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Structure and function of *Rhodotorula gracilis* D-amino acid oxidase 2. Site-directed mutagenesis of arginine 285 and pH effects

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Introduction

The enzyme D-amino acid oxidase (EC 1.4.3.3, DAAO) from the yeast *Rhodotorula gracilis* (RgDAAO) belongs to the large class of flavoprotein oxidases that catalyze the oxidation of amino or α-hydroxy acids. A fundamental question remains within this class of enzymes regarding the mechanism by which a proton and two electrons are transferred from the substrate α-carbon to the flavin N(5) position during the reductive half-reaction. Substrate oxidation might be accomplished by hydride transfer from the α-carbon to the flavin N(5). A carbanion mechanism has also been proposed in which an enzyme base removes the α-proton and so forms a carbanion intermediate (see (1) for a recent review). The latter would thus rapidly attack the N(5) locus of the flavin. The involvement of an (essential) arginine in the active site of DAAO was inferred by a number of chemical modification studies (for a review see Curti *et al.*,(2)).

To gain insight into the question regarding the mechanism of substrate dehydrogenation (an enzyme base for α-proton abstraction is essential for the carbanion mechanism), we are currently studying the active site of RgDAAO using different experimental approaches. We report here on the pH dependence of the reaction catalysed by DAAO and on the mutagenesis of arginine 285, the positively charged residue bound to the α-COO^- group of the substrate (see Umhau *et al.*, this volume).

Materials and Methods

R285K, R285A, R285Q and R285D mutants were expressed and purified as holoenzymes from BL21(DE3)pLysS *E. coli* cells using the pT7-DAAO expression system. Kinetic data were acquired in a stopped-flow instrument, at 25 °C. The pH dependence for a rate constant which is perturbed but not eliminated by an ionisation, is described by the following equation:

$$k_{obs} = (k_{acidic}[H^+] + K_a k_{basic})/([H^+] + K_a)$$

We refer to (3) for rules on the interpretation of parameters derived from pH analyses.

Table 1. Binding Properties, Steady State Coefficients and Reductive Half-Reaction (RHR) Rate Constants of Wild-type, R285K and R285A RgDAAOs.

	Binding		Steady state			RHR	
	Benzoate	Sulfite	k_{cat}	$K_{m,Ala}$	$K_{m,O2}$	k_2	$K_{d,app}$
	K_d (mM)		(s^{-1})	(mM)	(mM)	(s^{-1})	(mM)
Wild-type (4)	0.9	0.12	350	2.6	2.3	345	2.8
R285K	350	860	0.8	800	0.06	0.6	1000
R285A	1000	1000	0.05	310	0.08	0.035	200

Binding measurements were made in 10 mM HEPES, pH 7.5, 10 % glycerol, 5 mM 2-mercaptoethanol, at 15 °C. Kinetic experiments in 50 mM sodium pyrophosphate buffer, pH 8.5, 1 % glycerol, 0.3 mM EDTA, 0.5 mM 2-mercaptoethanol, at 25 °C.

Results

R285 Mutants

Visible spectra of the R285 mutants in the oxidized state are similar to that of wt RgDAAO. Anaerobic addition of an excess of D-alanine resulted in slow enzyme reduction of all mutants, with a final spectrum similar to that of the reduced wt DAAO. This result demonstrates that all the R285 mutants of RgDAAO are competent in catalysis. With the only exception of R285D (which does not produce the semiquinone form), the mutants produced ~70 % of the red, anionic flavin semiquinone by photoreduction under kinetically stabilised conditions. After addition of benzyl viologen as equilibrating agent, the mutants yielded ~20 % semiquinone, compared to 65 % for the wt, which thus reflects the extent of thermodynamic stabilisation.

The redox potentials of the R285K mutant at pH 7.5 and 15 °C show a 16 mV separation between the potential for transfer of the first and of the second electron (*vs.* 140 mV in the wt). Although the mutant has a E_m potential about 30 mV more positive than the wild-type DAAO (-97 mV *vs.* -130 mV), this change should have little influence on catalysis. The > 1000-fold weaker binding of sulfite observed for the mutants agrees with the changes in redox potentials of R285K. Loss of the guanidinium group of R285 in the mutants drastically reduces the binding of several carboxylic acids (*e.g.* benzoate, Table 1) also when the positive charge is conserved.

The catalytic properties of R285A and R285K were measured by enzyme monitored turnover experiments (while the reaction of R285Q and R285D was too slow to give reasonable results and thus was not investigated). Also, as a conservative a mutation as R285K resulted in a k_{cat} reduced ≈ 500-fold and in a 1000-fold larger K_m for D-alanine (Table 1). The spectral changes of the RHR of both mutants are essentially monophasic. This is consistent with the rate of product dissociation from the reduced enzyme (k_3 of Scheme 1) being significantly faster than the rate of flavin reduction. The reduction rate, k_2, and $K_{d,app}$ for R285K and R285A are very close to the k_{cat} and $K_{m,Ala}$ values determined under steady state conditions (Table 1), suggesting that mutation of R285 does not result in a change in the rate-limiting step: k_2 is still the

slow step in catalysis. The estimated value of $K_{d,app} \approx 1$ M is > 400 fold greater for R285 mutants than for wt RgDAAO.

pH Effects

In order to assess the involvement of acid/base catalysis in RgDAAO, the pH dependence of the RHR was measured from pH 5 to 10. Anaerobic mixing of oxidized enzyme with an excess of D-alanine resulted in biphasic kinetics:

$$E_{ox} + AA \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} E_{ox}{:}AA \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} E_{red}{:}IA \xrightarrow{k_3} E_{red} + IA \qquad \text{(Scheme 1)}$$

The first phase involves reduction of the enzyme to form a reduced enzyme:iminopyruvate complex (steps k_1, k_2), and the second, slow phase involves release of imino acid, resulting in the spectrum of the free fully reduced DAAO (k_3).

The pH dependence of the reduction rate, k_2, with D-alanine is depicted in Fig. 1: its value levels off at both low and high pH, with a $pK_{a,app} = 7.8$. The plateau at low pH is inconsistent with the existence of an essential base in RgDAAO. Base catalysis requires that k_2 extrapolates to zero at low pH, with a slope of one (or some higher integer) on the pk_2 *vs.* pH plot. This perturbation with pH is recapitulated for all the determined parameters (Harris *et al.*, manuscript in preparation), *i.e.* the slow phase in the RHR (product dissociation from the E_{red}:IA complex) shows a $pK_{a,app}$ of 9.5 that has been attributed to the bound complex.

The solvent kinetic isotope effect (KIE) on k_2 is also pH dependent (Fig. 1). At low pH the solvent KIE is 3.8, while at high pH it is only 1.2. The $pK_{a,app}$ is 7.0 (± 0.2). Any pH dependence of k_2 can be assigned to an E:S complex. We assign the ionisation on k_2 to the α-amino group of D-alanine, due to the lack of other exchangeable protons that could exert such a pH-dependent solvent KIE in the absence of an essential enzyme acid or base.

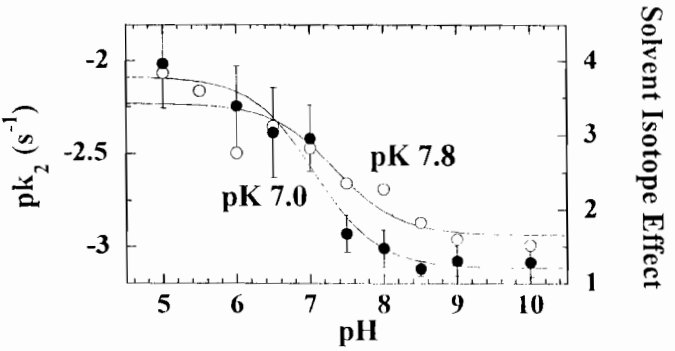

Figure 1: pH Dependence of pk_2 (O), the rate of flavin reduction of RgDAAO with D-alanine and of the solvent isotope effect (●) (these values represent the ratio of k_2, obtained in H_2O to those in D_2O at different pH values).

At pH 6.0 a very large substrate primary KIE of 10.2 ± 2.5 was measured, consistent with a symmetric transition state involving rupture of the C-H bond. The primary KIE dropped drastically to 1.15 ± 0.23 at pH 8. At pH 6, the double KIE is ~ 43 (k_2 with [2-^{1}H]-alanine in H_2O divided by k_2 with [2-^{2}H]-alanine in D_2O). This is clearly a multiplicative effect. At pH 8 and 10, both the individual solvent and primary KIE diminish, and interpretation between additive and multiplicative effects is indistinct. The double KIE of 43 observed at pH 6.0 is strong evidence in favour of a concerted (hydride transfer) over a stepwise (carbanion) mechanism.

Discussion

Our results argue against the presence of an active functional group capable of acid/base catalysis. Along this line R285 can be excluded from playing a chemical role *e.g.* as the active site base required by a carbanion mechanism. The effect of R285 substitution on RgDAAO catalysis can be satisfactorily explained in terms of the recently proposed mechanism in which orbital steering interactions are the predominant or the single most important factors in the transfer of redox equivalents. The structural perturbations in the R285 RgDAAO mutants probably affect the substrate-cofactor orientation. Alteration of the reaction trajectory thus would yield large changes in the reaction velocity (k_{red} and k_{cat}). Substitution of R285 also profoundly modifies some flavin properties reflected in a low degree of stabilization of the flavin semiquinone form and a change in the redox properties of the free enzyme. We suggest that, in this condition, the arginine interacts electrostatically with the flavin at least in the unliganded enzyme form. The pH effect on RgDAAO reduction also confirms that no essential base is present. In addition, solvent and primary kinetic isotope effects strongly support a concerted hydride transfer mechanism of substrate dehydrogenation.

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