Mechanism of catalysis by tyrosine phenol lyase from *Erwinia herbicola*. Multiple kinetic isotope effects for the reactions with adequate substrates

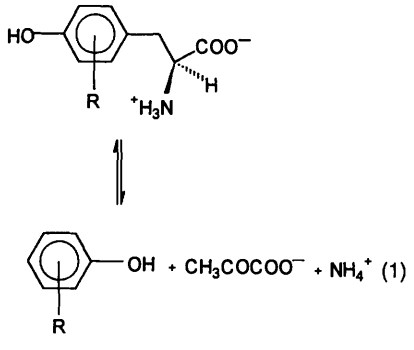
Nikolai G. Faleev,*[a] Svetlana N. Spirina,[a] Tatyana V. Demidkina[b] and Robert S. Phillips[c]

[a] *The Institute of Food Substances, Russian Academy of Sciences, 28 Vavilov Street, Moscow 117813, Russia*
[b] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov Street, Moscow 117984, Russia*
[c] *Departments of Chemistry and Biochemistry, School of Chemical Sciences, University of Georgia, Athens, Georgia 30602, USA*

The generally accepted mechanism of catalysis by tyrosine phenol lyase (TPL) includes three principal chemical transformations of the substrate: (1) abstraction of the α-proton in the external aldimine; (2) tautomerization of the aromatic moiety to convert it into a good leaving group and (3) β-elimination of the leaving group. The relative significance of these stages has been elucidated for the reactions of TPL from *Erwinia herbicola* with suitable substrates, L-tyrosine and 3-fluoro-L-tyrosine. The three stages are susceptible to different kinetic isotope effects (KIEs). To determine the respective KIEs the kinetics of TPL reactions with normal and α-deuteriated 3-fluorotyrosine in water and in ^2H_2O, and with β,β-dideuteriated substrate in water, were examined. The KIE values that were found (α-KIE = 3.4 in water and 2.0 in ^2H_2O; solvent KIE = 1.7; β-KIE = 1.1) are in good agreement with the classic stepwise (not concerted) mechanism of α-proton transfer to the leaving group. The solvent KIEs for the reactions of 3-fluorotyrosine and tyrosine are the same. This result and the low absolute values of the solvent KIEs allow the assumption that the tautomerization stage is at equilibrium. A reaction mechanism is suggested where the tautomerization stage does contribute significantly to the total free-energy barrier although the highest maximum on the free-energy profile corresponds to the subsequent β-elimination stage.

Introduction

Enzymes have already become a useful tool in organic chemistry, and so it is important to understand their reaction mechanisms, especially when they are used to catalyse reactions which cannot be performed by purely chemical means. Microbial tyrosine phenol lyase (TPL; EC 4.1.99.2) is an interesting enzyme of this kind. Being a pyridoxal-5-phosphate (PLP)-dependent lyase with a broad substrate specificity, it catalyses the α,β-elimination of L-tyrosine and its ring-substituted analogues to give, respectively, phenols and ammonium pyruvate. It also effectively catalyses the reverse synthetic reaction [reaction (1)].[1,2]



R = H, OH, alkyl, halide

Due to the favourable position of the equilibrium the direct synthesis of tyrosine-type amino acids (including 3,4-dihydroxyphenyl-L-alanine) from the corresponding phenols and ammonium pyruvate is possible with the use of TPL.[1,2]

The generally accepted mechanism of TPL catalysis[3-5] includes three principal chemical transformations of the substrate: (1) abstraction of the α-proton in the external aldimine; (2) tautomerization of the aromatic moiety to convert it into a good leaving group and (3) β-elimination of the leaving group (Scheme 1).

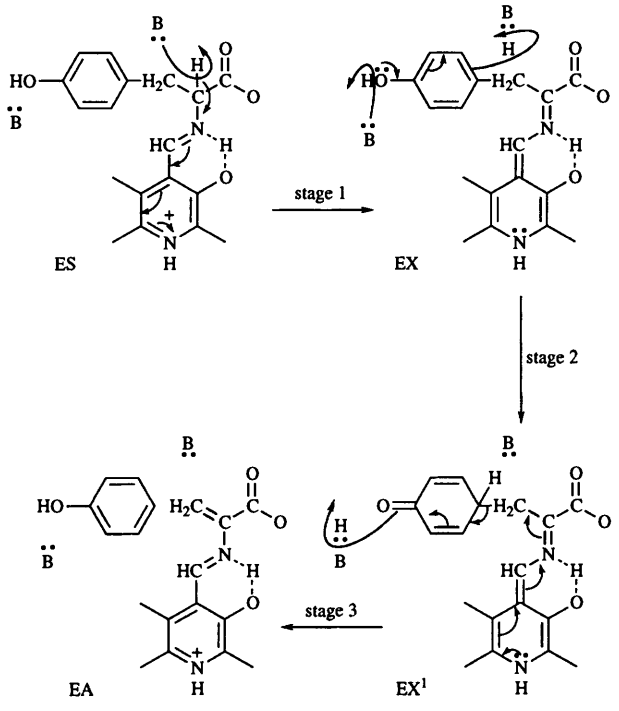
The aim of the present work was to estimate the relative significance of the respective stages. To reach this end we used multiple kinetic isotope effects (KIEs) as each stage is susceptible to a different KIE: stage (1) to α-deuteriation of the substrate; stage (2) to a solvent KIE on going from water to ^2H_2O; stage (3) to β-deuteriation of the substrate. In the present work these KIEs were examined for the reactions of TPL from *Erwinia herbicola* with suitable substrates, L-tyrosine and 3-fluoro-L-tyrosine.

Results and discussion

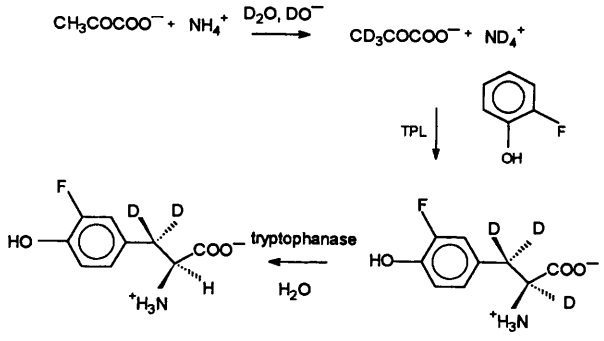
To shed light on the relative significance of the different elementary stages in the TPL catalysis mechanism, multiple KIEs were compared in this work for the reactions of TPL with suitable substrates, tyrosine and 3-fluorotyrosine. Synthesis of [β,β-$^2H_2$]-3-fluoro-L-tyrosine included three principal steps: (i) isotope exchange of pyruvate in ^2H_2O; (ii) synthesis of α,β,β-trideuteriated substrate under the action of TPL in ^2H_2O and (iii) isotope exchange of the α-deuterium for a proton under the action of tryptophanase in water (Scheme 2). 3-Fluorotyrosine binds with the enzyme even better than the natural substrate, L-tyrosine, the value of k_{cat} for 3-fluorotyrosine being only a

Table 1 Kinetic isotope effects on the reactions of *Erwinia herbicola* TPL with tyrosine and 3-fluorotyrosine

Substrates compared	Solvents compared	KIE (V_H/V_D)	Standard error (SE)
L-Tyrosine and [α-^{2}H]-L-tyrosine	H_2O	α-KIE 2.9(3)	0.2
L-Tyrosine	H_2O and ^2H_2O	Solvent KIE 1.66	0.08
3-Fluoro-L-tyrosine and [α-^{2}H]-3-fluoro-L-tyrosine	H_2O	α-KIE 3.4	0.2
3-Fluoro-L-tyrosine and [α-^{2}H]-3-fluoro-L-tyrosine	^2H_2O	α-KIE 2.0	0.2
3-Fluoro-L-tyrosine	H_2O and ^2H_2O	solvent KIE 1.7	0.2
3-Fluoro-L-tyrosine and [β,β-$^2H_2$]-3-fluoro-L-tyrosine	H_2O	β-KIE 1.1	0.04



Scheme 1



Scheme 2

factor of 1.5 less than k_{cat} for tyrosine.[6] The preparation of TPL used in the present work was obtained from the cells of *E. herbicola* by a new method,[6] which differs from the traditional one[7] by using a more simple composition of culture medium for the cell growth and fewer steps in the purification procedure. For the enzyme thus obtained the values of the maximal rate, V_{max}, were found to be independent of pH for both tyrosine and 3-fluorotyrosine, which simplified the choice of pH (or pD) for the determination of solvent KIE. In previous work[3] it was shown that the reaction with L-tyrosine is slowed down by a considerable degree by the dissociation of the substrate, which leads to a decrease in KIE values for V_{max} and the Michaelis constant, K_m. Bearing this in mind, we considered the determination of KIE on V_{max} as our main task, and we examined the kinetics at high concentrations of the substrate (from 3.5 K_m to 50 K_m). The results are summarized in Table 1. The KIE on the ratio V_{max}/K_m could not be determined accurately in these experimental conditions, so their values are not given.

Although the abstraction of the α-proton in TPL-complexes with different substrates, including 3-fluorotyrosine, is well documented, and the direct measurements of deprotonation and reprotonation rates of normal and α-deuteriated substrates have been reported,[8] the question of the kinetic competence of the quinonoid intermediate has not previously been examined. The generally accepted mechanism (Scheme 1) includes proton abstraction from the C_α position of the substrate, followed by the protonation at C_1 of the phenolic ring. For aspartate aminotransferase, an example has been described[9] where the analogous transfer from the C_α to the C_4 position of the cofactor occurs as a concerted 1,3-prototropic shift, while the quinonoid, which does actually form, is a dead-end intermediate. To distinguish between the stepwise and concerted proton transfers we used the double kinetic isotope criteria as described in ref. 9. The experimental results (see Table 1) show that the α-KIE and solvent KIE are mutually dependent which provides evidence for a classic stepwise mechanism of α-proton transfer,[9] where α-proton abstraction and protonation of the phenol moiety are performed as two successive distinct stages. So, for the reaction of *E. herbicola* TPL, the quinonoid intermediate is definitely kinetically competent.

Changing the solvent from water to ^2H_2O should mainly influence the rate of tautomerization of the phenolic ring into a cyclohexadienone ring. This process consists of protonation of the C_1 atom of the ring by a general acid catalyst (possibly Tyr71),[10] and deprotonation of the phenolic hydroxy group by the other base. The protons of both the group performing protonation and the phenolic hydroxy group are exchangeable with the solvent; consequently, the process should be slowed down in ^2H_2O. Taking into account the known values of the bond strengths and stabilization energies[11] the ΔH values for the tautomerization stage may be estimated as approximately 67 kJ mol^{-1} (16 kcal mol^{-1}) which corresponds to the equilibrium constant value of 10^{-12}. Thus, from a purely chemical point of view the tautomerization stage seems to be the most difficult stage. No examples of direct measurements of the tautomerization stage velocity are known, thus the determination of the solvent KIEs is the only way to estimate its relative

significance in the mechanism of TPL reactions. Rather unexpectedly, the observed values of the solvent KIEs are considerably smaller than the α-KIEs in water, both for tyrosine and 3-fluorotyrosine.

The β-elimination stage is accompanied by regeneration of aromaticity in the leaving group which makes it energetically favourable, so at first sight, it seems less important than the preceding stages. However, the data of Axelsson *et al.*,[12] obtained from the study of the $^{11}C/^{14}C$-KIE for reaction of tyrosine with *Citrobacter* TPL, provided evidence that this stage should also be considered as partially rate-limiting. In the present work we have used a simpler technique and determined the secondary KIE for the β,β-dideuteriated substrate. While interpreting the β-KIE, the two stages involved in the catalytic act should be taken into consideration: (i) the β-elimination stage leading to α-aminoacrylate formation and (ii) subsequent protonation of this intermediate, which begins the aminoacrylate hydrolysis, and also is effected enzymatically.[13] The rate of the latter stage should be the same for all the substrates of TPL, irrespective of the leaving group, so it obviously cannot be rate-determining for the slow reacting substrates. Meanwhile, for rapidly reacting substrates, like tyrosine or 3-fluorotyrosine, its role may be considerable. The elimination stage is accompanied by rehybridization of the β-C atom from sp^3 to sp^2 and consequently is slowed down on passing from the normal to β-deuteriated substrate.[14,15] The protonation stage results in rehybridization from sp^2 to sp^3, and should in the case of deuteriated substrate proceed faster. The observed value of the β-KIE (1.1) is comparable with the normally observed secondary KIE in other reactions with sp^3 to sp^2 hybridization changes,[14,15] and therefore it should be concluded[12] that for 3-fluorotyrosine, β-elimination is also at least partially rate-limiting, especially when the probable compensating effect of the following α-aminoacrylate protonation stage is taken into account. The obtained KIE values, at first sight, provide evidence that the proton abstraction stage is a more important contributor to the total energy barrier than the subsequent tautomerization stage. However, a comparison of the results for the reactions of 3-fluorotyrosine and tyrosine allows a different conclusion to be drawn. The electron withdrawing fluorine substituent in 3-fluorotyrosine is placed at the *meta*-position with respect to the C atom which is protonated during the tautomerization stage. This should result in the retardation of this stage ($\sigma_m^+ = 0.35$),[16] consequently its role as the rate-limiting stage should be increased. Meanwhile, the values of solvent KIE as well as α-KIE are practically the same for tyrosine and 3-fluorotyrosine. Based on this result and the low absolute values of solvent KIEs, it may be assumed that in fact the tautomerization stage is at equilibrium. In this case its real contribution to the energy-barrier may be quite significant (Fig. 1), although the highest maximum on the free-energy profile should correspond to the subsequent stage. This explains the role of the β-elimination stage as rate-limiting. In the framework of the suggested scheme the α-proton abstraction stage should also be at equilibrium. Arguments in favour of this assumption were given in ref. 8. Some additional comments are needed, however, on the high values of the α-KIEs which seem incompatible with this concept. If the α-proton abstraction is an equilibrated stage [eqn. (2)], the α-KIE reflects the isotope

$$ES \underset{k_r}{\overset{k_a}{\rightleftharpoons}} EX \xrightarrow{k_f} P \qquad (2)$$

effect on the steady-state concentration of the kinetically competent quinonoid intermediate, which is given by the ratio: $k_a/(k_a + k_r + k_f)$, where k_a is the rate of isotope-sensitive α-proton abstraction, k_r is the rate of the reprotonation of the quinonoid and k_f is the effective rate, reflecting the stages following the quinonoid formation which eventually lead to the formation of the reaction product (pyruvate).

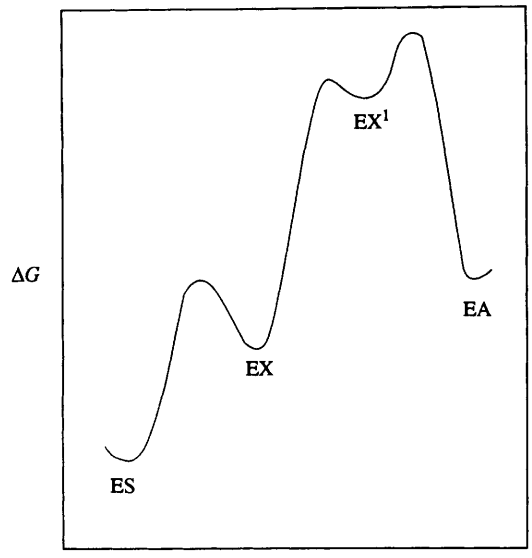


Fig. 1 Suggested free-energy profile for the reactions of TPL with suitable substrates

The considerable values of observed α-KIEs mean that the following conditions are satisfied: (i) k_a is considerably less than $k_r + k_f$ and (ii) k_r is not sensitive to α-deuteriation of the substrate. The latter is possible when the degree of internal return of the abstracted proton is negligible due to fast exchange with the solvent. In this case the decrease in the α-KIE observed on going from water to ^2H_2O may be the result of a decrease in the k_r value, which partially compensates the α-KIE on k_a.

Experimental

Materials

Apparently homogeneous TPL with specific activity of 1.1–1.3 tyrosine units mg^{-1} was obtained from the cells of *Erwinia herbicola* (ATCC 21434) by a modified method, which will be described in detail elsewhere.[6] The lactate dehydrogenase (LDH) from rabbit muscle, PLP and NADH were purchased from United States Biochemical Co. Tryptophanase was obtained as described in ref. 17. 3-Fluoro-L-tyrosine was prepared by enzymatic synthesis.[18] [α-^{2}H]-3-Fluoro-L-tyrosine was made by performing the enzymatic synthesis in ^2H_2O, as previously described for L-tyrosine.[3] To prepare [α,β,β-$^2H_3$]-3-fluoro-L-tyrosine, a solution of 2.18 mmol sodium pyruvate and 7.48 mmol NH_4Cl in 20 ml ^2H_2O adjusted to pD 8.4 by adding KOH solution in ^2H_2O, was incubated at 30 °C for 20 h. 3-Fluorophenol (2 mmol) and lyophilized cells of *Citrobacter intermedius* containing 30 units of TPL were added and the mixture was incubated with stirring for another 20 h. The product was then isolated as described in ref. 19. [α,β,β-$^2H_3$]-3-Fluoro-L-tyrosine was obtained in 40% yield. This product (0.26 mmol) was then dissolved in 20 ml of 0.1 M potassium phosphate buffer, pH 8.0 in water, 20 units of tryptophanase were added and the solution was incubated with stirring at 30 °C for 18 h. It was then heated at 90 °C for 5 min, the denatured enzyme was separated by centrifugation and the product was isolated on a column with Dowex-50 in the H^+ form, and additionally purified by HPLC as described for L-tyrosine.[20] [β,β-$^2H_2$]-3-Fluoro-L-tyrosine has been obtained, which according to ^{1}H NMR data contained no more than 2.5% of deuterium at the α-position, while the degree of deuteriation at the β-position was 81%.

Kinetic measurements

The rates of pyruvate formation from tyrosine, 3-fluoro-L-tyrosine and their deuteriated analogues were measured at 30 °C using a coupled assay with LDH and NADH as described in ref. 3.

Isotope effects were determined in 0.1 M potassium phosphate buffers at pH (pD) = 8.6. Values of pH were measured potentiometrically. To determine pD for buffers in ^2H_2O, an allowance was made for the isotope effect of the glass electrode (0.4). To ensure the accurate measurement of KIE on V_{max}, the kinetics were observed at high concentrations of the substrates (from 3.5 K_m to 50 K_m), the data obtained were fitted to eqn. (3), where v = the observed reaction rate, $VKI = V/K$

$$v = V \cdot S/[K(1 + I \cdot VKI) + S(1 + I \cdot VI)] \quad (3)$$

(isotope effect) − 1, $VI = V$ (isotope effect) − 1, S = substrate concentration and I = content of deuterium, by using the FORTRAN programs of Cleland[21] adapted to run on IBM-compatible personal computers.

Acknowledgements

This research was partially supported by grants from International Science Foundation to N. G. F. (M6G000 and M6G300), from the NIH to R. S. P. (GM42588) and from the Fogarty International Centre (TW00106) to R. S. P. and T. V. D.

References

1 H. Yamada and H. Kumagai, *Adv. Appl. Microbiol.*, 1975, **19**, 249.
2 T. Nagasawa, T. Utagawa, J. Goto, C.-J. Kim, Y. Tani, H. Kumagai and H. Yamada, *Eur. J. Biochem.*, 1981, **117**, 33.
3 D. M. Kiick and R. S. Phillips, *Biochemistry*, 1988, **27**, 7333.
4 N. G. Faleev, A. E. Lyubarev, N. S. Martinkova and V. M. Belikov, *Enzyme Microb. Technol.*, 1983, **5**, 219.
5 M. M. Palcic, S.-J. Shen, E. Schleicher, H. Kumagai, S. Sawada, H. Yamada and H. G. Floss, *Z. Naturforsch. C: Biosci.*, 1986, **420**, 307.
6 N. G. Faleev, S. N. Spirina, V. S. Ivoilov, T. V. Demidkina and R. S. Phillips, *Z. Naturforsch. C: Biosci.*, in the press.
7 H. Kumagai, N. Kashima, H. Torii, H. Yamada, H. Enei and S. Okumura, *Agric. Biol. Chem.*, 1972, **36**, 472.
8 H. Chen, R. S. Phillips and P. Gollnick, *Eur. J. Biochem.*, 1995, **229**, 540.
9 D. A. Julin and J. F. Kirsch, *Biochemistry*, 1989, **28**, 3825.
10 H. Chen, T. V. Demidkina and R. S. Phillips, *Biochemistry*, 1995, **34**, 12276.
11 T. L. Cottrell, *The Strengths of Chemical Bonds*, Butterworth, London, 2nd edn., 1958.
12 S. B. Axelsson, P. Bjurling, O. Matsson and B. Langstrom, *J. Am. Chem. Soc.*, 1992, **114**, 1502.
13 H. Kumagai, H. Yamada, S. Sawada, E. Schleicher, K. Mascara and H. Floss, *J. Chem. Soc., Chem. Commun.*, 1977, 85.
14 A. Streitwieser, Jr., R. M. Jagow, R. C. Fahey and S. Suzuki, *J. Am. Chem. Soc.*, 1958, **80**, 2326.
15 R. W. Murray, D. L. Shiang and M. Shing, *J. Org. Chem.*, 1991, **56**, 3677.
16 K. Johnson, *Hammet equation*, Mir, Moscow, 1977.
17 L. N. Zakomirdina, I. S. Sakharova and Y. M. Torchinsky, *Mol. Biol. Moscow*, 1988, **22**, 187.
18 R. S. Phillips, J. G. Fletcher, R. L. Von Tersch and K. L. Kirk, *Arch. Biochem. Biophys.*, 1990, **276**, 65.
19 N. G. Faleev, S. B. Ruvinov, T. V. Demidkina, I. V. Myagkikh, M. Yu. Gololobov, V. I. Bakhmutov and V. M. Belikov, *Eur. J. Biochem.*, 1988, **177**, 395.
20 R. S. Phillips, K. Ravichandran and R. L. Von Tersch, *Enzyme Microb. Technol.*, 1989, **11**, 80.
21 W. W. Cleland, *Methods Enzymol.*, 1979, **63**, 103.

Paper 6/00674D
Received 29*th January* 1996
Accepted 17*th April* 1996