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Note

Stereochemistry of 2-Phenylethylamine Oxidation Catalyzed by Bacterial Copper Amine Oxidase

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The stereochemical course of the reaction catalyzed by a copper amine oxidase from *Arthrobacter globiformis* has been investigated using 2-phenylethylamine stereospecifically deuterium-labeled at the C1 position. Measurements of deuterium content in the product, phenylacetaldehyde, by gas chromatography-mass spectrometry revealed stereospecific abstraction of the *pro-S* hydrogen during the enzymatic oxidation, as predicted from the structure modeling for the enzyme-bound substrate.

Key words: 2-phenylethylamine; copper-containing amine oxidase; topa quinone; stereospecifity; deuterium-labeled substrate

Copper amine oxidase (EC 1.4.3.6) catalyzes oxidation of various primary amines, producing the corresponding aldehydes, hydrogen peroxide, and ammonia.[1)] An organic redox cofactor, topa quinone (TPQ), contained in the active site of the enzyme is post-translationally generated by self-processed modification of a specific tyrosine residue in the presence of cupric ion and molecular oxygen.[2)] The cupric ion is essential for both TPQ generation and catalysis. The catalytic cycle of the enzyme is divided into the reductive and oxidative half-reactions depending on the redox state of TPQ.[3)] In the former reductive half-reaction, the C5 carbonyl group of TPQ reacts with the amino group of the substrate to form the substrate Schiff-base, which is then converted to the product Schiff-base through stereospecific proton abstraction from the substrate C1 position by an invariant aspartic acid residue (Scheme 1, A). In the latter oxidative half-reaction, the reduced TPQ is re-oxidized by dioxygen, during which the cupric ion serves as a binding site for $1e^-$ and $2e^-$-reduced dioxygen species to be efficiently protonated and released.[3)]

The stereospecificity of proton abstraction from the C1 position of the substrate has been studied for enzymes from bacteria, plants, and animals.[1,4)] Interestingly, the specificity is variable depending on the enzyme sources and substrate amines used. For example, the *pro-S* proton of dopamine is abstracted in the reaction of pea seedling enzyme, while the *pro-R* proton of the same amine is abstracted in the reaction of porcine plasma enzyme.[4)] On the other hand, the bovine plasma enzyme is non-stereospecific for dopamine but is *pro-S* specific for another substrate, benzylamine.[4)] In view of the highly conserved active-site structures of this enzyme family,[5,6)] the origin of the inconsistent stereospecificity remains to be settled. Toward unequivocal elucidation of the molecular basis for the stereospecific proton abstraction, we have investigated the stereochemical course of the reaction catalyzed by the enzyme from *Arthrobacter globiformis* (AGAO), for which the X-ray crystallographic structure had already been solved.[6)] We report here that the *pro-S* proton of 2-phenylethylamine is stereospecifically abstracted during the AGAO reaction and this stereospecificity is consistent with the structure model for the substrate Schiff-base intermediate.

To structurally predict the stereospecificity of proton abstraction, 2-phenylethylamine bound to the C5 carbonyl group of TPQ in the substrate Schiff-base form was modeled in the AGAO crystal structure (PDB entry 1IU7) using a commercial software package Insight II, version 98 (Accelrys Inc., San Diego, CA). After the two prochiral hydrogen atoms at the C1 position of 2-phenylethylamine were generated using the Builder module, energy minimization was done with the Discover_3 module for the zone within 3 Å from TPQ under a constant valance force field. In the final model thus obtained (Fig. 1), several residues around the phenyl ring of substrate (*e.g.*, Tyr302, Phe407, and Phe105) slightly changed their conformations to accommodate the phenyl ring in

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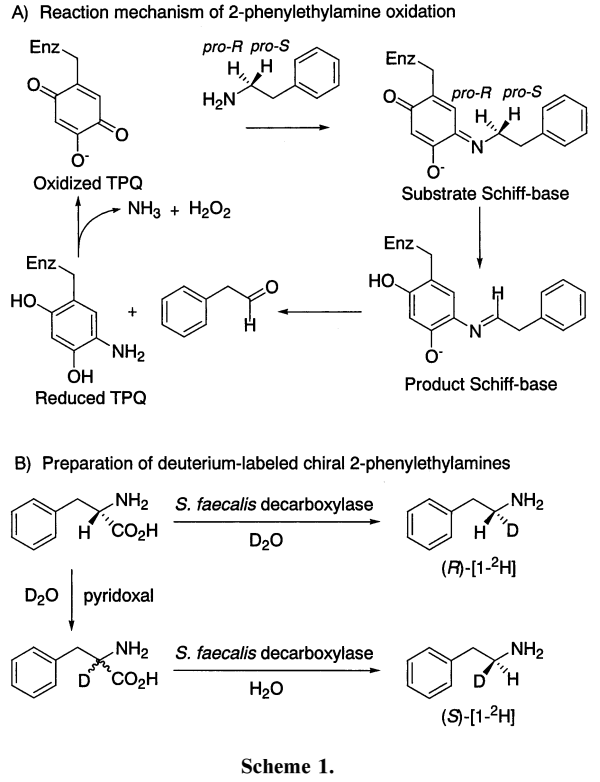
Abbreviations: TPQ, topa quinone; GC-MS, gas chromatography-mass spectrometry; t_R, retention time; AGAO, *Arthrobacter globiformis* amine oxidase

the hydrophobic pocket; after the modeling other residues did not move from the initial positions. Most importantly, the distance between one of the oxygen atoms of the side chain carboxyl group of the putative catalytic base, Asp298,[6] and the two prochiral hydrogen atoms is shorter for the *pro-S* hydrogen (3.56 Å) than for the *pro-R* hydrogen (5.20 Å). In addition, the *pro-S* hydrogen is oriented in a more advantageous direction to be abstracted by Asp298 than the *pro-R* hydrogen. In this way, the structure modeling suggests the *pro-S* specific proton abstraction.

Two enantiomers of mono-deuterated 2-phenylethylamine were prepared from L-phenylalanine by using tyrosine decarboxylase from *Streptococcus faecalis*, which catalyzes decarboxylation of aromatic amino acids with retention of configuration,[7] as shown in Scheme 1, B. To obtain (*R*)-[1-^{2}H]-2-phenylethylamine, sodium phosphate buffer (400 mM, pH 5.5, 5 ml) was evaporated and the residue and L-phenylalanine (330 mg) were dissolved in D_2O (5 ml), followed by evaporation of the solution again. After dissolving the residue in D_2O (10 ml), tyrosine decarboxylase (12.5 units) and pyridoxal 5′-phosphate (1 mg) were added to the solution and the mixture was incubated at 37°C for 3 days. The reaction mixture was neutralized with 15% NaOH and product amine was extracted with $CHCl_3$ to give (*R*)-[1-^{2}H]-2-phenylethylamine (29 mg, 12%); ^{1}H NMR (270 MHz, $CDCl_3$) δ: 1.3 (*s*, 2H), 2.74 (*d*, $J=6.6$, 2H), 2.95 (*m*, 1H), 7.2–7.3 (*m*, 5H). The content of the mono-deuterated amine was >97% by gas chromatography-mass spectrometry (GC-MS) [retention time (t_R), 10.7 min].

For synthesizing (*S*)-[1-^{2}H]-2-phenylethylamine, a mixture of L-phenylalanine (1.3 g), pyridoxal hydrochloride (0.16 g), and D_2O (10 ml)-NaOD (16 mmol) was refluxed for 2 h. After the mixture was freeze-dried, the residue was dissolved in D_2O (10 ml) and refluxed again for 2 h. When the mixture was neutralized with 3 N HCl (pH ~ 5) with ice-cooling, DL-[2-^{2}H]-phenylalanine precipitated as crystals. The crystals were collected by filtration and washed with cold water and methanol. The product was dissolved in 3 N HCl and treated with a small amount of activated charcoal. After the filtrate was neutralized with 15% NaOH, addition of methanol with ice-cooling afforded DL-[2-^{2}H]-phenylalanine (0.63 g, 47%); ^{1}H NMR (270 MHz, D_2O) δ: 2.82–2.98 (*AB-system*, $J=13.5$, 2H), 7.3–7.4 (*m*, 5H). The non-labeled phenylalanine was not detected by ^{1}H NMR. Decarboxylation of DL-[2-^{2}H]-phenylalanine (0.33 g, 2 mmol) in H_2O by the



Scheme 1.

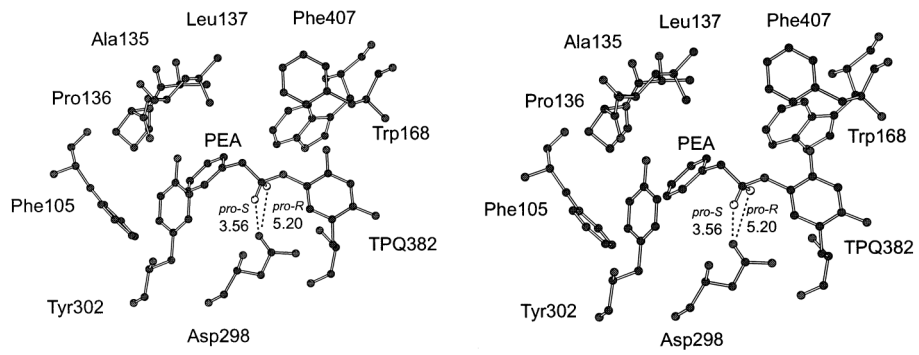


Fig. 1. Stereo Diagram of Structure Model for Substrate Schiff-base Complex.

Active-site residues of AGAO and 2-phenylethylamine bound to the C5 carbonyl group of TPQ with the two prochiral hydrogen atoms at the C1 position of substrate in the final model obtained by energy minimization are shown in ball and stick models. The distances between the two prochiral hydrogen atoms and one of the carboxyl oxygen atoms of Asp298 (catalytic base) are also shown in Å. This diagram was prepared using MolScript.[9]

Table 1. Oxidation of Deuterium-labeled Chiral Amines by AGAO

Configuration of starting amine	substrate/product	Relative peak intensity at m/z					Major component (%)[b]
		119	120	121	122	123	
Non-labeled	amine	—	0.118	1.000[a]	0.098	—	
	aldehyde	0.021	1.000[a]	0.095	—	—	
(*R*)-[1-^{2}H]	amine	—	—	0.101	1.000[a]	0.125	R-CHDNH$_2$ (>97)
	aldehyde	—	<0.001	1.000[a]	0.088	—	R-CDO (>99)
(*S*)-[1-^{2}H]	amine	—	—	0.085	1.000[a]	0.110	R-CHDNH$_2$ (>99)
	aldehyde	0.033	1.000[a]	0.087	—	—	R-CHO (>99)

[a] Molecular ion peak, M^+.
[b] Percent contents of the major component shown in parentheses were calculated from the mass number of the molecular ion peak and the peak intensity, which was rectified with the concomitant M−1 and M+1 peaks. R, $C_6H_5CH_2$.

same enzymatic procedure as described above afforded (*S*)-[1-^{2}H]-2-phenylethylamine (30 mg, 13%); ^{1}H NMR (270 MHz, $CDCl_3$) δ: 1.3 (s, 2H), 2.74 (*d*, *J*= 6.6, 2H), 2.95 (*m*, 1H), 7.2–7.3 (*m*, 5H). The content of the mono-deuterated amine was >99% by GC-MS (t_R = 10.7 min).

GC-MS analyses were done with a Shimadzu GC-17A equipped with DB-5MS (J & W Scientific, Inc.) and Shimadzu GCMS-QP5050A (starting column temperature, 50°C, maintained for 5 min; increasing rate, 10°C/min). The enantiomeric purities of both the labeled amines were also assessed by converting them to the diastereomeric amides of (−)-camphanic acid, according to Parker.[8] In ^{1}H NMR, the α-proton of the (*R*)-[1-^{2}H]-amide appeared at 3.29–3.34 (1.00H) and 3.10–3.14 (<0.006H), from which the purity of the (*R*)-[1-^{2}H]-amine was calculated to be >99%. Similarly, the (*S*)-[1-^{2}H]-amine was found to have a purity of >92% after converting to the (*S*)-[1-^{2}H]-amide, showing δ values of 3.10–3.14 (1.00H) and 3.29–3.34 (<0.08H). Before the stereochemical analysis, both of the substrate amines were converted to the corresponding hydrogen sulfates by treating with sulfuric acid in methanol.

The enzyme reaction was done in 200 mM HEPES buffer (pH 6.8) containing 0.5 mM substrate amine and 0.6 μg/ml AGAO in a total volume of 1 ml. The reaction mixture was incubated for 60 min at room temperature and the product phenylacetaldehyde was extracted with 0.5 ml ethyl acetate. A sample of the extract was directly analyzed by GC-MS (t_R = 9.6 min) and the contents of the deuterium-labeled amines and aldehydes were rectified with the relative intensities of the concomitant M−1 and M+1 peaks observed for the non-labeled amines and aldehydes. As summarized in Table 1, the AGAO oxidation gave [1-^{2}H]-aldehyde with a deuterium content of >99% from (*R*)-[1-^{2}H]-amine with a deuterium content of >97%. This result clearly indicates that the *pro-S* hydrogen has been abstracted during the reaction. This stereochemical course is also supported by the experiment with (*S*)-[1-^{2}H]-amine where the non-labeled aldehyde (content, >99%) was yielded from the labeled amine (content, >99%). Because this process suffers from a primary isotope effect, a considerable amount of the labeled aldehyde should be detected if the stereospecificity is incomplete. Thus, it is concluded that the AGAO oxidation of 2-phenylethylamine proceeds in a highly stereospecific manner, exclusively abstracting the *pro-S* hydrogen at the C1 position of substrate amine, as predicted from the modeling. The modeling also suggests that the stereospecificity could change by the binding mode of the distal part of amine substrate in the hydrophobic pocket, composed of residues that are not conserved among copper amine oxidases;[5,6] rotations of the phenyl ring of substrate around the C1–C2 bond would bring the *pro-R* hydrogen closer to Asp298. To examine this hypothesis, further studies of the stereospecificity of AGAO with other substrates are under way.

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