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Identification and development of amino acid oxidases

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Amino acid oxidases are an important class of enzymes that mostly participate in the oxidation of amino acids using FAD as a cofactor. Many of them function in the catabolism of amino acids with wider substrate specificities. On the other hand, based on the recent, successful use of the enzymes for diagnoses with new cofactor and mechanism, highly selective enzymes have been screened from Nature, and many new enzymes have been discovered and further characterized by X-ray crystallography. As a result of the screening for amino acid oxidases with biosynthetic or antibiotic functions, L-Trp oxidase, L-Lys oxidases, and Gly oxidase have been found. The pyridoxal phosphate-dependent L-Arg oxidase has the intriguing new activity of hydroxylating unactivated C–C bonds. A new amine oxidase was created by the protein engineering of D-amino acid oxidase. Recent developments in the characterization of amino acid oxidases and their applications are summarized.

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Introduction

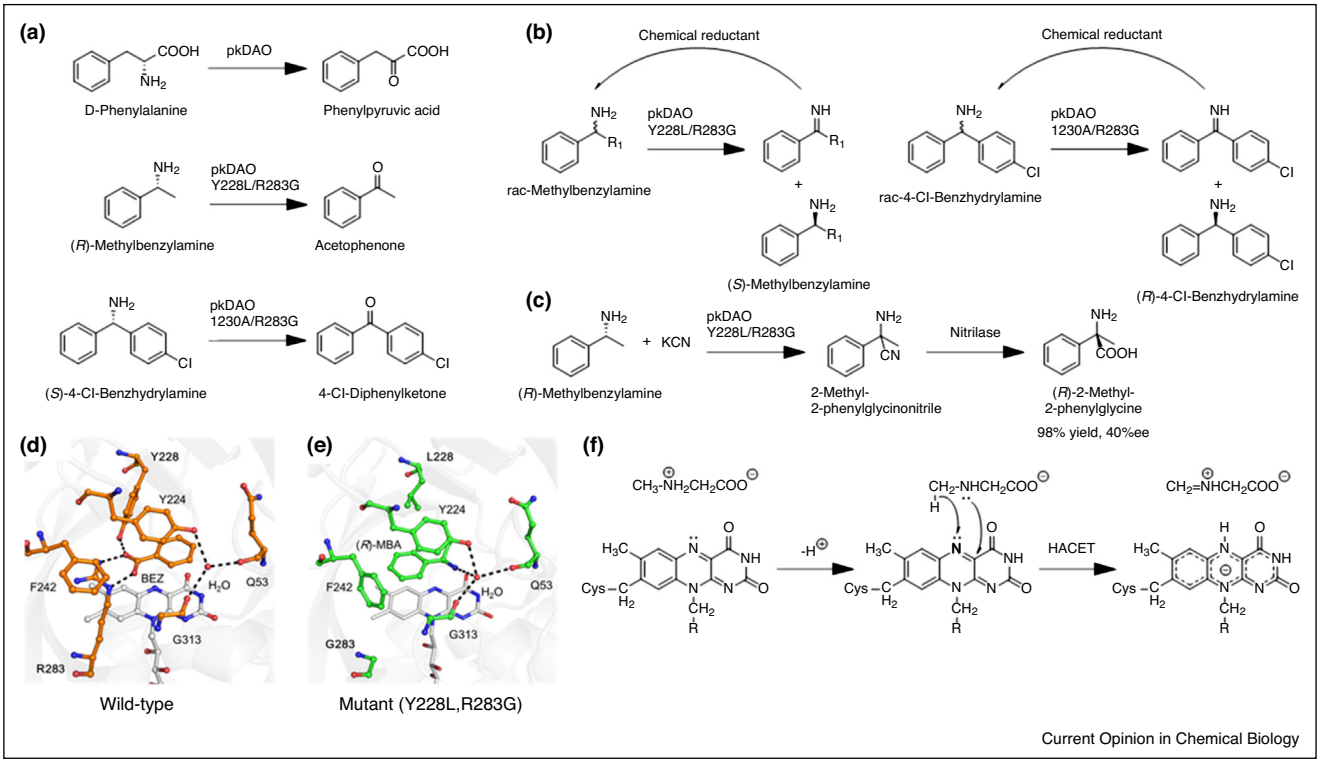
With the successful, practical use of oxidoreductases for the micro-determination of blood amino acids, such as the NAD^+-dependent L-Phe dehydrogenase for phenylketonuria among more than several millions of neonates in Japan [1], many enzymes with higher substrate specificities have been screened and discovered from Nature. This review article focuses on amino acid oxidases that are renewing the common sense of classical biochemistry: L-Lys oxidases, sarcosine oxidase, amino acid oxidase/monooxygenase, L-Trp oxidase, L-Arg oxidase, and Gly oxidase, all of which show high selectivities. They also show the potential for amino acid determination in biological samples, and some of them can be used practically. The X-ray crystallographic structures of some of the enzymes have been solved, and their reaction mechanisms are discussed herein. Some of the amino acid oxidases have been developed for the production of useful chemicals, such as chiral amines, α-keto acid and so on. It is interesting that D-amino acid oxidase, useful for the micro-determination of D-amino acids, has been modified by protein engineering into a new *R*-specific amine oxidase, which has been designed for the enzymatic production of chiral amines and branched cyanoamino acids. Furthermore, the structure of a new PLP-dependent L-Arg oxidase and its mechanism of hydroxylation of L-Arg and those of new L-Lys oxidases are presented.

Amino acid oxidase

Asano *et al.* evolved an *R*-stereoselective amine oxidase from the porcine kidney D-amino acid oxidase (pkDAO), and the new enzyme was subsequently used for the deracemization of racemic amines. The engineered pkDAO (Y228L/R283G) (PDB 4YJD, apo form) displayed a markedly changed substrate specificity toward (*R*)-amines, such as α-methylbenzylamine (MBA), resulting in the complete loss of activity toward D-amino acids (Figure 1(a)). The mutant was used to synthesize not only (*S*)-amines through deracemization (Figure 1(b)) [2••,3•], but also (*R*)-2-methyl-2-phenylglycine (40% *ee*) was synthesized in combination with nitrilase AY487533 *via* 2-methyl-2-phenylglycinonitrile prepared from (*R*)-MBA and KCN by an oxidative cyanation reaction. Unnatural α-amino acids, such as 2-ethylphenylglycine and *p*-fluoro-2-methylphenylglycine from α-ethylbenzylamine, and 4-fluoro-α-MBA, respectively, were similarly synthesized in combination with the nitrilase (Figure 1(c)) [4•]. Fitzpatrick *et al.* studied the catalytic mechanism of pkDAO (Y228L/R283G) and demonstrated that the mutant utilizes the same mechanism as the wild-type pkDAO [5].

Further expansion of the substrate specificity of pkDAO (Y228L/R283G) was successfully carried out, based on X-ray crystallographic analyses of mutants (Figure 1(d) and (e)). A pkDAO variant (I230A/R283G) was characterized as able to accommodate (*S*)-4-Cl-benzhydrylamine (CBHA), and it was efficiently used for the synthesis of (*R*)-CBHA in 96% *ee* from racemic CBHA by a deracemization reaction in the presence of a reducing agent, such as $NaBH_4$ in water (Figure 1(b)). X-ray crystallographic analysis of the new variant complexed with (*S*)-CBHA, clearly provided insight into the understanding of the structure-activity relationship of pkDAO (PDB 5WWV) [6•]. Four crystal structures of the substrate-bound protein (PDB 3WGT, (*R*)-MBA), (PDB 4YJH,

Figure 1



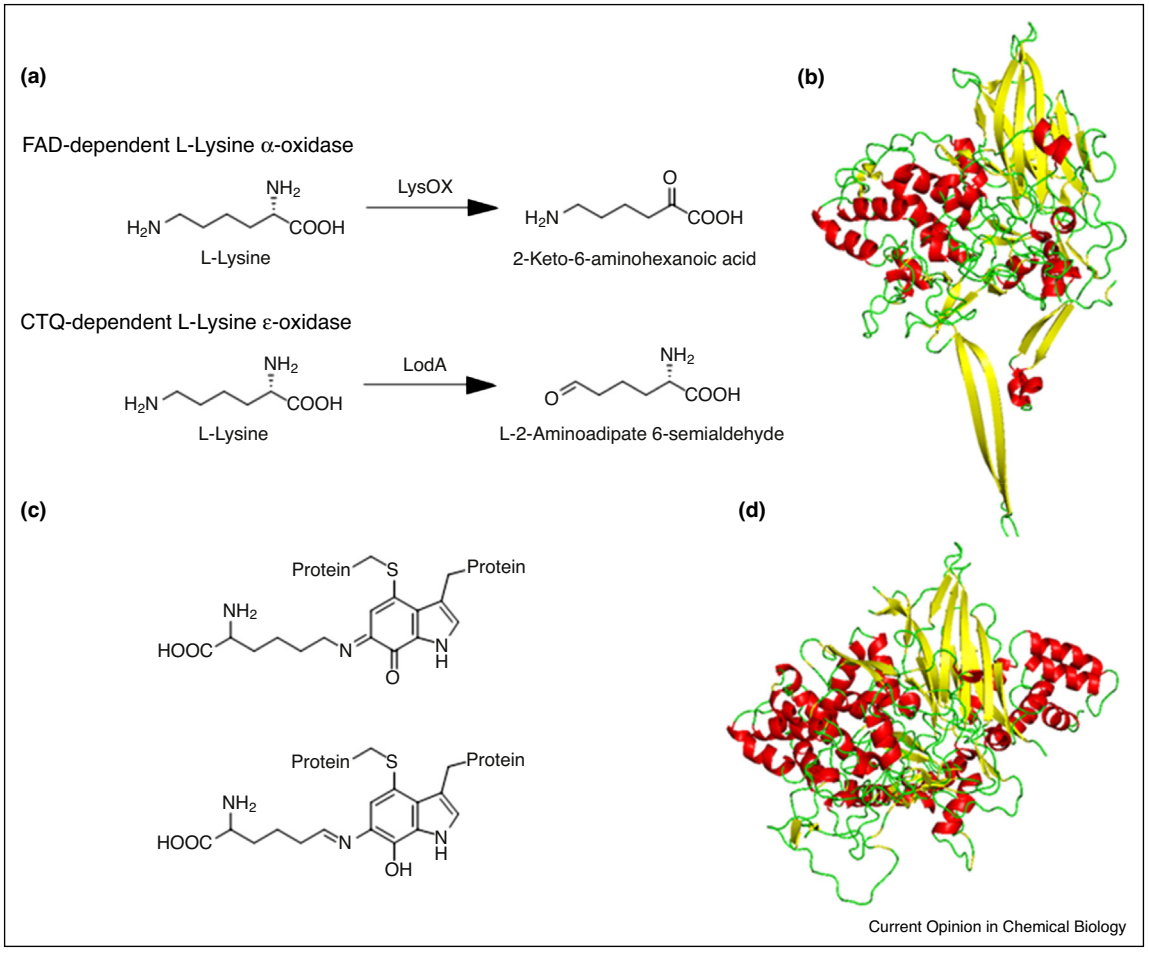
D-Amino acid oxidase (pkDAO) **(a)–(e)**; and **(f)** HACET mechanism.
(a) The reactions catalyzed by the wild-type pkDAO (upper), Y228L/R283G variant (middle) and I230A/R283G variant (lower). (b) Deracemization of amines catalyzed by pkDAO (Y228L/R283G) (right side) and I230A/R283G (left side). (c) One-pot synthesis of (*R*)-2-methylphenylglycine (40% *ee*) from (*R*)-MBA by two enzyme system using pkDAO (Y228L/R283G) and nitrilase AY487533. (d) The active site of wild type pkDAO with bound *o*-aminobenzoate (PDB 1AN9). (e) The active site of the pkDAO (Y228L/R283G) with bound (*R*)-MBA (PDB 3WGT). (f) A proposal of the 'Hydrogen Atom-Coupled Electron-Transfer' (HACET) reaction mechanism for sarcosine oxidase.

((*R*)-2-phenylpyrrolidine), (PDB 4YJG, (*R*)-3-amino 1-phenylbutane), and (PDB 4YJF, ((*S*)-MBA) and first-principles calculations, based on the correlated fragment molecular orbital (FMO), indicated that two aromatic residues, Tyr224 and Phe242, form a stable π–π stacking interaction with the substrates. The FMO analysis indicated that the mutant forms an approximate 13 kcal/mol more stable interaction with (*R*)-MBA than with (*S*)-MBA; the energy difference contributes to the specific recognition of (*R*)-MBA in the racemate, showing that (*S*)-MBA is not an apparent inhibitor of the mutant [7•].

Abe *et al.* studied the reaction mechanism of sarcosine oxidase catalyzed reaction. They examined the reaction mechanism using FMO and mixed quantum mechanics/molecular mechanics (QM/MM) methods (PDB 1EL5). They found that hydride transfer is the most energetically favorable mechanism, among the long discussed single-electron transfer, hydride-transfer, and polar mechanisms. Based on a detailed theoretical analysis of the calculated reaction pathway, they proposed a 'Hydrogen Atom-Coupled Electron-Transfer' (HACET) mechanism, in which hydrogen is transferred in a hydrogen atom ($H^{\bullet}$) but not in hydride state (H^{-}). First, sarcosine amine H atom is deprotonated and the anionic form of sarcosine moves toward the flavin ring, and then the H atom of sarcosine C_4 and one electron of sarcosine amine are transferred to the flavin ring (Figure 1(f)). A mechanism similar to HACET may be operating in many long discussed flavoprotein-catalyzed amine oxidations [8••].

The L-Lys α-oxidase (LysOX) gene from *Trichoderma viride* was heterologously expressed in *Streptomyces lividans* TK24. The enzyme shows antitumor effect on human cancer cells. Besides L-Lys, the enzyme showed activities toward L-Orn (18.3%), L-Arg (6.9%), and less than 2% toward L-Phe and L-Tyr (Figure 2(a) upper). The crystal structure of LysOX at 1.9 Å resolution (PDB 3 × 0V) revealed that the overall structure is similar to that of snake venom L-amino acid oxidase (PDB 2IID) [9]. L-Lys ε-oxidase and glycine oxidase from a marine bacterium *Marinomonas mediterranea* form a novel class of amino acid oxidases 'LodA-like proteins' that contain quinone as a cofactor instead of FAD; these are generated

Figure 2



L-Lysine α-oxidase **(a)**, and LodA-like proteins: L-Lys ε-oxidase **(b)–(c)**; Gly oxidase **(d)**.
(a) L-Lys α-oxidase and L-Lys ε-oxidase catalyzed oxidation reaction. (b) Crystal structure of LodA (PDB 3WEV). (c) Possible chemical structure of the L-Lys-CTQ substrate Schiff-base (upper) and the L-Lys-CTQ product Schiff-base (lower). (d) Crystal structure of plGoxA (PDB 6BYW).

by the post-translational modification of residues in the same protein by another oxidative LodB-like proteins [10,11]. L-Lys ε-oxidase (LodA, EC 1.4.3.20) catalyzes the oxidative deamination of L-Lys into L-2-aminoadipate 6-semialdehyde, ammonia and H_2O_2, thus we utilized the high selectivity for micro-determination of L-Lys (Figure 2(a) lower) [12]. The X-ray crystal structure of L-Lys ε-oxidase in its native and L-Lys-complex forms were determined at 1.94-Å and 1.99-Å resolution, respectively (PDB 3WEV) (Figure 2(b)) [13••]. The electron densities indicated the presence of cysteine tryptophylquinone (CTQ) previously identified in quinohemoprotein amine dehydrogenase [14,15]. In the L-Lys-complex, an electron density corresponding to the bound L-Lys showed that its ε-amino group is attached to the C6 carbonyl group of CTQ, suggesting the formation of a Schiff-base intermediate (Figure 2(c)). Heterologous production of LodA in *Escherichia coli* was made possible by mutation [16•], screened with an expression of a protein (LodB) acting in posttranslational modification of LodA [17,18].

In addition to L-Lys ε-oxidase as described above [10,11,12,13••,14,15,16•], *M. mediterranea* produces GoxA (Gly oxidase) with a CTQ cofactor formed by post-translational modifications, catalyzed by the modifying enzyme GoxB. It was revealed that Phe237 of the enzyme is critical for the cooperative allosteric behavior toward the substrate and homodimer stabilization. The mutations at Phe237 significantly affected the *k*cat and *K*m values for the substrates. Two active site residues, Asp547 and His466, were also shown to be critical for CTQ biogenesis [19]. A Gly oxidase from *Pseudoalteromonas luteoviolacea* (PlGoxA) has been studied in order to place it in a newly designated subgroup (group IID) of the LodA-like proteins. The crystal structure of PlGoxA revealed that it is a homotetramer (PDB 6BYW) (Figure 2(d)) [20•].

An amino acid oxidase/monooxygenase (L-AAO/MOG) produced by *Pseudomonas* sp. AIU 813 catalyzes the oxidation of L-Lys, L-Orn, and L-Arg [21]. The enzyme exhibits L-Lys 2-monooxygenase, as well as oxidase, activities to produce the 5-amino valeric acid amide and 2-keto-6-amino hexanoic acid, respectively. The oxidase activity increased with *p*-chloromercuribenzoate (*p*-CMB) to a level five-fold higher than that of the untreated enzyme. An L-AAO/MOG C254I mutant found from a series of mutants generated by the saturation mutagenesis of five cysteine residues showed oxidase-specific activity that was 5 times higher than that of the wild type. The key residue, Cys254, was located near the aromatic cage (Trp418, Phe473, and Trp516) based on X-ray crystallographic analysis (PDB 3WE0). Although the location of Cys254 indicates that it is not directly involved in substrate binding, chemical modification by *p*-CMB and the C254I mutation suggested that a slight difference in the binding position of a substrate can dictate the oxidase and monooxygenase activities [22•]. Furthermore, the crystal structures of L-AAO/MOG complexed with L-Lys (PDB 5YB6), L-Orn (PDB 5YB7), and L-Arg (PDB 5YB8) were solved, and the catalytic and kinetic features of the mutant enzymes at the residue responsible for the binding of the ε-amino group were investigated. The conformational changes and channel entrances for the substrates that connect the active site and solvent were suggested [23].

L-Phe oxidase (EC 1.13.12.9) from *Pseudomonas* sp. P-501 catalyzes both the oxidative deamination and oxygenative decarboxylation to produce 0.2 mol each of phenylpyruvate, ammonia, and H_2O_2 and 0.8 mol each of phenylacetamide and carbon dioxide under aerobic conditions [24]. The structure of L-Phe oxidase was solved [25] and the mechanism of L-Phe oxidase reactions is discussed (PDB 2YR6) [26]. The flavoprotein L-Trp 2-monooxygenase catalyzes the oxidative decarboxylation of L-Trp to yield indole-3-acetamide [27]. L-AAO/MOG, L-Phe oxidase, and L-Trp 2-monooxygenase seem to share the same reaction mechanism, in which subtle changes in their structures are influencing the balance between oxidase and oxygenase reactions.

A novel enzyme, which catalyzes decarboxylation of L-Lys into cadaverine with release of carbon dioxide and oxidative deamination of L-Lys into L-2-aminoadipic 5-semialdehyde with release of ammonia and H_2O_2, was discovered from a newly isolated *Burkholderia* sp. AIU 395 [28•]. The enzyme contains one mol of PLP per subunit as a prosthetic group. Since the enzyme was specific to L-Lys, total L-Lys was assayed by an additional putrescine oxidase from *Micrococcus rubens* [29].

To obtain an L-Trp-specific L-amino acid oxidase (AAO), we focused on bis-indole antibiotic biosynthesis in *Streptomyces* sp. TP-A0274. A putative L-AAO from StaO, which is involved in staurosporine biosynthesis, was heterologously expressed, biochemically characterized, and shown to serve as a selective L-Trp oxidase. Furthermore, another L-AAO, VioA, which is involved in violacein biosynthesis in *Chromobacterium violaceum*, was also characterized. StaO and VioA share similar properties, such as a narrow substrate specificity and high affinity for L-Trp, although they have many differences in their primary structures [30]. A stable mutant of VioA (C395A) from *C. violaceum* was generated and utilized for L-Trp quantification in human plasma samples. Fuller *et al.* reported the X-ray crystallographic structure of VioA (PDB 5G3T) [31], and those of VioA (C395A) complexed with FAD and L-Trp and with FAD alone were solved at 1.8 Å resolution (PDB 5DZB) [32•].

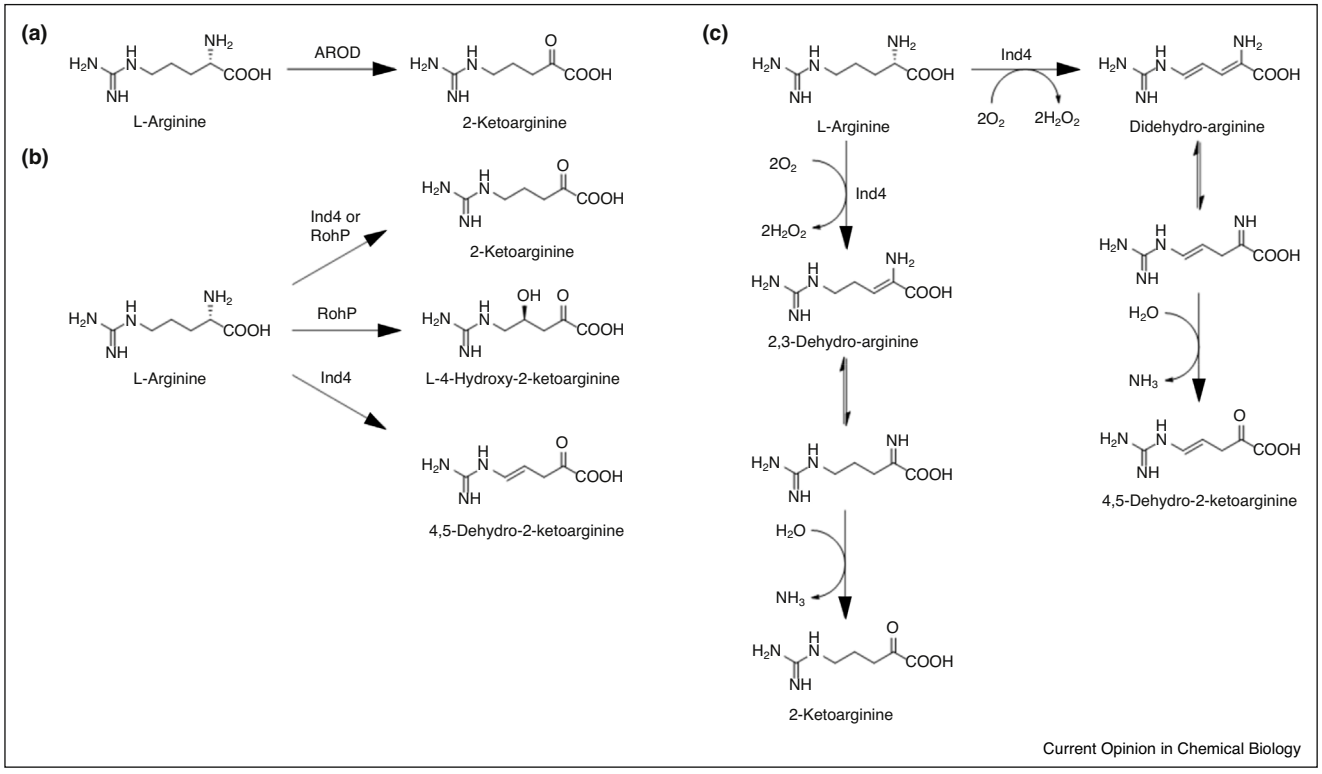
L-Arg oxidase (AROD) was discovered in the newly isolated *Pseudomonas* sp. TPU 7192. The FAD-dependent enzyme catalyzed the oxidative deamination of L-Arg to 2-ketoarginine, which was non-enzymatically converted into 4-guanidinobutyric acid in the presence of H_2O_2 that formed from L-Arg oxidation (Figure 3(a)). A simple enzymatic method for the determination of L-Arg was established using the enzyme [33•].

A new PLP-dependent enzyme, Ind4 or RohP, which catalyzes the oxidation of an unactivated carbon-carbon (C–C) of L-Arg and participates in the biosynthesis of indolmycin in *Streptomyces griseus* ATCC 12648, has been characterized [34•,35]. The enzyme is classified as a fold-type I PLP-dependent enzyme. The enzyme catalyzes the two electron oxidation of L-Arg to give 2-ketoarginine, similar to *Pseudomonas* AROD. This enzyme also carries out the four-electron oxidation of L-Arg, including the oxidation of an unactivated C–C bond to give didehydro-arginine (2-amino-5-guanidinopenta-2,4-dienoate). In the presence of RohP, the didehedro-arginine is hydrated in water to give L-4-hydroxy-2-ketoarginine (Figure 3(b)). The imine compound is hydrolyzed to 4,5-dehydro-2-ketoarginine (Figure 3(c)). Structures (1.5 Å resolution) of the intermediates along the catalytic cycle were obtained (PDB 6C3A, 6C3B, 6C3C, 6C3D) [36••]. Figure 4(a) shows a possible structure of RohP with a Schiff-base (quinoid intermediate) complex having a double bond between Cβ and Cγ (PDB 6C3C). Figure 4(b) shows the active site with the residues surrounding the quinoid intermediate. Structure of a similar L-Arg hydroxylase/deaminase MppP from *Streptomyces wadayamensis* involved in the biosynthesis of L-enduracididine was solved (PDB 5BK7) and a mechanism was proposed with an incorporation of ^{18}O from $H_2{}^{18}O$ [37,38••].

Applications of amino acid oxidases (L-Asp oxidase, L-Glu oxidase, and L-Phe oxidase)

L-Asp oxidase gene from *Thermococcus litoralis* DSM 5473 was expressed in the soluble fraction of *E. coli*,

Figure 3



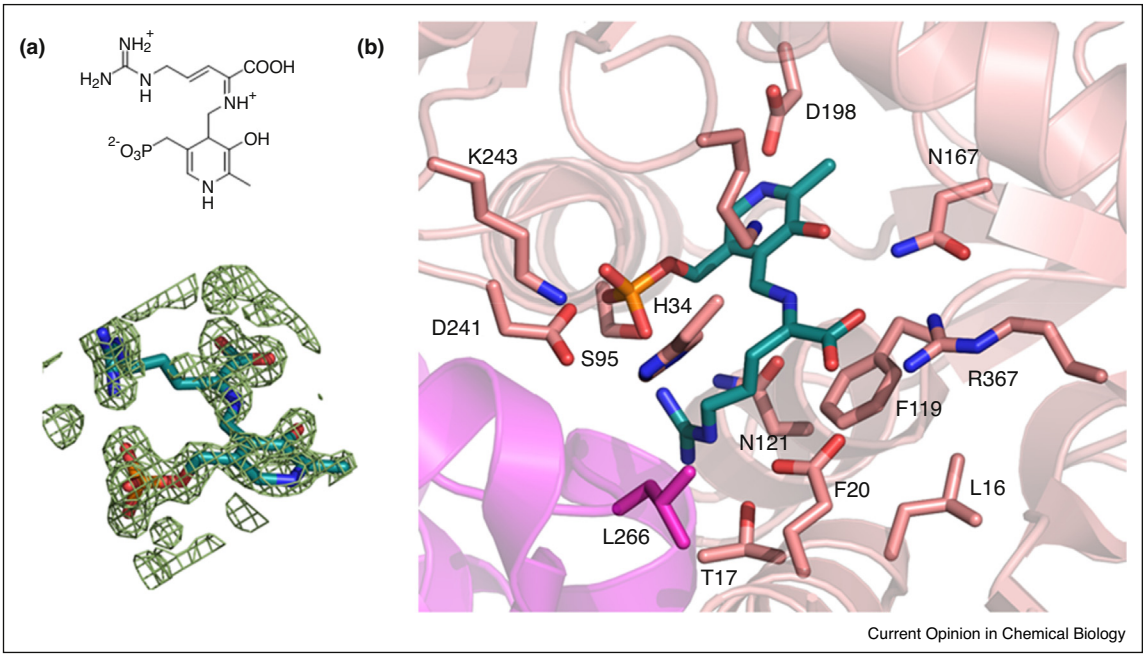
FAD-dependent **(a)** and PLP-dependent L-Arg oxidases (b,c).
FAD-dependent L-Arg oxidase-catalyzed reaction. **(b)** PLP-dependent L-Arg oxidase Ind4 and RohP-catalyzed reactions. **(c)** Ind4-catalyzed transformation of L-Arg.

and the gene product showed L-Asp oxidase activity. The quaternary structure was homotrimeric with a subunit molecular mass of 52 kDa. The enzyme was highly specific for L-Asp [39]. An L-Glu oxidase was isolated, and its gene was cloned and characterized from *Streptomyces diastatochromogenes*. The enzyme exhibited strict specificity for L-Glu and good thermostability [40]. Another gene for L-Glu oxidase from *Streptomyces ghanaenis* ATCC14672 expressed well in *Pichia pastoris*. The recombinant enzyme displayed an optimal temperature of 40 °C, suitable for the transformation of L-Glu to α-ketoglutaric acid [41]. The gene for a new L-Phe oxidase from a mushroom, *Coprinopsis cinereus*, was cloned and expressed in *E. coli*. It can be used for the conversion of L-Phe to phenylpyruvate and ammonia [42]. Review articles on the importance of α-keto acids as intermediates of metabolic engineering [43•] and the biotechnological production of α-keto acids have appeared [44]. Since L-amino acids can be produced by fermentative methods from raw materials, deaminated products from amino acids, α-keto acids have potentials as starting materials. They are transformed into aliphatic alcohols via aldehydes, and carboxylic acids, by decarboxylation and redox reactions, respectively. Bifunctional compounds such as diamines, diacids and diols can be produced from α-keto acids containing polar groups. Aromatic α-keto acids are key precursors to styrene, phenolic acids and polyphenols.

L-Amino acid deaminase

The crystal structure of a membrane-bound L-amino acid deaminase from *Proteus vulgaris* has been solved. The FAD-containing enzyme catalyzes the deamination of L-amino acids to the corresponding α-keto acids and ammonia without H_2O_2 production, because the electrons of the reduced cofactor are transferred to a membrane-bound cytochrome. Structural studies of the enzymes from *Proteus myofaciens* (PDB 5FJN) [45•] and *P. vulgaris* (PDB 5HXW) [46,47] showed that in addition to a FAD-binding and a substrate-binding domain, it also possesses an N-terminal putative transmembrane α-helix and a small α and β subdomain that is strictly interconnected to the substrate binding domain. The tertiary structure resembles those of FAD-binding oxidoreductases, such as the β subunit of L-Pro dehydrogenase from *Pyrococcus horikoshii* (PDB 1Y56), Gly oxidase from *Bacillus subtilis* (PDB 1C0I), and sarosine oxidase from *Corynebacterium* sp. U-96. (PDB 3ADA) [48].

Figure 4



A new PLP-dependent L-Arg oxidase.
(a) A possible structure of RohP with a Schiff-base (quinoid intermediate) complex with dehydro-L-Arg having a double bond between Cβ and Cγ (upper), electron densities supporting the structure (lower) (PDB 6C3D). **(b)** The active site with the residues surrounding the quinoid intermediate.

Use of the enzyme for phenyllactate synthesis is demonstrated by co-expressing genes for D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus delbrueckii* subsp. *bulgaricus* and formate dehydrogenase from *Mycobacterium vaccae* [48]. There are good review articles on L-amino acid deaminase which is present in the genera *Proteus*, *Providencia* and *Morganella* [47,49,50].

Conclusion

Many examples of intriguing new enzymes acting on several among twenty kinds of simple amino acids were introduced. The details of the X-ray crystallographic structures and the mechanisms could be traced from the PDB IDs and the literatures, respectively. There was a mother of invention along with curiosities of the researchers for the development of the science in this area. It has been necessary in the title area to develop very selective oxidases and dehydrogenases acting on L-amino acids for diagnosis following our success on the development of selective NAD^+-dependent L-phenylalanine dehydrogenase, which have been utilized to analyze the blood samples of more than 5,400,000 neonates in Japan against Phenylketourea. Most of the selective enzymes are patented and their applications seem to be under development. Recent discussion and advancement on enzymology are associated not only with the primary, secondary and tertiary structural information, but also with the first-principles FMO calculations, in addition to the 'activities' and 'substrate specificities' which have been used to discuss the properties of the enzymes. We saw such modern example in the case of the design and development of a new *R*-amine oxidase created from D-amino acid oxidase. There are emerging possibilities in the enzymatic production of α-keto acids from L-amino acids, and the practical uses of the oxidative enzymes *in vivo* and *in vitro* are expected.

Conflict of interest statement

Nothing declared.

Acknowledgements

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