Replacement of Lysine 269 by Arginine in *Escherichia coli* Tryptophan Indole-lyase Affects the Formation and Breakdown of Quinonoid Complexes*

(Received for publication, February 20, 1991)

Robert S. Phillips‡ and Ines Richter
From the Departments of Chemistry and Biochemistry, School of Chemical Sciences, University of Georgia, Athens, Georgia 30602

Paul Gollnick§
From the Department of Biological Sciences, Stanford University, Stanford, California 94305

Peter Brzovic and Michael F. Dunn
From the Department of Biochemistry, University of California, Riverside, California 92521

Lysine 269 in *Escherichia coli* tryptophan indole-lyase (tryptophanase) has been changed to arginine by site-directed mutagenesis. The resultant K269R mutant enzyme exhibits $k_{cat}$ values about 10% those of the wild-type enzyme with S-(a-nitrophenyl)-L-cysteine, L-tryptophan, and S-benzyl-L-cysteine, while $k_{cat}/K_m$ values are reduced to 2% or less. The pH profile of $k_{cat}/K_m$ for S-benzyl-L-cysteine for the mutant enzyme exhibits two $pK_a$ values which are too close to separate, with an average value of 7.6, while the wild-type enzyme exhibits $pK_a$ values of 6.0 and 7.8. The $pK_a$ for the interconversion of the 335 and 412 nm forms of the K269R enzyme is 8.3, while the wild-type enzyme exhibits a $pK_a$ of 7.4. Steady-state kinetic isotope effects on the reaction of $[\alpha^2H]^2$S-benzyl-L-cysteine with the K269R mutant enzyme ($\delta k_{cat} = 2.0; \delta (k_{cat}/K_m) = 3.9$) are larger than those of the wild-type enzyme ($\delta k_{cat} = 1.4; \delta (k_{cat}/K_m) = 2.9$). Rapid scanning stopped-flow kinetic studies demonstrate that the K269R mutant enzyme does not accumulate quinonoid intermediates with L-alanine, L-tryptophan, or S-methyl-L-cysteine, but does form quinonoid absorption peaks in complexes with S-benzyl-L-cysteine and oxindolyl-L-alanine, whereas wild-type enzyme forms prominent quinonoid bands with all these amino acids. Single wavelength stopped-flow kinetic studies demonstrate that the $\alpha$-deprotonation of S-benzyl-L-cysteine is 6-fold slower in the K269R mutant enzyme, while the intrinsic deuterium kinetic isotope effect is less for the K269R enzyme ($\delta k = 4.2$) than for the wild-type ($\delta k = 7.9$). The decay of the K269R quinonoid intermediate in the presence of benzimidazole is 7.1-fold slower than that of the wild-type enzyme. These results demonstrate that Lys-269 plays a significant role in the conformational changes or electrostatic effects obligatory to the formation and decomposition of the quinonoid intermediate, although it is not an essential basic residue.

Pyridoxal 5'-phosphate (PLP)1-dependent enzymes perform a wide variety of biologically important transformations of amino acids and amines. The PLP cofactor is held in the active sites of these enzymes through a Schiff base linkage with the $\alpha$-amino group of a lysine residue. In some of these enzymes, the amino acid residue immediately preceding this lysine is another basic amino acid. For example, in *Escherichia coli* and *Salmonella typhimurium* tryptophan synthase $\beta$-subunit, His-86 precedes Lys-87, which holds the PLP (Crawford et al., 1980). Replacement of this histidine in *S. typhimurium* tryptophan synthase by leucine using site-directed mutagenesis resulted in a partially active protein, indicating that the His-86 is not essential (Miles et al., 1989). This is consistent with the x-ray crystallographic structure of *S. typhimurium* tryptophan synthase, which shows that His-86 is interacting with the PLP (Hyde et al., 1988). In addition, in aromatic amino acid decarboxylase from pig kidney (Bossa et al., 1977) and in histidine decarboxylase from *Morganella morganii* (Vaaler et al., 1986), the lysine which forms a Schiff base with the PLP is preceded by a histidine residue. In contrast to the results obtained with tryptophan synthase, replacement of His-231 in histidine decarboxylase by site-directed mutagenesis indicated that the histidine is structurally or catalytically important, since only mutation to glutamine and asparagine gave partially active enzymes (Vaaler and Snell, 1989).

Tryptophan indole-lyase (tryptophanase)2 is another PLP-dependent enzyme that catalyzes the reversible hydrolytic cleavage of L-tryptophan to give indole and ammonium pyridine.
We have recently demonstrated that Cys-298 is likely to be in ruvate (Equation 1). In this enzyme, the PLP is held by Lysine-270, and there is another lysine residue at position 269.

\[ \text{L}-\text{Tryptophan} + \text{H}_2\text{O} \rightarrow \text{indole} + \text{pyruvate} + \text{ammonium} \]  

We have been probing the role of specific amino acid residues in tryptophan indole-lyase by site-directed mutagenesis, and we have recently demonstrated that Cys-298 is likely to be in or near the PLP-binding site (Phillips and Gollnick, 1989). We have also demonstrated that the 2 tryptophan residues, Trp-248 and Trp-330, are not catalytically essential since they can be replaced by phenylalanine without loss of activity (Phillips and Gollnick, 1990). We have now replaced Lys-269 by arginine using site-directed mutagenesis, and we find that the resultant K269R enzyme has altered catalytic and spectroscopic properties. The replacement of Lys-269 by arginine significantly reduces the steady-state catalytic efficiency of the K269R mutant enzyme. Furthermore, the rates and extents of formation of quinonoid complexes with amino acid substrates and inhibitors, as measured by rapid-scanning and single-wavelength stopped-flow spectrophotometry, are diminished in the K269R enzyme. These results suggest that Lys-269 plays an important role in the conformational changes or electrostatic effects associated with formation and breakdown of quinonoid intermediates in the reaction of tryptophan indole-lyase.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—L-Tryptophan and S-benzyl-L-cysteine were purchased from United States Biochemical Corporation and were recrystallized from hot water before use. [\( \alpha^3\text{H} \)] S-BenzyL-L-cysteine (Phillips, 1989), oxidindolyl-L-alanine (Phillips et al., 1984), and S-(o-nitrophenyl)-L-cysteine (SOFC) (Phillips et al., 1989) were prepared as previously described. PLP and NADH, disodium salt, were obtained from United States Biochemical Corporation. All enzyme solutions and buffers were prepared from distilled deionized water.

**Preparation of Enzymes**—Wild-type tryptophan indole-lyase was purified from tryptophan-induced cells of E. coli JM101 containing plasmid pHDR (Deley and Yanofsky, 1981), as previously described (Phillips and Gollnick, 1989). The K269R mutation was prepared by site-directed mutagenesis of the tnaA gene, using the appropriate mutagenic oligonucleotide, as described elsewhere (Phillips and Gollnick, 1989). The presence of the desired mutation was confirmed by sequencing using the dideoxy method (Sanger et al., 1975). The molecular weight of 5.2 \( \times \) 10^4 Da.

**RESULTS**

Activity of K269R Mutant Tryptophan Indole-lyase—The K269R mutant enzyme exhibits much lower activity in steady-state assays than the wild-type enzyme, when measured with SOPC, S-benzyl-L-cysteine, or L-tryptophan as the substrate for the \( \beta \)-elimination reaction (Table 1). The \( k_{cat} \) values for these substrates are about 10% those for the wild-type, while \( k_{cat}/K_m \) values are about 2% of the wild-type for SOPC, L-tryptophan, and S-benzyl-L-cysteine. We determined the pH dependence of \( k_{cat} \) and \( k_{cat}/K_m \) for both forms of the enzyme using S-benzyl-L-cysteine as the substrate (Fig. 1). The \( k_{cat} \) values in the \( k_{cat}/K_m \) profile for the wild-type enzyme, with values of about 7.79 ± 0.04 and 6.0 ± 0.16, are the same \( k_{cat} \) values we previously reported for the reaction with L-tryptophan (Kuck and Phillips, 1988). However, the K269R enzyme exhibits two \( k_{cat} \) values that cannot be separated but which titrate with an average value of 7.56 ± 0.02. This indicates that the \( k_{cat} \) values of 6.0 in the wild-type enzyme is perturbed about 1.6 units to higher pH in the K269R mutant enzyme. The values of \( k_{cat} \) for both enzymes are essentially pH independent.

**Table I**

| Enzyme          | \( k_{cat} \) | \( k_{cat}/K_m \) | \( \Delta k_{cat} \) | \( \Delta k_{cat}/K_m \) |
|-----------------|-------------|----------------|------------------|---------------------|
| **SOPC**        |             |                |                  |                     |
| Wild-type       | 44 s\(^{-1}\) | 5.1 \times 10^4 M\(^{-1}\) s\(^{-1}\) |                  |                     |
| K269R           | 2.9 ± 0.2 s\(^{-1}\) | 7.7 ± 0.5 \times 10^4 M\(^{-1}\) s\(^{-1}\) |                  |                     |
| **L-Trp**       |             |                |                  |                     |
| Wild-type       | 6.8 s\(^{-1}\) | 3.0 \times 10^4 M\(^{-1}\) s\(^{-1}\) |                  |                     |
| K269R           | 0.64 ± 0.03 s\(^{-1}\) | 2.8 ± 0.1 \times 10^2 M\(^{-1}\) s\(^{-1}\) |                  |                     |
| **S-Benzyl-L-cysteine** |             |                |                  |                     |
| Wild-type       | 5.2 ± 0.4 s\(^{-1}\) | 8.1 ± 0.3 \times 10^4 M\(^{-1}\) s\(^{-1}\) | 1.4 ± 0.1 s\(^{-1}\) | 2.9 ± 0.4 M\(^{-1}\) s\(^{-1}\) |
| K269R           | 0.51 ± 0.02 s\(^{-1}\) | 1.9 ± 0.1 \times 10^2 M\(^{-1}\) s\(^{-1}\) | 2.0 ± 0.1 s\(^{-1}\) | 4.0 ± 0.4 M\(^{-1}\) s\(^{-1}\) |

\( \Delta k_{cat} \) and \( \Delta k_{cat}/K_m \) refers to isotope effects measured with [\( \alpha^3\text{H} \)] S-benzyl-L-cysteine. From Phillips and Gollnick (1989).
(data not shown), as we found previously for the reaction of the wild-type enzyme with L-tryptophan and S-methyl-L-cysteine (Kiick and Phillips, 1988). Thus, the lower activity of the K269R mutant enzyme is not due to a loss of a catalytically essential residue.

The steady-state reaction of tryptophan indole-lyase with [α-2H]L-tryptophan exhibits significant primary kinetic isotope effects (Kiick and Phillips, 1988). In the present study, we determined the steady-state kinetic isotope effects for the wild-type and K269R enzymes with [α-2H]S-benzyl-L-cysteine. For the wild-type enzyme, the value of $k_{cat}/K_{m}$ is similar to the value that we previously reported for L-tryptophan ($k_{cat}/K_{m} = 2.8$), while the value for the K269R enzyme is significantly higher (Table 1). In contrast, $k_{cat}$ = 1.4 for S-benzyl-L-cysteine with the wild-type enzyme, a value which is significantly lower than that of L-tryptophan ($k_{cat} = 2.5$) (Kiick and Phillips, 1988). However, the K269R mutant enzyme exhibits a somewhat larger $k_{cat}$ with S-benzyl-L-cysteine than does the wild-type enzyme.

Effects of pH on the Spectrum of K269R Enzyme—Wild-type tryptophan indole-lyase exhibits absorption peaks at about 420 and 337 nm due to the bound PLP. The peak at 420 nm is favored at low pH, and the 337-nm peak is predominant at high pH; the interconversion of the two spectral forms exhibits an apparent $pK_a$ of about 7.4 (Morino and Snell, 1967). The spectrum of the K269R enzyme is similar to that of the wild-type enzyme at low pH, with a slight blue-shift of the $\lambda_{max}$ to about 412 nm (Fig. 2A). In contrast to the wild-type enzyme, the peak at 412 nm does not disappear completely at high pH (Fig. 2A). A plot of the absorbance at 412 nm for the K269R enzyme as a function of pH is presented in Fig. 2B; these data fit a titration curve with an apparent $pK_a$ of 8.3. Thus, replacement of Lys-269 by arginine shifts the ionization controlling the PLP spectrum by 0.9 pH units. This effect is similar to the shift we observed previously in the C298S mutant of tryptophan indole-lyase (Phillips and Gollnick, 1989).

Rapid Kinetic Studies of K269R Enzyme—Tryptophan indole-lyase forms complexes with substrates and substrate analogues that exhibit strong absorption peaks at about 500 nm. These peaks are attributed to the PLP Schiff's base of the amino acid α-carbanion, or “quinonoid” complex (Morino and Snell, 1967). We examined the interaction of a number of amino acid substrates and analogues with the K269R enzyme using rapid-scanning stopped-flow spectrophotometry. L-Alanine, L-tryptophan, and S-methyl-L-cysteine form prominent quinonoid absorption bands with the wild-type enzyme (Morino and Snell, 1967). However, the K269R mutant enzyme forms much smaller amounts of quinonoid complexes with these amino acids (Fig. 3A–C). S-Benzyl-L-cysteine and oxindolyl-L-alanine do exhibit prominent quinonoid bands when complexed with the K269R enzyme, but they are, however, significantly reduced in intensity relative to the wild-type enzyme (Fig. 3, D and E). The $\lambda_{max}$ of these intermediates are located at 506 nm for oxindolyl-L-alanine and 512 nm for S-benzyl-L-cysteine. These values are identical with the $\lambda_{max}$ of the corresponding intermediates formed from the wild-type enzyme. All these substrates and inhibitors accumulate intermediates absorbing at 400–420 nm (probably
Trp Indole-lyase K269R Mutant

FIG. 3. Rapid scanning stopped-flow spectra of the reaction of K269R tryptophan indole-lyase with amino acids in 0.02 M potassium phosphate, pH 8.0, 0.16 M KCl. Panel A, reaction with 0.1 M L-alanine. Scans were collected at 8.54 ms (curve 1), 17.08 ms (curve 2), 25.62 ms (curve 3), 34.16 ms (curve 4), 42.70 ms (curve 5), 106.42 ms (curve 6), 427.00 ms (curve 7), 939.40 ms (curve 8), 1.708 s (curve 9), 4.270 s (curve 10), 5.978 s (curve 11), and 18.788 s (curve 12). Curve 0 is the spectrum of the enzyme in the absence of L-alanine. Panel B, reaction with 10 mM L-tryptophan. Scans were collected at the same time intervals as in the reaction with L-alanine (panel A). Curve 0 is the spectrum of the enzyme in the absence of L-tryptophan. Panel C, reaction with 50 mM S-methyl-L-cysteine. Scans were collected at 85.4 ms (curve 1), 17.08 ms (curve 2), 25.62 ms (curve 3), 34.16 ms (curve 4), 42.70 ms (curve 5), 111.02 ms (curve 6), 213.50 ms (curve 7), 384.30 ms (curve 8), 640.50 ms (curve 9), 1.281 s (curve 10), 2.220 s (curve 11), and 4.270 s (curve 12) after mixing. Curve 0 is the spectrum of the enzyme in the absence of S-methyl-L-cysteine. Panel D, reaction with 1 mM oxindolyl-L-alanine. Scans were collected at the same time intervals as in the reaction with L-alanine (panel A). Curve 0 is the spectrum of the enzyme in the absence of oxindolyl-L-alanine. Panel E, reaction with 3 mM S-benzyl-L-cysteine. Scans were collected at 8.54 ms (curve 1), 17.08 ms (curve 2), 25.62 ms (curve 3), 34.16 ms (curve 4), 42.70 ms (curve 5), 76.86 ms (curve 6), 128.10 ms (curve 7), 230.58 ms (curve 8), 384.30 ms (curve 9), 666.12 ms (curve 10), 1.025 s (curve 11), and 1.708 s (curve 12) after mixing. Curve 0 is the spectrum of the enzyme in the absence of S-benzyl-L-cysteine.
an external aldimine) that are also seen with wild-type tryptophan indole-lyase. Both L-alanine (Fig. 3A) and L-tryptophan (Fig. 3B) form intermediates with peaks at 330–340 nm, which may be gem-diamines or PLP Schiff base in the enolimine tautomeric form.

Single wavelength stopped-flow kinetic experiments were then performed with S-benzyl-L-cysteine. The apparent rate constant, \( k_{\text{obs}} \), for the formation of the quinonoid intermediate at 512 nm was previously found to exhibit a hyperbolic dependence on the concentration of S-benzyl-L-cysteine (Phillips, 1989); similar behavior was observed with the K269R mutant enzyme (Fig. 4). From these data, the rate constants for deprotonation, \( k_a \), and reprotonation, \( k_r \), of the \( \alpha \)-carbon of S-benzyl-L-cysteine were calculated by fitting the concentration dependence to Equation 5 (Table II). There is no significant difference between the wild-type and K269R enzymes in \( k_{\text{cat}} \), the equilibrium constant for external aldimine formation, but there is a 2.5-fold decrease in the rate constant for deprotonation and a 2.7-fold decrease in the rate constant for reprotonation (Table II). Furthermore, the intrinsic isotope effect was estimated by comparing the rate constants of normal and \( \alpha \)-deuterated S-benzyl-L-cysteine at 3.2 mM. Assuming the deuteriation has no effect on the binding equilibrium or on \( k_a \), as we found previously (Phillips, 1989), then the isotope effect for formation of the quinonoid intermediate of \([\alpha-\text{H}]\)S-benzyl-L-cysteine is reduced from 7.9 for the wild-type to 4.1 for the K269R enzyme.

We next determined the rate constant for breakdown of the quinonoid intermediate of S-benzyl-L-cysteine with the K269R mutant enzyme using an active intermediate trapping reaction with benzimidazole. When wild-type tryptophan indole-lyase is mixed with S-benzyl-L-cysteine in the presence of benzimidazole there is a rapid increase in absorbance at 512 nm, followed by a slower decline \( (k_{\text{obs}} = 10 \, \text{s}^{-1}) \), which occurs only in the presence of benzimidazole (Phillips, 1991). A new intermediate with \( \lambda_{\text{max}} \) at 345 nm in the presence of benzimidazole, which is likely to be an \( \alpha \)-aminoacylate complex (Phillips, 1991). When the K269R enzyme is reacted under these conditions, the decay of the quinonoid intermediate is considerably slower with a rate constant of 1.4 s\(^{-1}\). These results suggest that there is a 7.1-fold decrease in the rate of elimination of phenylmethanethiolate from the quinonoid intermediate of S-benzyl-L-cysteine in the K269R mutant of tryptophan indole-lyase.

**Inhibition by Oxindolyl-L-alanine**—With a \( K_i \) value of about 3 \( \mu \)M (Phillips et al., 1984; Kiick and Phillips, 1988), oxindolyl-L-alanine is a potent competitive inhibitor of wild-type tryptophan indole-lyase. The inhibition constant of oxindolyl-L-alanine for the K269R mutant enzyme was found to be 100 ± 10 \( \mu \)M. This 33-fold increase in the \( K_i \) is comparable to the 30–100-fold decrease in the \( k_{\text{cat}}/K_m \) values observed for substrates. This observation is consistent with our proposal that oxindolyl-L-alanine is a transition-state or reaction intermediate analogue for tryptophan indole-lyase (Phillips et al., 1984). Bartlett and Marlowe (1983) demonstrated that \( K_i \) values for transition-state analogue inhibitors are correlated with the \( K_i/k_{\text{cat}} \) values for the corresponding structurally analogous substrates.

We then examined the reaction of oxindolyl-L-alanine using single wavelength stopped-flow measurements at 506 nm. The time courses were measured at concentrations of oxindolyl-L-alanine from 0.05 to 2.5 mM, and the apparent rate constants for the reaction were fit to Equation 5. The rate of deprotonation of this analogue by the K269R enzyme is about 50-fold slower than that of the wild-type enzyme. However, the equilibrium constant for binding is not significantly affected (Table II), nor is the rate constant for reprotonation of the quinonoid anion \( (k_r) \) affected by the mutation (Table II). Thus, the decreased \( K_i \) for inhibition of the K269R enzyme by oxindolyl-L-alanine is primarily due to the reduction in the rate constant for quinonoid intermediate formation.

**DISCUSSION**

Tryptophan indole-lyase (tryptophanase) has been one of the most extensively investigated PLP-dependent enzymes. Chemical modification experiments have suggested a wide range of possible catalytically or structurally important residues, including cysteine (Raibaud and Goldberg, 1977; Watanabe and Snell, 1977; Honda and Tokushige, 1985; Nihira et al., 1985; Phillips and Gollnick, 1989), histidine (Nihira et al., 1979), arginine (Kazarinoff and Snell, 1977), tyrosine (Nihira et al., 1981), methionine (Oda and Tokushige, 1988),

![Graph](chart.png)

**FIG. 4.** Concentration dependence of quinonoid intermediate formation from S-benzyl-L-cysteine at 512 nm. The reactions were performed in 0.02 M potassium phosphate, pH 8.0, 0.16 M KCl, with concentrations of S-benzyl-L-cysteine ranging from \( 8 \times 10^{-5} \) M to \( 3.6 \times 10^{-3} \) M. Filled squares, wild-type tryptophan indole-lyase; filled circles, K269R mutant tryptophan indole-lyase.

**Table II**

| Enzyme                  | \( K_i \) | \( k_a \) | \( k_r \) | \( k_{\text{cat}}/K_m \) |
|-------------------------|----------|----------|----------|--------------------------|
| S-Benzyl-L-cysteine     |          |          |          |                          |
| Wild-type               | 2.3 ± 0.5 \times 10^{-4} M | 47.6 ± 4.7 s\(^{-1}\) | 15.7 ± 5.0 s\(^{-1}\) | 7.9 ± 1.5\(^{b}\) |
| K269R                  | 3.0 ± 0.4 \times 10^{-4} M | 7.7 ± 0.2 s\(^{-1}\) | 5.9 ± 0.3 s\(^{-1}\) | 4.1 ± 1.2\(^{b}\) |
| Oxindolyl-L-alanine     |          |          |          |                          |
| Wild-type               | 4.85 ± 0.30 mM\(^{c}\) | 84.6 ± 0.1\(^{d}\) | 0.2 ± 0.09\(^{d}\) |                           |
| K269R                  | 3.5 ± 1.65 mM\(^{c}\) | 1.6 ± 0.4 | 0.2 ± 0.02 |                           |

\(^{a}\) Isotope effect measured with \([\alpha-\text{H}]\)S-benzyl-L-cysteine.

\(^{b}\) From Phillips (1989).

\(^{c}\) From Phillips et al. (1990).

\(^{d}\) From Phillips et al. (1984).
and tryptophan (Tokushige et al., 1979; Tokushige and Idaka, 1985). However, there is no x-ray structure of the enzyme available at the present time, and the only amino acid whose role is firmly established is Lys-270, the residue which forms a Schiff base with the PLP in the resting form of the enzyme. There is an adjacent lysine residue at position 269 (Deeley and Yanofsky, 1981; Tokushige et al., 1989), and it has been found that many PLP-dependent enzymes have histidine, another basic amino acid, in the position immediately preceding the active site lysine. To probe the role of Lys-269 in tryptophan indole-lyase, we have changed this lysine to arginine using site-directed mutagenesis. This replacement is conservative, in that the positive charge is retained, but the higher basicity of the guanidine moiety might be expected to have dramatic effects on the acid-base properties of the mutant enzyme, especially if the lysine functions as an acid-base catalyst.

Replacement of Lys-269 by Arg Perturbs the pH Dependence of \( k_{\text{cat}}/K_m \) —The K269R mutant tryptophan indole-lyase exhibits much lower steady-state catalytic activity than does the wild-type enzyme (Table I). However, the pH dependence of \( k_{\text{cat}}/K_m \) for the K269R enzyme exhibits two \( pK_a \) values, as does the wild-type enzyme. Consequently, we conclude Lys-269 cannot be one of the essential basic catalytic groups. Nevertheless, we find that replacement of Lys-269 with arginine perturbs one of these \( pK_a \) values from a value of 6.0 in wild-type enzyme to about 7.6 in the mutant enzyme. A similar shift in the pH profile of \( k_{\text{cat}}/K_m \) was found for the C998S mutant of tryptophan indole-lyase (Phillips and Gollnick, 1989). We found previously that the pH profile of \( k_{\text{cat}}/K_m \) for the reaction of the wild-type enzyme with L-tryptophan exhibits two \( pK_a \) values of 6.0 and 7.6, while the reaction of S-methyl-L-cysteine exhibits only one \( pK_a \) of 7.8 (Kiick and Phillips, 1988). We attributed this difference to the possible formation of a hydrogen bond between the indole NH and the catalytic group with \( pK_a \) = 6.0. However, while the reaction of S-benzyl-L-cysteine also shows the \( pK_a \) of 6.0, the benzyl ring is incapable of hydrogen bonding. Thus, it is possible that protonation of the catalytic group with \( pK_a \) of 6.0 results in an alteration of charge which affects the interaction of substrates with the aromatic ring-binding pocket.

The Transition State for \( \alpha \)-Proton Abstraction Is Altered by the K269R Mutation —The minimum kinetic mechanism for the reaction of tryptophan indole-lyase with amino acid substrates is given in Equation 6, where \( k_3 \) is the isotope-sensitive \( \alpha \)-proton abstraction step.

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_4} EQ \xrightarrow{k_6} E + P
\]  

(6)

Since thiols are poor nucleophiles for the \( \beta \)-substitution reaction catalyzed by this enzyme (Watanabe and Snell, 1977), for S-alkyl cysteines, \( k_6 \approx 0 \), and therefore the reverse commitment can be neglected. Thus, \( \nu_1(k_{\text{cat}}/K_m) \) can be represented by Equation 7 (Northrup, 1975).

\[
\nu_1(k_{\text{cat}}/K_m) = 1 - \frac{k_4}{1 + C_i}
\]  

(7)

In the case of S-benzyl-L-cysteine, the stopped-flow data (Table II) give a minimum estimate of the intrinsic isotope effect, \( k_i/k_0 \), for both the wild-type and the K269R mutant enzyme are 2.6 and 0.10, respectively. The reduction in \( C_i \) for the K269R mutant enzyme indicates that \( k_0 \) must be significantly slower. However, the low values of \( k_{\text{cat}}/K_m \) for both the wild-type and K269R mutant enzymes indicate the C-H bond breakage is not the slowest step, even in the mutant enzyme. This conclusion is consistent with our pre-steady-state kinetic studies, demonstrating that the elimination of thiols from the quinonoid intermediates (\( k_i \)) is the rate-determining step in the steady-state reaction of S-alkyl-L-cysteines with tryptophan indole-lyase (Phillips, 1991). The rapid-scanning and single-wavelength stopped-flow spectrophotometric experiments clearly demonstrate that the K269R mutant enzyme forms quinonoid intermediates more slowly than does the wild-type tryptophan indole-lyase. In fact, with most of the amino acids examined, the K269R enzyme does not accumulate significant amounts of quinonoid intermediates (see Fig. 3). S-Benzyl-L-cysteine was the only substrate examined that was found to form a prominent quinonoid absorption band, albeit weaker than that of the wild-type enzyme. From the variation of \( k_{\text{cat}} \) with S-benzyl-L-cysteine, we were able to determine that the rate constant for quinonoid intermediate formation is reduced 6.2-fold in the K269R mutant enzyme, compared with the wild-type enzyme (Table II). In addition, the primary kinetic isotope effect is diminished from 7.9 for the wild-type enzyme to 4.1 for the K269R mutant enzyme (Table II). Furthermore, the rate constant for deprotonation of a potent inhibitor of the wild-type enzyme, oxindolyl-L-alanine, is reduced by more than 50-fold (Table II). This finding suggests that the transition state for the \( \alpha \)-proton abstraction has been altered by the Lys to Arg mutation, moving from symmetrical bond cleavage to bond formation to a later transition state, where there is no \( C-H \) bond breakage. Alternatively, the lower isotope effect could be due to a less rate-determining proton abstraction, possibly the consequence of a slower conformational change coupled with deprotonation. In addition, we find that the breakdown of the quinonoid intermediate formed from S-benzyl-L-cysteine by the K269R mutant enzyme is reduced by 7.1-fold. In contrast, the lysine to arginine mutation seems to have little effect on the transaldimination process, since the equilibrium constants for external aldimine formation from S-benzyl-L-cysteine and oxindolyl-L-alanine (\( K_e \) in Table II) are not significantly altered in the K269R mutant enzyme.

The pre-steady-state kinetic parameters for the reactions of wild-type and the K269R mutant enzymes with S-benzyl-L-cysteine are illustrated in the form of a free energy profile.
in Fig. 5. The mutation of Lys-269 to arginine can affect the internal rates but not the external thermodynamic equilibrium for the reaction. This mutation results in a displacement of the free energy profile to higher energy both for the ground state of the quinonoid intermediate and for the transition states leading to and from it. For other substrates, this displacement must be even greater, since the accumulation of quinonoid intermediates is much lower (Fig. 3, B and C). The \( \Delta G^2 \) value calculated from the difference in S-benzyl-L-cysteine \( k_{\text{cat}}/K_m \) values of wild-type and K269R enzymes in Table I is \(-2.22 \text{ kcal/mol}\), in reasonable agreement with the difference of \(-1.84 \text{ kcal}\) between the highest barriers in Fig. 5. Since the \( \beta \)-elimination reaction of S-alkyl cysteines is thermodynamically reversible (Kumagai et al., 1974), the free energy difference for the reaction must be relatively small, and the free energies of the substrate and product in this graph have been arbitrarily set at zero.

Lys-269 Plays an Important Role in the Modulation of Conformational Change during Catalysis—The dramatic redistribution of covalent intermediates observed with the K269R mutant enzyme suggests that Lys-269 is critically involved in conformational changes and/or electrostatic effects associated with formation and breakdown of the quinonoid intermediate, but does not play a role in the formation of the external aldimine or \( \text{gem} \)-diamine intermediates. We note that the effect of the mutation is solely on the rates and equilibria of distribution of interconverting species, and there is no discernible effect on the \( \lambda_{\text{max}} \) or band shape of the quinonoid intermediates. However, there is a notable blue shift in the \( \lambda_{\text{max}} \) of the internal aldimine, from 420 nm for wild-type enzyme to 412 nm for the K269R mutant enzyme (Fig. 2). It will be of interest to examine the effects of mutation of Lys-269 to other basic (e.g. histidine), neutral (e.g. glutamine), or acidic (e.g. glutamic acid) residues.

REFERENCES

Bartlett, P. A., and Marlowe, C. K. (1983) Biochemistry 22, 4618-4624

Bossa, F., Martini, F., Barra, D., Borri Voltattorni, C., Minelli, A., and Turano, C. (1977) Biochem. Biophys. Res. Commun. 78, 177-184

Cleland, W. W. (1979) Methods Enzymol. 63, 103-138

Although formation of S-alkyl-L-cysteines from thiols, pyruvate, and ammonium is thermodynamically feasible, tryptophan indole-lyase does not catalyze this reaction efficiently.