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Modulating D-amino acid oxidase substrate specificity: production of an enzyme for analytical determination of all D-amino acids by directed evolution

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Recent research on the flavoenzyme D-amino acid oxidase from *Rhodotorula gracilis* (RgDAAO) has revealed new, intriguing properties of this catalyst and offers novel biotechnological applications. Among them, the reaction of RgDAAO has been exploited in the analytical determination of the D-amino acid content in biological samples. However, because the enzyme does not oxidize acidic D-amino acids, it cannot be used to detect the total amount of D-amino acids. We now present the results obtained using a random mutagenesis approach to produce RgDAAO mutants with a broader substrate specificity. The libraries of RgDAAO mutants were generated by error-prone PCR, expressed in BL21(DE3)pLysS *Escherichia coli* cells and screened for their ability to oxidize different substrates by means of an activity assay. Five random mutants that have a 'modified' substrate specificity, more useful for the analytical determination of the entire content of D-amino acids than wild-type RgDAAO, have been isolated. With the only exception of Y223 and G199, none of the effective amino acid substitutions lie in segments predicted to interact directly with the bound substrate. The substitutions appear to cluster on the protein surface: it would not have been possible to predict that these substitutions would enhance DAAO activity. We can only conclude that these substitutions synergistically generate small structural changes that affect the dynamics and/or stability of the protein in a way that enhances substrate binding or subsequently catalytic turnover.

Keywords: directed evolution/flavoprotein/function–structure relationships/substrate recognition

Introduction

In the past few decades, D-amino acid oxidase (EC 1.4.3.3) (DAAO) was one of the most extensively studied flavoproteins; thus it is considered as a paradigm of the dehydrogenase-oxidase class. DAAO catalyzes the dehydrogenation of D-amino acids (D-AA) to yield α-imino acids and, upon subsequent hydrolysis, α-keto acids and ammonia. Oxygen, the terminal redox acceptor, reoxidizes the reduced FAD cofactor to give hydrogen peroxide (for reviews, see Curti *et al*., 1992; Pilone, 2000). The reaction catalyzed by this flavoprotein is exploited in biotechnological applications (Pilone and Pollegioni, 2002), for example, for the two-step conversion of cephalosporin C in 7-aminocephalosporanic acid, to produce α-keto acids from essential D-AA, to resolve the racemic mixtures of amino acids and to detect D-AA analytically. DAAO from the yeast *Rhodotorula gracilis* (RgDAAO) exhibits a very high turnover number (compared with DAAO from other sources and to this class of enzymes) (Curti *et al*., 1992; Pilone, 2000), is stable and has an active site large enough to accommodate even substrates of considerable size, e.g. cephalosporin C (Pollegioni *et al*., 2004). DAAOs are strictly stereospecific for substrate and are mainly active on neutral and polar D-AA (Pollegioni *et al*., 1992, 1993): the apparent catalytic efficiency on basic and acidic D-AA is only 5 and 0.02%, respectively, compared with D-Ala (see below).

In recent years, it has become an important research goal in various fields to be able simply and accurately to determine and quantify D-AA in biological samples owing to the increasing interest in the significance of the presence of these D-isomers when present in various eukaryotic tissues or fluids, and also in food. In the latter case, D-AA (especially D-Ala, D-Asp and D-Glu) are formed during food processing and also originate from microbial sources, including water, soil and other environments, and may become part of the human diet. Both the D-AA content and the D/(D + L) ratio have been proposed as reliable molecular markers of ripening and as indexes for the assessment of food product quality (Gandolfi *et al*., 1990; Marchelli *et al*., 1997). D-AA have been found in milk and cheeses, fruits and vegetables, processed foods, eggs, honey, infants' formula, fish meal, wine, beer, etc. (Friedman, 1999). The occurrence of D-AA in foods is normally associated with a decrease in protein digestibility, thus affecting the bioavailability of essential amino acids, facilitating the formation of nutritionally antagonistic and toxic products and ultimately impairing the nutritional quality of food. Traditional analytical methods (such as gas chromatography, capillary electrophoresis and high-performance liquid chromatography) are not suitable for the simple and accurate determination and quantification of D-AA in foods and for on-line application (Brückner *et al*., 1995; Brückner and Westhauser, 2003). Many research groups have proposed enzyme sensors for the specific detection of D-isomers of amino acids based on the detection of hydrogen peroxide or ammonia produced by the DAAO reaction (even coupled to horseradish peroxidase or fungal peroxidase) (Kacaniklic *et al*., 1994; Riklin *et al*., 1995; Sarkar *et al*., 1999). The activity of these sensors is based on DAAO from pig kidney and they are essentially active on neutral D-AA. However, their application was initially limited by the low turnover of the mammalian enzyme and the inactivity on acidic D-AA. The first problem was overcome by using a sensitive biosensor produced by adsorbing RgDAAO on a graphite electrode (Sacchi *et al*., 1998); the second was recently solved by producing the M213R RgDAAO mutant active on D-Asp (Sacchi *et al*., 2002). This mutant enzyme was produced by combining the structural and functional information available on RgDAAO, given by its crystal structure (Umhau *et al*., 2000; Pollegioni *et al*., 2002) and mutagenesis studies (Harris *et al*., 1999; Molla *et al*., 2000;

Boselli *et al*., 2002) and on D-aspartate oxidase (the flavoenzyme that selectively catalyzes the oxidation of acidic D-AA) with the simulated annealing docking of D-Asp at the active site of both enzymes and investigation on substrate recognition (Sacchi *et al*., 2002). An ~100-fold increase in the catalytic efficiency on D-Asp (expressed as the V_{max}/K_m ratio) was observed for the M213R single-point mutant, in which a correct orientation of D-Asp in the active site is achieved. In contrast, the catalytic efficiency on neutral D-AA suffered a dramatic decrease (~17-fold) and the DAAO mutant was practically inactive on basic D-AA (Sacchi *et al*., 2002). Even so, and because of their substrate specificity, neither wild-type nor M213R RgDAAO are useful for determining the total concentration of D-AA in food samples or for distinguishing between different D-AA.

Directed evolution is a powerful tool for reshaping the functional and structural features of proteins, including their solubility, enzymatic proficiency and specificity. In the past few years there have been reports of the successful alteration of the substrate specificity of several enzymes (Arnold *et al*., 2001; Zhao *et al*., 2002). Here, we report on the application of directed evolution in achieving DAAO variants with different substrate specificities. The ultimate goal is the generation of a family of DAAO proteins for the development of a pilot biosensor (to detect and quantify the total amount of D-AA, and also to determine the amount of neutral and acidic D-AA) that can rapidly determine the amount of D-AA in foods. In addition to obtaining enzyme variants increasingly more suitable for biotechnological applications, the combination of results from structural studies (Umhau *et al*., 2000; Pollegioni *et al*., 2002), rational design (Sacchi *et al*., 2002) and directed evolution experiments should give us insight into the modulation of substrate specificity in this class of (flavo)enzymes and can be used to explore the limits of protein function.

Materials and methods

Reagents

Restriction enzymes were purchased from New England Biolabs, Taq DNA polymerase from Promega Life Sciences and AmpliTaq DNA polymerase from Applied Biosystem. T4 DNA ligase was obtained from Promega Life Sciences and from Roche. Horseradish peroxidase was purchased from Roche and D-AA, ligands and all other compounds from Sigma. Kinetic experiments were performed in 100 mM sodium pyrophosphate pH 8.5; ligand binding experiments were carried out in 50 mM HEPES pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol and 0.3 mM EDTA.

PCR-based random mutagenesis

For the first round of error-prone PCR, the pT7-DAAO plasmid (Molla *et al*., 1998) was used as template and the whole sequence encoding DAAO was chosen as the target of mutagenesis. Two oligonucleotides, 5′-TATAGGGAGACCACAACGGTTTCC-3′ and 5′-CTCATGTTTGACAGCTTATCATCGATAAG-3′, were used as 5′ and 3′ primers. The amplified fragments contained *Nde*I and *Hin*dIII restriction sites so that the PCR products could be ligated with the pT7 vector that was digested with the same enzymes (see below). The first round of error-prone PCR was performed using 20 ng of template DNA in 100 µl of reaction mixture under three different sets of conditions: (1) 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM $MnCl_2$, 20 µM each dNTPs, 0.1 µM each of the 5′ and 3′ primers and 2.5 U AmpliTaq; (2) 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 10% DMSO, 1.5 mM $MgCl_2$, 50 µM each dNTPs, 0.5 µM each of the 5′ and 3′ primers and 2.5 U *Taq* DNA polymerase; (3) 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 7.5 mM $MgCl_2$, 200 µM each dNTPs, 0.1 µM each of the 5′ and 3′ primers and 2.5 U *Taq* DNA polymerase. In all cases, PCR amplification was carried out at 94°C for 40 s, 60°C for 30 s and 72°C for 90 s, 40 cycles in total. The second round of error-prone PCR was performed starting from clone 1-7 (Q144R mutant) resulting from the first round of mutagenesis (see below). The cDNA coding for Q144R DAAO mutant was thus sub-cloned into the pT7-HisTag vector (Fantinato *et al*., 2001) and used as template for amplification with the oligonucleotides 5′-GGTGCCTCACTGATTAAGCATTGGTA-3′ and 5′-CAGCTGCCGATTCGAACTTCTCGATT-3′ as 5′ and 3′ primers. The amplified fragments contained *Not*I and *Xba*I restriction sites at which the PCR products could be ligated with the pT7-HisTag vector digested with the same enzymes. The PCR was performed using 20 ng of template DNA in 25 ml of reaction mixture under two different sets of conditions: (4) as under the first set of conditions above, but using 0.2 mM dGTP and dATP, 1 mM dCTP and dTTP and 0.4 µM each of the primers (25 cycles); and (5) as under the third set of conditions (40 cycles). In all cases, the size and yield of the amplified DNA fragments was determined by gel electrophoresis.

Construction of a random mutants library

Enzymatic DNA modifications were carried out according to the manufacturer's instructions and following standard protocols as described by Sambrook *et al*. (1989). The PCR-generated DAAO fragments were purified and digested with *Nde*I and *Hin*dIII (first round of mutagenesis) and *Not*I and *Xba*I (second round of mutagenesis) restriction enzymes. Digested fragments were separated by gel electrophoresis, purified and ligated to a *Nde*I/*Hin*dIII-digested pT7 vector (first round of mutagenesis) (Molla *et al*., 1998) and to a *Not*I/*Xba*I-digested pT7-HisTag vector (second round of mutagenesis) (Fantinato *et al*., 2001). The ligation products were used to transform JM109 and DH5α *E.coli* cells, respectively, according to a modification of original procedure by Inoue *et al*. (1990). All colonies on the transformation plates were then collected for DNA extraction; 20 ng of the extracted DNA were used to transform competent BL21(DE3)pLysS *E.coli* expression cells. Single colonies from LB plates containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol were used to inoculate fresh LB medium and then incubated overnight at 37°C for the screening procedure and to determine the DNA sequence.

Screening for enhanced DAAO substrate specificity

A saturated *E.coli* culture (300 µl) was induced by adding 1 mM IPTG and then incubating it at 30°C for 2 h; 50 µl of the induced cells were then transferred to three wells (one for each substrate tested). Cells lysis was performed by adding 150 µl of lysis buffer (50 mM sodium pyrophosphate buffer pH 8.5, 1 mM EDTA, 100 mM NaCl, 40 µg/ml lysozyme and 1 µg/ml DNase I) and incubating the plates at 37°C for 30 min. Oxidase activity was assayed on the crude extracts (200 µl) by adding 100 µl of 90 mM substrate (D-Ala, D-Asp or D-Arg), 0.3 mg/ml *o*-dianisidine and 1 U horseradish peroxidase in 100 mM sodium pyrophosphate buffer pH 8.5. The product of this

enzymatic reaction has a strong extinction coefficient at 440 nm. The time course of the absorbance change was followed at room temperature by using a microtiter plate reader (Biotrak II, Amersham Biosciences) and comparing it with that of cells expressing wild-type RgDAAO (for the first round of mutagenesis) and wild-type and Q144R HisDAAOs (for the second round of mutagenesis) as control. Each clone was evaluated in independent trials and the average was calculated; the mutants that outperformed the control were selected and used for further analysis.

Expression and purification of DAAO mutants

The cDNA of DAAO variants selected from the first round of mutagenesis was excited by *Eco*RI digestion from the plasmid DNA of selected mutants and subcloned into the pT7-HisTag vector that contained an additional sequence, thus yielding a protein with an N-terminal six-histidine tag (Fantinato *et al*., 2001) and transferred to BL21(DE3)pLysS *E.coli* cells. The pT7-HisDAAO variants selected from the second round of mutagenesis and screening procedure were instead directly transferred to the *E.coli* expression strain. The cells were grown overnight at 37°C in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and induced at different OD_{600} values (0.8 or 2.5) by adding 0.6–1 mM IPTG. After the induction, the cells were cultivated at different temperatures (25 or 30°C) for 3 h and then collected by centrifugation. The crude extract was prepared and the DAAO mutants were subsequently purified as previously reported for wild-type His-DAAO (Fantinato *et al*., 2001). The expression of the mutants was also investigated by means of western blot analysis, using an immunostaining procedure (Molla *et al*., 1998). The molecular mass of the random mutants was determined by gel permeation chromatography on a Superdex 200 column (Amersham Pharmacia Biotech) using 50 mM sodium pyrophosphate pH 8.5, 5% glycerol, 250 mM NaCl as elution buffer.

Activity assay and spectral experiments

DAAO activity was assayed with an oxygen electrode at pH 8.5 and 25°C with 28 mM D-Ala as substrate and at air oxygen saturation ($[O_2] = 0.253$ mM) (Molla *et al*., 1998). One DAAO unit is defined as the amount of enzyme that converts 1 µmol of D-Ala per minute at 25°C. Substrate specificity was investigated by means of the same polarographic assay, employing different concentrations of various D-AA as substrate. The previously developed amperometric biosensor (Sacchi *et al*., 1998) works on a kinetic assay basis, i.e. the current response is directly dependent on the hydrogen peroxide production by the enzymatic oxidation of D-AA. Thus, and in order to identify the DAAO mutants most suitable to quantify the entire content of D-AA, we measured v_0, the initial rate of oxygen consumption (that for wild-type DAAO depends strongly on the composition of the assay mixture) (Pollegioni *et al*., 1992), using D-AA solutions (10 mM final concentration) containing different proportions 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). The protein concentration of purified random mutants was determined using the extinction coefficient of wild-type DAAO at 455 nm (12 600 M^{-1} cm^{-1}) (Molla *et al*., 1998).

The dissociation constant for ligands was measured spectrophotometrically by adding small volumes of concentrated stock solutions to a sample containing 900 µl of ~10 µM enzyme at 15°C (Harris *et al*., 1999). The change in absorbance on adding ligand was plotted as a function of ligand concentration, after correcting for any volume change. Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter.

Results

Mutant libraries

The amount of amplification product recovered by mutagenic PCR was usually lower than that obtained under optimal conditions. In particular, in the presence of 0.25 mM $MnCl_2$ (condition 1), a yield of ~25% in comparison with native conditions was obtained, whereas under conditions 2 and 3 a higher figure (~50%) was reached. Interestingly, in the second round of mutagenesis using the pT7-HisDAAO-Q144R as template and error-prone PCR conditions 4 and 5, a higher amplification yield was obtained (~80% compared with native conditions). For each set of conditions tested, a library of ~10^4 clones was generated and up to 1200 independent clones obtained for each PCR condition were screened for DAAO activity on different substrates (D-Ala, D-Asp and D-Arg, at a final concentration of 30 mM). In order to estimate roughly the frequency of mutations, three independent clones for each condition were sequenced: frequencies of mutation of 0.77, 0.37, 0.17, 0.15 and 0.25% were determined for conditions 1–5, respectively. If the mutation frequency is too high, most of the enzymes generated will be inactive; if it is too low, the wild-type background will be too high and diversity too low. The observed mutational frequencies resulted in 2–7 base pair mutations per mutant in the first round and 1–5 base pair mutations per mutant in the second round of error-prone PCR. Transition and transversion occurred with equal frequency for all conditions tested during the first round of mutagenesis, whereas the strongest bias towards transition (e.g., A–G substitutions) was observed for conditions used in the second round of mutagenesis (Table I). Levels of 91, 27 and 17% of inactive mutants resulted from the first round of mutagenesis under PCR conditions 1, 2 and 3, respectively, whereas the second round of mutagenesis under PCR conditions 4 and 5 gave rise to 63 and 77% of inactive mutants, respectively.

Selection of DAAO variants with altered substrate specificity

Directed enzyme evolution requires the rapid and sensitive selection of the properties of interest. The colorimetric assay we used (based on the coupling of the H_2O_2 produced by DAAO with the reaction of horseradish peroxidase and *o*-dianisidine) is adequate for determining the alteration of the substrate specificity of RgDAAO. Among the DAAO mutants generated during the first round of mutagenesis under PCR condition 1, a significantly higher activity on D-Ala with respect to the wild-type enzyme was detected in the crude lysate of an *E.coli* culture expressing mutant clone 1-7 (Figure 1A). Furthermore, three mutants (clones 2-12, 2-41 and 2-381) derived under the second set of PCR conditions and one mutant derived under the third set of conditions (clone 3-382) showing an altered substrate specificity were isolated. As shown in Figure 1B and with respect to the wild-type DAAO, mutants 1-7 and 2-41 retained similar activity on D-Ala but different activity on D-Arg, whereas mutants 2-12 and 2-381 showed lower activity on all substrates tested. In contrast, the activity of the latter mutants on D-Asp and D-Arg is

Table I. DNA and amino acid substitutions in evolved DAAO mutants (obtained by random mutagenesis and screening for enhanced substrate specificity)

Clone	Base	Base substitution	Position in codon	AA	PCR condition	AA substitution	Secondary element	Location
First round of error-prone PCR starting from wild-type DAAO								
1-7	270	G → A	3	84	1	Silent		
	449	A → G	2	144		Q → R	α-Helix (αF2)	Surface
	543	G → A	3	175		Silent		
	684	C → T	3	222		Silent		
2-12	137	A → G	2	40	2	D → G	Loop (βF2–αI1)	Surface
	699	A → G	3	227		Silent		
	718	T → C	1	234		C → R	β-Sheet (βI7)	Buried
2-41	69	G → A	3	17	2	Silent		
	743	A → T	2	242		D → V	Loop (βI7–αI3)	Surface
	776	A → G	2	253		Q → R	α-Helix (αI3)	Dimerization area
	834	C → T	3	272		Silent		
	888	C → T	3	290		Silent		
	929	A → T	2	304		D → V	Loop (βF5–βF6)	Dimerization area
2-381	137	A → G	2	40	2	D → G	Loop (βF2–αI1)	Surface
	422	T → A	2	135		L → H	β-Sheet (βI3)	Buried
	1034	A → T	2	339		Q → L	α-Helix (αF5)	Active site
3-382	371	T → A	2	118	3	L → H	Loop (βI2–αI2)	Surface
	516	G → A	3	166		Silent		
Second round of error-prone PCR starting from clone 1-7 (in addition to the mutation Q144R)								
4-903	60	T → A	3	14	4	Silent		
	196	A → G	1	60		T → A	Loop (βF2–αI1)	Surface
	472	A → G	1	152		K → E	α-Helix (αF2)	Surface
5-249	614	G → A	2	199	5	G → D	β-Sheet (βI4)	Active site
	686	A → G	2	223		Y → C	β-Sheet (βI6)	Active site
	927	C → T	3	303		Silent		
	1004	A → G	2	329		H → R	β-Sheet (βF6)	Surface/buried

The amino acid numeration refers to the sequence of the wild-type DAAO (Faotto *et al.*, 1995; Umhau *et al.*, 2000) and does not consider any N-terminal tag.

higher than the value determined on D-Ala (compare Figure 1B and C).

The cDNAs coding for the isolated mutants contained 2–6 mutations (Table I) and resulted in a single point substitution for mutants 1-7 (Q144R) and 3-382 (L118H), two substitutions for mutant 2-12 (D40G/C234R) and three substitutions for mutants 2-41 and 2-381 (D242V/Q253R/D304V and D40G/L135H/Q339L, respectively). The location in the structure of RgDAAO of the mutations introduced by the first round of error-prone PCR in these five random mutants is depicted in Figure 2A.

Starting from the HisDAAO-Q144R mutant, a second round of random mutagenesis was performed. By means of the activity assay, one mutant clone was selected for each set of PCR conditions: clone 4-903 (condition 4) and clone 5-249 (condition 5) expressed DAAO variants exhibiting a higher activity on at least two of the three substrates tested compared with wild-type and Q144R DAAOs (see Figure 1D). The DNA sequence analysis showed the presence of three mutations in clone 4-903 and four nucleotidic mutations in clone 5-249, yielding two (T60A/K152E) and three (G199D/Y223C/H329R) additional amino acid substitutions, respectively (Table I). The position of the mutations introduced in the structure of RgDAAO by the second round of mutagenesis and of the R144 residue (the amino acidic substitution present in the parent clone used) is depicted in Figure 2B.

Expression and purification of DAAO random mutants

Selected DAAO variants identified in the first round of mutagenesis were expressed and purified as reported by Fantinato *et al.* (2001). However, whereas 6 and 3.6 mg of purified protein/liter fermentation broth were obtained for D242V/Q253R/D304V (clone 2-41) and Q144R (clone 1-7) mutant, respectively, a 24-fold lower yield was obtained for the L118H (clone 3-382) mutant (0.25 mg of purified DAAO mutant/liter fermentation broth) and no enzyme was purified following HiTrap chelating chromatography from the crude extracts of D40G/C234R (clone 2-12) and D40G/L135H/Q339L (clone 2-381) mutants. Western blot analysis indicates that the low expression yield of these latter mutants is probably due to the formation of inclusion bodies: 80–90% of the expressed mutant DAAO protein was in the insoluble fraction after cell lysis and no DAAO proteolytic products were detected in the crude extracts.

A significant increase in the expression yield of the L118H DAAO mutant (clone 3-382) was obtained when the cells were grown overnight at 37°C, induced at $OD_{600} = 2.5$ by adding 1 mM IPTG and then cultivated for up to 3 h at 25°C. As a result, 3.6 mg of purified enzyme per liter of fermentation broth were produced.

An increase in enzyme expression for clone 2-381 was obtained by growing the cells at 15°C for up to 48 h. A significantly higher amount of immunoreactively expressed protein was detected in the crude extract by means of western blot analysis; however, in the meantime multiple bands at lower molecular mass appeared, suggesting a susceptibility of the expressed mutant to proteolysis (data not shown). The change in the expression conditions did not improve the enzyme recovery for D40G/C234R and D40G/L135H/Q339L DAAO variants (clone 2-12 and 2-381, respectively), hence further characterization was not feasible.

Concerning the DAAO mutants identified in the second round of mutagenesis, the T60A/K152E/Q144R mutant (clone 4-903) was efficiently expressed under the conditions reported above for wild-type (Fantinato *et al.*, 2001): a figure of 5.5 mg of pure DAAO/liter of fermentation broth was obtained.

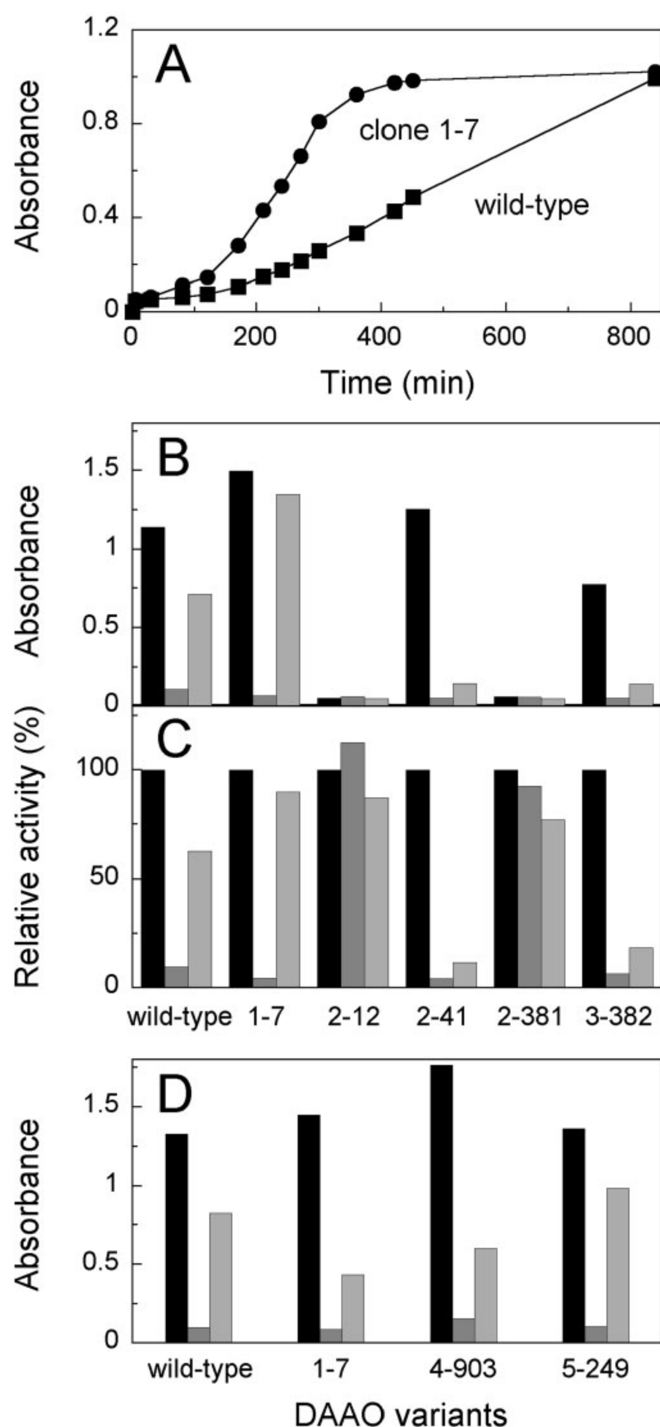

Fig. 1. (**A**) Time courses of the absorbance at 440 nm of wild-type (squares) and Q144R (circles) DAAOs on 30 mM D-Ala as substrate using the horseradish peroxidase assay and *o*-dianisidine, at 25°C and pH 8.5. Oxidase activity was measured on crude extracts obtained as detailed in the Materials and methods section. (**B**) Comparison of DAAO activity of wild-type and random mutants of DAAO identified from the first round of random mutagenesis (using the pT7 vector) and determined on D-Ala (black), D-Asp (gray) and D-Arg (light gray) as substrates (30 mM final concentration) using the horseradish peroxidase assay and *o*-dianisidine as in (A). The values represent the absorbance change at 440 nm measured after 19 h of incubation. (**C**). Comparison of the relative activity of wild-type and random mutants of DAAO identified from the first round of random mutagenesis. The absorbance values determined on D-Ala with each enzyme form [see (B)] were taken as 100%. (**D**) Comparison of DAAO activity of wild-type, clone 1-7 (the parent clone) and random mutants of DAAO identified from the second round of random mutagenesis (using the pT7-HisTag vector) and determined on D-Ala (black), D-Asp (gray) and D-Arg (light gray) as substrates (30 mM final concentration), as reported in (B).

On the other hand and although different expression conditions were analyzed, the Q142R/G199D/Y223C/H329R mutant (clone 5-249) was not expressed as a soluble and active enzyme. Significant expression of this latter mutant DAAO was only obtained when the His-tag sequence was absent and the protein expressed as detailed by Molla *et al*. (1998) (up to 2.1 mg of pure DAAO/liter of fermentation broth). This result suggests a specific interference of the His-tag in the folding process of this mutant DAAO.

Like the wild-type RgDAAO (Molla *et al*., 1998; Fantinato *et al*., 2001), all purified DAAO mutants were >95% homogeneous (as indicated by the E_{280}/E_{455} ratio of ~8.3 for the absorbance spectrum and SDS–PAGE analysis), were dimeric holoenzymes (according to gel permeation chromatography using 1 mg/ml protein samples) and were stable for several months when stored at −20°C. Except for mutant 5-249 (which shows a more pronounced ellipticity of the ~220 nm peak), far- and near-UV CD spectra of all purified mutants are superimposable.

Kinetic and binding properties of 'evolved' DAAO enzymes

To investigate the substrate specificity of the mutants generated by error-prone PCR and identified by the screening procedure, we determined the activity of wild-type and DAAO variants on different amino acids, measuring the oxygen consumption with a Clark-type electrode at pH 8.5 and 25°C (Molla *et al*., 1998). The apparent kinetic parameters V_{max} and K_m on different D-AA measured at a fixed (21%) oxygen concentration are summarized in Table II: D-Ala was chosen as the reference substrate and D-Asp and D-Arg as models of an acidic and a basic D-AA, respectively.

Among the RgDAAO mutants identified in the first round of error-prone PCR, the Q144R DAAO shows a significant increase in catalytic efficiency on D-Asp (3.6-fold, see Figures 3 and 4). All the evolved mutants exhibit a higher catalytic efficiency than wild-type RgDAAO on the acidic D-AA, since the substitutions resulted in an increase in the apparent affinity for D-Asp (Table II and Figure 4). Concerning the alteration of the catalytic efficiency on D-Ala, it is mainly due to a change in substrate affinity: for L118H mutant and with respect to the wild-type RgDAAO, a 2-fold decrease in $K_{m,app}$ was observed for the amino acid. The most significant change in catalytic efficiency on D-Arg was observed for the L118H variant; in any case, both L118H and D242V/Q253R/D304V mutants exhibit an increase in $V_{max,app}$ on D-Arg (see Table II).

Concerning the DAAO mutants identified in the second round of mutagenesis (and which are progeny of clone 1-7), the significantly (~7-fold) higher catalytic efficiency on D-Asp compared with wild-type DAAO was mainly due to an increased substrate affinity. With the neutral substrate D-Ala, the most significant change is the 3-fold increase in catalytic efficiency and the 1.5-fold increase in $V_{max,app}$ observed for the Q144R/G199D/Y223C/H329R mutant (Table II).

In order to identify the DAAO variants more appropriate to detect the entire content of D-AA, we determined v_0, the initial velocity of the enzyme reaction (expressed as μmol of oxygen consumption/mg protein) on D-AA mixtures (10 mM final concentration) containing different ratios of D-Ala, D-Glu, D-Lys, D-Gln and D-Met, since this parameter is highly dependent on the mixture composition for wild-type DAAO (see Materials and methods). When compared with the value measured on 10 mM D-Ala, the mutant Q114R/G199D/Y223C/H329R

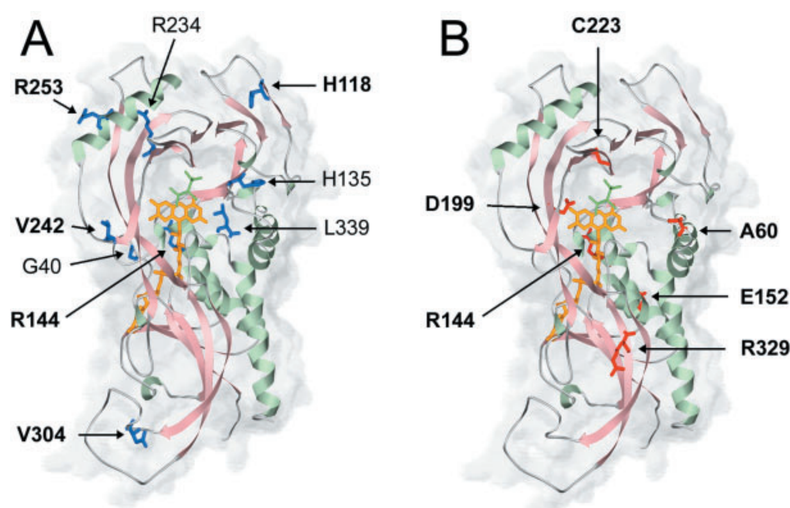

Fig. 2. Structural mapping of amino acid substitutions introduced in RgDAAO (Umhau *et al*., 2000). Overview of the positions mutated in the DAAO mutants isolated during the first (**A**) and during the second (**B**) round of error-prone PCR (the residues mutated in the purified DAAO variants are in bold). The flavin cofactor is in yellow and the ligand trifluoro-D-alanine is in green.

Table II. Comparison of the apparent steady-state kinetic parameters on D-Ala, D-Asp and D-Arg as substrates, obtained for wild-type, M213R and purified DAAO variants obtained by random mutagenesis and containing a His-tag sequence at the N-terminus (with the only exception of M213R and Q144R/G199D/Y223C/H329R DAAOs)

	D-Alanine			D-Aspartate			D-Arginine		
	V_{max} (min^{-1})	K_m (mM)	V_{max}/K_m	V_{max} (min^{-1})	K_m (mM)	V_{max}/K_m	V_{max} (min^{-1})	K_m (mM)	V_{max}/K_m
Wild-type	3900[a]	0.9[a]	4330[a]	40	33.1	1.2	640	2.8	228
M213R[b]	630	17.8	35	235	2.0	118	Below detection limit		
First round of error-prone PCR									
Q144R (1-7)	2685	0.8	3200	56	12.8	4.3	470	3.5	135
L118H (3-382)	3620	0.4	8415	31	12.8	2.4	880	2.1	410
D242V/Q253R/D304V (2-41)	4555	2.3	1980	40	16.3	2.4	920	4.6	198
Second round of error-prone PCR starting from His-Q144R									
T60A/Q144R/K152E (4-903)	3660	0.6	5800	53	7.9	6.7	745	3.4	210
Q144R/G199D/Y223C/H329R (5-249)	5740	0.4	13660	71	9.8	7.2	880	2.4	366

All measurements were performed at 25°C, in 100 mM sodium pyrophosphate buffer pH 8.5, at air saturation ($[O_2]$ = 0.253 mM), using an oxygen electrode (Molla *et al*., 1998).
[a]Fantinato *et al*. (2001).
[b]These values were determined on the purified RgDAAO mutant lacking of the His-tag (Sacchi *et al*., 2002).

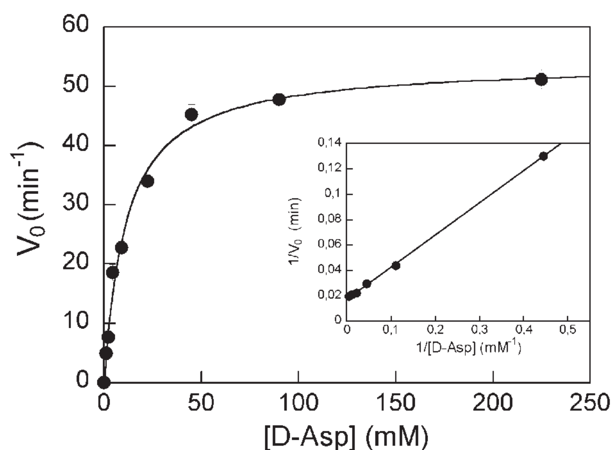

Fig. 3. Michaelis–Menten plot of the kinetics of Q144R DAAO mutant on D-Asp as substrate, at pH 8.5 and 25°C. All measurements were performed at air saturation ($[O_2]$ = 0.253 mM) using the oxygen-consumption assay and 3 μg of pure mutant DAAO per assay. Inset: double-reciprocal plot of the same data as in the main figure.

showed significantly lower v_0 values with all the D-AA mixtures containing a D-Ala concentration <5 mM, while the M213R DAAO showed appreciably lower activity on all mixtures not containing D-Glu. Among the DAAO variants available, the L118H and T60A/Q144R/K152E mutants exhibited the lowest variability of response as function of D-AA composition: a mean value of v_0 corresponding to 87 ± 19 and 76 ± 15% of the value determined on 10 mM D-Ala was determined, respectively. These values compare with 63 ± 15, 69 ± 14, 45 ± 11 and 95 ± 47% determined for wild-type, Q144R, Q144R/G199D/Y223C/H329R and M213R DAAOs, respectively. The L118H DAAO mutant appears to be the best choice to quantify the entire content of D-AA since its response shows a limited dependence on the mixture composition.

Binding experiments were performed on the purified mutants in an attempt to substantiate the contribution of mutated residues to substrate/ligand recognition. Dissociation constants were measured by following the perturbation of the visible spectrum of the flavin coenzyme upon the formation of the enzyme–ligand complex using two classical DAAO

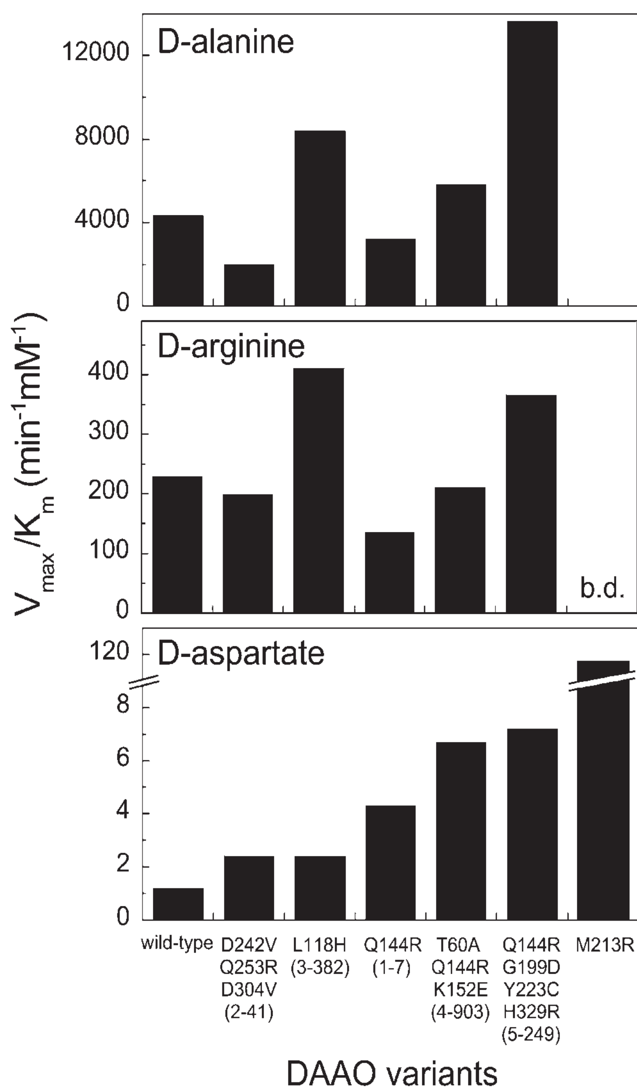

Fig. 4. Catalytic efficiency of the wild-type, M213R and mutant DAAOs obtained by random mutagenesis on D-Ala, D-Arg and D-Asp as substrates. The catalytic efficiencies, V_{max}/K_m ratio of the purified, mutated DAAOs, were calculated from the data given in Table II. The vertical axis in the bottom panel is broken to illustrate more clearly the differences in catalytic efficiencies of all mutants.

inhibitors, i.e. benzoate and anthranilate. For all random mutants, the spectral modifications induced by benzoate and anthranilate were identical with those observed for the binding to the wild-type RgDAAO (Molla *et al*., 1998; Harris *et al*., 1999). The binding of benzoate to L118H DAAO mutant represents the only exception: in such a case, a second spectral change at benzoate concentrations >50 mM (increase in the 420–500 nm wavelength range and loss of the isosbestic points at 465 and 360 nm) was evident. Such a spectral change ($K_d \approx$ 280 mM) suggests that a second molecule of benzoate binds close to the isoalloxazine ring in this mutant DAAO. The K_d values summarized in Table III indicate that modest effects in binding were observed for all mutants with the ligands tested: a significantly tighter binding of benzoate (up to 3-fold) was only determined for the Q144R and Q144R/G199D/Y223C/H329R DAAO mutants. Taken together, these results indicate that the substitutions do not introduce gross perturbations of the mode of ligand/substrate binding.

Table III. Binding of benzoate and anthranilate competitive inhibitors to wild-type and mutants of RgDAAO

	K_d (mM)	
	Benzoate	Anthranilate
Wild-type[a]	0.9	1.9
M213R[b]	6.3	7.5
Q144R (1-7)	0.4	2.8
L118H (2-382)	0.7	2.9
D242V/Q253R/D304V (2-41)	0.7	2.8
T60A/Q144R/K152E (4-903)	0.8	3.1
Q144R/G199D/Y223C/H329R (5-249)	0.3	2.2

All measurements were made in 50 mM HEPES buffer pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, at 15°C. Wavelengths used to calculate the ligand binding were 499 nm for benzoate and 540 nm for anthranilate.
[a]Harris *et al*. (1999).
[b]Sacchi *et al*. (2002).

Discussion

In an attempt to employ a directed evolution approach to modulate the substrate specificity of RgDAAO, the cDNA of wild-type DAAO was randomly mutated by error-prone PCR under different reaction conditions. Our strategy was to use a sequential generation of random mutagenesis and a colorimetric screening procedure, in which libraries of enzyme variants are generated that differ from the parent by a limited (and controlled) number of amino acids. Five mutants displaying an altered substrate specificity were selected from the three libraries generated by the first round of error-prone PCR. Two of the mutants that possessed the same substitution at position 40 of the protein sequence (D40G/C234R and D40G/L135H/Q339L corresponding to clone 2-12 and 2-381, respectively) gave a very low expression yield. Western blot analysis clearly showed that both of these recombinant DAAO variants are largely present in the insoluble fraction, suggesting that the D40G substitution could affect protein folding. Interestingly, D40 belongs to loop βF2–αI1 and, besides D191, it is the only aspartic residue conserved in all DAAO (Faotto *et al*., 1995). The inspection of the 3D structure of native RgDAAO shows that D40 is 8 Å away from the coenzyme and interacts with the side chain of R159 (see Figure 2) (Umhau *et al*., 2000). Our experimental observation, together with its conservation in all DAAO sequences known, provides strong evidence in favor of a specific role of this residue in the enzyme folding. Concerning the other substitutions introduced in clones 2-12 and 2-381, Q339 is the only residue mutated in the first round of error-prone mutagenesis belonging to the active site. Q339-Oε, together with N54-Nδ, is involved in the binding of W72, the only active site water molecule (Umhau *et al*., 2000): this water molecule forms a tight H-bond with the $\alpha - NH_3^+$ group of the substrate at pH ≤ 8 and with the $\alpha = NH_2^+$ of the product. Recently, a RgDAAO mutant obtained by site-directed mutagenesis of Q339 (which was mutated in Glu and Asn) was only successfully expressed in *E.coli* when the cells were grown at 15°C after the induction of DAAO expression (L.Pollegioni, unpublished results). Even mutation of this residue contributed negatively to protein expression.

Although none of the mutations introduced by error-prone PCR in the three DAAO mutants isolated in the first round of mutagenesis (Q144R, L118H and D242V/Q253R/D304V) is located in the vicinity of the active site (see below), these DAAO mutants exhibited increased catalytic efficiency on

some of the substrates tested (Table II and Figure 4). Among these mutants, a 3.6-fold higher V_{max}/K_m ratio was determined for Q144R on D-Asp and an ~2-fold higher figure for L118H on D-Arg and D-Ala in comparison with the wild-type (see Table II). The L118H mutant exhibited increased efficiency on all substrates tested. Interestingly, the residues modified in these DAAO mutants are mostly located on the protein surface (see Figure 2A). Two (Q253R and D304V) of three of the substitutions introduced in clone 2-41 belong to the wide interaction area between the two monomers (3049 Å^2) (Pollegioni *et al.*, 2002). In particular, D304 is located on the long loop connecting β-strands F5 and F6 (residues P302–E322) and appears to play an important role in monomer–monomer interaction: deletion of part of this long loop transformed RgDAAO from a dimeric protein into a stable monomer (Piubelli *et al.*, 2002, 2003). In any case, its substitution is not sufficient to destabilize the monomer–monomer interaction and therefore in solution this mutant is still a dimer.

Starting from the Q144R mutant, a second round of error-prone PCR was performed, still using different amplification conditions (see Table I). The two identified RgDAAO mutants show a significant change in kinetic properties with all the substrate tested (Table II). In particular, increases in $V_{max,app}$ and catalytic efficiency on D-Ala as substrate were observed for clone 5-249; this mutant also shows a higher efficiency on D-Asp and D-Arg as substrate. Among the mutations introduced in clone 5-249, two substitutions of active site residues are evident (see Figure 2B): Y223C and G199D. Y223 is involved in substrate anchoring by forming an H-bond with its α-carboxylate (Umhau *et al.*, 2000) and has also been proposed as the group (having $pK_a \approx 9.3$) governing product release (Harris *et al.*, 1999; Pollegioni *et al.*, 2001). Our results indicate that the function in substrate binding exerted by Y223 can be performed even by a cysteine residue at this position, facilitating at the same time the binding of all (neutral, acidic and basic) D-AA. Concerning G199, it is 3.2 Å from the side chain of R285, the residue involved in the two-point electrostatic interaction with the substrate α-carboxylate. The introduction of an aspartic acid at position 199 should favor the interaction with the positively charged side chain of R285, optimizing the orientation of this active site residue with respect to the bound substrate. A model of the active site of the 5-249 mutant was built using the 3D coordinates of RgDAAO (Umhau *et al.*, 2000): a prediction of D-Asp binding in the active site of the mutant was then obtained using AutoDock 3.0 software (Goodsell and Olson, 1990; Morris *et al.*, 1996; Sacchi *et al.*, 2002). Among the theoretical DAAO–D-Asp complexes obtained for wild-type and Q144R/G199D/Y223C/H329R enzymes, the percentage of substrate molecules possessing the orientation consistent for catalysis was identical, but a significantly lower energy conformation ($\Delta\Delta G \approx 9.5$ kcal/mol) was estimated for the mutant than for wild-type DAAO, supporting the experimentally observed stabilization of D-Asp binding with the 5-249 mutant DAAO (Table II).

Recently, by a rational design approach, an RgDAAO mutant (M213R) was obtained that showed a catalytic efficiency (expressed as V_{max}/K_m ratio) on D-Asp and D-Glu similar to that determined for beef kidney DASPO (Sacchi *et al.*, 2002). The appearance of appreciable activity on acidic D-AA in M213R DAAO mutant did not abolish the activity on neutral and polar D-AA, although a dramatic decrease in catalytic efficiency was observed on these substrates (an ~170-fold lower V_{max}/K_m ratio value was determined on D-Ala) and no activity on basic D-AA was detectable using the polarographic assay (Table II) (Sacchi *et al.*, 2002). This 'new' enzymatic activity is therefore not suitable for the proposed application (see also the Introduction). The substitutions introduced by the directed evolution approach modified the V_{max}/K_m ratios on the substrates tested to a more limited extent, but allowed the isolation of DAAO variants (e.g. L118H and T60A/Q144R/K152E) active on all D-AA (and with a remarkably improved catalytic efficiency on the acidic and the basic substrates tested—which are poor substrates of wild-type DAAO—as well as on D-Ala) and that are therefore useful for the analytical determination of the total content of D-AA in biological samples.

The substrate specificity of DAAO could be modified with both engineering approaches but, intriguingly, different structural determinants were involved. The crystal structure of an evolved aminotransferase containing up to 13 amino acid substitutions (Oue *et al.*, 1999) showed that mutations in residues distant from the active site cause significant changes in the higher order structure of the enzyme, which influence substrate and cofactor binding. If the enzyme activity is modified, the active site must be remodeled as the result of changes in the backbone flexibility, domain motion or subunit rearrangement. Our results confirm the observation that random mutagenesis is useful in modulating substrate specificity by substitution of residue(s) far from the active site. Concerning the effect of these substitutions and in the absence of detailed structural data, it is difficult to explain the mechanism(s) by which some of these amino acid substitutions modify the substrate specificity of the evolved DAAOs. With the only exception of Y223 and G199, none of the effective amino acid substitutions lie in segments predicted to interact directly with the bound substrate. The substitutions appear to cluster on the protein surface (not far from the active site entrance): it would have been impossible to predict that these substitutions would enhance DAAO activity, apart from concluding that they synergistically generate small structural changes that affect the dynamic and/or stability of the protein in a way that enhances substrate binding or subsequently catalytic turnover. In conclusion, because of the large amount of information available on RgDAAO, its substrate specificity could be successfully modulated in a predictable and effective way by 'rational design' (Sacchi *et al.*, 2002); however, an enzyme active on all D-AA could only be obtained using the directed evolution approach.

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