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Role of Arginine 285 in the Active Site of *Rhodotorula gracilis* D-Amino Acid Oxidase

A SITE-DIRECTED MUTAGENESIS STUDY*

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Arg^{285}, one of the very few conserved residues in the active site of D-amino acid oxidases, has been mutated to lysine, glutamine, aspartate, and alanine in the enzyme from the yeast *Rhodotorula gracilis* (RgDAAO). The mutated proteins are all catalytically competent. Mutations of Arg^{285} result in an increase (≈300-fold) of K_m for the D-amino acid and in a large decrease (≈500-fold) of turnover number. Stopped-flow analysis shows that the decrease in turnover is paralleled by a similar decrease in the rate of flavin reduction (k_2), the latter still being the rate-limiting step of the reaction. In agreement with data from the protein crystal structure, loss of the guanidinium group of Arg^{285} in the mutated DAAOs drastically reduces the binding of several carboxylic acids (*e.g.* benzoate). These results highlight the importance of this active site residue in the precise substrate orientation, a main factor in this redox reaction. Furthermore, Arg^{285} DAAO mutants have spectral properties similar to those of the wild-type enzyme, but show a low degree of stabilization of the flavin semiquinone and a change in the redox properties of the free enzyme. From this, we can unexpectedly conclude that Arg^{285} in the free enzyme form is involved in the stabilization of the negative charge on the N(1)-C(2)=O locus of the isoalloxazine ring of the flavin. We also suggest that the residue undergoes a conformational change in order to bind the carboxylate portion of the substrate/ligand in the complexed enzyme.

D-Amino acid oxidase (EC 1.4.3.3, DAAO)[1] catalyzes the dehydrogenation of D-isomer of amino acids to give the corresponding α-imino acids and, after subsequent hydrolysis, α-keto acids and ammonia. New interesting findings (as a role of the enzyme in modulation D-serine level in brain) shed light on the debated role of DAAO in mammalian organisms (1, 2). The precise mechanism of substrate dehydrogenation of this well studied enzyme (3) has not yet been solved, even if recently two groups have reported the crystal structure of the enzyme purified from pig kidney (pkDAAO) at a resolution of 2.6 and 3.0 Å, respectively (4, 5). Over the years, three main different mechanisms have been proposed for the reaction catalyzed by this flavoenzyme (see Mattevi *et al.* (6) for a recent review). (i) The hypothesis that the reductive half-reaction of DAAO involves the initial formation of a carbanion by abstraction of the α-H of the substrate as a proton comes from the elimination of halide from β-chloro-D-alanine (7). (ii) The observation of a transfer of α-hydrogen of the substrate to the C(5) position of the enzyme reconstituted with 5-deaza-FAD provides evidence in favor of a direct hydride-transfer mechanism (8). (iii) Finally a concerted mechanism (consistent with the experimental evidence for a carbanion mechanism) in which α-H^+ abstraction is coupled with the transfer of a hydride from the amino group of the substrate has been put forward (9).

As a model for DAAO to have a better understanding of this crucial issue, we have used the enzyme from the red yeast *Rhodotorula gracilis* (RgDAAO). Actually, RgDAAO possesses peculiar properties, as the high catalytic efficiency and the tight binding with the coenzyme FAD, which distinguish it from the mammalian enzyme (10–12). These properties are most probably related to its physiological role (yeast can metabolize D-amino acids and use them as the sole nitrogen and carbon source) and to an evolutionary drive. From comparison of the primary sequences of the known DAAOs, it is evident that only three residues, among those identified in or near the active site (13), are conserved (namely two tyrosines and one arginine). The presence of an arginine residue located at the active site and directly involved in DAAO catalysis was in fact previously proposed by various chemical modification studies (for a review, see Ref. 3 and references therein). The possibility that the arginine residue could be involved in substrate binding by electrostatic interaction with the substrate was inferred by Nishino *et al.* (14) from 2,3-butanedione modification of the mammalian enzyme, followed by reaction with dansylchloride. On the other hand, the reactivity with sulfite and the spectral properties of the native and cyclohexandione-modified pkDAAO reconstituted with 8-mercapto-FAD suggested that the active site arginine could act as the positively charged group near the flavin N(1)-C(2)=O locus and responsible for stabilization of anionic flavin forms (15). This basic residue has been identified in the primary sequence of RgDAAO, by irreversible inhibition using phenylglyoxal (16), although the question regarding its role was not solved. More recently, the resolution of

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[1] The abbreviations used are: DAAO, D-amino acid oxidase (EC 1.4.3.3); RgDAAO, *Rhodotorula gracilis* D-amino acid oxidase; pkDAAO, pig kidney D-amino acid oxidase; EFl_{ox}, oxidized enzyme; EFl_{seq}, enzyme flavin semiquinone; EFl_{red}, reduced enzyme; CF_3-alanine, 3,3,3-trifluoro-D-alanine.

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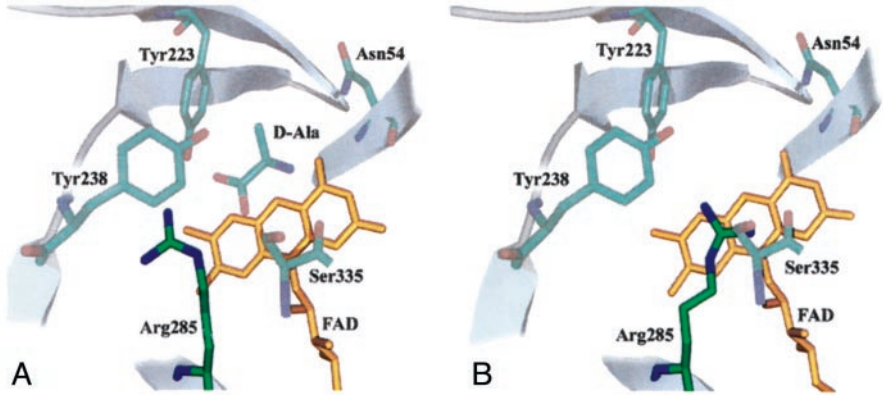

FIG. 1. **Proposed model of stabilization of the negative charge on the N(1)-C(2)=O locus of FAD in the free form of RgDAAO.** *A,* the guanidinium group of Arg^{285} is at ~7 Å distance from the N(1)-C(2)=O position of FAD in the structure of RgDAAO complexed with a ligand or substrate (accession code 1c0p). *B,* proposed position of the side chain of Arg^{285} in the active site of the free form of RgDAAO (rotation brings the ϵ-amino group of the arginine at ~3 Å from the flavin N(1)-C(2)=O position).

the crystal structure of pkDAAO in complex with benzoate and anthranilate (4, 5) showed that Arg^{283} interacts with the carboxylate group of the ligand and that the negative charge on the N(1)-C(2)=O locus of the flavin is stabilized by the dipole of α-helix F5. This result was apparently in contrast with the previous reports (see above).

The crystal structure of RgDAAO has been recently solved at very high resolution (up to 1.2 Å), as a basis for the interpretation of the mechanistic studies and to find a rationale of its high catalytic efficiency.[2] The structure of oxidized enzyme in complex with the quasi-substrate CF_3-alanine and of reduced enzyme in complex with the substrate D-alanine, revealed the mode of substrate binding (Fig. 1*A*). The α-carboxylic group of the D-amino acid interacts electrostatically with the γ- and ϵ-amino groups of Arg^{285} (at ~2.8 Å) and it is H-bonded with the hydroxyl groups of Tyr^{223} and Tyr^{238}. The substrate α-amino group is H-bonded symmetrically with the backbone C=O group of Ser^{335} and the active site water molecule H_2O^{72}, while the substrate side chain is oriented toward the hydrophobic binding pocket of the active site (see Fig. 1*A*).

At the same time, we are substantiating the role of the active site residues of RgDAAO by site-directed mutagenesis of each single residue. In a previous paper we reported the effect of substitution of Tyr^{223} with a phenylalanine and a serine (18). The characterization of the corresponding mutant RgDAAO's allowed us to exclude that Tyr^{223} can act as an active-site base, and highlighted its importance for the correct orientation of the bound substrate, as required for an efficient catalysis. We report here on the characterization of four single points mutants obtained by substitution of Arg^{285}. The combination of the information derived from the site-directed mutagenesis studies and from the crystal structures confirms the involvement of Arg^{285} in substrate binding, but put forward a possible role in stabilization of the negative charge on the flavin N(1)-C(2)=O locus in the free enzyme.

EXPERIMENTAL PROCEDURES

Reagents—Restriction enzymes and T4 DNA ligase were from Roche Molecular Biochemicals. Site-directed mutagenesis reactions were performed using the Altered Sites™ II Kit (Promega). D-Amino acids, xanthine, xanthine oxidase, and all other compounds were purchased from Sigma. 5-Deazaflavin was a generous gift of Dr. Sandro Ghisla (University of Konstanz, Germany). Kinetic experiments were performed in 50 mM sodium pyrophosphate, pH 8.5, 1% glycerol, 0.3 mM EDTA, and 0.5 mM 2-mercaptoethanol and at 25 °C. The other experiments in 10 mM HEPES, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol at 15 °C, except where stated otherwise.

Site-directed Mutagenesis and Enzyme Expression—DNA extraction and purification were performed using FlexiPrep™ DNA Extraction Kit and Sephaglass BandPrep™ Kit from Amersham Pharmacia Biotech. Enzymatic DNA modifications were carried out according to the manufacturer's instructions. Polymerase chain reaction was performed using *Taq* DNA polymerase essentially as described in Sambrook *et al.* (19). The mutant R285K was generated by mutagenic polymerase chain reaction. The cDNA for the wild-type RgDAAO was subcloned into the unique *Eco*RI site of the pCRII vector™ (InVitrogen). A 335-base pair region of the cDNA was then amplified using the ARG1 primer (GAACGATCGAAGGCATCGAGGTCCTCCGCCACAACGTCGGCTTGAAACCTGC, 52-mer) which carries 2 mutations that converted the CGA codon (Arg) in the AAA codon (Lys) as upstream oligonucleotide and the 3′ primer (CTTGTAGATGCCCGCAATACAG, 22-mer, which anneals to the 3′ nontranslated region of the RgDAAO cDNA) as downstream oligonucleotide. The amplification product was cloned into the pCRII vector and the recombinant plasmids were checked by restriction analysis and sequenced to confirm for the presence of the desired mutation. The sequenced fragment was then subcloned into the *Eco*RI site of the pKK223-3 expression vector (Amersham Pharmacia Biotech). The construct was then digested with *Pvu*I and the excised fragment was substituted to the corresponding wild-type portion of the pKK-DAAO expression vector (20).

The remaining Arg^{285} mutants were generated by site-directed mutagenesis (Altered Sites™ II Kit, Promega). The cDNA coding for the wild-type RgDAAO was subcloned in the *Eco*RI site of the pALTER-1™ vector (pALT-DAAOwt), which carries two genes for antibiotic resistance: the *tet*^{r} gene for tetracycline resistance (functional) and the β-lactamase gene for ampicillin resistance (inactivate). The single stranded recombinant DNA, obtained by phage infection, was used as the template for the *in vitro* mutagenesis reactions. The second strand of DNA was synthesized in the presence of two oligonucleotides: the mutagenic oligonucleotide (carrying the desired mutation) and the ampicillin repair oligonucleotide that restored the ampicillin resistance. This procedure allowed the selection of the clones carrying plasmids derived from the newly synthesized strand (potentially mutated). The products of each mutagenesis reaction were used to transform ES1301 *mutS* and successively JM109 *Escherichia coli* cells. Desired mutations were identified by restriction analysis using the appropriate enzymes since the mutagenic oligonucleotides were designed to insert or delete a restriction site.

Mutagenic oligonucleotides: R285Q (CAACGTCGGCTTG*CAG*CCTGCACGACGAGG, 30-mer, eliminates a *Bsp*MI site), R285A (CCACAACGTCGGCTTGGCGCCTGCACGACGAGG, 33-mer, inserts an *Ehe*I site) and R285D (CCACAACGTCGGCTTGGATCCTGCACGACGAGG, 33-mer, inserts a *Bam*HI site). Positive clones were then sequenced to confirm for the presence of the desired mutation. Finally, the mutant cDNAs were subcloned into the *Eco*RI restriction site of the pT7.7A expression vector (pT7-DAAO mutants) and expressed as reported in Molla *et al.* (21) and Harris *et al.* (18).

Activity Assay, Gel Electrophoresis, and Western Blot Analysis—DAAO activity was assayed with an oxygen electrode at pH 8.5 and 25 °C, using 28 mM D-alanine as substrate (10). One DAAO unit is defined as the amount of enzyme that converts 1 μmol of D-alanine per min, at 25 °C. Analytical SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (22). Due to the low activity of the DAAO mutants, the presence of the enzyme in the eluate fraction during purification was determined by Western blot and dot blot analysis, using the immunostaining procedure reported in Ref. 20.

Spectral Experiments—The extinction coefficients for the mutant DAAO enzymes were determined by measuring the change in absorbance after release of the flavin by heat denaturation (an extinction

[2] S. Umhau, L. Pollegioni, G. Molla, K. Diederichs, W. Welte, M. S. Pilone, and S. Ghisla, submitted for publication.

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coefficient of 11,300 M^{-1} cm^{-1} at 450 nm for free FAD was used). Photoreduction experiments were carried out using an anaerobic cuvette containing 7.5 μM enzyme, 5 mM EDTA, and 0.5 μM 5-deazaflavin, made anaerobic by alternative cycles of vacuum and O_2-free argon. The enzyme was photoilluminated as described previously (18, 23), following the progress of the reaction spectrophotometrically. The thermodynamic stability of the semiquinone was determined by the addition of 5 μM benzyl viologen from a side arm of the cuvette after the photoreduction was complete. Disproportionation of the semiquinone was then followed until equilibration was reached (for up to 24 h) at 15 °C (24).

Redox Potentials—Redox potentials for the EFl_{ox}/EFl_{seq} and EFl_{seq}/EFl_{red} couples of the R285K mutant was determined by the method of dye equilibration (25) using the xanthine/xanthine oxidase reduction system, at 15 °C (26). The reaction was initiated by addition of 10 nM xanthine oxidase to anaerobic cuvette containing 7 μM enzyme, 0.2 mM xanthine, 5 μM benzyl viologen, and 1.5–10 μM of the appropriate dye (18, 26). The amount of oxidized and reduced dye was determined at a wavelength where the enzyme has no absorbance (>550 nm) and the amount of oxidized and reduced enzyme was determined either at an isosbestic point for the dye or by subtraction of the dye contribution in the 400–470 nm region. Data were analyzed as described by Minnaert (25).

Ligand Binding—Dissociation constants for ligands were measured spectrophotometrically by addition of small volumes (1–10 μl) of concentrated stock solutions to samples containing 1 ml of ~10 μM enzyme, at 15 °C. The change in absorbance was plotted as a function of ligand concentration, after correction for any volume change (18). Wavelengths used were: 497 nm for sodium benzoate and sodium crotonate, 456 nm for sodium sulfite, 540 nm for sodium anthranilate, and 485 nm for CF_3-alanine.

Stopped-flow Measurements—The experiments were performed at 25 °C in a thermostated stopped-flow spectrophotometer which has a 2-cm path length cell and which is equipped with a diode array detector as described previously (18, 27). The reactions were routinely recorded in the 300–650 nm wavelength range (with an acquisition time of 0.8 ms/spectrum and a resolution of 1 nm). Steady-state kinetic analysis was performed using the enzyme-monitored turnover method by mixing air-saturated enzyme (10 μM final concentration) with air-saturated solutions of D-alanine at 25 °C. Traces at 456 nm were analyzed as described previously (28) using the Kaleidagraph program (Synergy Software). For reductive half-reaction experiments, the stopped-flow instrument was made anaerobic by overnight equilibration with sodium dithionite solutions, as detailed previously (18). Reaction rates were calculated by extracting traces at individual wavelengths and fitting them to a sum of exponential equations using Program A (developed by Dr. David P. Ballou, University of Michigan).

RESULTS

Enzyme Expression and Purification—The pT7-R285A, -R285Q, -R285D, and -R285K plasmids were used to transform BL21(DE3) and BL21(DE3)pLysS *Escherichia coli* cells (Novagen). The best induction conditions were found to be the same of those reported for the wild-type RgDAAO (21). These conditions were used for the fermentation of a 10-liter culture, yielding 20–70 mg of pure enzyme with a specific activity ≤0.1 unit/mg of protein (compared with 180 mg of pure enzyme with a specific activity of 8.8 units/mg protein obtained for the wild-type DAAO) (21). The 2–8-fold lower expression of the mutants is not due to protein instability or proteolytic process, since no proteolysis products have been detected on Western blot of crude extracts. In addition, comparison by Western blot analyses of the amount of DAAO presents in the whole cell paste and in the crude extracts excluded that DAAO forms inclusion bodies.

Spectral Properties—All the Arg^{285} RgDAAO mutants were purified to homogeneity as holoenzymes, having the typical spectrum of the FAD-containing flavoproteins, and were stable when stored at −20 °C for several months. In comparison to the wild-type RgDAAO, the visible spectra of the Arg^{285} mutant enzymes (shown in Fig. 2) reveal the emergence of a shoulder at 495 nm and a red shift of the 380 nm peak. These changes are reminiscent of the spectral perturbation caused by the benzoate binding to wild-type DAAO (10), and might be indicative of

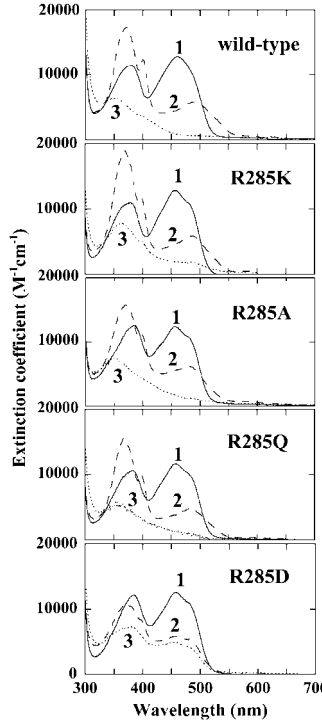

FIG. 2. **Spectral properties of wild-type and Arg^{285} mutants of *R. gracilis* D-amino acid oxidase.** *1,* oxidized enzyme in 10 mM HEPES buffer, pH 7.5, containing 10% glycerol and 5 mM 2-mercaptoethanol, at 15 °C; *2,* the semiquinone form generated by photoirradiation in the presence of 5 mM EDTA and 0.5 μM 5-deazaflavin; *3,* the fully reduced enzyme was from anaerobic reaction with up to 315 mM D-alanine.

a FAD microenvironment that has been rendered more apolar by the single residue substitution. All mutants, in their oxidized state, show an extinction coefficient at 455 nm of ≈12,700 M^{-1} cm^{-1} and a ratio $A_{274}/A_{455} \approx 8.7$. Anaerobic addition of an excess of D-alanine resulted in slow enzyme reduction of all mutants, with a spectrum like that of the reduced wild-type (*lines 3* in Fig. 2) (10). R285D is the only exception, since it cannot be completely reduced by the substrate (Fig. 2). The anaerobic reduction demonstrates that all the Arg^{285} mutants of RgDAAO are competent in catalysis.

Stabilization of the anionic semiquinone is typical for D-amino acid oxidases and for the family of flavoprotein oxidases (29). The amount of semiquinone formed with each mutant was determined by anaerobic photoreduction (24) until the concentration of the flavin semiquinone (EFl_{seq}) reached a maximum (*lines 2* in Fig. 2): this species in the wild-type DAAO represents near-complete formation of EFl_{seq} (≈95%). With the only exception of R285D (which does not produce the semiquinone form), the other mutants produced ~70% of the flavin semiquinone by photoreduction. In contrast to the wild-type DAAO, the anionic semiquinone species of the Arg^{285} mutants is unstable. On the anaerobic addition of benzyl viologen, the semiquinone form of Arg^{285} DAAO mutants slowly disproportionated to the oxidized and reduced forms with the end point containing the thermodynamically stabilized amount of semiquinone (24). After 24 h, when eventually the equilibrium was reached, ~20% of the photoreduced R285K and R285A remained in the semiquinone form (and ~0% for the R285D and R285Q, see Table I). The stability of the semiquinone form profoundly distinguishes the Arg^{285} mutants from the wild-type RgDAAO. The lower stability of the flavin semiquinone in the R285K DAAO has been also confirmed in the presence of benzoate: no semiquinone form was detected (with the mutant enzyme), in

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TABLE I
Semiquinone formation and stabilization, and pK_a of the flavin N(3)H position for wild-type and R285 mutants of D-amino acid oxidase

Experimental conditions: 10 mM HEPES buffer, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol.

	Semiquinone measured		pK_a N(3)H
	Maximum[a]	Equilibrium[b]	
	%		
Wild-type	≥95	65	10.6 ± 0.2[c]
R285K	73	16	10.7 ± 0.1
R285Q	75	0	10.3 ± 0.1
R285A	64	27	10.5 ± 0.1
R285D	5	~0	10.5 ± 0.1

[a] Represents the maximal percentage of semiquinone form obtained by anaerobic photoreduction.
[b] Represents the percentage of semiquinone form measured after equilibration with benzyl viologen, *i.e.* thermodynamically stabilised form.
[c] Ref. 30.

TABLE II
Comparison of redox potentials of wild-type and R285K D-amino acid oxidases

	$E°_1$	$E°_2$	E_m
		mV	
Wild-type	−60[a]	−200[b]	−130
R285K	−105[c]	−89[b,c]	−97

The measurements were made in HEPES buffer at pH 7.5 at 15 °C in the presence of [a] Methylene blue (−29 mV) as redox standards and the xanthine/xanthine oxidase system as source of reducing equivalents (26).
[b] Indigo disulfonate (−124 mV) as redox standards and the xanthine/xanthine oxidase system as source of reducing equivalents (26).
[c] Indigo trisulfonate (−80 mV) as redox standards and the xanthine/xanthine oxidase system as source of reducing equivalents (26).

comparison to a 20% figure for the wild-type DAAO.

The pK_a value for the deprotonation of the flavin N(3)H position can be determined from the pH dependence of the visible spectrum of oxidized enzyme. For wild-type RgDAAO, a pK_a ≈ 10.6 was determined, a value higher than the corresponding value for the free FAD (10.0) and for the pkDAAO (9.4) (30). This change was interpreted by assuming different microenvironments affecting the properties of the flavin position N(1)-C(2)=O and N(3)H; the existence of such differences has been confirmed by the comparison of crystal structure of *R. gracilis*[2] and mammalian DAAO (4, 5). The pK_a values for the deprotonation of the N(3)H position of FAD have been determined with all the mutants (see Table I) and do not show a significant change in comparison to the wild-type DAAO, indicating that the substitution of Arg^{285} does not affect the deprotonation of this flavin position.

Redox Potentials—The redox potentials of the R285K mutant were measured in order to assess changes in the thermodynamic properties of the flavin center caused by the mutation. When the xanthine oxidase-mediated reduction of R285K mutant was monitored in the absence of a reference dye, the percentage of semiquinone formed during the reduction is only ≈17% (95% for the wild-type enzyme), *i.e.* the potentials for transfer of each single electron are quite close to each other (Table II). The separation between the potentials (31) is only 16 mV. The potentials of the oxidized/semiquinone and semiquinone/reduced forms have been determined by using indigo trisulfonate and indigo disulfonate as reference dye. The redox potential difference with respect to the dye could be calculated plotting the log (oxidized/reduced) of the dye as a function of log (oxidized/reduced) of the enzyme (25). The redox potentials E_1 and E_2 are −105 and −89 mV, respectively. In this case, *i.e.* when E_2 is more positive than E_1, the transfer of the second electron, and therefore the bielectronic transfer, is favored in respect to the first electron. A similar situation is also observed for the wild-type enzyme in the presence of a ligand as benzoate.[3] The mutant has a midpoint redox potential E_m (31) about 30 mV more positive than the wild-type (Table II). This change should have little influence on catalysis, since the midpoint potential of −97 mV makes enzyme reduction by D-alanine (rate-limiting for wild-type enzyme) slightly more thermodynamically favorable. Retardation of reoxidation is not an issue because the O_2/H_2O_2 couple is more positive (+300 mV at pH 7) than the $FAD/FADH_2$ couple (26).

Ligand Binding—The contribution of residue Arg^{285} to substrate recognition has been determined by measuring the dissociation constants of Arg^{285}-DAAO mutants for several ligands. Binding was measured by the perturbation of the visible spectrum of the FAD upon formation of the bound complex. Drastic effects on binding were observed for all the Arg^{285} mutants and with all the ligands tested (Table III). Loss of the positively charged guanidinium group of Arg^{285} in the mutants drastically reduced the binding of these carboxylic acids. The observed 3 order of magnitude change in binding constant corresponds to ~3–4 kcal mol^{-1}, a value close to the energy of a hydrogen bond. Interestingly, with benzoate, the spectral perturbation was opposite to those observed for the binding to the wild-type DAAO (Fig. 3).

The observed >1000-fold reduction of sulfite binding in the mutants agrees with the lower separation in redox potentials (see Tables II and III). This result confirms a strict correspondence between a lower stability of the red, anionic flavin semiquinone and a higher K_d for binding of sulfite (as a general rule, the nucleophilic attack to the N(5) position of FAD by sulfite is facilitated by the localization and stabilization of a negative charge on the flavin N(1)-C(2)=O locus) (32).

Steady-state Kinetics—The ability of the R285A and R285K mutants to catalyze D-alanine/oxygen turnover was measured by enzyme-monitored turnover experiments (28). The reaction of R285Q and R285D was too slow to give reasonable results and thus their catalytic properties have not been investigated. Air-saturated solutions of enzyme and D-alanine were mixed in the stopped-flow spectrophotometer and the absorbance spectra were recorded continuously in the 350–650 nm wavelength range at 25 °C. As reported in Fig. 4, after mixing the absorbance at 456 nm shows an initial rapid decrease followed by a steady-state phase and then by further decrease to reach the final reduced state, confirming that both the R285A and R285K mutants are competent catalysts. During turnover the enzyme is largely present in the oxidized form, indicating that the overall process of reoxidation of reduced DAAO with oxygen is always faster than the reductive half-reaction. The corresponding Lineweaver-Burk plots show a set of parallel lines with both DAAO mutants (data not shown). A similar pattern in the secondary plots was found for the wild-type DAAO (12), consistent with a limiting case of a ternary complex mechanism where some specific rate constants (*i.e.* k_{-2}, see Scheme 1) are sufficiently small. Also in the case of the most conservative mutation (R285K), k_{cat} resulted reduced about 500-fold and K_m for D-alanine 300-fold increased, whereas K_m for oxygen is slightly reduced (Table IV). The drastic change in kinetic parameters with D-alanine was also confirmed by the results with R285A mutant (Table IV).

Reductive Half-reaction—The reductive half-reaction of R285K and R285A with D-alanine was measured to assess the influence of the mutation on individual rate constants. An

[3] L. Pollegioni, D. Porrini, G. Molla, and M. S. Pilone, manuscript in preparation.

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TABLE III

Binding of aromatic and aliphatic competitive inhibitors and of sulfite to wild-type and Arg^{285} mutant D-amino acid oxidase

All measurements were made in 10 mM HEPES buffer, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, at 15 °C.

Compound	K_d (mM)				
	Wild-type	R285K	R285Q	R285D	R285A
Benzoate	0.9	~350	550 ± 50	~450	~1000
Anthranilate	1.9	100 ± 30	NB[a]	NB	24 ± 14
Crotonate	0.4	NB	NB	15 ± 4	60 ± 25
Sulfite	0.12	860 ± 130	>1200	>1200	~1000

[a] NB, no binding observed (K_d » 1 M).

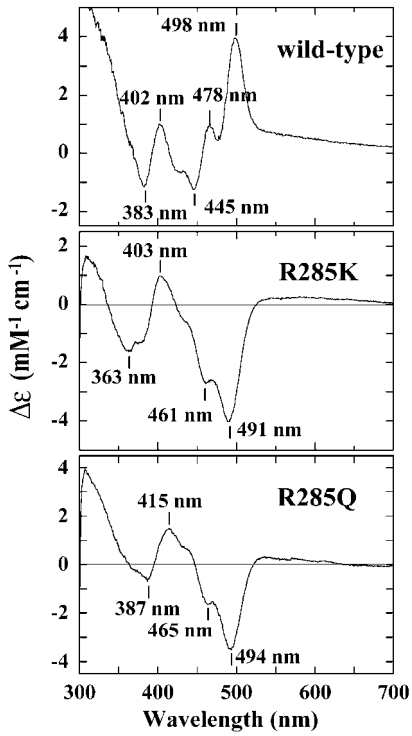

FIG. 3. **Differential spectra for benzoate binding to wild-type, R285K, and R285Q D-amino acid oxidases.** The differential spectra were obtained by subtraction of the absorbance spectrum of the free oxidized form of the wild-type, R285K, and R285Q DAAOs (~24 μM in 10 mM HEPES buffer, pH 7.5, containing 10% glycerol, and 5 mM 2-mercaptoethanol) to the spectrum of the same enzyme after addition of 0.2, 1.4, and 1.5 M sodium benzoate, respectively, at 15 °C.

anaerobic solution of each enzyme was mixed anaerobically with solutions containing varying concentrations of D-alanine, such that a pseudo-first order condition was maintained with respect to the substrate. In the absence of oxygen, the enzyme undergoes only one-half of a normal catalytic cycle,

$$E_{ox} + AA \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} E_{ox}{:}AA \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} E_{red}{:}IA \overset{k_3}{\longrightarrow} E_{red} + IA$$

SCHEME 1

For the wild-type RgDAAO, upon mixing with D-alanine, the oxidized enzyme is converted to the reduced enzyme-iminopyruvate complex (phase 1), followed by decay of the spectral intermediate (phase 2) resulting in a spectrum consistent with free, reduced enzyme (12). Like the wild-type RgDAAO, in the reductive half-reaction of both mutants there is no observed spectral change associated with formation of the oxidized enzyme-D-alanine complex. On the other hand, the absorbance traces at 456 nm for the mutants are essentially monophasic and thus were fit to a single exponential decay. In fact no significant change at 530 nm was detected (see Fig. 5 for R285K

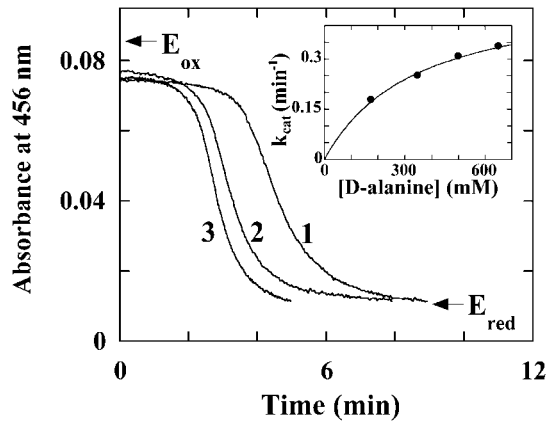

FIG. 4. **Time courses of turnover of R285A mutant RgDAAO followed in the stopped-flow spectrophotometer.** The changes in absorbance were monitored at 456 nm after mixing of 13.6 μM R285A mutant enzyme with 175, 350, and 500 mM D-alanine (all final concentrations) at pH 8.5 and 25 °C. *Inset,* plot of the turnover data *versus* D-alanine concentration determined from the enzyme monitored turnover traces of R285A, as those reported in the main graph.

TABLE IV

Comparison of the steady-state coefficients and reductive half-reaction specific rate constants obtained from stopped-flow experiments of wild-type, R285K, and R285A D-amino acid oxidases

The $K_{d,app}$ was obtained from the slope divided by the intercept in the double-reciprocal plot of the rate of reduction *versus* D-alanine concentration. All measurements were made in 50 mM sodium pyrophosphate, pH 8.5, 1% glycerol, 0.3 mM EDTA, and 0.5 mM 2-mercaptoethanol.

	Steady-state			Reductive half-reaction	
	k_{cat}	$K_{m,Ala}$	$K_{m,O2}$	k_2	$K_{d,app}$
	s^{-1}	mM		s^{-1}	mM
Wild-type[a]	350	2.6	2.3	345	2.8
R285K	0.8	800	0.06	0.6	~1000
R285A	0.05	310	0.08	0.033	200

[a] Wild-type DAAO data have been determined in 60 mM sodium pyrophosphate, pH 8.5, 1.5% glycerol, 0.3 mM EDTA, and 0.75 mM 2-mercaptoethanol (12).

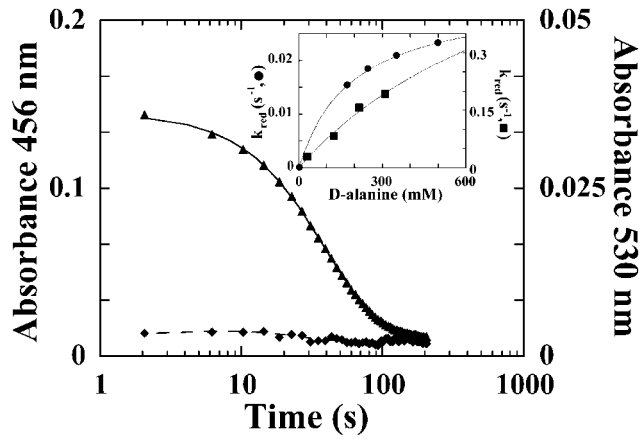

FIG. 5. **Time course of anaerobic reduction of R285K DAAO using D-alanine as substrate followed in the stopped-flow instrument.** Time course of anaerobic reduction of 26.5 μM R285K mutant reacted with 250 mM D-alanine, at pH 8.5 and 25 °C, and followed at two different wavelengths: at 456 nm (▲, ——) the traces reflect the conversion of oxidized to fully reduced enzyme, at 530 nm (◆, - - -) the formation and decay of reduced enzyme-product complex (see Scheme 1). *Inset*: dependence of the observed rate for the anaerobic reduction of R285K (■) and R285A (●) on the concentration of D-alanine.

DAAO). This result is consistent with the rate constant of product dissociation from the reduced enzyme (k_3 of Scheme 1, ~7.7 s^{-1} for the wild-type enzyme) (12) being significantly

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faster than the rate constant of flavin reduction (k_2).

Both Arg^{285} mutants show an hyperbolic dependence of the observed reduction rate as a function of D-alanine concentration (see Fig. 5, *inset*) and, analytically according to Strickland *et al.* (33), it describes a first-order reaction of a binary complex ($k_2 + k_{-2}$), that follows a second-order complex formation. Furthermore, since the data are best fit with a rectangular hyperbola that intersects the origin, these data indicate that the reduction step is essentially irreversible ($k_{-2} \approx 0$). A double reciprocal plot of these data does clearly indicate a positive y intercept. Using such a model, k_2 and $K_{d,app}$ for R285K and R285A have been determined and their values are very close to the k_{cat} and $K_{m,D\text{-}Ala}$ determined under steady state conditions (Table IV). The estimated value of $K_{d,app} \approx 1$ M is >400-fold greater for R285K mutant than for wild-type RgDAAO. Numerically, the value of $K_{d,app}$ is equal to $(k_{-1} + k_2)/k_1$ (33). For wild-type RgDAAO, it has been shown that D-alanine is a "sticky" substrate ($k_2 \gg k_{-1}$), and thus $K_{d,app} = k_2/k_1$ (12). The increase in $K_{d,app}$ for Arg^{285} mutants, together with the large decrease in k_2, possibly reflects a change in binding involving a significant decrease on k_1. Simulations of the absorbance traces at 456 nm using Program A allows an estimation of the minimal values for the k_1 and k_{-1} rate constants involved in substrate binding. The estimated value of k_1 is $\approx$1200 M^{-1} s^{-1}, significantly slower than the same value determined for wild-type enzyme, 1.2×10^5 M^{-1} s^{-1} (12). From this it is evident that k_2 is $\ll k_{-1}$ (0.6 s^{-1} *versus* 1000 s^{-1}), *i.e.* $K_{d,app} = K_d = k_{-1}/k_1$. This change in $K_{d,app}$ for the mutant parallels a corresponding increase in the K_m value for D-alanine, as demonstrated from the mathematical treatment of the known equation of $K_{m,D\text{-}Ala}$ for wild-type DAAO (10) when $k_2 \ll k_{-1}$ and $\ll k_4$,

$$K_{m,D\text{-}Ala} = \frac{k_4 \cdot (k_{-1} + k_2)}{k_1 \cdot (k_2 + k_4)} = \frac{k_4 \cdot k_{-1}}{k_1 \cdot k_4} = \frac{k_{-1}}{k_1} = K_d \cong 800 \text{ mM} \quad \text{(Eq. 1)}$$

where k_4 is the rate constant of product dissociation from the (re)oxidized enzyme form. The calculated value of $K_{d,app}$ (~1000 mM) for R285K is in reasonable agreement with the measured K_m for D-alanine (800 mM, Table IV). The fine correspondence of our estimates of k_2 with k_{cat} values suggests that mutation of Arg^{285} does not result in a change in the rate-limiting step: k_2 is still the slow step in catalysis.

DISCUSSION

The results presented in this paper demonstrate that the conserved Arg^{285} plays different roles at the active site of RgDAAO. We successfully expressed in *E. coli* four RgDAAO mutants at position Arg^{285} using the pT7-DAAO expression system (21) and purified them to homogeneity as holoenzyme with a good yield (≥20%). The mutation of Arg^{285} results in no gross perturbation or loss of FAD, thus the observed changes are due to only specific and local structural modifications. The main role of this residue in substrate binding has been substantiated by the known three-dimensional structure of mammalian DAAO (4, 5) and, recently, from the structure at high resolution of RgDAAO.[2] In the latter, the structure of the reduced enzyme-D-alanine complex at 1.2-Å resolution reveals the mode of substrate binding (Fig. 1*A*): the carboxylate position of the amino acid is electrostatically bound via a two-point interaction with the γ- and ϵ-amino group of Arg^{285} (at a distance of 2.79 Å).[2] The large decrease in substrate affinity (and ligand binding) observed with the mutant enzymes is well explained by the involvement of this arginine residue in binding and fixation (Fig. 1*A*). The ligand-binding experiments demonstrate that the overall substrate binding pocket is largely altered even when a conservative mutation, as in the case of the R285K mutant, is present (Table III).

The change in flavin redox potentials of R285K and the thermodynamic instability of the semiquinone form in all mutants profoundly distinguish them from the wild-type DAAO. At the moment, only the three-dimensional structure of RgDAAO and pkDAAO in complex with a substrate or a ligand is available (4, 5).[2] In this structure, the isoalloxazine ring is located in a hydrophobic environment making contacts with the side chain of different residues but, differently from pkDAAO and from other flavoproteins (6), no basic residue(s) or α-helix dipole is properly located to interact with the flavin N(1)-C(2)=O position.[2] A (partial) positive charge in proximity of this flavin locus is required to stabilize several anionic flavin derivatives (*e.g.* the N(5) covalent adduct with sulfite). Since the ϵ-amino group of Arg^{285} in RgDAAO is ~7 Å far away from the N(1)-C(2)=O flavin position (in the EFl_{red}-D-alanine complex), it cannot stabilize the negative charge on the flavin.[2] We suggest that in the free enzyme form (*i.e.* in the absence of a ligand) the side chain of Arg^{285} is able to rotate to a distance of ~3 Å from the N(1)-C(2)=O flavin locus (Fig. 1*B*). Therefore, the lack of the guanidinium group of Arg^{285} in the mutants determines the absence of the counterion required for stabilization of flavin semiquinone. This lower stabilization is further supported by the ~10^4-fold increase in K_d for binding of sulfite (Table III). Moreover, we are tempted to conclude that a similar function is exerted by Arg^{283} in the mammalian DAAO, and thus to give a rationale of the results obtained from chemical modification studies (15, 34). In fact, the inferred interaction of Arg^{283} with the flavin N(1)-C(2)=O locus in the free form of pkDAAO could explain the observation that the chemical modification of this residue destroyed the ability of pkDAAO to stabilize the benzoquinoid form of 8-mercapto-FAD and to form an N(5)-adduct with sulfite (15).

The crystal structure of oxidized DAAO in complex with L-lactate and of reduced enzyme complexed with D-alanine shows that the side chain of Arg^{285} is at >5 Å from the α-CH group of the substrate. Thereby, in this position Arg^{285} is far way from the reactive α-hydrogen and cannot be the active site base required by a carbanion mechanism for substrate dehydrogenation. In fact, all the Arg^{285} mutants we produced possess appreciable dehydrogenase activity (they can be anaerobically reduced by the substrate D-alanine). On the other hand, the large decrease in the rate of flavin reduction (and therefore in turnover) is quite surprising. The k_{cat} for R285K and R285A are decreased by about 450- and 7000-fold, respectively, in comparison to the wild-type RgDAAO (12); this change is accompanied by a parallel decrease in the rate of flavin reduction (Table IV). Since k_2 is still rate-limiting, we can rule out a change in the kinetic mechanism. A main kinetic difference with respect to the wild-type DAAO is that, due to the large decrease in k_2, $k_3 \gg k_2$ for the Arg^{285} mutants and thus the reductive half-reaction is essentially monophasic. The effect of Arg^{285} substitution on RgDAAO catalysis can be explained in terms of the recently proposed mechanism in which "orbital steering/interactions are the predominant or the sole important factors in catalysis."[2] The perturbation of the active site in the Arg^{285} mutants modifies the precise substrate alignment: alteration of the reaction trajectory results in a large change in the reaction velocity (k_{red} and k_{cat}). A similar large effect has been also reported for the enzyme isocitrate dehydrogenase, as a consequence of small trajectory changes via substrate modification and metal co-ordination (17). Actually, a second possibility has also to be taken into account, namely that an equilibrium form of the enzyme-substrate complex exists, in which the guanidinium side chain of Arg^{285} is no longer in contact with the carboxylate but it reaches a position in which it could abstract the proton from the α-carbon of the substrate.

The mechanism by which substrate dehydrogenation occurs

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in DAAO cannot be solved solely on the basis of structural data, thus new investigations are required to rule out definitively the possibility that Arg^{285} could be the acid/base residue required by a carbanion-type mechanism. Our results support the concept that precise binding and orientation of the substrate is a main quantitative factor in RgDAAO catalysis. Albeit the lack of the free enzyme crystal structure, the novel hypothesis on a conformational swing of the side chain of Arg^{285} from the position (seen in the structure) in which it binds the carboxylate anion, to a different one (next to the flavin N(1)-C(2)=O locus) in the free enzyme form, fits with the interpretation of the whole body of data. Moreover, the model we have proposed permits a reconciliation of the apparently contradictory conclusions that arose from the three-dimensional structure and from chemical modification studies of pkDAAO (4, 5, 14, 15).

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Role of Arginine 285 in the Active Site of *Rhodotorula gracilis* d-Amino Acid Oxidase: A SITE-DIRECTED MUTAGENESIS STUDY

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