PH-Dependent Enantioselectivity of D-amino Acid Oxidase in Aqueous Solution

Qingju Liu1, Li Chen1, Zhikun Zhang1, Bibai Du1, Yating Xiao1, Kunhao Yang1, Lingling Gong1, Li Wu1, Xiangjun Li1 & Yujian He1,2

D-amino acid oxidases (DAAO) are stereospecific enzymes which are generally almost inactive towards L-enantiomer in neutral solution when L-, D-amino acids are supplied as substrates. In this paper, the D-amino acid oxidase can catalytically oxidize L-amino acids by modulating pH of aqueous solution. With L-Pro as substrate, the catalytic rate ($k_{cat}$) and the affinity ($K_m$) of DAAO were 6.71 s$^{-1}$ and 33 mM at pH 8.0, respectively, suggesting that optimal pH condition enhanced the activity of DAAO towards L-Pro. Similar results were obtained when L-Ala (pH 9.8), L-Arg (pH 6.5), L-Phe (pH 9.0), L-Thr (pH 9.4), and L-Val (pH 8.5) were catalyzed by DAAO at various pH values. The racemization of the L-amino acids was not found by capillary electrophoresis analysis during oxidation, and quantification analysis of L-amino acids before and after catalytic reaction was performed, which confirmed that the modulation of enantioselectivity of DAAO resulted from the oxidation of L-amino acids rather than D-amino acids by changing pH. A mechanistic model was proposed to explain enhanced activity of DAAO towards L-amino acids under optimal pH condition.

D-amino acid oxidase (DAAO) is the prototype of the FAD-dependent flavoprotein, which catalyzes oxidative deamination of a majority of D-amino acids rather than L-amino acids, producing the corresponding α-keto acid and ammonia1–3. DAAO is a ubiquitous enzyme which presents in numerous eukaryotic organisms4 and plays a crucial role in acting as a detoxifying agent of accumulated D-amino acids in the kidney and liver, maintaining the necessary levels of D-serine in different brain tissues5, and producing of 7-Aminocephalosporanic acid from the natural antibiotic cephalosporin C in a two-enzyme process6. Once enantioselectivity of DAAO is disturbed, the metabolic systems might be disordered, which might be found as precipitating factors of chiral disease.

DAAO has been extensively investigated over the past 80 years, but most issues focused on determining the structure of its binding to targeting protein7, 8, increasing its catalytic activity9 as well as exploring the probably correlation between the DAAO and the human diseases10. Recent advancements in utilization of DAAO include as biocatalysts for resolving racemic amino acid mixtures, as a tool for biosensing, a new mechanism of herbicide resistance11–13 and enzymatic synthesis of chiral amine14, 15. However, altering enantioselectivity of DAAO has been regarded as one of the most remarkable topics in enzymatic reactions, since the stereochemical outcome of a given enzyme-substrate reaction is predetermined in specific target environment. It is demonstrated that the alteration of enzyme enantioselectivity can be achieved by directed evolution16, site-directed mutagenesis17–19 and reaction temperature20–22. They require either a time-consuming enzymatic preparation or bring about undesirable deviation from the initial substrate. Additionally, the variation of the nature of the solvent greatly affected the enzyme enantioselectivity. For example, Margolin23 discovered that a facile method for enzymatic preparation of diverse peptides containing D-amino acids by radically altering enantioselectivity of enzymes in organic solvents. Cerovsky et al.24 found a complete reversal of enantioselectivity of the transesterification catalyzed by Aspergillus oryzae protease with 18 anhydrous solvents. These studies have clearly shown that the changes of enzyme enantioselectivity can be achieved by using organic solvents, whereas little attention has been focused on the exploitation of aqueous solution, which is more important for the living systems.

1School of Chemistry and Chemical Engineering, University of Chinese Academy of Sciences, Huabei Village, Huabei Town, Huairou District, Beijing, 101408, China. 2State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, 38 Xueyuan Road, Haidian District, Beijing, 100191, China. Correspondence and requests for materials should be addressed to L.W. (email: wuli@ucas.ac.cn) or X.L. (email: lixiangj@ucas.ac.cn) or Y.H. (email: heyujian@ucas.ac.cn)

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Water is convenient to maintain the conformation of the enzyme catalytic center via the formation of hydrogen bonds, hydrophobic bond and Van der Waals force. In aqueous solution, pH has a predominant influence on the enzymatic catalysis and can be easily controlled and manipulated. What's more, appropriate pH conditions are essential for the ionic groups around the enzyme active center to achieve the best ionic state that enzyme catalytic reaction required. Up to date, much of researches have focused on the influence of pH on the activity and stability of enzyme, while few studies have looks at the issue of pH-dependent enantioselectivity. Just one typical example is reduction reaction of 2-butanone catalyzed by horse liver alcohol dehydrogenase, which exhibits the changes of pH-induced enantioselectivity and determine how the discrimination of substrates was affected. Furthermore, Francesco developed a theoretical model based on differential catalytic commitments and to explain effect of pH on the reaction catalyzed by horse liver alcohol dehydrogenase and secondary alcohol dehydrogenase from thermoanerobacter ethanolicus.

Enzyme plays an important role in metabolism. In view of strict enantioselectivity and broad substrate specificity of DAAO to amino acids, a pH screening along with both enantiomers of amino acids was performed to study the influence of pH on enantioselectivity of the enzyme. Here, aqueous solution was used in enzymatic reaction. The results demonstrated that DAAO activities towards some neutral and basic L-amino acid were obviously improved by increasing or decreasing pH values when compared with their catalytic activities in neutral aqueous solution. Also, the DAAO activity towards acidic L-Asp was initiated and became quite similar towards both enantiomers of the acidic amino acids. Furthermore, catalytic oxidation of L-amino acids by DAAO was further confirmed by excluding the configuration inversion of L-amino acids and quantifying consumption of L-amino acids in the reaction process. Molecular modeling and optimization suggested that the enhanced catalytic activity of DAAO towards L-Ala at pH 9.8 may be due to the easy transpositions of amino group and methyl group of L-Ala binding to DAAO when positively charged amino group was changed into electrically neutral amino group under alkaline conditions. Such a study is necessary to understand the structure of active center, especially the state of charge, contributing alteration of stereospecificity of DAAO, which may provide a positive guidance for asymmetric catalytic oxidation of chiral amino acids by DAAO in aqueous solution.

### Results

**Effect of pH on activity of DAAO with neutral and basic L-amino acids as substrate.** It’s acknowledged that DAAO was highly enantioselective which showed a strong preference for the D-amino acids and possessed a negligible catalytic efficiency on L-amino acids under physiological conditions. DAAO could dehydrogenate D-amino acids yielding hydrogen peroxide, which could interact with DHBS and 4-AAP yielding purple red semiquinone. According to this detection method, the catalytic activities of DAAO with various L-amino acids as substrate were studied in a pH range from 6.0 to 10.5, as shown in Supplementary Figures S1–S17, and the ratios of UV absorbance of L-amino acids and D-amino acids were derived for easy observation, as shown in Table 1.

Water is convenient to maintain the conformation of the enzyme catalytic center via the formation of hydrogen bonds, hydrophobic bond and Van der Waals force. In aqueous solution, pH has a predominant influence on the enzymatic catalysis and can be easily controlled and manipulated. What's more, appropriate pH conditions are essential for the ionic groups around the enzyme active center to achieve the best ionic state that enzyme catalytic reaction required. Up to date, much of researches have focused on the influence of pH on the activity and stability of enzyme, while few studies have looks at the issue of pH-dependent enantioselectivity. Just one typical example is reduction reaction of 2-butanone catalyzed by horse liver alcohol dehydrogenase, which exhibits the changes of pH-induced enantioselectivity and determine how the discrimination of substrates was affected. Furthermore, Francesco developed a theoretical model based on differential catalytic commitments and to explain effect of pH on the reaction catalyzed by horse liver alcohol dehydrogenase and secondary alcohol dehydrogenase from thermoanerobacter ethanolicus.

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It was found that varying degrees of oxidation deamination of L-Pro occurred with the change of pH values. DAAO displayed a slight catalytic activity towards L-enantiomers, and the ratio of UV absorbance of L-Pro to D-Pro at 512 nm was less than 2.7 when pH of the solution ranging from 6.0 to 7.0. Unexpectedly, with increasing pH value to 7.5, the ratio of relative activity raised to 6.8, and it reached to a plateau of activity when pH value was 8.0. Then, the catalytic activity of DAAO towards L-Pro instead decreased when increasing...
K8.0. Also, there was a tremendous increase in affinity of DAAO to substrate at pH 8.0, as evidenced by a 9.59-fold increase (9.59-fold for pH 7.5 and 16.78-fold for pH 8.5) in catalytic activity of DAAO towards L-Pro at pH 8.0. However, there was a significant increase in affinity of DAAO towards D-pro slightly increased at pH 8.0 based on the results were shown in Table 1 and Supplementary Tables S1 and S2. Obviously, pH variation strikingly accelerates the reaction of DAAO with L-Arg, L-Phe, L-Thr and L-Val, in addition to L-Pro and L-Ala, and even the lower reactivity of its D-counterpart.

Comparison of kinetic parameters of DAAO to D-Pro and L-Pro under different pH values.

| pH  | D-Pro  | L-Pro  | k_{cat}(s^{-1}) | K_{m}(mM) | k_{cat}(s^{-1}) | K_{m}(mM) | k_{cat,D-Pro}/k_{cat,L-Pro} |
|-----|--------|--------|-----------------|-----------|-----------------|-----------|-----------------------------|
| 7.5 | 50.20±1.20 | 0.38±0.02 | 0.70±0.06 | 252.00±10.00 | 71.42 | |
| 8.0 | 107.38±9.10 | 0.33±0.01 | 6.71±0.51 | 20.00±1.73 | 15.99 | |
| 8.5 | 43.30±3.40 | 0.85±0.06 | 0.40±0.03 | 1800.00±90.50 | 108.25 | |

Table 2. Comparison of kinetic parameters of DAAO to D-Pro and L-Pro under different pH values.

pH value from 8.0 to 10.5. Obviously, catalytic activity of DAAO towards L-Pro was most evident at pH 8.0 and the ratio of UV absorbance of D-Pro to D-Pro was up to 17.2 (Table 1). The determination of DAAO activities at various pH values by coupling method was using peroxidase, whose activity could also be influenced by pH. Therefore, 2,4-dinitrophenylhydrazine was used to further confirm the activities, and the results were shown in Supplementary Figures S18–S34 and Table S2. The catalytic activities DAAO towards the amino acids at various pHs using 2,4-dinitrophenylhydrazine agree with their corresponding results by a coupling method using horseradish peroxidase, indicating that horseradish peroxidase was undisturbed by pH within the test range.

Next, the kinetic parameters (k_{cat} and K_{m}) of DAAO with both enantiomers of Pro as substrate were determined in a range from pH 7.5 to pH 8.5, as shown in Table 2, where k_{cat} and K_{m} reflected the ability to catalyze substrate and affinity of enzyme, respectively. When D-Pro was used as substrate, k_{cat} of DAAO at pH 8.0 was 107.30 s^{-1}, increased 2.2-fold and 2.47-fold in catalytic ability compared with pH 7.5 and pH 8.5, respectively. The affinity of DAAO towards D-pro slightly increased at pH 8.0 based on K_{m} values. However, there was a significant increase (9.59-fold for pH 7.5 and 16.78-fold for pH 8.5) in catalytic activity of DAAO towards L-Pro at pH 8.0. Also, there was a tremendous increase in affinity of DAAO to substrate at pH 8.0, as evidenced by K_{m} values changing from 252 mM (pH 7.5) and 1800 mM (pH 8.5) to 20.00 mM (pH 8.0). Clearly, the increase in catalytic rate of DAAO to L-Pro was greatly different from that of DAAO to D-Pro when pH was changed into 8.0, thereby leading to only 15.99-fold drop in the catalytic activity according to k_{cat,D-Pro}/k_{cat,L-Pro}, while it occurred 71.42-fold and 108.25-fold drop at pH 7.5 and pH 8.5, respectively. Similarly, the relative catalytic ability (k_{cat,D-Ala}/k_{cat,L-Ala}) of DAAO to Ala tremendously decreased from 1680.00 at pH 7.0 to 14.18 at pH 9.8 (Table 3). However, it was noted that the initial rate of enzymatic deamination for D-amino acids was found to exceed that for L-amino acids by 3000 to 4000-fold under physiological condition. Our results indicated that the changes of pH significantly enhanced the catalytic rate of DAAO towards L-Pro and L-Ala. Moreover, the observed relaxation of enantioselectivity of DAAO induced by pH was due to a much higher reactivity of the L-Pro rather than a much lower reactivity of its D-counterpart.

To check the phenomenon was common occurred for all amino acids, all L-amino acids except for L-Tyr were applied to check DAAO activity due to L-Tyr’s low solubility. Enzyme samples were prepared by dissolving the purified DAAO in different buffer solutions containing various concentrations of L-amino acids and activity was evaluated under standard assay conditions. Similar phenomena were observed under different pH conditions and the results were shown in Table 1 and Supplementary Tables S1 and S2. Obviously, pH variation strikingly accelerated the reaction of DAAO with L-Arg, L-Phe, L-Thr and L-Val, in addition to L-Pro and L-Ala, and even the k_{cat} is up to 9.4 s^{-1} for L-Ala under optimum pH conditions (Supplementary Table S1). In contrast, the influence of pH on DAAO activity was modest in presence of L-Leu, L-Asn, and L-Gln. However, in case of L-Ile, L-Ser, L-Lys, L-His, L-Cys, L-Met and L-Trp, the activities were negligible and thus the ratio in absorbance of L-amino acid and UV absorbance of L-Pro to D-Pro was up to 17.2 (Table 1). The determination of DAAO activities at various pH values by coupling method was using peroxidase, whose activity could also be influenced by pH. Therefore, 2,4-dinitrophenylhydrazine was used to further confirm the activities, and the results were shown in Supplementary Figures S18–S34 and Table S2. The catalytic activities DAAO towards the amino acids at various pHs using 2,4-dinitrophenylhydrazine agree with their corresponding results by a coupling method using horseradish peroxidase, indicating that horseradish peroxidase was undisturbed by pH within the test range.

Effect of pH on activity of DAAO with acidic L-amino acid as substrate. As we know, DAAO possesses very poor or no activity towards D-Asp and D-Glu under normal physiological conditions since D-aspartate oxidase oxidizes D-enantiomer of acidic amino acids in more specific fashion. However, it’s wondered whether pH variation would increase DAAO activity when acidic L-amino acids were used as substrates.

The enantiomers for Glu and Asp were parallelly assayed under the various pH conditions and the results were shown in Table 4. As inactive as DAAO went, the catalytic efficiency of DAAO to both D-Glu and L-Glu was unnoticeable regardless of what kind of pH condition. Nevertheless, DAAO exhibited activity towards not only L-Asp but also D-Asp when pH was changed into 8.0. Moreover, DAAO possessed a similar catalytic activity (k_{cat,L-Asp} = 0.89) for L-Asp compared to with D-Asp (k_{cat,D-Asp} = 0.95), as well as similar affinity (K_{m,L-Asp} = 43 and K_{m,D-Asp} = 45). These results confirmed that pH variation could initiate activity of DAAO towards certain acidic L-amino acid.

| pH  | D-Pro  | L-Pro  | k_{cat}(s^{-1}) | K_{m}(mM) | k_{cat}(s^{-1}) | K_{m}(mM) | k_{cat,D-Pro}/k_{cat,L-Pro} |
|-----|--------|--------|-----------------|-----------|-----------------|-----------|-----------------------------|
| 7.0 | 151.20±1.20 | 30.50±1.50 | 0.09±0.01 | 362.00±10.00 | 1680.00 | |
| 9.8 | 133.30±3.40 | 38.20±1.20 | 9.40±0.39 | 43.11±3.61 | 14.18 | |

Table 3. Comparison of kinetic parameters of DAAO to D-Ala and L-Ala under different pH values.
Confirmation for catalytic oxidation of L-amino acids in the catalytic process. Since racemization of amino acids is easy to occur in alkaline solution or when heated, it’s obviously impossible to distinguish whether DAAO catalyzed the oxidative deamination of L-amino acids under special conditions or DAAO catalyzed the oxidative deamination of D-amino acids arising from the racemization of L-amino acids during the experiments. If the racemization occurred during the reaction, how much contamination of D-amino acid over L-amino acid would result in visualizing the oxidative deamination of L-amino acid by DAAO. Thus, to exclude racemization of L-amino acid, we chose some L-amino acids able to be catalyzed by DAAO (Fig. 1 and Supplementary Figure S35).

After 30 minutes incubation at 37 °C, the solution of L-Pro (pH 8.0) was derivatized by the FMOC and then analyzed on CE, which was capable of differentiating the derivatives of D- and L-amino acids with high resolution and efficiency. The derivative of D-Pro was not detected in the solution of derivatized L-Pro after 30 min incubation in the detection limit range (10^{-9} mol/L), according to the comparation of L-Pro solution and the mixture solution of L-Pro and D-Pro after derivatization (Fig. 1), which indicated that the L-Pro was not transformed to its mirror-image configuration at pH 8.0 and DAAO indeed catalyzed the oxidation of L-Pro during the reaction time. Similarly, solutions of the other L-amino acids solutions such as Ala, Phe, Gln, Arg, Leu, Thr, Asp, Asn and Val were also analyzed under their optimal pH conditions, and it was found that D-enantiomers were not detected within reaction time (Supplementary Figure S35).

Although the initial activity of DAAO to L-amino acids and D-amino acids was determined by UV (Supplementary Figure S1–S34) and HPLC analysis (Supplementary Figure S36), the quantitative analysis of catalytic oxidation of L-amino acids by DAAO was further performed. The contents of L-amino acids before (S_{before})
Gly313 and Gln536, which would be vital for the binding and orientation of the substrate. which is designated pkDAAO. The active site of pkDAAO is made up of five amino acids: Tyr224, Tyr228, Arg283, structures of DAAO in complex with D- and L-enantiomers of an amino acid were deduced by molecular opti-

Supplementary Figure S37a The interesting phenomena was consistent with experimental data that much activity DAAO solution increased to 10.5, suggesting a relative larger change for secondary structure formed, as shown in

D- and L-amino acids, such as substrate binding, hydrophobic binding and reactive potential of active sites33. The rates of the L-amino acids were different from each other, due to different interactions between enzyme and vari-
sous the L-amino acids, such as substrate binding, hydrophobic binding and reactive potential of active sites33. The reduction in content of L-amino acids with catalytic oxidation of DAAO was also an evidence for the increasing of DAAO activity to L-amino acids under optimal pH conditions.

Discussion
Change of pH might induce conformational variations of DAAO as well as other effects such as protonation state of amino acid residues at the active site and interaction between active site of DAAO and substrate. Thus, a structural understanding is helpful to explain relaxation of enantioselectivity of DAAO. We first investigated the conformation of DAAO solution in various pH conditions by CD analysis, since CD spectrum analysis was utilized to test whether pH induced a disruption of the overall protein structure or just indicative of local unfold-
ing. The stability studies of DAAO were characterized by the CD signal around 215 nm (taken as a reporter of the change in secondary structure)34 and depicted in Supplementary Figure S37. DAAO is a type of [βα]3 protein under physiological condition, as evidenced by a clear negative peak around 215 nm35. Moreover, CD signal at 215 nm became slightly stronger with pH of DAAO solution increasing from 6.5 to 10.0, but no shift in wave-
length was observed (Supplementary Figure S37b), indicating that there occurred a minor alteration from α to β for DAAO conformation in the pH range. However, CD signal at 215 nm shifted to 222 nm when pH of the DAAO solution increased to 10.5, suggesting a relative larger change for secondary structure formed, as shown in

Supplementary Figure S37a The interesting phenomena was consistent with experimental data that much activity to L-Ala at pH 9.8 but not completely at pH 10.5 (Supplementary Figure S2).

To further explain why DAAO’s enantioselectivity is pH-dependent as shown in Tables 1,2 and 3, the plausible structures of DAAO in complex with D- and L-enantiomers of an amino acid were deduced by molecular optimi-

ization. The DAAO utilized in the experiments is a domain from porcine kidney with 347 amino acid residues, which is designated pkDAAO. The active site of pkDAAO is made up of five amino acids: Tyr224, Tyr228, Arg283, Gly313 and Gln53, which would be vital for the binding and orientation of the substrate.

The model of binding of D-Ala to pkDAAO under physiological conditions is presented in Fig. 2a and Supplementary Figure S38. Also make special note that the amino group of Ala is positively charged in near-neutral pH solution, and the interaction between DAAO and D-enantiomer is described as follows36,37: (a) The −COO− of the substrate makes a salt bridge with the cationic guanidine group of Arg283 and one of its oxygen atoms interacts with the hydroxyl group of the two tyrosine residues (Tyr224 and Tyr228); (b) The NH3+ of substrate is hydrogen bond with the backbone carbonyl of Gly313, with a tendency to ionic bonding, and also bound to Gln53 by a water molecule in between, whereas the water molecule has well-defined electron density in all eight subunits present in the asymmetric unit, which helps H2O to attack α-amino group during deam-
imization. Rather, D-Ala is tightly bound to the active site of the enzyme by noncovalent interaction, forming a quite stable transient intermediate. However, L-Ala, as mirror image of D-Ala, has both NH3+ and CH3 but in the reversed position, which are placed into the binding site cavity in terms of same steric bulk36,37. The L-enantiomer of Ala binds to the enzyme in a fashion shown in Fig. 2b. The −COO− substituents at the chiral carbon bind to the Arg283, Tyr224 and Tyr228 the same way as those of its D-counterpart, whereas the amino group faces away from the carbonyl moiety of Gly313 and Gln53, thereby making the nucleophilic attack impossible, which would explain why the pkDAAO is high stereospecific to D-Amino acids under optimal pH conditions.

When pH of the near-neutral solution rised to 9.8, the cationic amino group could be deprotonated to become neutral amino group-NH2, forming the hydrophobic environment surrounding. The function groups -NH2 and CH3 of L-Ala were isosteres, which shared similar electronegativities and biological activities. The

| pH | L-amino acid | Sbefore | Safter | (Sbefore − Safter)/Safter(%) |
|----|--------------|---------|-------|-----------------------------|
| 8.0 | L-Pro        | 38880.83| 33545.33 | 17.16                       |
| 9.8 | L-Ala        | 18084.72| 15603.50 | 13.72                       |
| 6.5 | L-Arg        | 32483.21| 27603.50 | 15.02                       |
| 9.0 | L-Phe        | 36132.83| 29223.80 | 9.12                        |
| 8.0 | L-Gln        | 17798.74| 17711.53 | 0.49                        |
| 9.8 | L-Leu        | 22968.91| 22819.61 | 0.65                        |
| 9.4 | L-Thr        | 11935.17| 11036.45 | 7.33                        |
| 8.0 | L-Asp        | 12307.99| 12251.37 | 0.46                        |
| 8.5 | L-Asn        | 9707.83 | 9652.49  | 0.57                        |
| 8.5 | L-Val        | 12108.88| 11728.66 | 3.14                        |

Table 5. Quantification of L-amino acids before and after reaction of DAAO by CE analysis.
Steric difficulties encountered by the D- or L-enantiomer will not be exacerbated, then either of Ala would be enough to fit into the pocket of enzyme active center. Thus, the productive binding between α-COO\(^{-}\) substituents and the amino acids (Arg283, Tyr224 and Tyr228) of enzyme active center will be unaffected for both D- and L-enantiomer in spite of an increase of pH, whereas the hydrogen binding formed by amino group's nucleophilic attack carbonyl of Gly313 weakened due to change of α-amino group of Ala from NH\(_3^+\) to NH\(_2\). Interestingly, there remains one hydrogen binding comes into being between H\(_2\)O and α-amino group for the L-enantiomer, in addition to hydrogen binding between the amino group and Gln53 through coupling with one H\(_2\)O for the D-Ala. It is reported that the reaction of Ala catalyzed by DAAO first dehydrogenates the amino acid to the corresponding imino acid, coupled with the reduction of FAD, then hydrolyzes to the α-keto acid and ammonia\(^1\). Thus, the attack of H\(_2\)O to α-amino group play a key role in catalyzed oxidization of amino acid. The weakened interaction between substrate and active site of enzymic cavity at pH 9.8 in comparision to the near-neutral condition, leads to easy rotation of Ala and thus increases attacking chances of H\(_2\)O to α-amino group of L-Ala (Fig. 2b and d). In addition, the efficient hydride transfer of substrate to FAD is also crucial for the dehydrogenation of amino acid. At high pH (≥8), the substrate amino acid binds in the “α-NH\(_2\)” form, that is suitable for immediate hydride transfer. After pH ≤ 7 (α-NH\(_3^+\) form), the H\(^+\) liberated during hydride transfer is proposed to be relayed to bulk solvent\(^40\).

Indeed, the catalytic activity (k\(_{\text{cat}}\)) of the DAAO to L-Ala at pH 9.8 increases from 0.09 s\(^{-1}\) to 9.40 s\(^{-1}\), and the binding affinity of the DAAO for L-Ala is relatively altered (Table 3). Clearly, the relaxation of stereospecificity of DAAO occurred from D- to L-enantiomer when the pH increases from 7.0 to 9.8. This has been verified experimentally: Upon transition from pH 7.0 to pH 9.8, (k\(_{\text{cat}}\))\(_{\text{L-Ala}}\) increases more than 104.4-fold, whereas (k\(_{\text{cat}}\))\(_{\text{D-Ala}}\) decreases only 1.1-fold, thereby leading to the overall 114.8-fold drop in enantioselectivity (Table 3).

In the case of Pro, the group –CH\(_2\)- and –NH- attached to chiral carbon atom are also isosteres like Ala, and further the five membered heterocyclic conformation containing chiral carbon at the side chain of Pro formed a compact and unencumbered structure so that both enantiomers of Pro would fit well with cavity of active center of DAAO, and thus the reason for relaxation of enantioselectivity of DAAO to Pro may be the same as the explanation for modeling of DAAO binding to Ala.

Previous studies have shown that the reaction catalyzed by DAAO proceeds without direct involvement of amino acid functional groups\(^13\). Most residues present at the active site are involved in substrate binding and precise orientation of the interacting orbitals\(^13\), the remarkable substrate specificity change mainly ascribes to new favorable electrostatic interactions between the guanidium group of the L-Arg and the negative charge of the active center. The ε-NH\(_3\) and -NH\(_2\)- of Arg is positively charged in neutral, basic and even most basic environments. The electrostatic interaction between ε-NH\(_3\) and -NH\(_2\)- and active center of enzyme in presence of pH 6.5 is stronger than in pH 7.5, thus electrostatic interactions may facilitate the orientation of L-Arg by DAAO.
resulting in activation to L-Arg. Conformational diversity of the active site and substrate is likely to contribute to acceptance of non-native compounds in many cases. Due to the flexibility of active site, the electrostatic interactions between the COO\(^-\) of side chain and enzyme help the recognition of L-Asp by DAAO. In contrast, the longer side chain of L-Glu displays increased steric exclusion from an active site.

In view of different pK\(_a\) and pK\(_c\) corresponding to different amino acids (Pro, Ala, Arg, Thr, Val, Tyr and Phe), the amino acids residues in the active center and the substrates are in different ionic and charge states. Therefore, the L-configuration of different amino acids could be recognized by DAAO under different pH conditions, exhibiting optimal activity at different pH values. However, for most amino acids, despite pH is able to change the protonated state of substrate, there is striking disparity in the function group of the side chain, thereby showing no activation by DAAO. One possible reason is that the reversed groups of amino acids experiences severe steric hindrances in fitting into the binding pocket, which agreed with the fact that the active sites of hydrophobic residues of pkDAAO formed a cavity roughly corresponding to the volume occupied by an amino acid with a four-carbon-atom side chain.

Conclusions

In summary, the study demonstrated that a biochemically useful process, an enzymatic enantioselectivity of amino acids with DAAO, could be controlled by easily adjusting the pH of aqueous solution, although the adjustment of pH was not always in good agreement with our predictive physiological conditions. The model that catalytic oxidation of DAAO to all the amino acids was developed and confirmed experimentally, and this phenomenon was further mechanistically explained by molecular optimization. In addition, it should be noted that pH control of enantioselectivity is not limited to DAAO. Actually, our preliminary study with aminoacyl-tRNA synthetase is to predict that its enantioselectivity in transesterification of amino acids also depends on the environmental factors, even though the catalyzed enantioselective reaction of DAAO towards amino acids is opposite to that of the aminoacyl transferase. On a practical note, these data (A pH screening along with a conventional biocatalyst) exhibit the usefulness and generality of incorporating the pH optimization step in the overall strategy of the biocatalytic asymmetric resolutions.

Methods

Materials. L- and D-amino acids, 9-fluorenylmethyl chloroformate (FMOC), sodium 3, 5-dichloro-2-hydroxybenzenesulfonate (DHBS), 4-aminoantipyrine (4-AAP) and 2,4-dinitrophenylhydrazine were purchased from J&K Scientific. D-Amino acid oxidase from porcine kidney (EC 1.4.3.3) and peroxidase (POD) were obtained from Sigma-Aldrich. All other chemicals were obtained from Aladdin.

Kinetics measurements at different pH values with L-amino acids as substrate. The activity of DAAO on L-amino acids was determined according to improved hydrogen peroxide determination via the coupled peroxidase assay. The effect of pH on the enantioselectivity of DAAO was determined by incubating the reaction mixture at pH values ranging from 6.0 to 10.5. In order to meet the desired range in pH, different buffers were utilized: 20 mM sodium phosphate buffer solutions (pH 6.0–8.0); 20 mM sodium borax buffer (pH 8.5–10.5). All buffers were adjusted to the desired pH at the given reaction temperatures and detected by PB-pH meter (Sartorius, German). All experiments were carried out for 30 min at 37°C in 1 mL reaction mixtures containing 228 U/L DAAO, 300 U/L POD, 0.275 nM 4-AAP, 5.5 mM DHBS, 0.1 mM FAD and various concentrations of L- or D-amino acids at different pH levels. Amino acids were consumed from control mixtures. Afterward, 200 μL 6% (w/v) ice-cold trichloroacetic acid was added to the incubated mixtures and centrifuged at 12000 rpm for 10 min. Then the supernatant was determined by UV spectrophotometer (Shimadzu UV-2550) and the absorbance of the supernatant was measured at 512 nm against a blank sample consisting of the same mixture without amino acids. The activity of DAAO in the mixtures was quantified against the standard DAAO curves (from 3–54 units/L, r > 0.97).

Quantification of L-amino acids before and after reaction. Capillary electrophoresis (CE) method conditions were carried out on Beckmann MDQ-3063847. 20 mM borax buffer solution (pH 9.2) containing 30 mM β-CD, 25 mM SDS and 17% IPA was used as the electrolyte. The effective length and total length of the capillaries were 47 and 60 cm, respectively. Detection at 210 nm was performed. Sample solutions were introduced at the cathodic end at a pressure of 20 KV.

Prior to sample introduction, the capillary was flushed with 100 mM NaOH, water and running electrolyte for 2 min, respectively. 1 mL reaction mixtures containing 228 U/L DAAO and 25 mM L-Pro were incubated for 30 min at 37°C. After derivatization with FMOC, an aliquot (10 μL) of the sample was diluted and injected into the CE apparatus for the quantification of L-amino acids before and after reaction. Derivatization of amino acids with FMOC was as described earlier. Briefly, 200 μL of 10 mM FMOC was added to 200 μL of amino acids in 20 mM borate buffer (pH 9.0). This mixture was kept for 2 min, and then extracted with 0.5 mL pentane to remove excess of reagent. After dilution ten times with water, the sample was ready for introduction.

Detection of racemization of amino acid. After incubation at 37°C for 30 min, 1 mL L-Pro solution (25 mM) prepared with sodium borax buffer (pH 8.0) was divided into two parts. One of them was for the CE analysis after derivatization with FMOC. D-Pro of the same concentration was added to the other part and was for the detection of racemization of amino acids after derivatization with FMOC. Derivatization of the amino acids was carried out according to the above methods. L-Ala (pH 9.8), L-Arg (pH 6.5) and L-Phe (pH 9.0) were tested under the same condition.

Circular dichroism (CD) spectroscopy. CD spectroscopic studies were performed using a Jasco-815 spectrophotometer (Jasco, Japan) equipped with a Peltier system for controlling the temperature. The sample was
pre-equilibrated at 25 °C for 15 min and the scan speed was fixed for adaptive sampling (error F 0.01) with a response time of 1 s and 1 mm bandwidth. The structures of DAAO were monitored by using 1.0 cm path length cuvette. The concentration DAAO was 0.4 mg/mL. Each sample spectrum was obtained by appropriate blank media without DAAO from the experimental enzyme spectrum.

**Structure modeling of DAAO.** Swissmodel (http://swissmodel.expasy.org) was used to predict structure by indentifying npDAAO structural homologues. Homology modeling was performed with use of the crystal structure of the DAAO (PDB code 1COP) as a template. The ligand geometries of the 3D structure were optimized by using MMFF forcefield and MMFF charges for the atoms, till a gradient of 0.001 kcal/mol/Å was reached, maintaining the template structure rigid during minimization. The modeling of the enzyme was performed in the presence and absence of two buffers (pH 7.0 and pH 9.8). Molecular graphics were constructed by PyMOL.

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Author Contributions
Y. H. and X. L. conceived the idea and directed the work. Q. L. performed the experiments. L. C. performed the CE analysis. Z. Z., B. D., Y. X., K. Y., & L. G. helped analyzed the results. Q. L. and L. W. analyzed the experiments and wrote the main manuscript text. All authors reviewed the manuscript.

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