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Analyzing the D-amino acid content in biological samples by engineered enzymes☆

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ABSTRACT

The aim of our present research is to produce mutant forms of D-amino acid oxidase from *Rhodotorula gracilis* in order to determine D-amino acid content in different biological samples. During the past few years, our group has produced yeast D-amino acid oxidase variants with altered substrate specificity (e.g., active on acidic, or hydrophobic, or on all D-amino acids) both by rational design and directed evolution methods. Now, the kinetic constants for a number of amino acids (even for unnatural ones) of the most relevant D-amino acid oxidase variants have been investigated. This information constitutes the basis for considering potential analytical applications in this important field.

1. Introduction

D-Amino acids are found in all organisms, from microrganisms (where they are involved in cell metabolism) to mammals, although protein synthesis uses just the L-amino acids. In recent years some very interesting findings have come to light: firstly, D-amino acids can be found in appreciable concentrations in some organisms [1]. Secondly, new, unexpected roles were discovered for these substances, giving way to novel perspectives: in human brain there is a significant concentration of D-serine, a neuromodulator generated by a specific racemase and degraded by the enzyme D-amino acid oxidase [2].

Taking into consideration all the important roles that D-amino acids play, it appears crucial to develop a rapid, robust, and sensitive method of analyzing and quantifying amino acids (even unnatural ones); this could provide a reliable tool for assessing complex biological samples. To address this issue, in recent years we have applied protein engineering methods to build up new protein variants that can oxidize selectively the D-amino acids, starting from a suitable protein scaffold. The elective protein was represented by the enzyme D-amino acid oxidase (DAAO, EC 1.4.3.3): DAAO catalyzes the oxidative deamination of D-amino acids in a strictly stereospecific reaction (L-amino acids are neither substrates nor inhibitors) and shows a broad substrate specificity (the best substrates are neutral or apolar D-amino acids, such as D-Met, D-Phe, D-Trp, and D-Ala, whereas the acidic ones are not oxidized). A reaction scheme is depicted in Fig. 1. DAAO is a FAD-dependent flavooxidase that has been studied widely and represents a paradigm for the dehydrogenase-oxidase class [3]. In particular, our group has extensively investigated the enzyme from the yeast *Rhodotorula gracilis* [4,5]: this enzyme shows properties that render it a good candidate for biotechnological applications, such as a high turnover number, stable FAD binding, and good thermal and operational stability [6–8]. Most importantly, we resolved the 3-D structure of *R. gracilis* DAAO some years ago (see Fig. 2) [9,10] and owing to this achievement we have been able to engineer the protein to obtain a better biocatalyst.

Most recently, we completed the investigation of the substrate specificity of engineered yeast DAAO variants with the ultimate goal of using them to analyze D-amino acids in biological samples. Remarkably, in this study we developed a novel, simple, fast, and robust method of analysis, the results of which quantitatively agree with those obtained by using 'classical' analytical methods (e.g., HPLC chromatography). The biosensor family based on DAAO variants clearly appears to represent an innovative approach for tackling a complex analysis.

Abbreviations: DAAO, D-amino acid oxidase (EC 1.4.3.3); DASPO, D-aspartate oxidase (EC 1.4.3.1); NMDA, N-methyl-D-aspartate.

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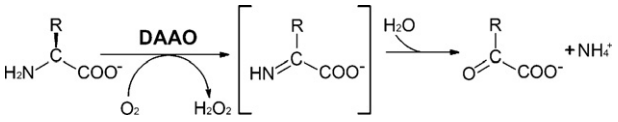

Fig. 1. Scheme of the reaction catalyzed by DAAO on D-amino acids to yield the corresponding α-keto acids, ammonia, and hydrogen peroxide.

2. Experimental

2.1. Proteins expression and purification

Wild-type, T60A/Q144R/K152E [11], M213R [12], and M213G [13] variants of DAAO were expressed as His-tagged recombinant proteins in BL21(DE3)pLysS *Escherichia coli* cells using the pT7-HisDAAO vector. These proteins were purified by Hitrap Chelating chromatography (GE Healthcare). All purified DAAO variants are >90% pure and yield a single band at 40 kDa on SDS–PAGE. Like the wild-type protein, all purified DAAO variants are dimeric holoenzymes, showing the classical visible absorbance spectrum of FAD-containing flavoenzymes (i.e., with peak maxima at ~455 and 374 nm). Protein concentration of the purified enzymes was determined using the extinction coefficient at 455 nm (~12.6 mM^{-1} cm^{-1} for wild-type DAAO) [14].

2.2. Activity assay and kinetic measurements

Standard DAAO activity was assayed by employing an oxygen electrode at air saturation (0.253 mM O_2) and 25 °C, using 28 mM D-Ala as substrate in 75 mM sodium pyrophosphate buffer, pH 8.5 [14,15]. One DAAO unit is defined as the amount of enzyme that converts 1 μmol of D-amino acid per minute at 25 °C. The substrate specificity was investigated by the same assay, employing different concentrations of various D-amino acids as substrate. The initial reaction rates at different substrate concentrations were taken to calculate the apparent kinetic parameters ($k_{cat,app}$ and $K_{m,app}$) using the KaleidaGraph software (Synergy Software). DAAO variants most suitable to quantify the total content of D-amino acids in complex mixtures were identified by measuring the initial rate of oxygen consumption using 10 mM solutions of D-amino acids containing different ratios of D-Ala (0–10 mM), D-Glu (0–8 mM), D-Lys (0–8 mM), D-Gln (0–3.3 mM), and D-Met (0–2 mM) and 0.05 units of DAAO enzymes [16,17].

2.3. Determination of D-amino acid content using a DAAO-based biosensor

Purified DAAO variants were covalently immobilized on an Amberzyme Oxirane support (Rohm and Haas Advanced Biosciences) by coupling the protein-free amino groups. The biosensor we developed consists of two plastic cells: a working electrode (graphite disk, diameter = 28 mm) and a reference electrode (Ag/AgCl); the electrochemical cells were fabricated by a screen-printing process (Specialities s.r.l.). The amperometric biosensor was connected to our data acquisition and processing system [17]. In this device the response represents the difference in signals between the working and the reference electrode that differ for the presence of the enzyme in the previous one. The electrode potential was set at +250 mV because at this value the optimal amplitude of the signal was coupled to a low operating potential that reduced interference. The amperometric measurements were carried out in a final 3 mL D-amino acid solution in 100 mM potassium phosphate buffer, pH 7.0, at room temperature and using a small amount of different, immobilized DAAOs (0.5–4.0 units added in the working cell). A calibration curve was set up using a D-Ala standard solution in the 0.25–5.0 mM concentration range.

The effect of the substrate composition on the amperometric response was evaluated using different immobilized DAAO variants and 0.5-mM D-amino acid solutions obtained mixing different ratios of the following D-amino acids: D-Ala (up to 0.5 mM), D-Glu (up to 0.4 mM), D-Lys (up to 0.4 mM), D-Gln (up to 0.16 mM), and D-Met (up to 0.1 mM).

D-Amino acid content in a cheese (Grana Padano) specimen was determined by preparing the samples as stated in [17]: 3 g of finely grated cheese was suspended in 27 mL of 100 mM potassium phosphate buffer, pH 7.0 (at a final concentration of ≈0.1 g mL^{-1}); this suspension was sonicated for 30 min and centrifuged at 3200 × *g* for 30 min and then the aqueous phase was centrifuged at 27,000 × *g* for 1 h. The resulting supernatant was added to both the working and reference cell of the biosensor.

2.4. Statistics

Kinetic data are expressed as mean ± standard deviation (S.D.); only for the values on different D-amino acid mixtures was the mean ± standard error of the mean (S.E.M.) used. Statistical tests were performed using KaleidaGraph (Synergy Software).

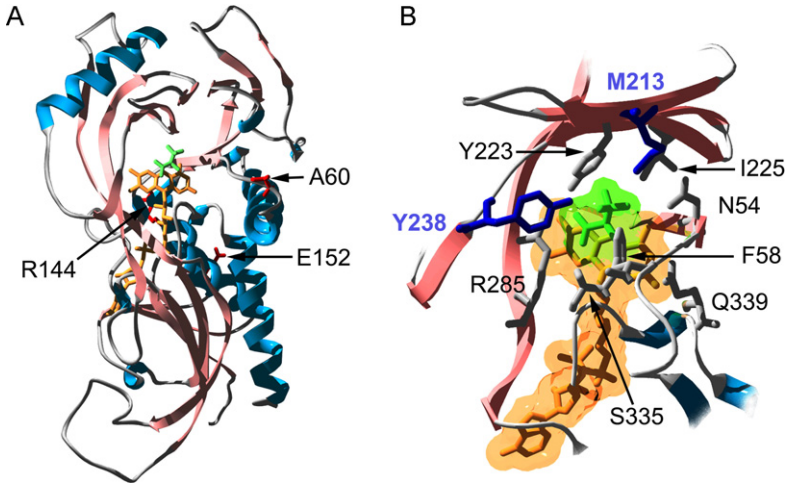

Fig. 2. Structural mapping of amino acid substitutions introduced in the yeast DAAO variants used in this work. (A) Overview of the positions mutated in the T60A/Q144R/K152E DAAO variant obtained by error-prone PCR [11]. (B) Detail of the active site of DAAO showing the position 213 and 238 [9,10]. The flavin cofactor is in yellow and the ligand trifluoro-D-alanine is in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results and discussion

3.1. DAAO variants active on all D-amino acids

The apparent kinetic parameters at 21% oxygen saturation ($k_{cat,app}$ and $K_{m,app}$ values) were determined for the wild-type enzyme, the T60A/Q144R/K152E (obtained by error-prone PCR) [11], and the M213G (obtained by site-directed mutagenesis) [13] DAAO variants on various D-amino acids using the oxygen consumption assay. For all these enzymes, the chemical nature of the substrates profoundly influenced the kinetic parameters (Table 1). The M213G variant showed a significantly (>10-fold) lower kinetic efficiency on D-Ala and a higher one on D-Glu as compared to wild-type and T60A/Q144R/K152E DAAOs. In contrast, the kinetic efficiency observed on D-Lys, D-Ser, D-Gln, D-Trp, and D-Phe was quite similar (≤3-fold).

In a previous paper, we determined the dependence of the activity value on the substrate composition for the wild-type, M213G, and T60A/Q144R/K152E DAAOs by measuring the initial velocity of the enzyme reaction on mixtures (10 mM final concentration) containing different ratios of five D-amino acids (Ala, Glu, Lys, Gln, and Met, see Section 2.2) as substrate [17]. The M213G variant showed an average response in the presence of all five D-amino acids that was closer to the value determined on 10 mM D-Ala (set as 100%) than on the wild-type DAAO, while the response obtained using the T60A/Q144R/K152E DAAO appeared only moderately dependent on the D-amino acid composition in the assay solution: the mean relative activity value is ~75 ± 10% (black bars, Fig. 3) [17]. Indeed, the same DAAO variants were used in immobilized form in an amperometric biosensor to test 0.5-mM solutions containing different ratios of the aforementioned D-amino acids: for both enzymes in the immobilized form the response, as compared to the one on 0.5 mM D-Ala set as 100%, was higher than for the corresponding free forms, combined with a limited dependence on the substrate composition (S.E.M. is ≤9%) for the M213G variant (white bars in Fig. 3) [17]. We thus speculate that the decrease in mean response as observed for wild-type DAAO following enzyme immobilization might be related to alterations in its (maximal) activity on different D-amino acids due to diffusion limits: this is most evident with wild-type DAAO since it shows the highest k_{cat} values among the enzyme variants studied (see Table 1).

A lower variability of this response as a function of D-amino acid composition was apparent with an enzyme combination obtained by mixing the same amount (in terms of enzyme units) of M213G and T60A/Q144R/K152E DAAOs: the average response (as compared to a substrate solution made up solely of D-Ala) is 92% and the S.E.M. of the response is only 7%. This approach identifies the latter mixture of immobilized DAAO variants as an optimal choice for determining the total D-amino acid content. In fact, the biosensors based on single DAAO variants (T60A/Q144R/K152E) [17] or a mixture (M213G and T60A/Q144R/K152E) were used to quickly and inexpensively monitor the D-amino acid content in Grana Padano cheese: the results (6.1 ± 0.5 and 6.0 ± 0.3 mM, respectively) are in good agreement with the amount estimated using time-consuming and complex (chiral) HPLC procedures (=5–8 mM in terms of D-Ala concentration, depending on ageing) [18].

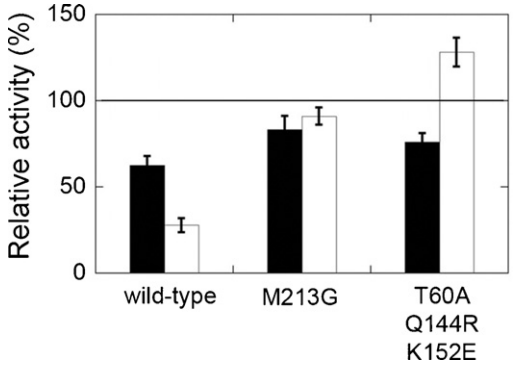

Fig. 3. Average of the activity values determined for free (black bars, 10 mM substrate concentration) or immobilized (white bars, 0.5 mM substrate concentration) wild-type, M213G, and T60A/Q144R/K152E DAAOs on D-amino acid solutions constituted by five different amino acids [17]. The activity value determined for each DAAO variant with a 10-mM (free enzymes) or 0.5-mM (immobilized enzymes) D-Ala solution was set as 100%.

3.2. A DAAO variant active on acidic D-amino acids

In previous work we demonstrated that wild-type yeast DAAO can oxidize acidic D-amino acids but that its kinetic efficiency (expressed as k_{cat}/K_m ratio) is almost 4000- to 8000-fold lower than with the reference substrate D-Ala (see Tables 1 and 2) [12]. On the other hand, a detectable trace is observed using a limited amount (2–6 μg) of the recombinant M213R variant in the polarographic assay of acidic D-amino acids [12]. This DAAO variant was produced by a rational design approach based on sequence homology between DAAO and D-aspartate oxidase (DASPO), molecular modeling, and simulated annealing docking analyses of D-Asp at the active site of the two oxidases [12]. We have now completed the characterization of the substrate preference of the M213R DAAO by determining the apparent kinetic parameters at 21% oxygen sat-

Table 1
Comparison of the apparent kinetic parameters obtained for wild-type, M213G, and T60A/Q144R/K152E DAAO variants on a number of D-amino acids.

	$k_{cat,app}$ (s^{-1})			$K_{m,app}$ (mM)			$k_{cat,app}/K_{m,app}$ (s^{-1} mM^{-1})		
	Wild-type	M213G	T60A/Q144R/K152E	Wild-type	M213G	T60A/Q144R/K152E	Wild-type	M213G	T60A/Q144R/K152E
Neutral									
D-Met	106 ± 29	59 ± 7	83 ± 11	0.4 ± 0.1	0.8 ± 0.1	0.30 ± 0.06	265	73.8	278
D-Ala	103 ± 19	16 ± 3	68 ± 7	0.8 ± 0.1	4.9 ± 1.0	0.6 ± 0.1	129	3.3	113
D-Pro	97 ± 19	9.4 ± 0.7[a]	39.6 ± 1.6[a]	21.5 ± 2.2	20.3 ± 5.1[a]	10.7 ± 1.7[a]	4.5	0.5[a]	3.7[a]
D-Ser	61 ± 10	6.7 ± 0.3[a]	30.5 ± 1.6[a]	13.7 ± 2.3	1.3 ± 0.3[a]	7.7 ± 2.4[a]	4.5	5.2[a]	4.0[a]
Charged									
D-Arg	20 ± 3		12.4 ± 1.7	18 ± 1.8		3.4 ± 1.1	1.1		3.65
D-Lys	13.3 ± 1.6	5.8 ± 0.7	17.8 ± 3.2	14.6 ± 3.0	8.0 ± 0.6	16.6 ± 2.3	0.90	0.72	1.07
D-Glu	1.2 ± 0.2	1.7 ± 0.1	2.0 ± 0.1	77 ± 2	5.1 ± 1.3	101 ± 14	0.016	0.32	0.02
Polar									
D-Gln	43 ± 19	57 ± 12	78 ± 16	5.0 ± 0.4	6.1 ± 1.2	2.5 ± 0.7	8.6	12.9	22.6
Hydrophobic									
D-Trp	160 ± 19	21.4 ± 0.6[a]	23.1 ± 0.8[a]	0.3 ± 0.1	0.13 ± 0.03[a]	0.14 ± 0.02[a]	530	165[a]	165[a]
D-Phe	105 ± 36	29.5 ± 1.9	39.8 ± 1.5[a]	0.3 ± 0.1	0.20 ± 0.02	0.19 ± 0.03[a]	358	148	210[a]

Conditions: pH 8.5; 25 °C; 21% O_2 saturation; oxygen electrode assay; data from [11–13,15,17,20].
[a] This paper.

Table 2
Comparison of the apparent kinetic parameters obtained for wild-type and M213R yeast DAAOs and DASPO on a number of D-amino acids.

	$k_{cat,app}$ (s^{-1})			$K_{m,app}$ (mM)			$k_{cat,app}/K_{m,app}$ (s^{-1} mM^{-1})		
	Wild-type	M213R	DASPO	Wild-type	M213R	DASPO	Wild-type	M213R	DASPO
Neutral									
D-Ala	103 ± 19	10.5 ± 1.9		0.8 ± 0.1	17.8 ± 2.4		129	0.59	
D-Pro	97 ± 19	27 ± 2.3	0.4	21.5 ± 2.2	1280 ± 145	0.9	4.5	0.02	0.44
D-Ser	61 ± 10	11.5 ± 0.6[a]		13.7 ± 2.3	13.7 ± 1.5[a]		4.5	0.83[a]	
Gly	9.5 ± 1.2	0.9 ± 0.2[a]		160 ± 29	425 ± 130[a]		0.06	0.002[a]	
Charged									
D-Arg	20 ± 3	b.d.[a]		18 ± 1.8	*b*.d.[a]		1.1	b.d.[a]	
D-Lys	13.3 ± 1.6	b.d.[a]		14.6 ± 3.0	*b*.d.[a]		0.90	b.d.[a]	
D-Asp	0.6 ± 0.1	3.9 ± 1.2	4.7	18 ± 3.4	2.0 ± 0.3	2.7	0.033	2.0	1.74
D-Glu	1.2 ± 0.2	16.5 ± 2.3	2.5	77 ± 2	27.0 ± 4.3	8.8	0.016	0.32	0.25
NMDA	Inactive[a]	1.3 ± 0.2	16.3	Inactive[a]	18.0 ± 2.1	0.2	Inactive[a]	0.073	81.5
Polar									
D-Asn	21.7 ± 2.0	1.8 ± 0.1		14.4 ± 1.5	11.0 ± 3.4		1.5	0.16	
Hydrophobic									
CephC	91 ± 18	3.6 ± 0.7		5.0 ± 0.9	13.0 ± 3.8		18	0.28	

Conditions: pH 8.5; 25 °C; 21% O_2 saturation; oxygen electrode assay; data from [12,13,15,17,19]; b.d.: below detection.
[a] This paper.

uration on a number of substrates: the kinetic parameters on the acidic D-amino acids are similar to those reported for beef DASPO [19] and a ~7-fold higher $k_{cat,app}$ value with D-Glu is also observed (Table 2). The activity on neutral and polar D-amino acids is not abolished in M213R DAAO but the kinetic efficiency is higher on the acidic ones (Table 2). Notably, the M213R variant is the only DAAO that is active on N-methyl-D-aspartate (NMDA).

Based on its kinetic properties, the M213R DAAO represents the most suitable biotool for developing a biosensor to determine neutral and acidic D-amino acid concentrations (e.g., in food samples). In fact, DAAO was previously adsorbed on the graphite electrode of a commercial biosensor: at an applied potential of +400 mV, a current was recorded in the system after adding ~0.2 units of M213R DAAOs on D-Ala or D-Asp solutions [12]. The current intensity increased with time, yielding a constant slope within 2–3 min: the slope and the final current reached depended on the D-amino acid concentration.

3.3. A DAAO variant active on aromatic natural D-amino acids

Substitution of Y238, the residue which controls substrate binding and product release (for details see [9,10]), was previously reported to modify to a limited extent the overall biochemical properties of yeast DAAO [20]. Substituting it with a Phe or a Ser residue only slightly decreased the activity of DAAO on the reference substrate D-Ala (~3-fold lower $k_{cat,app}$) but dramatically altered its substrate preference. We have now completed our investigation of the substrate preference of the Y238F and Y238S DAAO variants: the kinetic results showed that only for D-Phe and D-Trp is the kinetic efficiency > 100 $mM^{-1}s^{-1}$ (up to 200-fold higher than with the other substrates tested, Fig. 4). This, together with the very high apparent affinity for aromatic D-amino acids ($K_{m,app}$ is 0.04 and 0.07 mM on D-Phe and 0.3 and 0.2 mM on D-Trp for Y238F and Y238S, respectively), makes it possible to preferentially detect D-Phe and D-Trp according to DAAO variants at position 238.

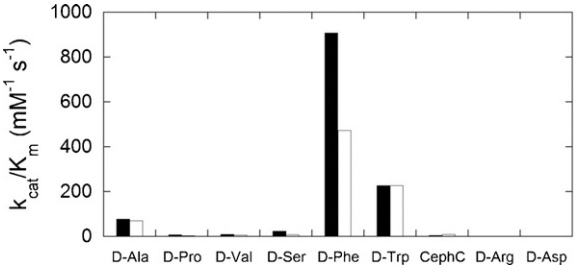

Fig. 4. Comparison of the apparent kinetic efficiency (k_{cat}/K_m ratios) of Y238F (black bars) and Y238S (white bars) variants of yeast DAAO on a number of natural D-amino acids (data from this investigation and from [15,20]). The values determined using wild-type DAAO are reported in Tables 1 and 2.

3.4. A DAAO variant active on aromatic unnatural D-amino acids

A comparison of the substrate specificity of wild-type DAAO on D-phenylalanine, D-phenylglycine, and another 10 aromatic unnatural amino acids is reported in Table 1 of [21]. As a general rule, the substrate affinity is excellent (the $K_{m,app} \leq 3.5$ mM, and frequently <0.1 mM) but a low turnover number (<9 s^{-1}) was

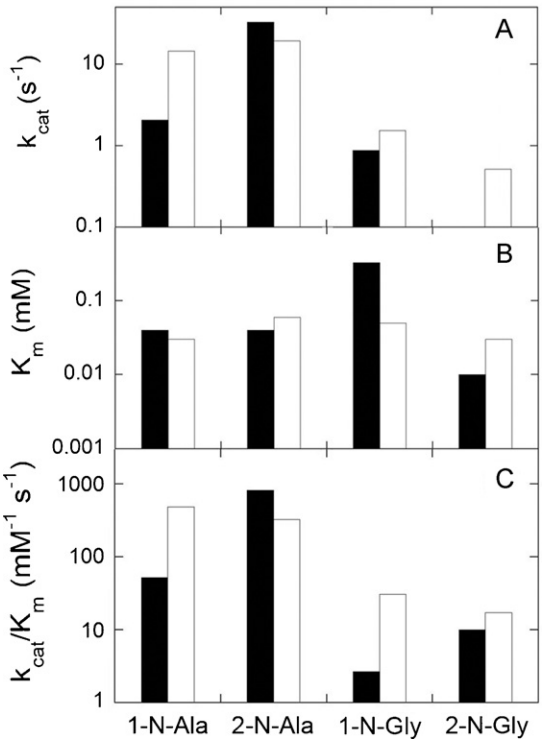

Fig. 5. Comparison of the apparent kinetic properties of wild-type (black bars) and M213G variant (white bars) of yeast DAAO on a number of unnatural naphthyl-D-amino acids [13,21]. 1-N-Ala: D-1-naphthylalanine; 2-N-Ala: D-2-naphthylalanine; 1-N-Gly: D-1-naphthylglycine; 2-N-Gly: D-2-naphthylglycine.

apparent, the latter yielding a low kinetic efficiency. The only exceptions are D-Phe and D-2-naphthylalanine ($k_{cat,app} \geq 33\ s^{-1}$). An increase in the maximal activity on D-1-naphthylalanine and D-1- and D-2-naphthylglycines is seen for the M213G DAAO variant (Fig. 5A) [13]. In the case of D-1-naphthyl amino acids, this change is also accompanied by an increased affinity for the substrate and significantly increases kinetic efficiency (up to 10-fold for D-1-naphthylalanine, Fig. 5C). Notably, the substrate inhibition effect with D-2-naphthylalanine is less evident for the M213G variant than for wild-type DAAO [13], thus favoring its application in the conversion, and for the analytical detection of unnatural aromatic D-amino acids. Indeed, this latter application is now of main interest since a number of pharmaceutical and agrochemical products contain enantiopure, unnatural amino acids (e.g., D-2-naphthylalanine is a component of the peptide drug Nafarelin).

4. Conclusions

The residue that appears to play a major role in modulating the substrate preference of yeast DAAO is M213, which belongs to the hydrophobic pocket of the active site (Fig. 2B) [9,10]. Perturbation of the active site in the M213R DAAO variant resulted in a large decrease in substrate affinity for neutral amino acids and in a modified reaction velocity (mainly due to the alteration in substrate alignment with respect to the N(5)-flavin position). On the other hand, interaction with D-Asp and D-Glu was better by using this substitution: the M213R DAAO variant shows a similar kinetic efficiency on neutral and acidic D-amino acids (Table 2). Notably, the catalytic efficiency on D-Asp and D-Glu of M213R DAAO is similar to that determined for beef kidney DASPO [19]. Indeed, the introduction of a glycine at position 213 also significantly altered the substrate preference of DAAO, producing two important consequences. Firstly, the M213G variant was less dependent on the substrate composition than the wild-type DAAO (see Fig. 3). Secondly, this DAAO variant showed a higher efficiency on unnatural naphthyl-D-amino acids (Fig. 5).

In addition, a biocatalyst for detecting the total content of D-amino acids was obtained by using a directed evolution approach. The k_{cat}/K_m ratios of the T60A/Q144R/K152E DAAO on the substrates tested were modified to a more limited extent than when a rational design approach was employed but, on the other hand, an enzyme variant active on all D-amino acids (and with a remarkably improved catalytic efficiency on the acidic and the basic substrates tested – which are poor substrates of wild-type DAAO) could be isolated by using random mutagenesis. This triple DAAO variant (or a mixture with M213G) is therefore useful in determining the total content of D-amino acids in biological samples and foodstuffs. Intriguingly, the substrate specificity of DAAO could be modified for analytical purposes with both engineering approaches by altering different structural determinants (Fig. 2).

In conclusion, we have been successful in attaining variants of yeast DAAO with a broader substrate specificity (which can be used to develop a biosensor for determining the total content of D-amino acids in food specimens, i.e., the M213G and T60A/Q144R/K152E DAAO variants), or an enzyme that efficiently oxidizes unnatural aromatic D-amino acids (M213G variant), or that preferentially acts on D-Phe and D-Trp (Y238F and Y238S variants), or is active on both neutral and acidic D-amino acids (M213R DAAO). These achievements open the door for a completely new and very sensitive tool for analyzing D-amino acids in complex biological mixtures, to determine both the entire content of these substances and to detect specific amino acids.

Acknowledgments

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