Formation in Vitro of Hybrid Dimers of H463F and Y74F Mutant *Escherichia coli* Tryptophan Indole-lyase Rescues Activity with L-Tryptophan†

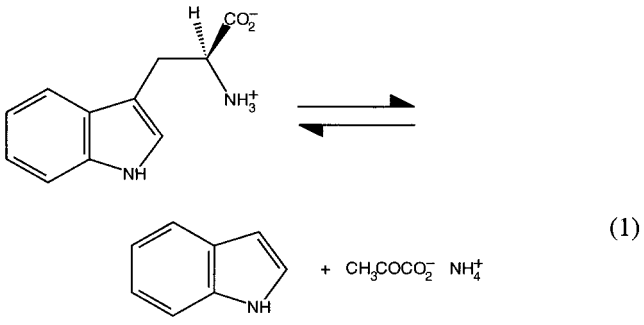
Robert S. Phillips,*,‡ Nancy Johnson,‡ and Ajith V. Kamath§

Departments of Chemistry and of Biochemistry and Molecular Biology and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602-2556, and Discovery Technology Center, Pfizer Inc., 620 Memorial Drive, Cambridge, Massachusetts 02139

Received October 8, 2001; Revised Manuscript Received January 31, 2002

ABSTRACT: Y74F and H463F mutant forms of *Escherichia coli* tryptophan indole-lyase (Trpase) have been prepared. These mutant proteins have very low activity with L-Trp as substrate (k_{cat} and k_{cat}/K_m values less than 0.1% of wild-type Trpase). In contrast, these mutant enzymes exhibit much higher activity with *S*-(*o*-nitrophenyl)-L-cysteine and *S*-ethyl-L-cysteine (k_{cat}/K_m values about 1−50% of wild-type Trpase). Thus, Tyr-74 and His-463 are important for the substrate specificity of Trpase for L-Trp. H463F Trpase is not inhibited by a potent inhibitor of wild-type Trpase, oxindolyl-L-alanine, and does not exhibit the pK_a of 6.0 seen in previous pH dependence studies [Kiick, D. M., and Phillips, R. S. (1988) *Biochemistry 27*, 7333]. These results suggest that His-463 may be the catalytic base with a pK_a of 6.0 and Tyr-74 may be a general acid catalyst for the elimination step, as we found previously with tyrosine phenol-lyase [Chen, H., Demidkina, T. V., and Phillips, R. S. (1995) *Biochemistry 34*, 12776]. H463F Trpase reacts with L-Trp and *S*-ethyl-L-cysteine in rapid-scanning stopped-flow experiments to form equilibrating mixtures of external aldimine and quinonoid intermediates, similar to those observed with wild-type Trpase. In contrast to the results with wild-type Trpase, the addition of benzimidazole to reactions of H463F Trpase with L-Trp does not result in the formation of an aminoacrylate intermediate. However, addition of benzimidazole with *S*-ethyl-L-cysteine results in the formation of an aminoacrylate intermediate, with λ_{max} at 345 nm, as was seen previously with wild-type Trpase [Phillips, R. S. (1991) *Biochemistry 30*, 5927]. This suggests that His-463 plays a specific role in the elimination step of the reaction of L-Trp. Refolding of equimolar mixtures of H463F and Y74F Trpase after unfolding in 4 M guanidine hydrochloride results in a dramatic increase in activity with L-Trp, indicating the formation of a hybrid H463F/Y74F dimer with one normal active site.

Tryptophan indole-lyase (Trpase,[1] EC 4.1.99.1) is a pyridoxal 5′-phosphate (PLP) dependent enzyme which catalyzes a reversible β-elimination reaction to form indole and ammonium pyruvate from L-Trp (eq 1). This enzyme is



(1)

found in a wide variety of Gram-negative bacteria, including *Escherichia coli* (*1*), *Proteus vulgaris* (*2*), *Haemophilus influenza* (*3*), and *Vibrio cholera* (*4*). In addition to the physiological reaction, the enzyme can also catalyze the β-elimination reactions of a wide range of amino acids with suitable leaving groups on the β-carbon in vitro, including *S*-(*o*-nitrophenyl)-L-cysteine (*5*), *S*-alkyl-L-cysteines (*6*), β-chloro-L-alanine (*6*), L-serine (*6*), and *O*-acyl-L-serines (*7*). A similar enzyme, tyrosine phenol-lyase (TPL, EC 4.1.99.2) catalyzes the β-elimination reaction of L-Tyr to give phenol and ammonium pyruvate (*8*). Comparison of the crystal structures of *Citrobacter freundii* TPL (*9*) and *P. vulgaris* Trpase (*10*) shows that the active site is at the interface of subunits in the dimer, and nearly all amino acid residues in the active site are conserved. The alignment of the *P. vulgaris* Trpase structure with that of *C. freundii* TPL shows that phenylalanine is found in TPL in place of His-458 (His-463 in the *E. coli* sequence). This phenylalanine to histidine substitution is also seen in the sequence alignments of all known Trpase and TPL sequences. In contrast, Tyr-72 in the structure of *P. vulgaris* Trpase (Tyr-74 in the *E. coli* sequence) is conserved in the TPL structure and in all Trpase and TPL sequences. Previously, we found that the homologous Tyr-71 in TPL is required for β-elimination activity

† This work was partially supported by a grant (GM42588) from the National Institutes of Health to R.S.P.

* To whom correspondence should be addressed at the Department of Chemistry, University of Georgia, Athens, GA 30602-2556. E-mail: rsphillips@sunchem.chem.uga.edu. Fax: (706) 542−9454.

‡ University of Georgia.

§ Pfizer Inc.

[1] Abbreviations: Trpase, tryptophan indole-lyase (tryptophanase; EC 4.1.99.1); TPL, tyrosine phenol-lyase (EC 4.1.99.2); PLP, pyridoxal 5′-phosphate; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine.

10.1021/bi015838t CCC: $22.00
Published on Web 03/03/2002

with L-tyrosine (*11*). Therefore, in the present work we performed site-directed mutagenesis of Tyr-74 and His-463 in *E. coli* Trpase to phenylalanine to determine the role of these residues in the catalytic mechanism and in the reaction specificity for L-Trp. The results suggest that both Tyr-74 and His-463 in *E. coli* Trpase are important for reaction of L-Trp, since the elimination activity is decreased at least 3000-fold by the mutations. Furthermore, we performed refolding experiments with mixtures of the Y74F and H463F mutant proteins and obtained proteins with near wild-type activity. This demonstrates that a hybrid dimer is formed during refolding, with one normal active site, that partially rescues the activity of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. Indole was purchased from Aldrich. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) was prepared as previously described (*12*). Lactate dehydrogenase (LDH) from rabbit muscle, L-tryptophan, *S*-ethyl-L-cysteine, PLP, and NADH were purchased from U.S. Biochemical Co. (USB).

Site-Directed Mutagenesis. The *E. coli tnaA* gene was PCR-amplified from pMD6 (*13*) using two primers. The 5′ primer also contained an upstream *Nco*I site, whereas the 3′ primer contained a *Bam*HI site after the stop codon. After PCR, the product was digested with *Nco*I and *Bam*HI and ligated into pET15b, which was digested with *Nco*I and *Bam*HI. The ligation mixture was used to transform *E. coli* DH5α and plated onto LB + ampicillin plates. Positive clones were identified by restriction digestion, and the plasmid DNA from a positive clone was used to transform *E. coli* BL21(DE3). A single colony was grown in LB + ampicillin at 37 °C. When the $OD_{600} = 0.7$, the cells were induced with 0.4 mM IPTG at 30 °C. After 3 h, the cells were harvested by centrifugation for 20 min at 15000 rpm. Samples were loaded onto 10% NuPAGE gels with MOPS buffer, and the induced cells showed a strong band at 52 kDa. The Y74F and H463F mutations were made in the *tna*A gene in the pET15b plasmid. The Y74F and H463F mutations were generated by polymerase chain reaction using primers containing the desired changes and *Pfu* DNA polymerase. The wild-type *tna*A in pET15b plasmid was used as a template. In the case of Y74F, the codon TAC was mutated to TTC, while in H463F, the codon CAC was changed to TTC. The mutant plasmids were sequenced to confirm that there were no other mutations in the gene. The mutant plasmids were used to transform *E. coli* BL21(DE3) with transposon *tn*5 in the genomic copy of *tna*A to avoid contamination of the mutant protein with wild-type enzyme.

Enzyme Purification. The enzymes were purified by hydrophobic chromatography on a column of Sepharose CL-4B, as described previously (*14*), except that a gradient of $(NH_4)_2SO_4$ from 40% to 20% saturation was used to elute the Trpase rather than a stepwise elution. Protein was determined in crude extracts by the method of Bradford (*15*) with purified Trpase as a standard. The concentration of purified Trpase was determined from the absorbance at 278 nm ($E^{1\%} = 9.19$) (*14*). The PLP content was determined by measuring the spectrum of the enzyme in 0.1 N NaOH, assuming $\epsilon^{390} = 6600\ M^{-1}\ cm^{-1}$ (*16*).

Enzyme Assays. Steady-state kinetic experiments were performed using a Cary 1E UV/vis spectrometer equipped with a 6 × 6 thermoelectric cell compartment. The β-elimination reactions of wild-type and mutant Trpase with L-Trp and *S*-alkyl-L-cysteines were generally measured using the coupled assay with lactate dehydrogenase and NADH, measured at 340 nm ($\Delta\epsilon = -6.22 \times 10^3\ M^{-1}\ cm^{-1}$), as previously described by Morino and Snell (*17*). Reaction mixtures contained, in a total volume of 0.6 mL, 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 50 μM PLP, 0.2 mM NADH, 0.02 mg of lactate dehydrogenase, and various amounts of amino acid substrate at 25 °C. The reaction was initiated by the addition of a 10 μL aliquot of enzyme solution. Enzyme activity during purification was routinely measured with 0.6 mM *S*-(*o*-nitrophenyl)-L-cysteine (SOPC) in 50 mM potassium phosphate, pH 8.0 at 25 °C (*5*), following the decrease in absorbance at 370 nm ($\Delta\epsilon = -1.86 \times 10^3\ M^{-1}\ cm^{-1}$). A unit of enzyme activity is defined as the amount of enzyme which produces 1 μmol of product/min. Determination of the kinetic parameters for SOPC was performed at 25 °C in 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, and 50 μM of PLP, with varying amounts of SOPC and appropriate dilutions of wild-type and mutant Trpase. Indole determination was performed with modified Ehrlich's reagent (*18*).

Protein Folding Experiments. The wild-type and mutant proteins were denatured in 4 M guanidine hydrochloride and refolded according to the procedure described by Mizobata and Kawata (*19*). The protein concentration in the 4 M guanidine solution was 200 μg/mL, and after dilution into the refolding buffer (0.05 M HEPES, pH 7.8, 5 mM DTT, 200 μM PLP), it was 8 μg/mL. The dilution into refolding buffer was performed into buffer preincubated at 0 °C, and the refolding was then allowed to proceed for 1 h at 37 °C. The refolded protein solution (50 mL) was then concentrated in an Amicon ultrafiltration cell over a YM-30 membrane to a volume of approximately 1 mL. This solution was used for activity assays after centrifugation to remove precipitated protein. The protein concentration in the refolded enzyme solutions was determined by the method of Bradford (*15*).

Stopped-Flow Reactions. Stopped-flow experiments were carried out using an RSM-1000 instrument from OLIS, Inc., equipped with a stopped-flow mixing chamber, as described previously (*20*). The stopped-flow mixer has a 10 mm path length and a dead time of about 2 ms. Absorbance spectra were collected over the wavelength range from 320 to 560 nm at a rate of 1000 scans s^{-1}. Enzymes for stopped-flow measurements were preincubated with excess PLP at 30° C for 30 min and then passed through a PD-10 gel filtration column equilibrated with 0.05 M potassium phosphate, pH 7.8, to remove excess PLP. The reactions were performed at 25° C with the syringes immersed in a bath controlled by an external circulating water bath.

Data Analysis. Steady-state kinetic values of k_{cat} and K_m were obtained by fitting the data (initial velocity versus substrate concentration) to the Michaelis−Menten equation (eq 2) using the program of Cleland, HYPERO (*21*). In eq

$$V = k_{cat}[E][S]/(K_m + [S]) \qquad (2)$$

2, k_{cat} is the turnover number of the enzyme, [S] is the substrate concentration, [E] is the total enzyme concentration, and K_m is the Michaelis constant. Generally, the data fit with standard errors of less than 10%. The dependence of k_{cat}/K_m

Table 1: Steady-State Kinetic Parameters for Wild-Type and Y74F and H463F Mutant Trpase

	SOPC		L-Trp		*S*-Et-L-Cys	
enzyme	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}
wild-type Trpase	38	5×10^5	6	3×10^4	6	9×10^3
Y74F	0.4	3.4×10^3	$<10^{-4}$		6.0×10^{-3}	5
H463F	5.1	1×10^5	1.8×10^{-3}	14	1.3	4.5×10^3

on pH for H463F Trpase was analyzed by fitting to a single-base (eq 3) or two-base (eq 4) mechanism with HABELL and DHABLL, respectively (*21*). The rapid-scanning stopped-flow spectra were analyzed by global analysis of all spectra at all wavelengths using the software provided by OLIS (*22*). The spectra were fitted to the minimum number of species and exponential processes to adequately describe the data on the basis of residuals and standard deviation, using eq 5, where A_t is the absorbance at a wavelength at time t, A_i is the absorbance at that wavelength for each phase, k_i is the rate constant for each phase, and A_∞ is the final absorbance at that wavelength of the reaction mixture. The figures show the resultant fitted spectra for each phase of the reaction.

$$\log V = \log C/(1 + [H^+]/K_a) \quad (3)$$

$$\log V = \log C/(1 + [H^+]/K_1 + [H^+]^2/K_1/K_2) \quad (4)$$

$$A_t = \sum A_i \exp(-k_i t) + A_\infty \quad (5)$$

RESULTS

Activity of H463F and Y74F Trpase. H463F and Y74F mutant Trpases from *E. coli* were expressed using a pET15b vector containing the *tna*A gene, in *E. coli* BL21(DE3) cells, with transposon *tn*5 in the genomic copy of *tna*A, to avoid contamination with background wild-type enzyme. Both H463F and Y74F mutant Trpases exhibit low (1−20%), but easily measurable, activity with a chromophoric synthetic substrate, SOPC (Table 1). However, Y74F Trpase has undetectable activity for L-Trp, $<10^{-4}$ compared to wild-type Trpase, while H463F Trpase has about 3000-fold lower activity with L-Trp than wild-type Trpase (Table 1). Y74F Trpase also exhibits k_{cat}/K_m with *S*-ethyl-L-cysteine about 1000-fold lower than wild-type enzyme, while H463F Trpase has a k_{cat}/K_m value for *S*-ethyl-L-cysteine 50% that of wild-type Trpase (Table 1). In constrast to wild-type Trpase, H463F Trpase is not significantly inhibited by 1 mM oxindolyl-L-alanine. The pH dependence of k_{cat}/K_m for H463F Trpase fits well to a single-base model, with pK_a of 8.24 ± 0.24 (Figure 1, solid line). Fitting the data to a two-base model results in pK_as of 8.14 ± 0.13 and 5.10 ± 0.59 (Figure 1, dashed line). Wild-type Trpase exhibits two pK_as of 7.8 and 6.0 in the pH dependence of k_{cat}/K_m (*23*); the predicted pH profile for H463F with two pK_a's of 8.14 and 6.0 is shown in Figure 1 as the dotted line.

Rapid-Scanning Stopped-Flow Spectrophotometry of H463F Trpase. H463F Trpase exhibits a strong absorbance peak at 420 nm, similar to wild-type Trpase, due to the presence of 1 mol of bound PLP/monomer. However, the 420 nm peak of H463F Trpase does not show a pH-dependent shift to 337 nm at high pH, as does wild-type Trpase (*1*, *24*). In contrast, Y74F Trpase as isolated has low absorbance at 420 nm,

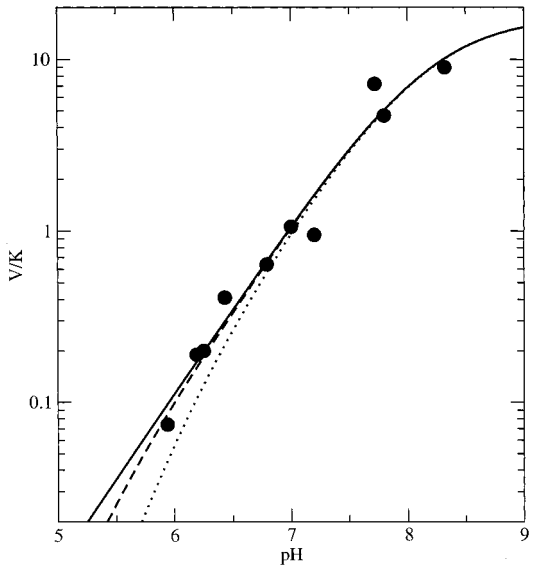


FIGURE 1: pH dependence of k_{cat}/K_m for the reaction of H463F Trpase with L-Trp: solid line, fitted curve for a single pK_a of 8.20; dashed line, fitted curve for two pK_as of 8.14 and 5.10; dotted line, calculated curve for two pK_as of 8.14 and 6.00.

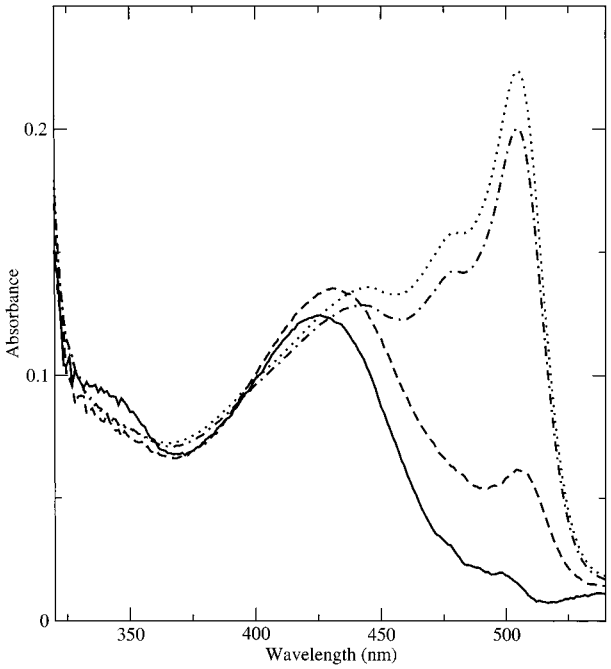


FIGURE 2: Fitted spectra for the reaction of H463F Trpase with 20 mM L-Trp in 0.02 M potassium phosphate, pH 7.8, and 0.16 M KCl: solid line, initial spectrum; dashed line, spectrum after the first exponential phase; dashed and dotted line, spectrum after the second exponential phase; dotted line, spectrum after the third exponential phase.

indicating a decreased affinity for PLP, and contains only about 0.2 mol of PLP/monomer as isolated. A similar result was obtained previously with the Y71F mutant of TPL (*11*). Since H463F Trpase binds PLP with high affinity, it was possible to perform rapid-scanning stopped-flow experiments. H463F Trpase reacts with both L-Trp and *S*-ethyl-L-cysteine to form equilibrating mixtures of external aldimine and quinonoid intermediates, with λ_{max} at about 425 and 505 nm, respectively (Figures 2 and 3). These spectra are qualitatively very similar to those previously observed with wild-type Trpase (*20*, *25*). The best global fit for the spectral data

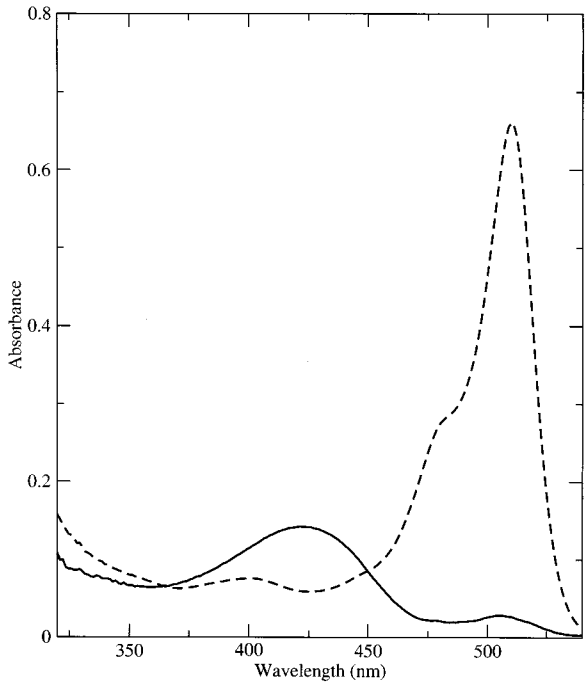


FIGURE 3: Fitted spectra for the reaction of H463F Trpase with 20 mM *S*-ethyl-L-cysteine in 0.02 M potassium phosphate, pH 7.8, and 0.16 M KCl: solid line, initial spectrum; dashed line, spectrum after the first exponential phase.

obtained in the reaction of H463F Trpase with 10 mM L-Trp was found to require four spectra and three exponentials (Figure 2). Thus, the minimal kinetic mechanism for the reaction is described by eq 6, where E_a is the external

$$E + \text{L-Trp} \overset{1/\tau_1}{\rightleftharpoons} E_a \overset{1/\tau_2}{\rightleftharpoons} E_{q1} \overset{1/\tau_3}{\rightleftharpoons} E_{q2} \qquad (6)$$

aldimine, E_{q1} is the first quinonoid intermediate, and E_{q2} is the second quinonoid intermediate. The fitted spectrum for the reaction immediately after mixing (solid line) shows a small peak at 340 nm that is not present in the enzyme spectrum alone and could indicate the formation of a *gem*-diamine intermediate in the dead time of the instrument. The first phase of the reaction is associated with an absorbance increase and red shift of the peak at 420 nm, with a rate constant of 180 s^{-1} (Figure 2, dashed curve). This corresponds to the formation of the external aldimine of L-Trp. This spectrum also exhibits a small peak at 505 nm, since the subsequent formation of the quinonoid intermediate is fast enough (see below) that the spectra cannot be completely separated. The formation of a *gem*-diamine intermediate was not observed previously with wild-type Trpase and L-Trp, and the rate constant for external aldimine formation was not measurable, as external aldimine formation is complete within the stopped-flow dead time (*25*). The formation of the quinonoid intermediate is biphasic; rate constants for formation of the quinonoid intermediate absorbing at 505 nm were found to be $1/\tau = 79$ s^{-1} for the fast phase and $1/\tau = 4.9$ s^{-1} for the slow phase (Figure 2, curve with dashes and dots and curve with dots). This compares with a rate constant of about 377 s^{-1} for the fast phase and 32 s^{-1} for the second phase of quinonoid intermediate formation from 10 mM L-Trp with wild-type enzyme (*20*, *25*, *26*). The spectra for the reaction of H463F Trpase with 10 mM L-Trp with 5 mM benzimidazole present are very similar to those shown in Figure 2. The rate constant for formation of the external aldimine intermediate of H463F Trpase in the presence of benzimidazole was found to be 140 s^{-1}, and the quinonoid intermediate formed with rate constants of 56 and 1.7 s^{-1}. These kinetic results are summarized in Table 2. However, when benzimidazole was included in the reactions of H463F Trpase, the quinonoid intermediate of L-Trp did not decay to form an aminoacrylate intermediate with λ_{max} at 345 nm (data not shown), in contrast to wild-type Trpase. The spectra of H463F Trpase with L-Trp either with or without benzimidazole present are stable for more than 3 h at 30 °C. Thus, the second phase of quinonoid intermediate formation from L-Trp for H463F Trpase is not the elimination of indole.

Table 2: Pre-Steady-State Kinetic Parameters for the Reaction of L-Trp and *S*-Et-L-Cys with the H463F Mutant Trpase[a]

	$1/\tau_1$ (s^{-1})		$1/\tau_2$ (s^{-1})		$1/\tau_3$ (s^{-1})	
substrate	−BZI	+BZI	−BZI	+BZI	−BZI	+BZI
L-Trp	180[b]	140[b]	79 (377)	56 (351)	4.9	1.7
S-Et-L-Cys	77 (68)	76 (68)	*c*	2.4 (6)		

[a] Numbers in parentheses are for the corresponding reaction in wild-type Trpase; data taken from refs *20* and *24*. +BZI is in the presence of 5 mM benzimidazole. [b] The reaction is too fast to measure for the wild type. [c] This reaction is not observed without benzimidazole present.

In contrast to L-Trp, only two spectra and one exponential were required to obtain an adequate global fit of the data from the rapid-scanning stopped-flow reaction of H463F Trpase and *S*-ethyl-L-cysteine (Figure 3). The rate constant for formation of the quinonoid intermediate, with λ_{max} at 510 nm, from 20 mM *S*-ethyl-L-cysteine was found to be $1/\tau = 77$ s^{-1}, compared to 68 s^{-1} previously observed for the reaction of wild-type enzyme with 20 mM *S*-ethyl-L-cysteine (*25*). Furthermore, the quinonoid intermediate of *S*-ethyl-L-cysteine of H463F Trpase decays rapidly in the presence of 5 mM benzimidazole to form an aminoacrylate intermediate with λ_{max} at 345 nm (Figure 4), with a rate constant of 2.4 s^{-1}, compared to 6 s^{-1} previously observed for wild-type Trpase (Table 2) (*25*). Thus, these data can be described by the kinetic mechanism shown in eq 7 , where E_a is the

$$E + \textit{S}\text{-Et-Cys} \overset{\text{fast}}{\rightleftharpoons} E_a \overset{1/\tau_1}{\rightleftharpoons} E_q \overset{1/\tau_2}{\rightleftharpoons} E_{ac} \qquad (7)$$

external aldimine, E_q is the quinonoid intermediate, and E_{ac} is the aminoacrylate intermediate.

Unfolding and Refolding Experiments with H463F and Y74F Trpase. Wild-type Trpase can be unfolded in 4 M Gdn-HCl, and refolded by dilution, as reported previously by Mizobata and Kawata (*19*). The recovery of activity from refolding is variable from about 10% to as high as 80%, depending on the protein concentration, temperature during dilution, or the addition of chaperone proteins. As reported by Mizobata and Kawata, we confirmed that the best recovery of wild-type activity in the absence of GroEL is obtained by dilution of the protein at low concentration (<10 μg/mL) into refolding buffer at 0 °C, followed by incubation at 37 °C for 1 h. We also performed unfolding and refolding experiments with H463F, Y74F, and an equal mixture of H463F and Y74F Trpases under the same conditions. There was no detectable elimination activity with L-Trp in either the H463F or Y74F samples after unfolding and refolding,

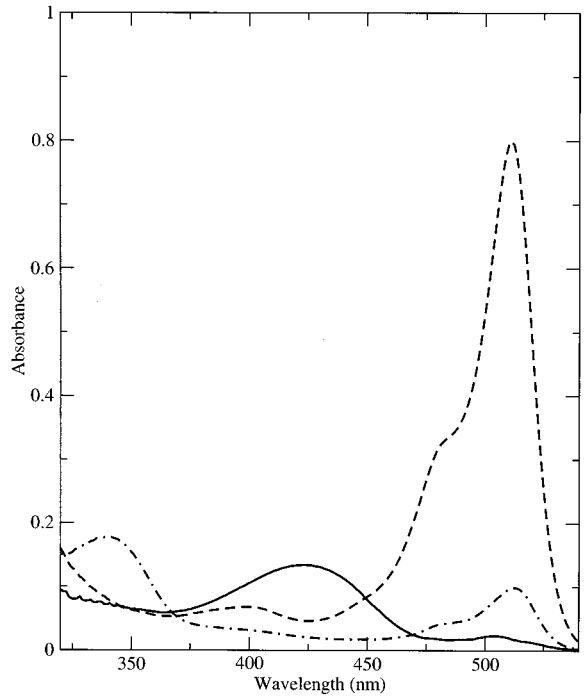


FIGURE 4: Fitted spectra for the reaction of H463F Trpase with 20 mM *S*-ethyl-L-cysteine in 0.02 M potassium phosphate, pH 7.8, and 0.16 M KCl with 5 mM benzimidazole: solid line, initial spectrum; dashed line, spectrum after the first exponential phase; dashed and dotted line, spectrum after the second exponential phase.

Table 3: Steady-State Kinetic Parameters for Refolded Enzymes

	SOPC		L-Trp	
enzyme	k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}\ s^{-1}$	k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}\ s^{-1}$
wild-type Trpase	23	1.1×10^5	3.2	3.0×10^4
H463F/Y74F	11	5.4×10^4	1	3.6×10^3

but the H463F/Y74F mixture exhibited very high activity after unfolding and refolding. The kinetic parameters obtained for the refolded enzymes are given in Table 3. The k_{cat} of the hybrid H463F/Y74F Trpase with L-Trp is 30% that of the refolded wild-type enzyme, close to the statistical value of 25% expected on the basis of 50% hybrid dimer formed containing a single competent active site. When equal amounts of H463F Trpase and wild-type TPL from *C. freundii* were mixed and treated in the same manner, no recovery of Trpase activity with L-Trp was found.

DISCUSSION

The mechanism of Trpase has been of considerable interest for some time, since it catalyzes the elimination of a formally unactivated carbon leaving group. We demonstrated previously that the indolenine tautomer of tryptophan is likely to be an intermediate or transition state, due to the potent inhibition of the enzyme by the L-Trp analogues, oxindolyl-L-alanine and dihydro-L-Trp (*27*, *28*). In pH dependence studies, we showed that there are two basic catalytic groups required in the mechanism, with pK_a values of 6.0 and 7.8 (*23*). The catalytic base with pK_a of 6.0 is required both for the reaction of L-Trp and for the binding of oxindolyl-L-alanine (*23*). The identity of this second catalytic base has been obscure. In the overlay of the crystal structure of Trpase from *P. vulgaris* (*10*) and the structure of TPL with a bound substrate analogue, 3-(4′-hydroxyphenyl)propionate (9), it can be seen that His-458 (corresponding to His-463 in the sequence of *E. coli* Trpase) is located in the substrate binding portion of the active site (Figure 5). This histidine residue is conserved in all Trpase sequences known, but it is invariably replaced by phenylalanine in the closely related enzyme, TPL, as seen in Figure 5. The extremely low activity of H463F Trpase for elimination of indole from L-Trp, the lack of inhibition by oxindolyl-L-alanine, and the pH dependence of k_{cat}/K_m (Figure 1) are consistent with the proposition that His-463 is the catalytic base with the pK_a of 6.0 observed in pH dependence studies. Alternatively, the mutation of His-463 to Phe could cause a perturbation of the base to shift the pK_a from 6.0 to about 5.

Furthermore, the stopped-flow kinetic studies with H463F Trpase demonstrate that the effect of the mutation on elimination activity with L-Trp is specifically on the formation of the aminoacrylate intermediate. Previous stopped-flow studies have shown that the mechanism of Trpase involves the formation of an external aldimine complex, absorbing at 425 nm, which is rapidly deprotonated at the α-carbon to form a quinonoid intermediate, which has an absorption maximum at 505 nm (Scheme 1). Elimination of indole takes place via an indolenine intermediate or transition state (*29*) to give an α-aminoacrylate intermediate, which has λ_{max} at 345 nm (Scheme 1). However, this intermediate is not observed in the reaction of wild-type Trpase with L-Trp, since it does not accumulate to any significant extent. Benzimidazole is an isoelectronic but nonnucleophilic analogue of indole that is an uncompetitive inhibitor of Trpase, which binds specifically to the α-aminoacrylate intermediate and hence increases its steady-state concentration (*25*). Addition of benzimidazole to stopped-flow reactions of wild-type Trpase results in a decrease in the absorbance of the 505 nm peak and concomitant formation of a peak at 345 nm (*20*, *25*, *26*). In contrast, addition of benzimidazole has no effect on the intensity of the 505 nm absorbance peak in the reaction of H463F Trpase with L-Trp. The H463F mutation has modest effects on other steps; it causes the rate constant for external aldimine formation to slow enough to be measured, and the subsequent formation of the quinonoid intermediate is about 5-fold slower than that of wild-type Trpase. However, the rate constant for aminoacrylate intermediate formation is reduced to a much greater extent than that for quinonoid intermediate formation, from 32 s^{-1} for wild-type Trpase to undetectable for H463F. In previous stopped-flow studies, we found that the rate of quinonoid intermediate formation from tryptophan analogues is dependent on the presence of the indole NH, presumably for H-bonding to a protein residue (*30*). Those results are consistent with the effect of the H463F mutation on quinonoid intermediate formation with L-Trp observed here. Since k_{cat} is 3000-fold slower for H463F Trpase, and all steps up to and including formation of the quinonoid intermediate are relatively fast in H463F Trpase, it is reasonable to conclude that elimination of indole has become rate-limiting for the H463F mutant enzyme. However, the reduction of the activity by only about 3000-fold in the H463F mutant Trpase suggests that His-463 functions in catalysis by participation in hydrogen bonding with the substrate indole NH, either directly or through a network of other residues and/or water, rather than by proton abstraction. This is consistent with the

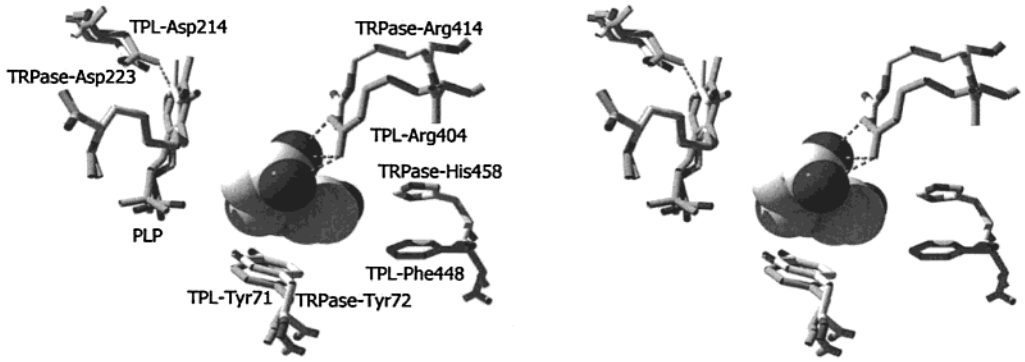
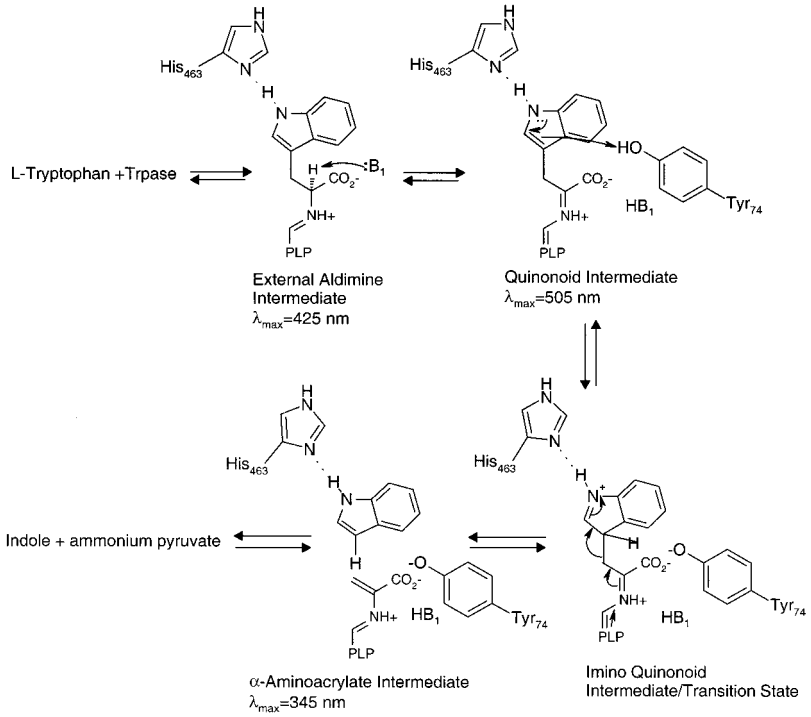


FIGURE 5: Stereoview of the overlay of active site residues in the structure of *C. freundii* TPL and *P. vulgaris* Trpase. The space-filling model in the center is bound 3-(4′-hydroxyphenyl)propionate in the active site of TPL. The coordinates were taken from the Protein Data Bank (2TPL and 1AX4, respectively).

Scheme 1



results of Woolridge and Rokita (*31*), who did not find evidence for deprotonation of the NH in the reaction of 6-(difluoromethyl)-L-Trp with Trpase.

In contrast to the reaction of L-Trp, *S*-ethyl-L-cysteine has values of k_{cat} about 25% and k_{cat}/K_m 50% of wild type for H463F Trpase (Table 1). The rapid-scanning stopped-flow spectra show that an aminoacrylate intermediate forms rapidly in the presence of benzimidazole, demonstrating that β-elimination takes place readily (Figure 4). Thus, His-463 does not appear to play a significant role in the reaction of nonphysiological substrates with suitable leaving groups on the β-carbon.

Tyr-74 is also located in the active site and is conserved in all sequences of Trpase as well as TPL. In the structure of the *P. vulgaris* holoenzyme, the corresponding Tyr-72 is hydrogen bonded to the phosphate oxygen of PLP through an intervening water molecule and is located near Glu-70 and the monovalent cation activator site (*10*). In the structure of TPL with bound 3-(4′-hydroxyphenyl)propionate, the homologous Tyr-71 is located near the aromatic ring of the substrate analogue, and Arg-404 forms an ion pair with the carboxylate of the analogue (Figure 5) (*9*). Arg-414 in *P. vulgaris* Trpase is in the same position in both the amino acid sequence and the structure (Figure 5). In both enzymes, the PLP is bound to a lysine ϵ-amino group, and there is an aspartate residue close to the pyridine nitrogen (Figure 5). The Y71F mutant form of TPL was previously found to have undetectable activity with L-tyrosine but was still measurably active with SOPC and *S*-alkyl-L-cysteines (*11*). We concluded that this tyrosine residue has a dual role in TPL, both in PLP binding and as a general acid catalyst for the elimination of phenol from L-tyrosine (*11*). In the present work, our results with Y74F Trpase mirror the previous results with Y71F TPL, suggesting a similar role of the homologous tyrosine in both enzymatic reactions. The activity of L-Trp and *S*-Et-L-Cys is affected more by the tyrosine to phenylalanine mutation than is the activity with SOPC, which has a good leaving group with pK_a of 5.2 (*5*). This is consistent with the postulated role of Tyr-74 as a general acid to donate a proton to the leaving group in the elimination reaction. Tyr-74 may be the essential tyrosine of Trpase which can be modified with tetranitromethane (*32*).

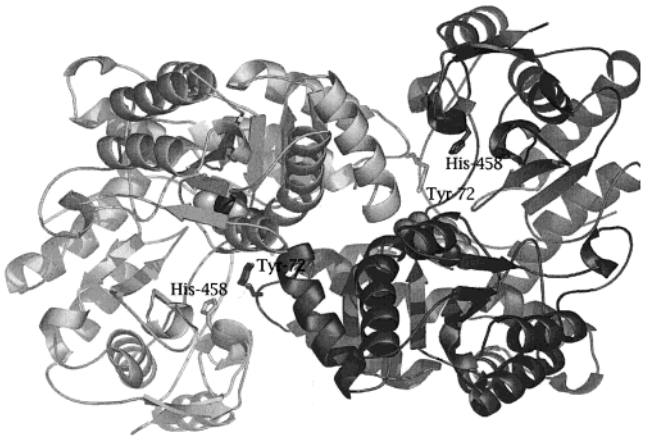


FIGURE 6: Ribbon diagram of the structure of *P. vulgaris* Trpase showing the position of Tyr-72 and His-458, which correspond to Tyr-74 and His-463 in the *E. coli* sequence, on different chains. The PLP is shown as the space-filling model. The coordinates were taken from the Protein Data Bank (1AX4).

The proposed mechanism of *E. coli* Trpase showing the postulated roles of Tyr-74 and His-463 is shown in Scheme 1.

Trpase is a tetrameric protein composed of four identical subunits, organized as pairs of dimers. The enzyme is a member of the α-family of PLP-dependent enzymes (*33*). Kawata and co-workers have studied the refolding of Trpase extensively (*19*, *34*). They found that the refolding must be performed at low concentration (5−10 μg/mL) in order to avoid aggregation to inactive precipitate (*19*). The recovery of activity can also be enhanced by the addition of GroEL, a chaperone protein (*34*). In addition, they found that the dilution of the guanidine solution into refolding buffer at 0 °C helps to retard aggregation, followed by incubation at 37 °C to re-form the active tetrameric holoenzyme. We used the latter protocol in our refolding experiments, and we confirmed that the recovery of activity of wild-type Trpase is much improved. We then used this procedure to prepare Trpase composed of hybrid dimers containing a Y74F and H463F mutant chain. Since the active site is located at the monomer−monomer interface of the dimer, as is characteristic of the α-family of PLP enzymes, and the Tyr-74 and His-463 residues come from different chains (Figure 6), we reasoned that a hybrid dimer could form one competent active site with activity toward L-Trp comparable to wild-type Trpase. Indeed, the formation of active enzyme from the inactive mutants by denaturation and refolding of a mixture could be taken as *prima facie* evidence for the presence of hybrid dimers. Our results confirmed this prediction, with the refolded hybrid showing k_{cat} for L-Trp about 30% that of refolded wild-type Trpase and more than 500-fold higher than H463F Trpase. Control experiments with the separated mutant proteins showed no detectable activity with L-Trp upon refolding. When we performed the same refolding experiment with a mixture of H463F Trpase and *C. freundii* TPL, no active enzyme was obtained. Thus, even though TPL is a highly homologous enzyme with a very similar three-dimensional structure (*10*), it does not appear to form a chimeric hybrid with Trpase which has activity toward L-Trp.

Our experiments demonstrate that formation of complementary hybrid dimers of inactive mutant monomers in vitro can rescue the activity of Trpase, in which the active site is located at a dimeric subunit interface. This technique was originally developed by Schachman for aspartate transcarbamoylase (*35*) and is useful to demonstrate the formation of hybrid dimers and/or to show that active sites are at a subunit interface. Surprisingly, there seem to be only a few other examples of this kind of rescue experiment in the literature. This complementation technique has been applied in vivo to other PLP-dependent enzymes in the α-family to demonstrate the presence of an active site at the subunit interface in aminocyclopropane carboxylase synthase (*36*), δ-aminolevulinate synthase (*37*), and ornithine decarboxylase (*38*). Adenylosuccinate synthase was found to undergo rapid subunit exchange to generate heterodimers without addition of chaotropic agents, allowing for complementation of inactive mutants (*39*). In the case of thymidylate synthase, in vitro refolding experiments of mixtures of inactive mutant enzymes were found to give near wild-type activity, demonstrating the location of the active site at a subunit interface before structural data became available (*40*).

ACKNOWLEDGMENT

We thank Professor Paul Gollnick of the Department of Biology, SUNY, Buffalo, NY, for the gift of *E. coli* BL21-(DE3) with *tn*5 inserted in *tna*A.

REFERENCES

1. Morino, Y., and Snell, E. E. (1967) *J. Biol. Chem. 242*, 2800.
2. Kamath, A. V., and Yanofsky, C. (1992) *J. Biol. Chem. 267*, 19978.
3. Martin, K., Morlin, G., Smith, A., Nordyke, A., Eisenstark, A., and Golomb, M. (1998) *J. Bacteriol. 180*, 107.
4. Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Umayam, L., Gill, S. R., Nelson, K. E., Read, T. D., Tettelin, H., Richardson, D., Ermolaeva, M. D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R. D., Nierman, W. C., and White, O. (2000) *Nature 406*, 477.
5. Suelter, C. H., Wang, J., and Snell, E. E. (1976) *FEBS Lett. 66*, 230.
6. Watanabe, T., and Snell, E. E. (1977) *J. Biochem. (Tokyo) 82*, 733.
7. Phillips, R. S. (1987) *Arch. Biochem. Biophys. 256*, 302.
8. Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H., and Ogata, K. (1970) *J. Biol. Chem. 250*, 1661.
9. Sundararaju, B., Antson, A. A., Phillips, R. S., Demidkina, T. V., Barbolina, M. V., Gollnick, P., Dodson, G. G., and Wilson, K. S. (1997) *Biochemistry 36*, 6502.
10. Isupov, M. N., Antson, A. A., Dodson, E. J., Dodson, G. G., Dementieva, I. S., Zakomirdina, L. N., Wilson, K. S., Dauter, Z., Lebedev, A. A., and Harutyunyan, E. H. (1998) *J. Mol. Biol. 276*, 603.
11. Chen, H., Demidkina, T. V., and Phillips, R. S. (1995) *Biochemistry 34*, 12776.
12. Dua, R. K., Taylor, E. W., and Phillips, R. S. (1993) *J. Am. Chem. Soc. 115*, 1264.
13. Deeley, M. C., and Yanofsky, C. (1981) *J. Bacteriol. 147*, 787.
14. Phillips, R. S., and Gollnick, P. D. (1989) *J. Biol. Chem. 254*, 10627.
15. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
16. Peterson, E. A., and Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169.
17. Morino, Y., and Snell, E. E. (1970) *Methods Enzymol. 17A*, 439.
18. Newton, W. A., and Snell, E. E., (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1431.

19. Mizobata, T., and Kawata, Y. (1995) *J. Biochem. (Tokyo) 117*, 384.
20. Sloan, M. J., and Phillips, R. S. (1996) *Biochemistry 35*, 16165.
21. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
22. Matheson, I. B. C., and DeSa, R. J. (1990) *Comput. Chem. 14*, 49.
23. Kiick, D. M., and Phillips, R. S. (1988) *Biochemistry 27*, 7333.
24. Ikushiro, H., Hayashi, H., Kawata, Y., and Kagamiyama, H. (1998) *Biochemistry 37*, 3043.
25. Phillips, R. S. (1991) *Biochemistry 30*, 5927.
26. Phillips, R. S. (1989) *J. Am. Chem. Soc. 113*, 727.
27. Phillips, R. S., Miles, E. W., and Cohen, L. A. (1984) *Biochemistry 23*, 6228.
28. Phillips, R. S., Miles, E. W., and Cohen, L. A. (1985) *J. Biol. Chem. 260*, 14665.
29. Phillips, R. S., Sundararaju, B., and Faleev, N. G. (2000) *J. Am. Chem. Soc., 122*, 1008.
30. Phillips, R. S., Bender, S. L., Brzovic, P., and Dunn, M. F. (1990) *Biochemistry 29*, 8608.
31. Woolridge, E. M., and Rokita, S. E. (1991) *Biochemistry 30*, 1852.
32. Nihira, T., Toraya, T., and Fukui, S. (1981) *Eur. J. Biochem. 119*, 273.
33. Alexander, F. W., Sandmeier, E, Mehta, P. K., and Christen, P. (1994) *Eur. J. Biochem. 219*, 953.
34. Mizobata, T., Akiyama, Y., Ito, K., Yumoto, N., and Kawata, Y. (1992) *J. Biol. Chem. 267*, 17773.
35. Wente, S. R., and Schachman, H. K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 31.
36. Tarun, A. S., and Theologis, A. (1998) *J. Biol. Chem. 273*, 12509.
37. Tan, D., and Ferreira, G. (1996) *Biochemistry 35*, 8934.
38. Tobias, K. E., and Kahana, C. (1993) *Biochemistry 32*, 5842.
39. Kang, C., Kim, S., and Fromm, H. J. (1996) *J. Biol. Chem. 271*, 29722.
40. Pookanjanatavip, M., Yuthavong, Y., Greene, P. J., and Santi, D. V. (1992) *Biochemistry 31*, 10303.

BI015838T