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Engineering the Substrate Specificity of Porcine Kidney D-Amino Acid Oxidase by Mutagenesis of the "Active-Site Lid"

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Comparison of the primary structures of pig kidney D-amino acid oxidase (DAO) and human brain D-aspartate oxidase (DDO) revealed a notable difference at I215–N225 of DAO and the corresponding region, R216–G220, of DDO. A DAO mutant, in which I215–N225 is substituted by R216–G220 of DDO, showed D-aspartate–oxidizing activity that wild-type DAO does not exhibit, together with a considerable decrease in activity toward D-alanine. These findings indicate that I215–N225 of DAO contributes profoundly to its substrate specificity. Based on these results and the crystal structure of DAO, we systematically mutated the E220–Y224 region within the short stretch in question and obtained five mutants (220D224G, 221D224G, 222D224G, 223D224G, and 224D), in each of which an aspartate residue is mutated to E220–Y224. All of the mutants exhibited decreased apparent K_m values toward D-arginine, *i.e.*, to one-seventh to one-half that of wild type DAO. The specificity constant, $k_{cat\ app}/K_{m\ app}$, for D-arginine increased by one order of magnitude for the 221D224G or 222D224G mutant, whereas that for D-alanine or D-serine decreased to marginal or nil.

Key words: D-amino acid oxidase, D-aspartate oxidase, directed evolution, mutagenesis, substrate specificity.

Abbreviations: DAO, D-amino acid oxidase; DDO, D-aspartate oxidase; DRO, DAO mutant with activity toward D-arginine; DXO, DAO-based DAO-DDO hybrid.

Two types of mammalian flavoenzyme are known to catalyze oxidative deamination of D-amino acids, *i.e.*, D-amino acid oxidase (EC 1.4.3.3, DAO) and D-aspartate oxidase (EC 1.4.3.1, DDO) (Scheme 1) (see Refs. *1* and *2* for recent reviews on DAO). The former has been regarded as an epitome of flavin-dependent oxidases and has been a target of numerous studies in various disciplines ranging from physical chemistry to enzymology and to medicine. Comparison of the primary structures of DAO and DDO from various sources has suggested that the two enzymes have evolved from a single ancestor and that they belong to the same protein family (*3*). However, DAO and DDO are mutually exclusive in the type of substrate that they are active on; the former exhibits optimal activity toward neutral D-amino acids but marginal activity toward basic D-amino acids, while the latter oxidizes only acidic D-amino acids. We and another research group have independently solved the three-dimensional structure of pig kidney DAO (*4*, *5*). However, the three-dimensional structure of DDO of either mammalian or any other origin is not available. We recently cloned and purified human brain DDO to homogeneity by utilizing an expression system of *Escherichia coli*, and demonstrated that the enzyme specimen exclusively oxidizes acidic D-amino acids and shows no activity toward either neutral or basic D-amino acids (*6*). Intrigued by the substrate specificities of these intrafamilial oxidases, we scrutinized their amino acid sequences for a better and clearer understanding of the unique specificity differences. Figure 1 depicts the sequence alignment of porcine kidney DAO and human brain DDO. Although the sequence homology or residue identity extends throughout their entire sequences, there is a unique difference between the short stretches of I215–N225 of DAO and R216–G226 of DDO, as highlighted in Fig. 1. The DAO I215–N225 stretch constitutes a part of the loop (T216–Y228) connecting two antiparallel β-strands in the pseudo-barrel domain (Fig. 2, left) (*4*, *5*). This loop has been postulated to act as an "active-site lid" that opens and closes upon substrate/product migration in and out of the active site (*7*). Moreover, the loop involves Y224 that covers the bound substrate, apparently protecting the hydrophobic environment of the active site from the aqueous bulk medium (*4*, *5*, *8*). The "active-site lid" of pig kidney DAO is unique among mammalian DAOs in that it is not conserved in all species and is absent in yeast DAO (*9*, *10*). That yeast DAO lacks the lid, particularly Tyr224, of pig DAO was correlated to the broader substrate specificity and less hydrophobic active site of yeast DAO than those of pig kidney DAO (*2*, *9*, *10*). Sacchi *et al*. have reported on the engineering of yeast DAO, one of their aims being broadening of the substrate specificity for analytical determination of all D-amino acids (*11*, *12*). Considering these notions and taking advantage of the "active-site lid" for narrower substrate specificity, we have systematically mutated the short stretch of I215–N225 within the lid for directed substrate specificity.

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Scheme 1.

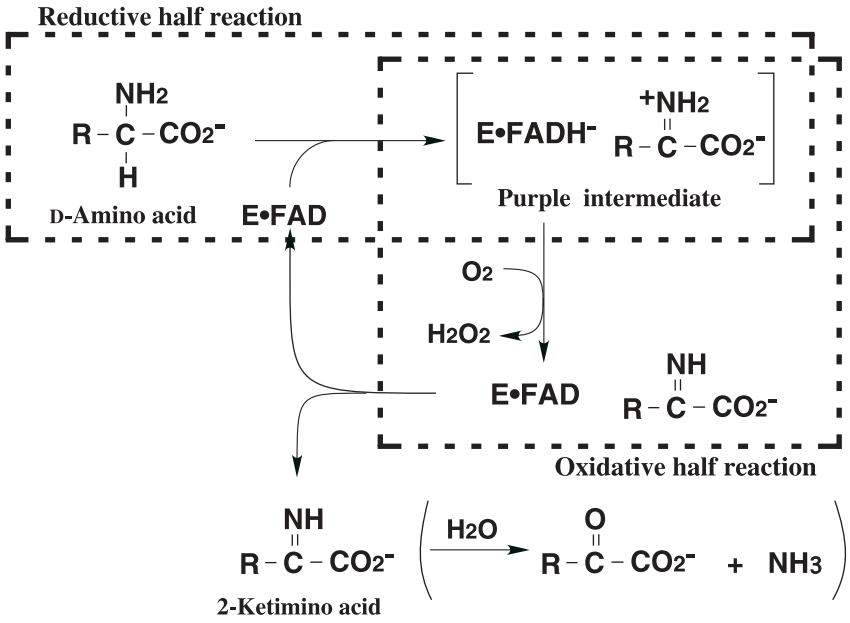

Fig. 1. **Sequence alignment of human brain DDO (*3*) with porcine kidney DAO (*5*)**. The highlighted stretches are those uniquely different from one another (see text for details).

MATERIALS AND METHODS

Wild-type DAO was obtained as previously described utilizing the *Escherichia coli* expression system (*13*). The concentrations of wild-type and mutant DAOs are based on the molar absorption coefficient of 11,300 M^{-1} cm^{-1} for the 455-nm band assuming that the molar absorption coefficient does not change upon the mutation described in the text. The chemicals used in the present study were of the highest grade available from commercial sources and used as supplied.

The DAO-based DAO-DDO hybrid (DXO) and DAO mutants with activity toward D-arginine (DRO) were constructed by a PCR method, using a system, Mutan-super express km (Takara Shuzo, Japan). The expression plasmid, pETDAO, previously described (*13*) was digested with *Xba*I and *Hin*dIII, and a 1.1 kb DNA fragment containing porcine DAO cDNA was subcloned into plasmid pKF18k (*14*) with dual amber in the *Km* gene. The DXO mutant was generated by three-step mutagenesis. First, an oligonucleotide 5′-TTGGCTGAAGAACTTCATTCGTGACCTAGAGAGAG-GCATCTAC-3′ was used to delete I215 and T216, and to replace H217 by arginine. Second, 5′-TTGGCTGAA-GAACTTCATTCGTGACGGGTCAGGCATCTACAACTC-TCCAT-3′ was used for deletion of L219, and replacement of E220 by glycine and R221 by serine. Third,

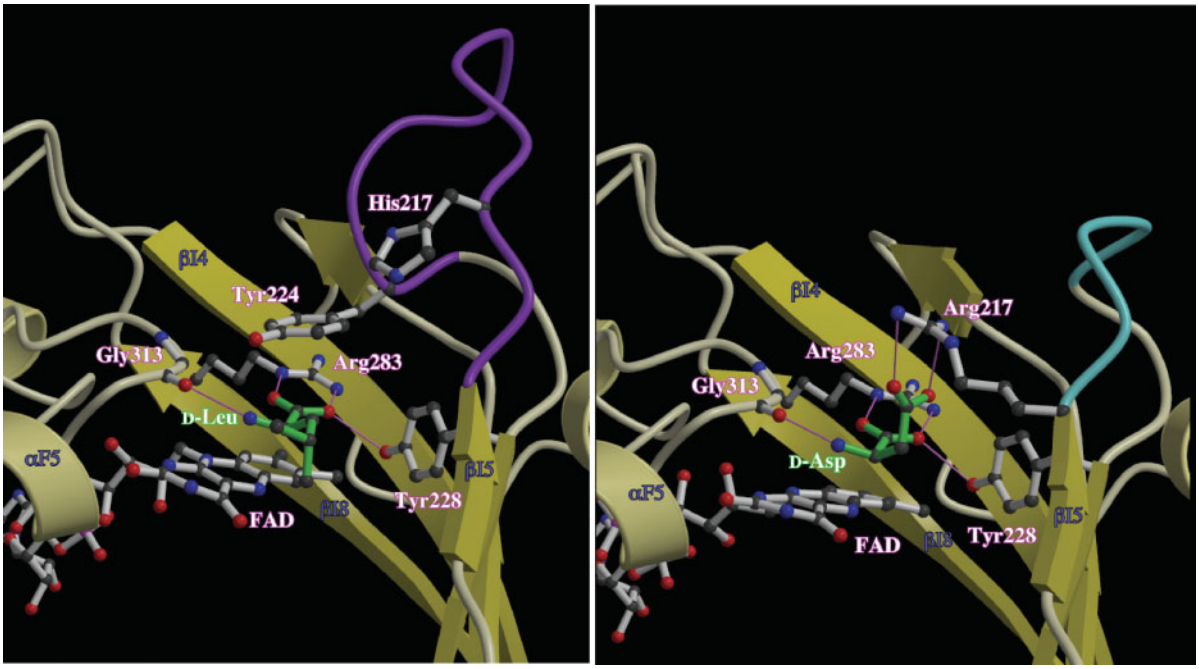

Fig. 2. **Left, the substrate-binding region of the DAO–D-leucine complex model (*6*)**. The short stretch, I215–N225, is highlighted in blue. **Right, the D-aspartate–binding model of DXO, as obtained by molecular mechanics simulation**. The mutated stretch is highlighted in cyan. The nomenclature for the secondary structures is according to Mattevi *et al*. (*5*).

5′-TTCATTCGTGACGGGTCAGGCTCTCCATACATCAT-TCCAGGGCTGCAG-3′ was used for deletion of I223, Y224 and N225. Finally, the amino acid residues (215-ITHDLERGIYN-225) of DAO covering the active site were substituted by the corresponding region (216-RDGSG-220) of DDO. For the PCR reaction to construct DRO mutants, oligonucleotides, 5′-ATCACCCATGACCTA**GAT**AGAGG-CATC**GGC**AACTCTCCATACATC-3′ (where **GAT** and **GGC** denote replacement of E220 and Y224 by aspartate and glycine, respectively), 5′-ACCCATGACCTAGAG-**GAT**GGCATC**GGC**AACTCTCCATACATC-3′ (**GAT** and **GGC**, replacement of R221 and Y224 by aspartate and glycine, respectively), 5′-CATGACCTAGAGAGA**GAC**ATC-**GAACTCTCCATACATCAT-3′ (GAC** and **GGC**, replacement of G222 and Y224 by aspartate and glycine, respectively), 5′-GACCTAGAGAGAGGC**GACGGC**AACT-CTCCATACATCAT-3′ (**GAC** and **GGC**, replacement of I223 and Y224 by aspartate and glycine, respectively), and 5′-GAGAGAGGCATC**GAC**AACTCTCCATACATC-3′ (**GAC**, replacement of Y224 by aspartate), were used to obtain 220D224G, 221D224G, 222D224G, 223D224G and Y224D, respectively. The mutant clones were selected as a kanamycin-resistant phenotype in MV1184 cells. By using the unique *Xba*I and *Hin*dIII restriction sites, the mutated DAO fragment was replaced into the wild-type vector. The final plasmids, pETDXO and pETDROs, were confirmed by DNA sequencing. *E. coli* BL21(DE3) harboring pETDXO or pETDRO was used to express the mutant enzyme. Cells were grown in an LB medium containing 50 μg/ml carbenicillin at 28°C for 48 h, and then were harvested. Purification of the enzyme specimens was performed according to the procedure described previously (*13*).

The activities of wild-type and mutant DAOs were measured as oxygen consumption with a Galvani-type oxygen electrode (Iijima Electronics Corp., Japan) fitted in a 1.5-ml reaction vessel thermostated at 25°C. The reaction was carried out in 50 mM sodium pyrophosphate buffer, pH 8.3, containing 20 μM FAD. The initial velocity was calculated taking the oxygen concentration to be 240 μM at 25°C. Visible absorption spectra were obtained with a Hitachi U-3200 spectrometer thermostated at 25°C in 50 mM sodium pyrophosphate buffer, pH 8.3.

RESULTS AND DISCUSSION

Construction and Characterization of a DAO-Based DAO-DDO Hybrid (DXO)—Sequence alignment of the primary structures of pig kidney DAO and human brain DDO, as shown in Fig. 1, revealed a unique difference localized in the short stretch of I215–N225 of DAO and the corresponding region, R216–G220, of DDO. Since the I215–N225 stretch of DAO overlaps the loop (T216–Y228) connecting two antiparallel β-strands, *i.e*., βI5 and βI6, according to Mattevi *et al*. (*5*), in the pseudo-barrel domain (Fig. 2, left) (*4*) and is located close to the substrate-binding site, it is expected to play a crucial role in substrate recognition. Moreover, this loop has been postulated to act as a lid that gives pig DAO a narrower substrate specificity and a more hydrophobic active site than those of yeast DAO (*2*, *9*, *10*). We thus mutated DAO by substituting the short stretch of I215–N225 of DAO with R216–G220 of DDO. We coined the acronym DXO for this DAO-based DAO-DDO hybrid. The enzymatic properties of DXO were ditermined and compared with those of DAO as well as DDO. When a molar excess of D-aspartate was added to DXO, its flavin absorption was bleached (Fig. 3, middle), indicating that DXO is capable of oxidizing D-aspartate.

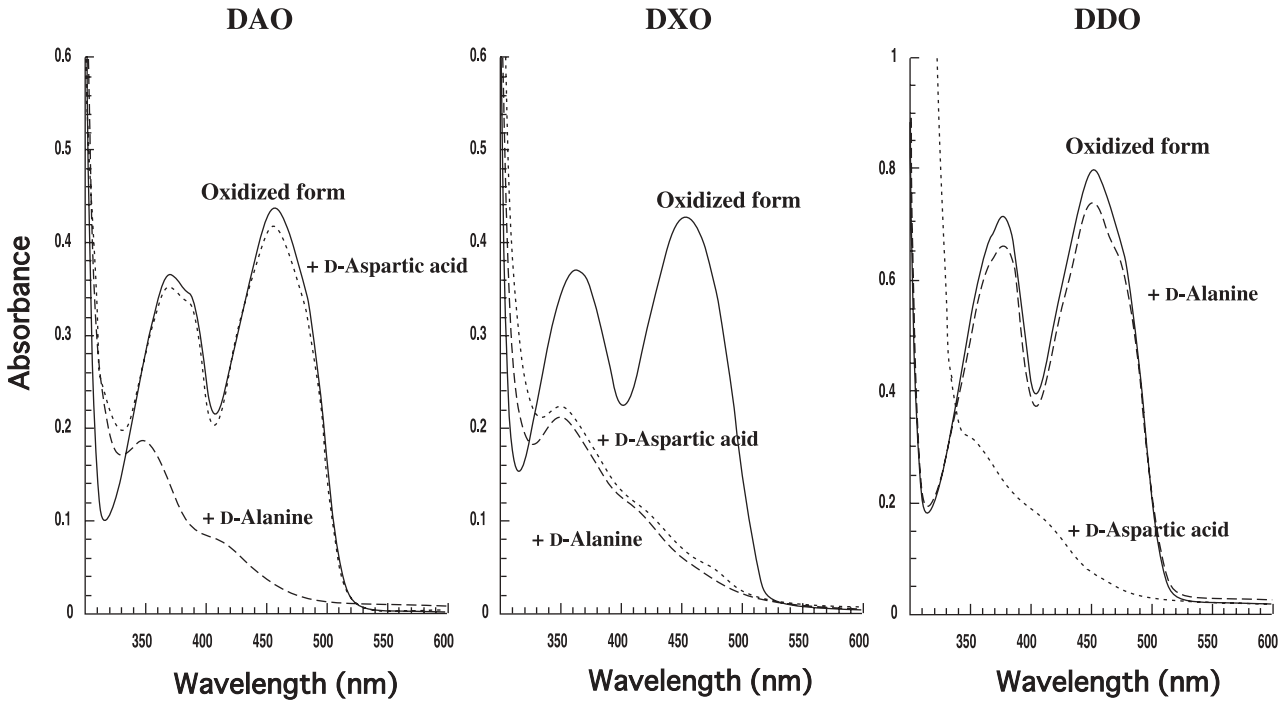

Fig. 3. **Spectral changes of DAO (left), DXO (middle), and DDO (right) upon addition of excess D-alanine and D-aspartate**. The concentrations were: DAO, 38.5 μM; DXO, 37.2 μM; DDO, 69.1 μM; D-alanine, 50.1 mM; D-aspartate, 45.5 mM.

Table 1. **Apparent steady-state kinetic properties of DAO, DXO, and DDO.**

Substrate	$k\text{cat}_{app}$ (min^{-1})			$K_{m\ app}$ (mM)			$K_{cat\ app}/K_{m\ app}$ ($mM^{-1}\ min^{-1}$)		
	DAO	DXO	DDO	DAO	DXO	DDO	DAO	DXO	DDO
D-Ala	510	53	n.d.	1.1	45	n.d.	470	1.2	n.d.
D-Arg	190	n.d.	n.d.	12	n.d.	n.d.	17	n.d.	n.d.
D-Asp	n.d.[a]	30	2,800	n.d.	26	2.2	n.d.	1.1	1,300

[a]n.d., not detectable.

Furthermore, when a molar excess of D-alanine was added, the flavin absorption was also bleached, indicating that DXO retains the D-alanine–oxidizing function. These results demonstrate that DXO exhibits enzymatic properties intermediate between those of DAO and DDO, and that the I215–N225 stretch of DAO profoundly contributes to its substrate specificity, in accordance with our expectation. The apparent steady-state kinetic parameters of DXO, in comparison with those of DAO and DDO, are summarized in Table 1. The substrate-binding model of DXO with D-aspartate was calculated by molecular-mechanics simulation using the DAO–D-leucine complex (*8*) as the initial template. The result, as shown in Fig. 2 (right), indicates clearly that the hydrophilic side chain of D-aspartate is orientated away from the hydrophobic pocket, which was occupied by the side chain of D-leucine in the wild-type DAO–D-leucine complex (Fig. 2, left), to a newly created hydrophilic environment provided by the R217 side chain exposed to the protein surface (Fig. 2, right).

Construction and Characterization of DAO Mutants with Activity toward D-Arginine—The properties of DXO described in the foregoing section convinced us that the I215–N225 stretch contributes to its substrate-recognizing specificity, we next systematically mutated this region taking its geometrical arrangement in the DAO–D-leucine complex model (Fig. 2, left) into consideration. Of the 11 residues comprising the stretch, E220, R221, G222, I223, and Y224 are more important than the other residues, because these positions are more favorably located for making close contact with the substrate-binding site or the flavin ring, and are accordingly better suited for changing of the substrate specificity by means of mutation. We thus substituted each of E220, R221, G222, I223, and Y224

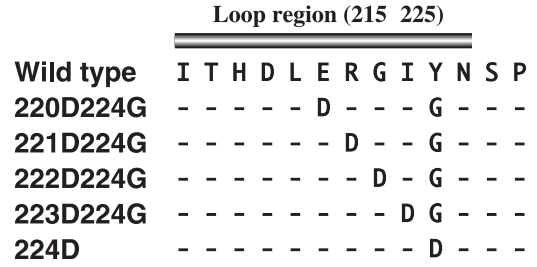

Fig. 4. **Illustrative representation of mutants 220D224G, 221D224G, 222G224G, 223D224G, and 224D**.

Table 2. **Comparison of the apparent k_{cat} (in min^{-1}) and K_m (in mM) values of the wild type and mutant pig DAOs for different substrates.**

Substrate	Wild type		220D224G		221D224G		222D224G		223D224G		224D	
	$K_{cat\ app}$	$K_{m\ app}$	$K_{cat\ app}$	$K_{m\ app}$	$K_{cat\ app}$	$K_{m\ app}$	$K_{cat\ app}$	$K_{m\ app}$	$K_{cat\ app}$	$K_{m\ app}$	$K_{cat\ app}$	$K_{m\ app}$
D-Arg	210	13	240	3.0	450	3.0	240	1.7	110	5.9	120	5.1
D-Lys	48	10	200	14	300	20	300	20	180	20	140	18
D-Met	410	0.65	630	0.67	540	0.67	150	0.61	80	0.77	83	0.77
D-Phe	1,000	1.4	3,200	1.4	780	0.77	210	0.57	180	1.3	190	1.0
D-Pro	620	0.56	260	4.1	230	7.7	210	22	n.d.[a]	n.d.	100	29
D-Val	150	0.63	63	3.1	45	3.7	15	5.1	n.d.	n.d.	26	22
D-Ala	390	0.77	180	44	85	46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
D-Ser	180	3.3	130	>250	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
D-Asp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a]n.d., not detectable.

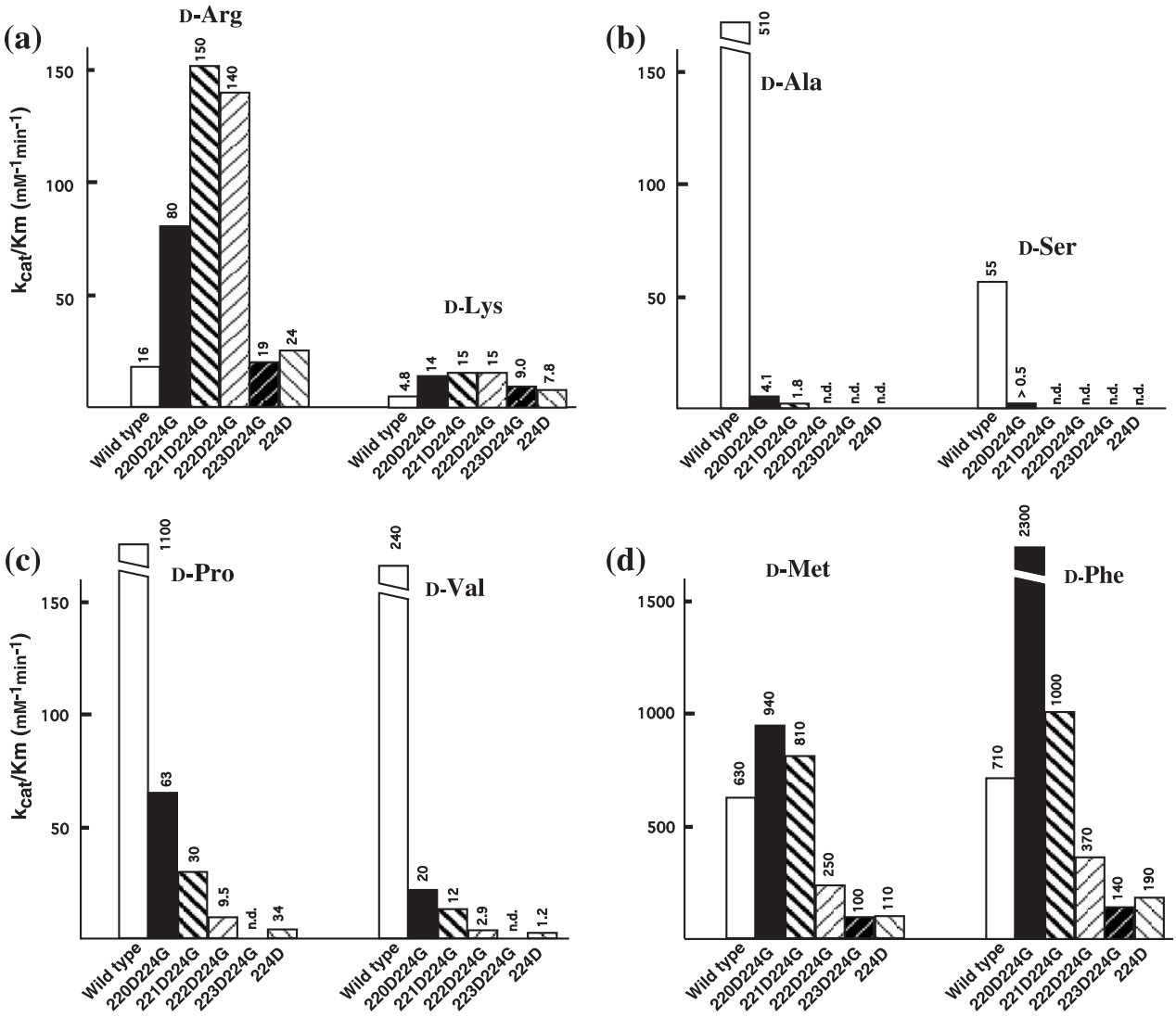

Fig. 5. **Comparison of the $k_{cat\ app}/K_{m\ app}$(in $mM^{-1}\ min^{-1}$) values of mutants 220D224G, 221D224G, 222D224G, 223D224G, and 224D with those of the wild type for various substrates**.

with aspartate in order to endow DAO with basic D-amino-acid–oxidizing activity. For the former four mutants, Y224 was also mutated to glycine for the following reason. Y224 covers the active site and ensures the hydrophobic environment for neutral D-amino acid side chains, and thus seems unfavorable for the hydrophilic side chains of basic D-amino acids. This is also in accordance with the substrate-binding model of DXO (Fig. 2, right) as

well as with the absence of Y224 in DDO (Fig. 1). It is known that Y224 is not necessarily conserved in DAO species from various sources (*2* for review) and thus is considered not essential for catalysis. Pollegioni *et al*. have probed the functional role of Y224 by mutation to phenylalanine (*15*). We designated the mutants as 220D224G, 221D224G, 222D224G, 223D224G, and 224D, respectively (see Fig. 4 for an illustrative representation of the mutants). The apparent steady-state kinetic parameters were determined and compared with those of the wild type (Table 2 and Fig. 5).

The $K_{m\ app}$ values of the mutants for D-arginine were considerably decreased, the minimum value being 1.7 mM with 222D224G compared to 13 mM with the wild type, whereas those for neutral D-amino acids with small side chains, *i.e*., D-alanine, D-serine, and D-proline, were significantly greater with the mutants. The catalytic efficiency of 221D224G and 222D224G as to D-arginine were increased by one order of magnitude compared to that of the wild type (Fig. 5a), and that of the four mutants as to D-alanine or D-serine was drastically decreased (Fig. 5b). Even though the catalytic efficiency of the mutants as to D-lysine is much lower than that as to D-arginine, it is appreciably greater than that of the wild type (Fig. 5a). Thus we have succeeded in designing mutant DAOs with specificity toward D-arginine. However, contrary to our expectation, the increase in catalytic efficiency of these mutants as to D-lysine was not more than moderate. The reason for the difference between D-arginine and D-lysine can be ascribed to the differences in length and shape of the side chain; the side chain of D-arginine is longer and bulkier and probably better fits within the newly created substrate binding site, while smaller D-lysine has more freedom, hence the greater $K_{m\ app}$ values. The same explanation also applies to the increased catalytic efficiency of 220D224G as to D-phenylalanine and to the decrease as to small D-amino acids (D-alanine and D-serine). The increase in efficiency as to D-phenylalanine, to roughly three-times that of the wild type, is purely due to that in $k_{cat\ app}$ (Table 2), which depends on the alignment of the substrate relative to the flavin ring allowing optimum electron transfer from the substrate to flavin.

We have designed the active-site architecture of pig DAO in a directed evolutionary manner for activities not exhibited by the wild type. It is remarkable that mutations as simple as those studied herein can alter the substrate specificity so drastically. The designing was based on careful scrutiny of the active-site structure revealed by X-ray crystallography, the enzyme-substrate complex modeled thereupon, and comparison of the primary structures of DAO and DDO. We have thus succeeded in designing a novel enzyme that can catalyze the oxidation of a basic D-amino acid, D-arginine, with very simple mutations. Sacchi *et al*. have engineered yeast DAO and obtained several mutants with unique substrate specificities (*11*, *12*). Whereas the sites of their mutation are not limited to any particular protein segment but extend over the entire protein region, our mutation sites in pig DAO are localized in a very short stretch within the active-site lid. The lack of this lid seems to give yeast DAO a broader substrate specificity, which makes yeast DAO a better candidate for an even broader specificity than pig DAO. In contrast, mammalian DAO with a narrower substrate specificity may be better suited for narrowing down of the specificity by mutation toward tailor-made enzymes for a particular substrate. Even though final evaluation of our strategy for designing mutants in this study must await elucidation of the three-dimensional structures of the mutants, preferably in a complex with a substrate (analog), we are convinced that the design of tailor-made enzymes with intended specificity can be attained by using the basic strategy we employed in this study. Attempts along these lines are currently in progress.

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