in energy (dotted line). However, if the mechanism were stepwise, then the isotope effects of α- and *β*-deuteration should not be multiplicative. Furthermore, for the deep-well model there should be no primary isotope effect of deuteration of indole at C_3 on the reaction with the α-aminoacrylate intermediate. Thus, a stepwise mechanism with a long-lived indolenine intermediate is not consistent with the data. The conclusion of these results is that elimination of indole from the quinonoid intermediate of Trpase is either a concerted process, with proton transfer to C_3 and C_3-C_β bond cleavage ocurring simultaneously (Chart 2, right) or the reaction coordinate has a very broad shallow well for the indolenine species, so that its lifetime may be limited to only a few bond vibrations (Chart 2, center). The stereospecificity of inhibition by (3*R*)-2,3-dihydro-L-tryptophan[13] suggests that this indolenine intermediate or transition state has the (R)-configuration (Scheme 1).

Chart 2



Why would Trpase utilize a concerted or very nearly concerted mechanism rather than the alternative chemically reasonable classical stepwise mechanism for indole elimination? In contrast, tryptophan synthase appears to catalyze the synthesis of tryptophan by the expected stepwise mechanism.[21] This mechanistic difference may explain the very weak activity of tryptophan synthase to catalyze the elimination of indole from L-tryptophan.[22] There is no apparent chemical precedent for a concerted S_E2 mechanism in similar enzymatic or nonenzymatic electrophilic aromatic substitution reactions. These considerations suggest that the indolenine structure in the reaction of Trpase is an intermediate which occupies a broad shallow well on the reaction coordinate and hence has a very short lifetime. However, S_E2 mechanisms have been proposed for the protonolysis of alkyl mercury compounds by bacterial organomercurial lyase,[23] and the organomercurial lyase also reacts readily with aryl mercury compounds. The electrophilic substitution reaction of indoles at C_3 is a very well-known chemical reaction, and it is generally accepted that indoleninium ions are intermediates in the mechanisms of these reactions.[24] However, these chemical transformations are typically carried out under moderately to strongly acidic reaction conditions. Indole is a rather weak base, with a pK_a for protonation at C_3 of about -2,[25] and 3-alkyl substituted indoles are even weaker bases than indole,[26] so indoleninium ions are extremely unlikely to form under neutral conditions, even in enzyme active sites. The neutral indolenine tautomer of indole is also highly unfavorable.[25] Since the range of pK_a's available for general acids in the active site of enzymes is restricted to values near neutrality, it is possible that the *ipso* proton transfer to C_3 of tryptophan is so unfavorable that the steady-state concentration of the indolenine intermediate in the deep-well model would be extremely small. If the activation free energies for the reactions leading to and from the indolenine are reduced to a greater extent than the free energy of indolenine formation, then the reaction coordinate with the deep well may readily become converted to that with the shallow well (Chart 2).

Conclusion. Pre-steady-state kinetic isotope effect studies on the elimination of indole catalyzed by Trpase have failed to provide any evidence for the existence of the expected indolenine intermediate in the reaction mechanism. Hence, Trpase apparently uses an unexpected mechanism with very nearly simultaneous proton transfer and carbon−carbon bond cleavage to achieve the efficient elimination of indole from L-tryptophan.

Experimental Section

Materials. L-Tryptophan was purchased from U.S. Biochemical Corp and was recrystallized from 50% aqueous ethanol before use. The concentration of L-tryptophan in solutions was determined by the absorbance at 280 nm ($\epsilon = 5500$ M^{-1} cm^{-1}). Benzimidazole was purchased from Aldrich Chemical Co. and was recrystallized from hot water, after treatment with charcoal, before use. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) for enzyme assays was prepared from L-cysteine and 2-fluoronitrobenzene as previously described.[27] Indole (Gold label) was obtained from Aldrich, and the concentration of aqueous solutions was standardized from the absorbance at 270 nm ($\epsilon = 5500$ M^{-1} cm^{-1}).

Deuterated Substrates. α-^{2}H-L-Tryptophan was prepared as previously described by Kiick and Phillips.[17] α,*β*,*β*-Tri-^{2}H-L-tryptophan was

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prepared by synthesis of L-tryptophan from ammonium pyruvate and indole catalyzed by Trpase in D_2O. Ammonium sulfate (1.50 g) and potassium pyruvate (1.29 g) were dissolved in 15 mL of D_2O (99.8%), and the stoppered solution was left for 1 week at room temperature. The solution was then lyophilized, and the resultant yellow solid was dissolved in 50 mL of D_2O. Indole (0.119 g), disodium EDTA (0.0378 g), pyridoxal-5′-phosphate (0.8 mg), and 2-mercaptoethanol (21.8 μL) were added, and the pD was adjusted to 8.35 with solid K_2CO_3. Trpase (16 mg) was then added to the reaction, which was stoppered, covered with Al foil, and left at room temperature. After 4 days, an additional 16 mg of Trpase was added. After a total of 2 weeks, the reaction mixture was acidified with acetic acid to pH 4, filtered through Celite, and extracted with 2 × 20 mL of toluene to remove indole. The aqueous solution was applied to a column of Dowex 50 (10 × 2.5 cm), washed with water, and eluted with 1 M aqueous NH_3. Evaporation of the ammonia eluate gave a yellow foam, which was dissolved in 5 mL of H_2O and applied to a column (1 × 20 cm) of reverse phase (C18) silica gel. The column was washed with 100 mL of H_2O and then 100 mL of 10% methanol. The fraction containing the tryptophan was evaporated to give 7.9 mg of white solid. MS (ESI) showed *m*/*z* 206 (no deuterium) (0%), 207 (monodeuterated) (20.2%), 208 (dideuterated) (26.5%), and 209 (trideuterated) (53.3%). ^{1}H NMR indicated that the α-H was >99% exchanged.

β,*β*-Dideuterio-L-tryptophan was prepared by the reaction of indole with α,*β*,*β*-trideuterio-*S*-ethyl-L-cysteine catalyzed by Trpase in water. α,*β*,*β*-Trideuterio-*S*-ethyl-L-cysteine was prepared from normal *S*-ethyl-L-cysteine by an isotope exchange reaction in D_2O catalyzed by methionine-*γ*-lyase. The lyophilized cells of *Citrobacter intermedius* containing methionine-*γ*-lyase were obtained as described[28] and used as a catalyst. Lyophilized cells (100 mg) were added to the solution of 150 mg of *S*-ethyl-L-cysteine in 0.1 M potassium phosphate buffer in D_2O, pD 8.8, in the presence of 0.1 mM PLP, and the mixture was incubated at 30 °C with stirring for 3 days. The cells were separated by centrifugation, and the solution was analyzed by NMR, which showed that, together with the partial decomposition of the starting S-ethyl-L-cysteine, extensive isotope exchange of its α,*β*,*β* protons occurred. The amino acid was isolated on a Dowex-50 column, and about 70 mg of a brown material was obtained. A portion (30 mg) of this material was dissolved in 10 mL of 0.1 M potassium phosphate buffer, pH 8.5, containing 0.1 mM PLP and 15 mg of indole, and 0.05 mL of Trpase solution (0.82 mg) was added. The mixture was incubated with stirring at room remperature for 24 h. The enzyme was denatured by heating at 100 °C for 5 min and removed by centrifugation. The unreacted indole was extracted with ether, and amino acids were isolated from the solution on a Dowex-50 column. The *β*,*β*-dideuterio-L-tryptophan was purified on a reverse phase (C 18) silica gel column as described above. Its identity was proven by TLC (single spot on a silica gel plate in *n*-butanol−acetic acid−water (4:1:1)) and NMR analysis. MS (ESI) analysis revealed the presence of 47% dideuterated, 38% monodeuterated, and 15% nondeuterated tryptophan. ^{1}H NMR analysis showed that all of the deuterium was on the *β*-carbon.

3-^{2}H-Indole was prepared by the reaction of Trpase in D_2O. L-Tryptophan (0.200 g), K_2HPO_4 (0.313 g), KH_2PO_4, pyridoxal-5′-phosphate (0.25 mg), and 20 mL of D_2O were combined, and 0.5 mL of Trpase (5 mg) was added. The flask was sealed with a serum stopped and incubated in a shaker bath at 37 °C for 2 days. The reaction mixture was then extracted with 2 20 mL portions of CH_2Cl_2. The extracts were dried over Na_2SO_4 and evaporated to give 14 mg of indole, as light yellow crystals. ^{1}H NMR of the indole indicated that it contained 93% ^{2}H at C_3.

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Enzyme and Assays. Tryptophan indole-lyase was purified from cells of *E. coli* JM101 containing plasmid pMD6, with the *E. coli tna*A gene under natural regulation, as described, except that the Sepharose treatment was performed in a column rather than batchwise.[29] Routine activity assays were performed with SOPC in 0.1 M potassium phosphate, pH 8.0, at 25° C, following the absorbance decrease at 370 nm ($\Delta\epsilon = -1860\ M^{-1}\ cm^{-1}$).[8] Enzyme concentrations were estimated from the absorbance of the holoenzyme at 278 nm ($A^{1\%} = 9.19$),[29] using a subunit molecular weight of 52 kDa.[30]

Kinetic Measurements. Single-wavelength stopped-flow kinetic measurements and rapid-scanning experiments were performed using an RSM spectrophotometer and a stopped-flow compartment with a 10 mm path length observation cell from OLIS, Inc. This instrument has a mixing dead time of about 2 ms; thus, a reaction with a rate constant of 350 s^{-1} will lose about half its amplitude during mixing. Rapid-scanning measurements were performed over the range from 325 to 550 nm at 1 kHz, while single-wavelength measurements for the concentration dependencies were collected at 4 kHz. Some of the single-wavelength measurements (Figure 4) were performed on a Kinetics Instruments stopped-flow mixer with a modified Cary 14 UV/vis spectrophotometer (OLIS), and preliminary rapid-scanning experiments were performed with a diode array detector from EG&G Princeton Applied Research, as previously described.[13] Prior to performing the rapid kinetics experiments, the stock enzyme was incubated with 0.5 mM PLP for 1 h at 37 °C and then separated from excess PLP on a short desalting column (PD-10, Pharmacia) equilibrated with 0.02 M potassium phosphate, pH 8.0, and 0.16 M KCl, and the reactions were performed in the same buffer. For stopped-flow solvent isotope effect studies, the enzyme was concentrated in an Amicon cell to about 0.1 mL, diluted with 2 mL of buffer in D_2O, concentrated to 0.1 mL, diluted again with 2 mL of buffer in D_2O, concentrated again to 0.1 mL, and then diluted with 1 mL of buffer in D_2O. The stopped-flow kinetic measurements were performed at 25 °C with the stopped-flow compartment thermostated by an external water bath. Generally, the enzyme solutions, in buffer with 10 mM benzimidazole, were mixed with 20 mM L-tryptophan or deuterated tryptophan in the stopped-flow instrument. In the reverse reaction, enzyme solutions in 0.5 M L-serine with 10 mM benzimidazole were mixed with solutions of indole in the same buffer. Time courses at selected wavelengths were analyzed by fitting with the SIFIT or LMFIT programs (eq 5) (OLIS, Inc.), which can fit

$$A_t = \sum_{i}^{1-3} a_i e^{-k_i t} + c \qquad (5)$$

up to three exponentials with amplitudes and an offset, where A_t is the absorbance at time t, a_i is the amplitude of each phase, k_i is the rate constant for each phase, and c is the final absorbance, if nonzero. Quality of fit was judged by analysis of the residuals and by the Durbin−Watson value.[31] The concentration dependencies of relaxations in Figure 5 were fit by linear regression to obtain the second-order rate constants. The reactions were replicated from 3 to 14 times, and the mean rate constants were determined. The standard deviations were generally 10% or less of the parameter mean values. The mean values were then used to calculate the isotope effects.

Acknowledgment. This work was partially supported by a grant from NIH (GM42588) to R.S.P.

JA991647Q

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