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# The phosphate of pyridoxal-5′-phosphate is an acid/base catalyst in the mechanism of *Pseudomonas fluorescens* kynureninase

Robert S. Phillips[1,2], Israel Scott[1], Riya Paulose[2], Akshay Patel[2] and Taylor Colt Barron[1]

1 Department of Biochemistry and Molecular Biology, University Georgia, Athens, GA, USA
2 Department of Chemistry, University Georgia, Athens, GA, USA

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**Correspondence**
R. S. Phillips, Department of Chemistry, University of Georgia, Athens, GA 30602-2556, USA
Fax: +1 706 542 9454
Tel: +1 706 542 1996
E-mail: plp@uga.edu

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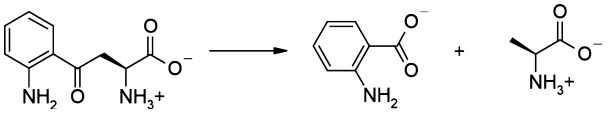
Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) catalyzes the hydrolytic cleavage of L-kynurenine to L-alanine and anthranilic acid. The proposed mechanism of the retro-Claisen reaction requires extensive acid/base catalysis. Previous crystal structures showed that Tyr226 in the *Pseudomonas fluorescens* enzyme (Tyr275 in the human enzyme) hydrogen bonds to the phosphate of the pyridoxal-5′-phosphate (PLP) cofactor. This Tyr residue is strictly conserved in all sequences of kynureninase. The human enzyme complexed with a competitive inhibitor, 3-hydroxyhippuric acid, showed that the ligand carbonyl O is located 3.7 Å from the phenol of Tyr275 (Lima, S., Kumar, S., Gawandi, V., Momany, C. & Phillips, R. S. (2009) *J. Med. Chem.* **52**, 389–396). We prepared a Y226F mutant of *P. fluorescens* kynureninase to probe the role of this residue in catalysis. The Y226F mutant has approximately 3000-fold lower activity than wild-type, and does not show the p$K_a$ values of 6.8 on $k_{cat}$ and 6.5 and 8.8 on $k_{cat}/K_m$ seen for the wild-type enzyme (Koushik, S. V., Moore, J. A. III, Sundararaju, B. & Phillips, R. S. (1998) *Biochemistry* **37**, 1376–1382). Wild-type kynureninase shows a resonance at 4.5 ppm in $^{31}$P-NMR, which is shifted to 5.0, 3.3 and 2.0 ppm when the potent inhibitor 5-bromodihydrokynurenine is added. However, Y226F kynureninase shows resonances at 3.6 and 2.5 ppm, and no change in the peak position is seen when 5-bromodihydrokynurenine is added. Taken together, these results suggest that Tyr226 mediates proton transfer between the substrate and the phosphate, which accelerates formation of external aldimine and *gem*-diol intermediates. Thus, the phosphate of PLP acts as an acid/base catalyst in the mechanism of kynureninase.

## Introduction

Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that catalyzes the hydrolytic $C_\beta$–$C_\gamma$ cleavage of L-kynurenine to alanine and anthranilic acid (Eqn 1) [1]:

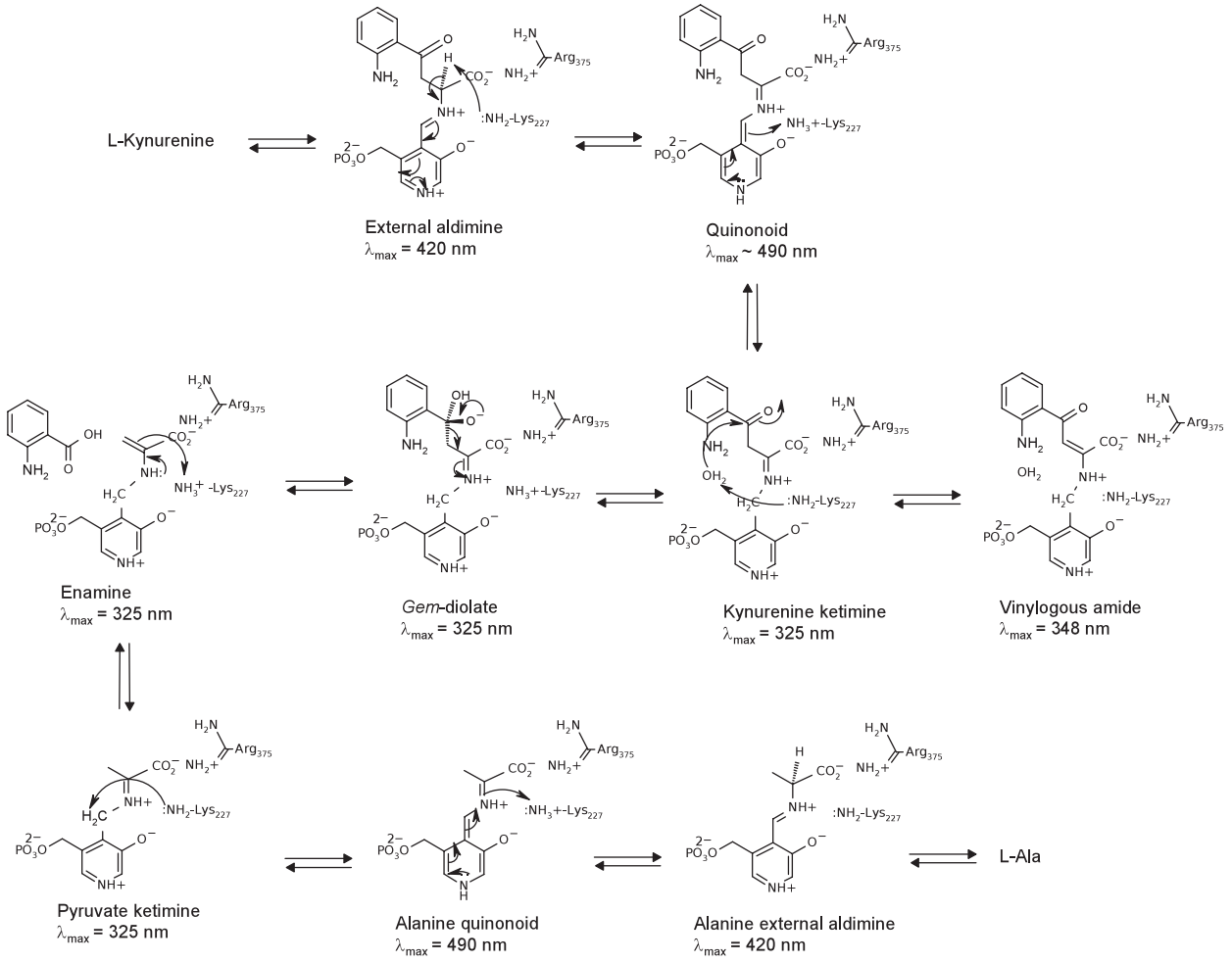



This is one of a small group of enzymes that catalyze a retro-Claisen reaction. The enzyme is part of the major eukaryotic pathway for tryptophan catabolism, and is also found in a number of bacteria. However, the eukaryotic enzyme has a reaction preference for 3-hydroxykynurenine, while most bacterial enzymes react preferentially with kynurenine [1].

**Abbreviation**
PLP, pyridoxal-5′-phosphate.

The reaction of kynureninase is relatively unusual among PLP-dependent enzymes in that it occurs with electrophilic rather than nucleophilic substitution at $C_\beta$. The proposed mechanism of kynureninase, shown in Scheme 1, requires extensive acid/base catalysis [2–6]. Initially, trans-aldimination of the substrate with the internal aldimine gives the external aldimine. Deprotonation of $C_\alpha$ then forms a quinonoid structure, which is reprotonated at C-4′ to give the kynurenine ketimine. Subsequent addition of water to the γ-carbonyl of the ketimine intermediate forms a *gem*-diol, which then undergoes $C_\beta$–$C_\gamma$ bond cleavage to give the carboxylic acid product and an enamine intermediate. Protonation of the enamine on $C_\beta$ gives a pyruvate ketimine, which is deprotonated on C-4′ to give the L-alanine quinonoid structure. Finally, protonation of the L-alanine quinonoid intermediate on $C_\alpha$ gives the L-alanine aldimine, which releases the L-alanine product.

The crystal structure of the complex of human kynureninase bound to a competitive inhibitor, 3-hydroxyhippuric acid, shows the carbonyl oxygen of the inhibitor located near the strictly conserved residues Tyr275 and Ser75, with 3.7 Å between the carbonyl O and the Tyr275 phenolic O [7]. Tyr275 also donates a hydrogen bond to the phosphate of the PLP [8], as does the homologous Tyr226 in *Pseudomonas fluorescens* kynureninase [9]. We hypothesized from the structural data that this Tyr may act as a shuttle to transfer a proton from PLP to the substrate γ-carbonyl oxygen, to accelerate formation of the *gem*-diol. Hence, we mutated Tyr226 in *P. fluorescens* kynureninase to phenylalanine to assess the role of this tyrosine in catalysis, and we performed $^{31}$P-NMR on wild-type and Y226F kynureninase to examine the ionization state of the phosphate in the resting enzyme and complexed with a potent inhibitor. The results of these experiments are reported here.




**Scheme 1.** Proposed mechanism for kynureninase.

## Results

### Kinetic properties of Y226F kynureninase

Y226F mutant kynureninase has very low catalytic activity compared to wild-type enzyme, with a $k_{cat}$ that is reduced approximately 2800-fold, and a $k_{cat}/K_m$ that is reduced approximately 376-fold (Table 1). The binding of PLP to Y226F kynureninase is not strongly affected, as the isolated enzyme contains 1 mol PLP/subunit. However, the apparent $K_m$ of Y226F kynureninase for PLP under assay conditions is 21 μM, compared with 0.6 μM for the wild-type enzyme [9]. Wild-type kynureninase normally shows no significant increase in initial rate with added PLP, with the function of the PLP being primarily to increase the length of the linear portion of the reaction because of competing abortive trans-amination of the L-alanine product [10]. The reaction of Y226F kynureninase shows no significant pH dependence for either $V_{max}$ or $V_{max}/K_m$ (Fig. 1). It was shown previously that wild-type kynureninase has a single base, with a p$K_a$ of 6.8, in the pH dependence of $k_{cat}$ [3]. The pH dependence of $k_{cat}/K_m$ for wild-type kynureninase has a bell-shaped curve, with a base with a p$K_a$ of 6.5 and an acid with a p$K_a$ of 8.8 [3]. The pH dependence of $k_{cat}/K_m$ for the reaction with 3-hydroxykynurenine, a slower substrate, gave a p$K_a$ of 6.8 for the basic group [3]; hence, kynurenine is a 'sticky substrate'. Although we attempted to fit the data in Fig. 1 to a pH dependence similar to wild-type kynureninase, the results were not reliable, and a simple linear regression fit is more reasonable, as may be seen from the lines in Fig. 1A,B. Thus, it may be concluded that the Y226F mutation strongly affects the pH dependence of the reaction, and the conjugate base with a p$K_a$ of 6.5 in wild-type kynureninase must be either Tyr226 or a group closely associated with it.

We also examined the reaction of wild-type and Y226F kynureninase with *O*-benzoyl-L-serine. *O*-benzoyl-L-serine is an alternate substrate for kynureninase, which undergoes a normal β-elimination reaction to give benzoate, pyruvate and ammonium, as previously shown for tyrosine phenol lyase and tryptophan indole lyase [11]. The formation of pyruvate was followed

**Table 1.** Kinetic parameters for wild-type and Y226F kynureninase.

| | Wild-type | | | Y226F | | |
|---|---|---|---|---|---|---|
| Compound | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | $K_i$ (nM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | $K_i$ (nM) |
| L-kynurenine | 16[a] | $2.0 \times 10^5$ [a] | – | $(5.8 \pm 0.3) \times 10^{-3}$ | 530 ± 85 | – |
| *O*-benzoyl-L-serine | 2.5 ± 0.2 | $(1.1 \pm 0.1) \times 10^4$ | – | 0.10 ± 0.02 | 41 ± 11 | – |
| *S*-(2-aminophenyl)-L-cysteine dioxide | – | – | 30[a] | – | – | 920 ± 240 |

[a] Data from reference 13.

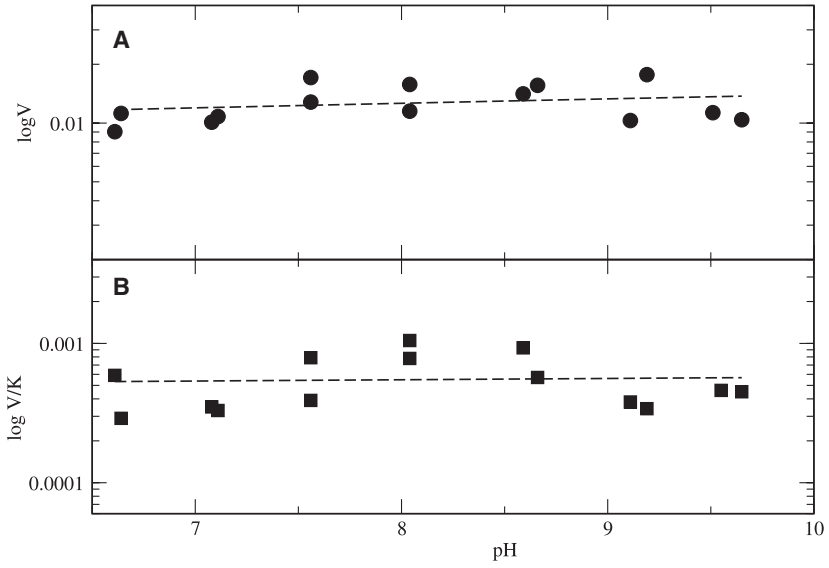



**Fig. 1.** pH dependence of Y226F kynureninase. (A) pH dependence of $V_{max}$ values. The line is a linear regression fit to the data. (B) pH dependence of $V_{max}/K_m$ values. The line is a linear regression fit to the data.

using a coupled assay with lactate dehydrogenase and NADH. β-substituted amino acids with good leaving groups, such as β-chloroalanine, were previously shown to undergo β-elimination reactions with kynureninase [12]. However, *O*-benzoyl-L-serine is a better substrate for kynureninase than the compounds studied previously, probably due to its closer structural similarity to kynurenine. The reaction of *O*-benzoyl-L-serine is less affected than L-kynurenine by the Y226F mutation, with the $k_{cat}$ value being reduced only 25-fold and $k_{cat}/K_m$ being reduced 280-fold (Table 1). Finally, binding of a potent transition state analog inhibitor, *S*-(2-aminophenyl)-L-cysteine *S*,*S*-dioxide [13,14], is weakened approximately 30-fold for the Y226F mutant kynureninase (Table 1).

### Spectrum of Y226F kynureninase

The UV-vis spectrum for Y226F kynureninase as isolated shows an absorbance peak at 416 nm (Fig. 2), i.e. slightly blue shifted compared to wild-type kynureninase. Addition of the product, L-alanine, to Y226F does not result in any immediate changes in the spectrum, in contrast to wild-type enzyme, but, upon incubation, pyridoxamine-5′-phosphate, absorbing at 325 nm, is formed slowly in an abortive trans-amination reaction (Fig. 2), as also seen with the wild-type enzyme. The rate constant for the abortive trans-amination, $7.3 \times 10^{-5}$ $s^{-1}$, is approximately 30-fold slower for Y226F than for the wild-type enzyme, which shows a $t_{½}$ of 3–5 min for the abortive trans-amination of alanine [10]. Addition of other substrates and inhibitors, i.e. kynurenine [4], β-benzoylalanine [5] and *S*-(2-aminophenyl)-L-cysteine *S*,*S*-dioxide [13,14] did not result in significant changes in the mutant enzyme spectrum, in contrast to wild-type kynureninase.

## $^{31}$P-NMR of kynureninase

The $^{31}$P-NMR spectrum of *P. fluorescens* kynureninase exhibits a peak at 4.6 ppm (Fig. 3A, solid line) consistent with a dianionic phosphate ester group in the resting enzyme, as is characteristic of PLP-dependent enzymes [15,16]. The line width is approximately 23 Hz, consistent with restricted motion of the phosphate around the C–O bond. The peak position is at a lower field than normal for PLP-dependent enzymes, but is quite similar to what is observed for aspartate aminotransferase and *O*-acetyl serine sulfhydrylase [15,17]. However, when a potent transition-state analog inhibitor, 5-bromodihydrokynurenine [13], is added, the spectrum becomes more complex, with peaks at approximately 2.0, 3.3 and 5.0 ppm (Fig. 3A, dashed line). The upfield shift of the 2 ppm peak in the ligand complex by approximately 3 ppm is consistent with formation of a monoanionic phosphate upon ligand binding [15,16]. In contrast, the $^{31}$P-NMR spectrum of Y226F kynureninase exhibits peaks at 2.5 and 3.6 ppm (Fig. 3B, solid line), which do not change significantly upon addition of 5-bromodihydrokynurenine (Fig. 3B, dashed line), although there is a change in relative intensity.

## Discussion

The mechanism proposed for kynureninase requires nucleophilic addition of water to the γ-carbonyl of the substrate (Scheme 1), so it is likely that the enzyme will

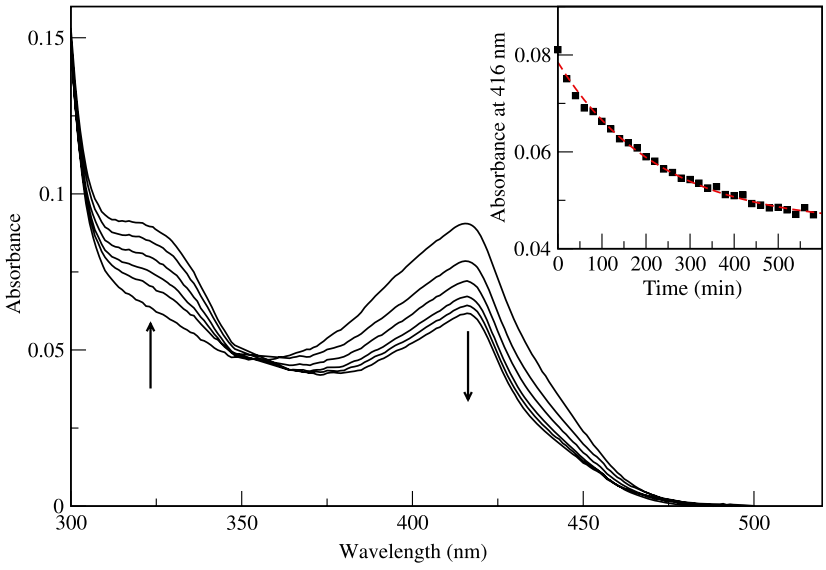



**Fig. 2.** Visible spectra for Y226F kynureninase. The solution contained 1 mg·mL$^{-1}$ Y226F kynureninase in 0.05 M potassium phosphate, pH 8.0, 100 mM L-alanine. Spectra were collected at 30 min intervals. Inset: Absorbance at 416 nm as a function of incubation time. The line is the result of fitting to a single exponential decay.

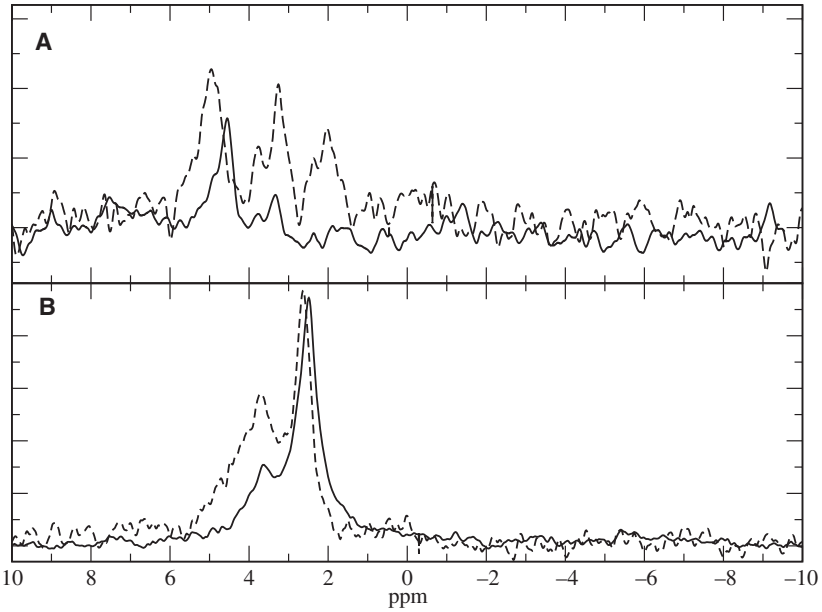



**Fig. 3.** $^{31}$P-NMR spectra for kynureninase. (A) Wild-type kynureninase. The concentration of the enzyme was 1 mM in 0.05 M bicine-Na, pH 8.0. Solid line, wild-type enzyme; dashed line, 1 mM wild-type enzyme with 2 mM 5-bromodihydrokynurenine. (B) Y226F kynureninase. The concentration of the enzyme was 1.1 mM in 0.05 M bicine-Na, pH 8.0. Solid line, Y226F mutant enzyme; dashed line, 1 mM Y226F mutant enzyme with 2 mM 5-bromodihydrokynurenine.

polarize the carbonyl to increase its reactivity. In the serine proteases, the reactive carbonyl of substrates is located in an 'oxyanion hole', which is a pocket of hydrogen bond donors that polarizes the carbonyl and stabilizes the tetrahedral intermediate [18]. The phenolic group of Tyr275 is located near the carbonyl oxygen of 3-hydroxyhippurate, a competitive inhibitor, that is bound at the active site of human kynureninase [7], with an O–O distance of 3.7 Å (Fig. 4). This tyrosine residue is located in the active site of kynureninase, in the sequence just prior to the PLP-binding lysine, is strictly conserved (Fig. 5), and was found to form a hydrogen bond with the PLP phosphate in the crystal structures of *P. fluorescens* and human kynureninase, with a tyrosine O–$PO_4$ distance of 2.6 Å (Fig. 4) [6,8]. This hydrogen bond is maintained in the structure of human kynureninase with 3-hydroxyhippuric acid bound, as may be seen from the position of the phenolic O of Tyr275 in Fig. 4, located between the phosphate O and the 3-hydroxyhippuric acid amide carbonyl O. Thus, we postulated that this tyrosine residue (Tyr226 in *P. fluorescens* kynureninase) may serve as part of an oxyanion hole to facilitate addition of water to the substrate γ-carbonyl, by analogy with serine proteases. To test this hypothesis, we prepared the Y226F mutant of *P. fluorescens* kynureninase, and evaluated its physical and catalytic properties. The *P. fluorescens* enzyme was chosen for these studies, rather than the human enzyme, because of its much higher expression in recombinant systems, ease of purification, and the fact that the majority of previous kinetic and mechanistic data have been obtained using it.

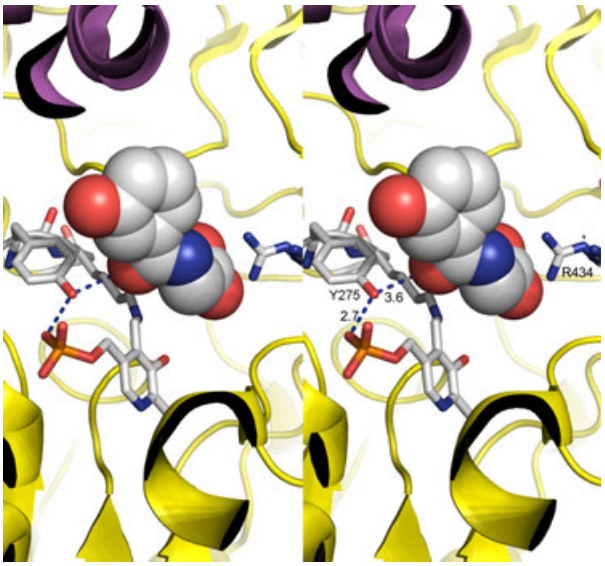



**Fig. 4.** 3-hydroxyhippurate bound in the active site of human kynureninase. The hydrogen bonds are indicated by blue dashed lines, and 3-hydroxyhippurate is shown as a space-filling model. The hydrogen bond distances in Å are indicated. The figure was created using PDB structure 3E9K with Pymol.

Y226F *P. fluorescens* kynureninase has low, but measurable, activity with L-kynurenine, with a $k_{cat}$ value almost 2800-fold lower and a $k_{cat}/K_m$ ratio almost 375-fold lower than those of the wild-type enzyme (Table 1). Thus, the effect of the Y226F mutation on $k_{cat}$ is approximately 7.5-fold greater. We have shown that the rate-determining step for wild-type kynureninase is release of the second product, L-alanine

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                                  #                                *
Xanthomonas      IVRLARAQGA AVGCDLAHAV GNIPLTLHDD GVDFAVWCNY KYLNAGPGAV
Cytophaga        ISQATHLAGA FVGFDLAHAI GNIPLHLHDW KVDFAVWCSY KYLNGGPGAV
Human            ITKAGQAKGC YVGFDLAHAV GNVELYLHDW GVDFACWCSY KYLNAGAGGI
Dog              ITRAGQAKGC FVGFDLAHAV GNVELHLHDW GVDFACWCSY KYLNSGAGGL
Rat              ITQAGHAKGC FVGFDLAHAV GNVELHLHDW DVDFACWCSY KYLNSGAGGL
Mouse            ITKAGHAKGC FVGFDLAHAV GNVELRLHDW GVDFACWCSY KYLNSGAGGL
Chicken          ITKAGQKKGC FVGFDLAHAV GNVELCLHDW GVDFACWCTY KYLNSGAGGL
Zebra fish       ITTAGHSKGC FVGFDCAHAV GNAELRLHDW NVDFACWCTY KYMNSGAGGL
Caenorhabditis   ITEAGHRKGC FVGFDLAHAF ANVPLHLHWW DVDFACWCSY KYGCTGAGSI
Dictyostelium    ITEVGHEIGA IVGWDLAHAA GNVELSLHDW NVDFACWCTY KYLNSGPGCI
Aspergillus      ITAHAQSKGI LVGWDCAHAA GNVDLQLHDW NVDFAAWCTY KYLNSGPGGT
P. fluorescens   LTALSHECGA LAIWDLAHSA GAVPVDLHQA GADYAIGCTY KYLNGGPGSQ
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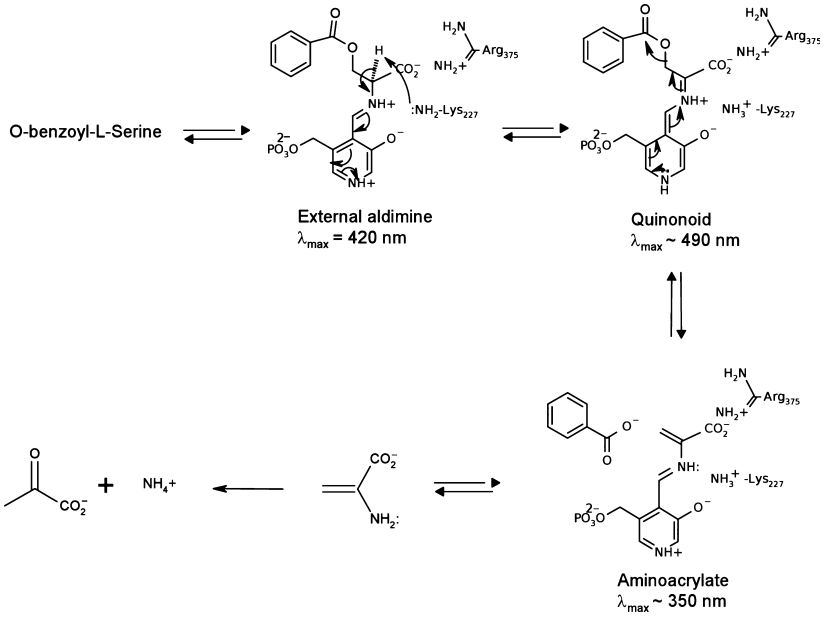
**Fig. 5.** Sequence alignments of kynureninases. The active site Lys227 is indicated by an asterisk, the PLP-binding Asp203 residue is indicated by a hash symbol (#), and the Tyr226 residue is shown with a black background.

[3,6], but based on the mechanism in Scheme 1, the mutation is more likely to affect the rate of formation of the first product, anthranilate, which occurs at 54 $s^{-1}$. If the rate-determining step for the Y226F mutant enzyme is anthranilate formation, then the effect of the mutation is an 8600-fold reduction. In contrast, the Y226F mutation has a much smaller effect on both PLP binding and alanine trans-amination. The effect of the Y226F mutation on $k_{cat}$ for the *O*-benzoyl-L-serine reaction is only 25-fold, approximately 100-fold less than the effect on kyurenine (Table 1). This is consistent with a different rate-determining step for the two substrates. However, the effect of the Y226F mutation on $k_{cat}/K_m$ for the elimination reaction of *O*-benzoyl-L-serine, a 268-fold decrease, is similar to that for L-kynurenine (Table 1). The mechanism for reaction of *O*-benzoyl-L-serine is simpler, as the enzyme needs only to form the external aldimine and quinonoid intermediates, as shown in Scheme 2.

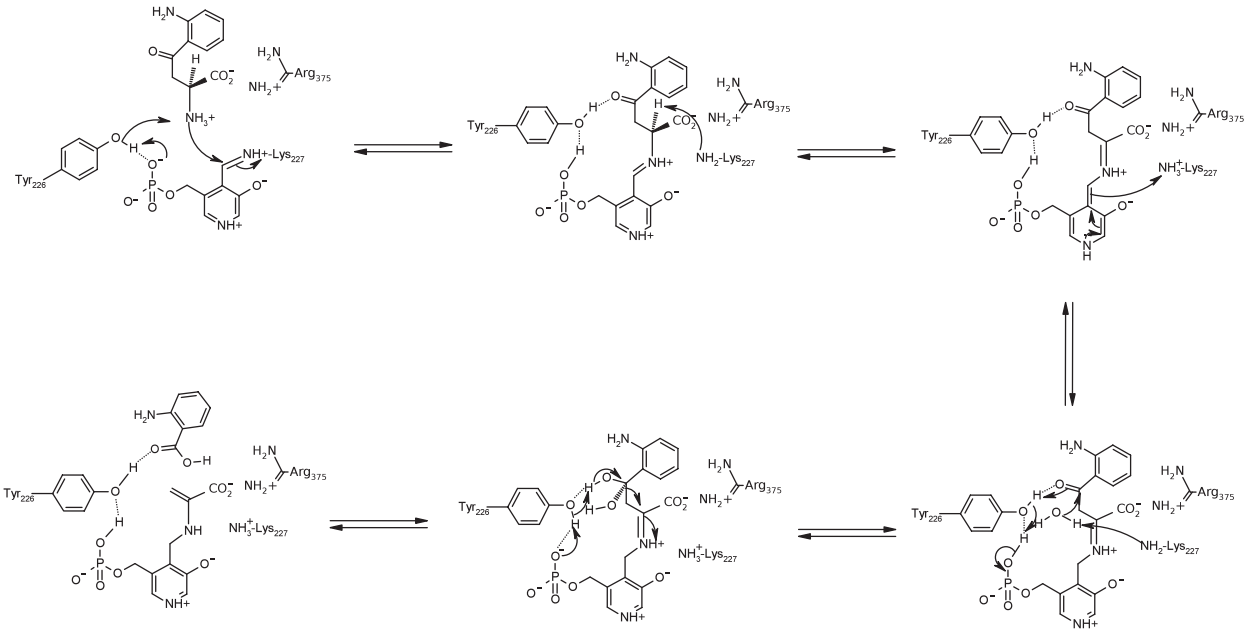
The ionization of a catalytic base with an apparent p$K_a$ of 6.5 in the pH dependence of $k_{cat}/K_m$ for kynurenine with wild-type kynureninase is not seen in the Y226F mutant (Fig. 1B). The real p$K_a$ of the enzyme is 6.8, as that is the p$K_a$ of the reaction with 3-hydroxykynurenine, a slow substrate. It seems unlikely that the Tyr226 phenol has a p$K_a$ of 6.8, as it is hydrogen-bonded to the dianionic phosphate, so we believe it is more likely that this ionization corresponds to that of the phosphate group of PLP. Furthermore, the wild-type enzyme shows no changes in the UV-vis absorption spectrum with pH, so the basic group is not the Schiff's base of the PLP. Phosphate esters, including PLP, are well known to exhibit p$K_a$ values close to pH 6.8. The $^{31}$P-NMR of wild-type kynureninase shows a peak of 5.0 ppm at pH 8.0, consistent with a dianionic phosphate, as is seen with all PLP enzymes [15]. We attempted to obtain the NMR spectrum of kynureninase at pH 6.0 to determine whether an upfield shift may be seen that may be attributed to a phosphate monoanion, but the enzyme precipitated. However, in the presence of a potent competitive inhibitor, 5-bromodihydrokynurenine, at pH 8, we observed two new peaks with an upfield shift in position, at 3.3 and 2.0 ppm (Fig. 3A). This 3 ppm upfield shift to




**Scheme 2.** Proposed mechanism for reaction of *O*-benzoyl-L-serine.

2.0 ppm suggests that ligand binding results in a complex with partial formation of the monoanionic phosphate ester even at pH 8. A change in the ionization state of the PLP phosphate on ligand binding has not been observed for other PLP-dependent enzymes [15]. The $^{31}$P-NMR spectrum of Y226F kynureninase shows resonances at 3.7 and 2.5 ppm, suggesting that the phosphate is in the monoanionic form, and there is no further shift upon addition of 5-bromodihydrokynurenine (Fig. 3B). The loss of basic ionization in the pH dependence and the effect of mutation on the $^{31}$P-NMR spectrum of the Y226F mutant enzyme suggests that Tyr226 plays a role in the protonation state of the PLP phosphate group. Furthermore, the short hydrogen bond between Tyr226 and the phosphate oxygen demonstrates that Tyr226 cannot be a proton donor independently of the phosphate.

We propose that Tyr226, which is hydrogen-bonded to the PLP phosphate in the free enzyme, accepts a proton from the incoming substrate ammonium group to initiate formation of the external aldimine (Scheme 3), and concomitantly releases a proton to the phosphate, resulting in the monoprotonated phosphate hydrogen-bonded to the phenol of Tyr226. This is also supported by the pH dependence of $k_{cat}/K_m$ for wild-type kynureninase, which shows an acidic group with a p$K_a$ of 8.8, which we assigned to the substrate zwitterion [3]. The lack of such ionization in the Y226F mutant enzyme suggests that both the zwitterionic and anionic forms of kynurenine may bind. There must be an alternative base for deprotonation of the zwitterion, possibly the PLP 3′-O, in the Y226F mutant enzyme. Furthermore, deprotonation of the amino acid substrate and subsequent trans-aldimination results in release of Lys227 as the free base, which is required in subsequent steps to form the quinonoid intermediate and the ketimine. The rate-limiting step in the reaction of wild-type kynureninase is deprotonation of C-4′ of the pyruvate ketimine [3]. Hence, it is likely that the basic group with a p$K_a$ of 6.8 seen in the pH dependence of $k_{cat}$ is that of Lys227, rather than the PLP phosphate. This ionization is not seen in the pH dependence of $k_{cat}$ of Y226F kynureninase, consistent with a change in the rate-limiting step. Lysine residues in enzyme active sites are often found to have p$K_a$ values reduced by 3–4 pH units, for example, in the case of acetoacetate decarboxylase, with a p$K_a$ of 5.9 [19]. At the kynurenine ketimine stage, Tyr226 donates a hydrogen bond to the γ-carbonyl oxygen of the substrate, resulting in polarization of the carbonyl, and mediates transfer of a proton to it from the phosphate when hydroxide attacks the carbonyl, assisted by Lys227 as a general base (Scheme 3). It is interesting that an off-pathway vinylogous amide intermediate, with $\lambda_{max}$ = 348 nm, accumulates during turnover, apparently arising from accidental deprotonation of the rather acidic β-carbon, located between a carbonyl and an iminium ion, either by Lys227 or by the nascent hydroxide, in competition with carbonyl hydration [5,6]. This intermedi-

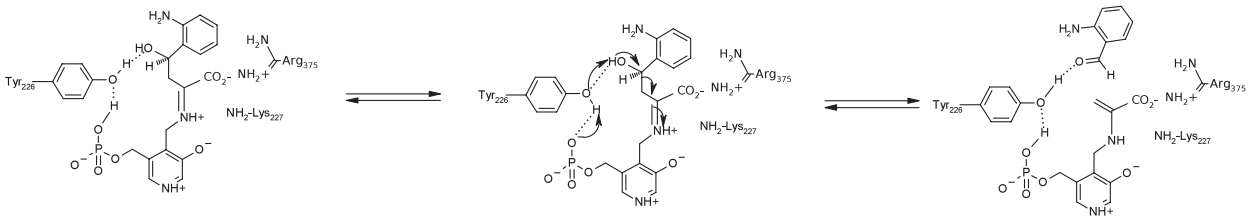


**Scheme 3.** Proposed role of Y226 in the mechanism of kynureninase.

ate is in rapid equilibrium with the ketimine, so it does not impair catalysis. The 30-fold weaker binding of the potent transition state analog inhibitor, *S*-(2-aminophenyl)-L-cysteine *S*,*S*-dioxide, to Y226F kynureninase (Table 1) may be due to loss of a hydrogen bond from the tyrosine to the sulfone oxygen, which structurally resembles the *gem*-diol intermediate. Furthermore, this mechanism is consistent with the stereochemical properties of the dihydrokynurenines, whereby the (αS,4R) diastereomer is a slow substrate for a retro-aldol reaction, and the (αS,4S) isomer is a potent competitive inhibitor [2,13]. Based on this mechanism, the low $k_{cat}$ of the (αS,4S) isomer of dihydrokynurenine may be due to the active form of the enzyme being the low-concentration dianionic protonation state of the phosphate, as proton abstraction from the alcohol is required to initiate the retro-aldol reaction, rather than proton donation (Scheme 4). In the reaction of kynurenine, the proton is donated to the carbonyl oxygen from the phosphate as the *gem*-diol is formed, through mediation of Tyr226, and then returned to the phosphate, initiating the retro-Claisen reaction resulting in $C_\beta$–$C_\gamma$ cleavage (Scheme 3).

If the mechanism in Scheme 3 is correct, how does the Y226F mutant enzyme have any remaining activity? There is another conserved tyrosine residue, Tyr228 in *P. fluorescens* and Tyr277 in human kynureninase, located just after the active-site lysine (Fig. 5). However, in the structure of human kynureninase with 3-hydroxyhippurate bound, the phenol of Tyr277 is 11.3 Å from the ligand carbonyl oxygen and 15.0 Å from the nearest phosphate oxygen, so Tyr228 is unlikely to be able to substitute in the Y226F mutant enzyme. In the Y226F mutant, it is possible that a water molecule may form a bridge between the phosphate and the substrate γ-carbonyl oxygen, replacing the Tyr OH and allowing reaction to proceed more slowly. Alternatively, the *gem*-diol intermediate may be partly stabilized by other residues. In this regard, it is interesting that there is another strictly conserved residue, Ser75 (Ser35 in *P. fluorescens*) with the γ-O located 4.5 Å from the 3-hydroxyhippurate carbonyl oxygen. Although the geometry of Ser35 is not ideal for hydrogen bonding in the 3-hydroxyhippurate complex, the distance may be reduced after trans-aldimination and the geometry may improve. Once the anthranilate product is released, Tyr226 appears to have little or no role in the subsequent enamine to ketimine conversion steps (Scheme 1). The principle of microscopic reversibility suggests that the proton on the phosphate is ultimately donated via Tyr226 back to the amino group of the L-alanine product as it leaves the active site, thus re-setting the enzyme in the proper ionization state for another catalytic cycle. This explains the low reactivity of Y226F in the abortive trans-amination with alanine.

There are very few examples of PLP-dependent enzymes that catalyze a reaction with amino acid substrates using the phosphate group of the cofactor as an auxiliary acid/base catalyst. The phosphate group of PLP is generally assumed to be only a 'handle' for the enzyme to hold the cofactor during catalysis, when the covalent attachment with the lysine is lost. Interestingly, for threonine synthase, the phosphate product from elimination of γ-phosphohomoserine was shown to be catalytically active in water addition to the aminocrotonate intermediate [20]. Glycogen phosphorylase contains PLP, and the phosphate group has been shown to be the catalytically essential part of the PLP molecule [21], and the pyridine ring provides the non-catalytic 'handle' for the enzyme. It is interesting that the phosphate group must be dianionic in glycogen phosphorylase, and recent evidence suggests that it does not function via simple acid/base catalysis [21]. It has been proposed on the basis of X-ray crystal structures that L-serine dehydratase [22] and D-serine dehydratase [23] use the phosphate of the PLP both as a base to facilitate external aldimine formation and as an acid to assist in elimination of water from the substrate. However, in contrast to this hypothesis, $^{31}$P-NMR studies of D-serine dehydratase bound to a substrate analog (isoserine) do not show formation of




**Scheme 4.** Proposed mechanism for reaction of dihydrokynurenine.

the phosphate monoanion [15], but instead show stabilization of the dianion, even at acidic pH values. Thus, the evidence supports the hypothesis that the reaction mechanism of kynureninase requires catalysis using both the electronic properties of the pyridine ring and the acid/base properties of the phosphate moiety of PLP.

## Experimental procedures

### Materials

5-bromodihydrokynurenine was prepared by $NaBH_4$ reduction of 5-bromo-L-kynurenine as previously described [13], and used as the obtained 60:40 mixture of diastereomers. *O*-benzoyl-L-serine [11] and *S*-(2-aminophenyl)-L-cysteine *S*, *S*-dioxide [14] were prepared as described previously. Buffers and reagents were purchased from Fisher Scientific Co (Waltham, MA, USA).

### Enzymes

Y226F mutant kynureninase was prepared using a modification of the QuikChange protocol (Stratagene, Santa Clara, CA, USA), using plasmid pTZKyn [22] as the template with mutagenic primers 5′-CTACGCCATCGGTTGCACTTTC AAAT-3′ (forward) and 5′-GACCGCCATTGAGGTATTT GAAAGTG-3 (reverse) (mutated positions are underlined). The resulting Y226F mutant plasmid was transformed into *Escherichia coli* TOP-10 cells (Invitrogen, Carlsbad, CA, USA), colonies were selected, and plasmids were isolated, sequenced to confirm the mutation, and transformed into *E. coli* DH5α cells for expression. Cell were grown, and wild-type and Y226F *P. fluorescens* kynureninases were purified by phenyl-Sepharose chromatography as described previously [24]. The concentration of enzyme was estimated from the absorbance at 280 nm, using a value for $A_{280}^{1\%}$ of 14.3 [25]. The PLP content was determined by the absorption of the protein at 388 nm in 0.1 M NaOH [26].

### Kinetic studies

Kinetics measurements were performed on a Cary 1 UV-vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 6 × 6 Peltier temperature-controlled cell compartment. The kinetic studies were performed at 37 °C in 40 mM potassium phosphate, pH 8.0, 50 μM PLP, with varying amounts of substrate, by following the absorbance decrease at 360 nm ($\Delta\varepsilon = -4500\ M^{-1}\ cm^{-1}$) as kynurenine is converted to anthranilic acid. For the reaction of *O*-benzoyl-L-serine, formation of pyruvate was followed at 340 nm by addition of 20 $\mu g \cdot mL^{-1}$ rabbit muscle lactate dehydrogenase (US Biochemicals, Cleveland, OH, USA) and 0.1 mM NADH ($\Delta\varepsilon = -6220\ M^{-1}\ cm^{-1}$). The kinetic parameters were obtained by fitting the data to Eqns (2,3), using HYPERO for $k_{cat}$ and $k_{cat}/K_m$ and COMPO for $K_i$ [27]:

$$V = V_{max}S/(K_m + S) \quad (2)$$

$$V = V_{max}S/(K_m(1 + I/K_i) + S) \quad (3)$$

The $k_{cat}$ and $k_{cat}/K_m$ parameters were obtained from $V_{max}$ and $V_{max}/K_m$ using the enzyme molecular weight of 47 kDa [21]. The pH dependence data were obtained in 0.1 M triethanolamine phosphate buffers adjusted to pH values ranging from 6.5 to 9.5. The actual pH of the reaction mixtures was measured at the end of data collection.

### $^{31}$P-nmr

Wild-type *P. fluorescens* kynureninase was passed through a PD-10 gel (GE Healthcare Life Sciences, Pittsburgh, PA, USA) filtration column equilbrated with 0.05 M bicine-Na, pH 8, to remove phosphate buffer, and concentrated using a Centricon centrifugal filter over a YM-30 membrane (Millipore, Billerica, MA, USA) to a final volume of 0.50 mL. $D_2O$ (50 μL) was then added for the field frequency lock. The final concentration of the enzyme was 1 mM (47 $mg \cdot mL^{-1}$). $^{31}$P-NMR data were collected at ambient temperature (approximately 20 °C) on a Varian Mercury 400 (Aligent Technologies, Santa Clara, CA, USA) instrument operating at 161.984 Mhz, with proton decoupling. A total of 20 000 transients were collected in 64 k data points with a spectral width of 40 486 Hz. The acquisition time was 0.809 s, with a 5 s relaxation delay. The data were multiplied with 20 Hz exponential line broadening before Fourier transformation. For the inhibitor complex, the enzyme was first exchanged into 0.05 M bicine-Na, pH 8.0, then diluted with 6 mL of 2 mM 5-bromodihydrokynurenine in 0.05 M bicine-Na, pH 8.0, and concentrated to 1 mM in a Centricon centrifugal filter over a YM-30 membrane, and 10% v/v $D_2O$ was added. In this experiment, a total of 30 000 transients were collected, and the data were multiplied with a 20 Hz exponential before Fourier transformation. The Y226F mutant kynureninase was prepared similarly to wild-type kynureninase. A total of 38 000 transients were collected in 64 k data points with a spectral width of 40 486 Hz, and the data were multiplied with 20 Hz exponential line broadening before Fourier transformation. For the Y226F–inhibitor complex, a total of 38 000 transients were collected, and the data were multiplied with a 20 Hz exponential before Fourier transformation. Chemical shifts are reported relative to an external standard of 85% $H_3PO_4$.

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