Optimization of \textit{d}-amino acid oxidase for low substrate concentrations – towards a cancer enzyme therapy

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Introduction

Chemotherapy, together with surgery and radiotherapy, is widely used for the treatment of malignant disease. Unfortunately, and as is widely known, the selectivity of most drugs for malignant cells remains insufficient. An insufficient therapeutic index, a lack of specificity and the emergence of drug-resistant cell sub-populations often lower the efficacy of these therapies. In particular, a number of specific difficulties are associated with the treatment of solid tumors, where the access of drugs to cancer cells is often limited by poor, unequal vascularization, and areas of necrosis [1]. The histological heterogeneity of the cell population within the tumor is another major drawback [2].

One recent approach to the treatment of solid tumors relies on the application of gene/enzyme therapy technologies. Enzyme-activated prodrug therapy is a two-step approach. First, a drug-activating enzyme is targeted to the tumor. Then, a nontoxic prodrug, a substrate of the exogenous enzyme, is administered systematically so that it can be converted to an active anticancer drug in tumors to yield high local concentrations [2,3]. Specifically, treatments have been

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D-\textit{Amino acid oxidase (DAAO)} has recently become of interest as a biocatalyst for industrial applications and for therapeutic treatments. It has been used in gene-directed enzyme prodrug therapies, in which its production of \textit{H}_2\textit{O}_2 in tumor cells can be regulated by administration of substrate. This approach is limited by the locally low \textit{O}_2 concentration and the high \textit{K}_m for this substrate. Using the directed evolution approach, one DAAO mutant was identified that has increased activity at low \textit{O}_2 and \textit{d}-\textit{Ala} concentrations and a 10-fold lower \textit{K}_m for \textit{O}_2. We report on the mechanism of this DAAO variant and on its cytotoxicity towards various mammalian cancer cell lines. The higher activity observed at low \textit{O}_2 and \textit{d}-\textit{Ala} concentrations results from a combination of modifications of specific kinetic steps, each being of small magnitude. These results highlight the potential \textit{in vivo} applicability of this evolved mutant DAAO for tumor therapy.

Abbreviations

DAAO, \textit{d}-amino acid oxidase (EC 1.4.3.3); E-Fl\textsc{ox}, oxidized enzyme form; E-Fl\textsc{red}, reduced enzyme form; E-Fl\textsc{ox}/C24S, oxidized enzyme form in complex with the substrate \textit{d}-alanine; E-Fl\textsc{red}/C24P, reduced enzyme–iminoacid complex; m-DAAO, S19G/S120P/Q144R/K321M/A345V \textit{d}-amino acid oxidase mutant; ROS, reactive oxygen species; wt-DAAO, wild-type \textit{d}-amino acid oxidase.
designed to produce reactive oxygen species (ROS) in tumors. ROS are potentially harmful byproducts of the cellular metabolism that directly affect cellular functions and survival, and cause mutations [4]. Over-production of ROS can initiate lethal chain reactions that involve oxidation and that also affect the integrity and survival of normal cells [1]. Among the ROS, \( \text{H}_2\text{O}_2 \) readily crosses cellular membranes and causes oxidative damage to DNA, proteins and lipids by direct oxidation [5,6]. Furthermore, \( \text{H}_2\text{O}_2 \) induces apoptosis of tumor cells in vitro via activation of the caspase cascade [7,8]. The use of ROS-generating enzymes such as xanthine oxidase and glucose oxidase as anticancer agents has been reported [9]. However, regulation of ROS production by exogenously administered glucose oxidase in tumors is problematic because the availability of its substrate cannot be significantly controlled. Similarly, the production of superoxide by xanthine oxidase cannot be regulated in vitro because of the promiscuity of the enzyme [10]. For a recent, general review on the use of oxidative stress for cancer therapy, see [1].

To overcome these limitations, we have proposed the use of \( \delta \)-amino acid oxidase (DAAO) from *Rhodotorula gracilis* (EC 1.4.3.3) for cancer treatment [11]. Subsequently, the strategy for cancer therapy based on oxyzstress and DAAO was implemented using, in addition, the enzyme from pig kidney [12,13]. The flavoenzyme DAAO catalyzes the oxidation of \( \delta \)-amino acids into the corresponding \( \alpha \)-keto acids, ammonia, and specifically \( \text{H}_2\text{O}_2 \) [14,15]. Yeast DAAO possesses a very high catalytic activity and undergoes a stable interaction with the FAD cofactor [14,15]; moreover, its substrates are not endogenously present at high concentrations, allowing easier regulation of enzyme activity in therapy in comparison with the enzymes previously used [9,10]. Unfortunately, the *in vivo* use of wild-type DAAO (wt-DAAO) for this application is limited by the low local \( \text{O}_2 \) concentration and the correspondingly high \( K_m \), which is in the millimolar range. In the present study, we report on the application of a directed evolution approach to obtain yeast DAAO variants with substantially increased activity at low \( \text{O}_2 \) and \( \delta \)-amino acid concentrations. This could lead to better efficacy in therapeutic applications.

**Results**

**Selection of DAAO variants with improved \( \text{O}_2 \) affinity**

A library of \(~10\,000\) clones was generated by error-prone PCR, starting from the cDNA encoding for the wild-type (first generation) and, subsequently, starting from the Q144R-DAAO mutant (second generation, see below). In order to estimate the frequency of mutations, five independent clones for each generation were sequenced: a frequency of mutation of 0.16% was found, with the strongest bias towards transitions (e.g. A→G substitutions). An 80% fraction of inactive mutants was obtained. For each generation, \(~1000\) independent clones were screened for DAAO activity at a 2.5% (30 \( \mu \text{M} \) \( \text{O}_2 \)) concentration. Among the DAAO mutants generated from wt-DAAO and compared with it, the supernatant of *Escherichia coli* cells expressing clone 7 (containing the Q144R substitution) shows increased activity in the specific test described in Experimental procedures that detects the formation of \( \text{H}_2\text{O}_2 \). We find it remarkable that the first stage in the mutagenesis procedure pulls out exactly the same mutant that was identified during a previous screening of the same library in a search for a DAAO with broader substrate specificity [16]. The two screening procedures (differing in \( \text{O}_2 \) concentration and the \( \delta \)-amino acids used) show a higher response for the same DAAO mutant, a result that can arise from alterations in kinetic properties and/or from different contributions (e.g. higher protein expression or higher stability).

Subsequently, a library generated by starting from the cDNA encoding for Q144R-DAAO was screened analogously. The crude extract from *E. coli* clone 305 shows increased production of \( \text{H}_2\text{O}_2 \) as compared with both wt-DAAO and Q144R-DAAO. The product of the cDNA coding for this DAAO mutant is abbreviated as m-DAAO; it contains the four amino acid substitutions S19G, S120P, K321M, and A345V in addition to the Q144R mutation. The position of these mutations is shown in Figure 1.

**Selected properties of DAAO mutants**

The purified mutants are homodimeric 80 kDa holoenzymes, as judged by gel permeation chromatography and spectral properties. The substitutions introduced in the two DAAO mutants do not affect the contents of secondary and tertiary structure of the protein, as the far-UV and near-UV CD spectra of both mutants and wt-DAAO are indistinguishable (not shown). Similarly, no differences in stability versus time or pH were observed with the mutants. The mutants in the oxidized state show the typical spectrum of FAD-containing flavoproteins, i.e. absorbance maxima at \(~455\) nm and \(~375\) nm, an \( A_{445\text{nm}}/A_{455\text{nm}} \) ratio of \(~8.5\), which is within the same error margin as found for wt-DAAO [15,17]. As
the type and amount of semiquinone formed correlates with different properties of the various flavoprotein classes, this parameter was studied for the mutants using the anaerobic photoreduction method [18]. In the present case, near-complete formation of the anionic semiquinone (≥ 95% on the basis of flavin content) was found for wt-DAAO, Q144R-DAAO and m-DAAO. Semiquinone stabilization is of a kinetic nature, as addition of the redox mediator benzyl violo-
gen resulted in dismutation to a thermodynamically determined mixture of oxidized, fully reduced and semiquinone forms. The DAAO mutants show a somewhat lower percentage of thermodynamic semiquinone stabilization than wt-DAAO (≤ 20% versus 40%, respectively) [15,17]. As shown by the work of Yorita et al. [19], the reduction potential of the flavin cofactor within a given flavoprotein is reflected by the $K_d$ for formation of a sulfite flavin N(5)-adduct. In the present case, this $K_d$ is lowered ~2-fold (from 110 to 51 μM for wt-DAAO and m-DAAO), corresponding to an increase of ~15 mV in reduction potential for m-DAAO.

Information about the active center can be derived from the spectral effects observed upon binding of specific ligands to DAAO [20]. Thus, typical spectral effects induced by benzoate binding reflect the polarity of the binding site cavity, whereas the charge transfer complexes observed upon binding of anthranilate are sensitive to the orientation of flavin cofactor and ligand [15,17]. The spectral effects observed with the DAAO mutants are identical to those found with wt-DAAO (not shown) [15,17]. A minor difference is an approximately three-fold tighter binding of benzoate to m-DAAO than to wt-DAAO ($K_d=0.30 \pm 0.02$ versus $0.9 \pm 0.1$ mM).

Wild-type DAAO, Q144R-DAAO and m-DAAO showed similar specific activities of 12.9, 10.2 and 12.6 U·mg$^{-1}$ protein in the polarographic assay under standard conditions (see Experimental procedures). However, significantly different activities were found when the activity was determined at low substrate concentrations, i.e. at 0.1 mM D-Ala and 2.5% (30 μM) $O_2$. Under these conditions, Q144R-DAAO and m-DAAO showed 35% and 50% of the activity found at 250 μM $O_2$, whereas wt-DAAO was practically inactive (see below).

**Kinetic properties**

**Steady-state measurements**

The dependence of the catalytic activity of the DAAO mutants on the oxygen and D-Ala concentrations was assessed using the enzyme-monitored turnover method and as detailed in Experimental procedures. Air-saturated solutions of DAAO and of D-Ala were reacted in the stopped-flow instrument, and absorbance spectra were recorded continuously in the 300–700 nm range at 15 °C. This temperature is lower than that used in

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**Fig. 1.** Overview of the positions mutated in the DAAO variants. Mutants were obtained from the first round (Q144R, bold) and the second round (S19G/S120P/Q144R/K321M/A345V) of error-prone PCR of yeast DAAO (A). The flavin cofactor is in yellow and the ligand CF$_3$-D-Ala is in red (Protein Data Bank code: 1c0l). (B) Structure of the dimeric form of yeast DAAO. Note that the mutated residues do not belong to the monomer–monomer interface region.
previous studies with yeast DAAO [21], and was chosen in order to better follow specific rapid steps. As shown in Fig. 2, during turnover the enzymes are largely present in the oxidized form, and the spectrum of reduced enzyme is observed only towards the end of the observation time, i.e. when the O2 concentration becomes very low. This is consistent with the steps involving oxidation of reduced DAAO by O2 being faster than those involved in reduction (Fig. 2A). In this context, the behavior of the DAAO mutants is not much different from that of wt-DAAO; that is, the ratios of steps involved in the oxidative and reductive half-reactions are not significantly different. However, comparison of the reaction profiles at 21% O2 (305 μM) with those at 5% (73 μM) O2 reveals striking differences: thus, whereas at air saturation the time profiles that reflect O2 consumption are essentially the same for wt-DAAO and m-DAAO, at 73 μM O2 m-DAAO consumes the available O2 in approximately half the time required by wt-DAAO (Fig. 2B). These traces confirm the higher activity of m-DAAO than of the wild-type enzyme at low concentrations of both O2 and D-Ala (see below). An accurate determination of steady-state parameters according to the method of Gibson [22] is, however, not possible at low O2 concentration, because the steady-state phase is too short (Fig. 2B).

The Lineweaver–Burk plots obtained from the primary data at 21% O2 saturation show a set of slightly converging lines with wt-DAAO and Q144R-DAAO and parallel lines with m-DAAO (not shown). The pattern observed for wt-DAAO has been demonstrated previously to be consistent with a limiting case of a ternary complex mechanism in which some specific rate constants (i.e. k_cat; see Scheme 1) are sufficiently small [21]. The parameters obtained from steady-state measurements at [O2] = 0.305 mM (Table 1) show that, whereas k_cat for m-DAAO is smaller than for wt-DAAO, its O2 affinity is significantly higher (~10-fold decrease in K_m,O2 value).

The reductive half-reaction

This was studied with wt-DAAO and m-DAAO using D-Ala under anaerobic conditions and at 15 °C, and the results are shown in Fig. 3A. Because the steady-state kinetic properties of Q144R-DAAO closely resemble those determined at 15 °C for wt-DAAO, and because selected experimental traces of the reduct-

\[ \text{Reducive half-reaction:} \]

A \[ \text{E-Fl}_{\text{ox}} + S \xrightarrow{k_1} \text{E-Fl}_{\text{red}} \]

B \[ \text{E-Fl}_{\text{red}} \xrightarrow{k_3} \text{E-Fl}_{\text{ox}} \]

C \[ \text{E-Fl}_{\text{ox}} \xrightarrow{k_5} \text{E-Fl}_{\text{red}} + P \]

\[ \text{Oxidative half-reaction:} \]

A \[ \text{E-Fl}_{\text{ox}} + P \xrightarrow{k_1} \text{E-Fl}_{\text{red}} \]

B \[ \text{E-Fl}_{\text{red}} + \text{O}_2 \xrightarrow{k_3} \text{E-Fl}_{\text{ox}} + P \]

C \[ \text{E-Fl}_{\text{ox}} \xrightarrow{k_5} \text{E-Fl}_{\text{red}} + P \]

\[ \text{Scheme 1. Kinetic steps in the reductive and oxidative half-reactions of the catalytic cycle proposed for yeast DAAO, adapted from [15,21,23].} \]

\[ \text{Fig. 2. Steady-state measurements of O2 consumption by wild-type DAAO and mutants. The experiments were carried out by monitoring the time dependence of the flavin oxidation state via its absorbance at 455 nm [21,23] and at pH 8.5 and 15 °C. (A) Wild-type DAAO or m-DAAO at 8.6 μM, O2 at 305 μM and D-Ala at 0.6 mM. The symbols are the experimental data points for wt-DAAO (|) and m-DAAO (x); the trace (—) represents the simulations performed as detailed in Experimental procedures, based on the sequence of kinetic steps of Scheme 1a–c and using the following rate constants. wt-DAAO: k_1 = 2.5 \times 10^5 M^{-1} s^{-1}; k_{-1} = 530 s^{-1}; k_2 = 395 s^{-1}; k_{-2} \leq 10 s^{-1}; k_3 = 2.7 \times 10^4 M^{-1}s^{-1}; k_4 \geq 2500 s^{-1}; k_5 \leq 1.5 s^{-1}; k_6 = 18 \times 10^3 M^{-1} s^{-1}. m-DAAO: k_1 = 4.6 \times 10^5 M^{-1} s^{-1}; k_{-1} = 750 s^{-1}; k_2 = 350 s^{-1}; k_{-2} \leq 10 s^{-1}; k_3 = 2.8 \times 10^6 M^{-1} s^{-1}; k_4 = 250 s^{-1}; k_5 \leq 1 s^{-1}; k_6 = 25 \times 10^3 M^{-1} s^{-1}. (B) Comparison of steady-state kinetic traces obtained analogously for the indicated DAAOs but under the following conditions: 6.1 μM DAAO, 73 μM O2, and 0.2 mM D-Ala. The symbols are the experimental data points for the indicated enzyme forms.} \]
Flavin reduction (not shown). The dependence of similar time courses, corresponding to similar rates of m-DAAO than for wt-DAAO (Fig. 3A). At higher substrate concentrations, the oxidized form of the enzyme dissociation from E-Fl red is rapidly converted to the reduced enzyme–iminoacid complex (E-Flred is rapidly converted to the reduced enzyme–iminoacid with wt-DAAO [21], the oxidized form of the enzyme.

Table 1. Comparison of steady-state kinetic parameters for wild-type DAAO and mutants with d-Ala as substrate and at 15 °C. Data were obtained in buffer A (50 mM sodium pyrophosphate buffer, pH 8.5, 1% glycerol, and 0.25 mM 2-mercaptoethanol). The values in parentheses are those calculated using Eqns (1) and (2) from the rate constants reported in Table 2 and in the legend of Fig. 3. Data are expressed as mean ± standard deviation; at least five experiments at each substrate concentration were analyzed.

<table>
<thead>
<tr>
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<th>Lineweaver–Burk plot behaviora</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$\Phi_{D,Ala}$ (M$^2$s$^{-1}$)</th>
<th>$K_{m,D,Ala}$ (mM)</th>
<th>$\Phi_{O_2}$ (Ms$^{-1}$)</th>
<th>$K_{m,O_2}$ (mM)</th>
<th>$\Phi_{D,Ala,O_2}$ (M$^2$s$^{-1}$)</th>
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<tr>
<td>wt-DAAO</td>
<td>Convergent</td>
<td>330 ± 30</td>
<td>0.8 ± 0.1</td>
<td>2.6 ± 0.4</td>
<td>5.0 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Q144R-DAAO</td>
<td>Convergent</td>
<td>370 ± 30</td>
<td>1.2 ± 0.1</td>
<td>4.7 ± 0.3</td>
<td>6.1 ± 0.2</td>
<td>2.0 ± 0.5</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>m-DAAO</td>
<td>~ Parallel</td>
<td>140 ± 25</td>
<td>1.3 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>0.22 ± 0.01</td>
<td>2.0 ± 0.2</td>
</tr>
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*a This refers to the lines obtained at different D-Ala concentrations in the $v/C_0$ versus 1/[O$_2$] plot.

Fig. 3. Reductive half-reaction of wt-DAAO and m-DAAO. (A) Comparison of time courses of flavin reduction followed at 455 nm [(|) symbols are the experimental data points]. The enzymes (~ 12 μM) were reacted under anaerobic conditions with 40 μM d-Ala, at pH 8.5 and 15 °C. The rate constants were obtained by fitting (continuous line) using a double exponential equation (see Experimental procedures): $k_{obs1} = 10.5$ and 15.7 s$^{-1}$ and $k_{obs2} = 0.55$ and 0.63 s$^{-1}$ for wt-DAAO and m-DAAO, respectively. (B) Dependence of the rate of the observed first phase of anaerobic reduction ($k_{obs1}$) for wt-DAAO (o) and m-DAAO (□) on the concentration of d-Ala. The line represents the fit of the wt-DAAO data points based on a hyperbolic equation. The reaction rates were determined from experiments such as those reported in (A).

tive half-reaction obtained for Q144R-DAAO are identical to those of wt-DAAO, a detailed kinetic investigation of this mutant DAAO was not carried out. As with wt-DAAO [21], the oxidized form of the enzyme is rapidly converted to the reduced enzyme–iminoacid complex (E-Flred→P in Scheme 1; phase 1, $k_{obs1}$). This species is subsequently converted at a lower rate into free, fully reduced enzyme (phase 2, $k_{obs2}$). Monitoring of the absorbance changes at 455 nm conveniently follows the time course of these processes. At very low d-Ala concentrations, the value of $k_{obs1}$ is larger for m-DAAO than for wt-DAAO (Fig. 3A). At higher substrate concentrations, wt-DAAO and m-DAAO show similar time courses, corresponding to similar rates of flavin reduction (not shown). The dependence of $k_{obs1}$ values (obtained as in Fig. 3A) on d-Ala concentration is shown in Fig. 3B. Therein, a curvature of the line intersecting the data points is apparent. A hyperbolic dependence of $k_{obs1}$ on d-Ala concentration has been amply described for various DAAOs [21,23,24]. It can be represented by a second-order process (formation of an initial enzyme–substrate complex) followed by a first-order reaction, as depicted in Scheme 1a [25]. As the data are satisfactorily fitted by a rectangular hyperbola that intersects close to the origin, this indicates that the reduction step is practically irreversible ($k_{-2} \leq 10$ s$^{-1} < k_2 \geq 200$ s$^{-1}$; see Scheme 1a). The rate for the observed second-phase $k_{obs2}$, which corresponds to product dissociation from E-Flred→P, step $k_5$ in Scheme 1, does not depend on d-Ala concentration, and its value is ~1 s$^{-1}$ for both wt-DAAO and m-DAAO. Thus, the same kinetic model derived for wt-DAAO [21] applies for m-DAAO, and the values of $k_2$, $k_5$ and $K_{L,app}$ are similar (Table 2).

The absolute values of the substrate-binding steps $k_1$ and $k_{-1}$ (Scheme 1) are outside the range accessible to
direct experimental verification. However, lower limits for the rates of these steps can be estimated by simulation of kinetic traces, as outlined in Experimental procedures, using the application specfit. The simulations were based on the sequential mechanism described in Scheme 1a, where the absorbance spectra of the oxidized enzyme in the free (E-Flox) and substrate-complexed (E-Flox/C24S) form were assumed to be identical ($\varepsilon_{455}$ nm = 12 600 M$^{-1}$ cm$^{-1}$) and were held fixed. Figure 3A depicts a typical comparison of experimental traces with simulation results at a specific d-Ala concentration. Therein, a value for $k_1$ of 4.6 $\times$ 10$^5$ M$^{-1}$ s$^{-1}$ was used for m-DAAO; this is approximately two-fold that required for simulations with wt-DAAO ($\sim$ 2.5 $\times$ 10$^5$ M$^{-1}$ s$^{-1}$). Similarly, for $k_{-1}$ a value of 750 s$^{-1}$ is required for m-DAAO, as compared with $\sim$ 530 s$^{-1}$ for wt-DAAO. The higher rate of substrate binding thus appears to be responsible for the observed higher rate of flavin reduction at low substrate concentration, as depicted in Fig. 3A.

### The oxidative half-reaction

The (re)oxidation of reduced DAAO forms by O$_2$ (see Scheme 1b,c) was studied using stopped-flow apparatus. For this, anaerobic solutions of free reduced enzyme were reacted with buffer equilibrated at different O$_2$ concentrations (Scheme 1c), and reoxidation was monitored by following the (re)appearance of the absorption of the oxidized flavin species. The time course of (re)oxidation at 455 nm is monophasic (representative results are shown in Fig. 4A). The same type of experiment was repeated, however, starting from reduced DAAO in the presence of high concentrations of ammonia and pyruvate, conditions that induce formation of E-Flred/P (see Scheme 1b): in this case, the time course of reoxidation is clearly biphasic. A fast phase with an amplitude corresponding to $\sim$ 50% of the overall absorbance change at 455 nm was followed by a slower one, the rate of which was the same as that observed with free reduced DAAO.

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**Table 2.** Rate constants estimated from rapid reaction methods, at 15 °C. For the reductive half-reaction, the parameters were obtained from stopped-flow experiments using D-Ala as substrate; for the oxidative half-reaction, the reoxidation was started from the free or the iminoacid complexed reduced enzyme species (Scheme 1c and Scheme 1b, respectively). The rate constants refer to those defined in Scheme 1.

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<th>Reductive half-reaction</th>
<th>Oxidative half-reaction</th>
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<tr>
<td></td>
<td>$k_{obs1}$ ($\sim$ $k_2$) (s$^{-1}$)</td>
<td>$k_5$ (K$^{-1}$/$k_1$) (mM)</td>
</tr>
<tr>
<td>wt-DAAO</td>
<td>$\geq$ 250</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Q144R-DAAO</td>
<td>2.0 ± 0.1</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>m-DAAO</td>
<td>$\geq$ 260</td>
<td>1.6 ± 0.1</td>
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$^a$ Buffer A containing 20 mM glucose, 20 mM pyruvate, and 400 mM NH$_4$Cl. $^b$ Buffer A containing 20 mM glucose. The rate constant of the second (slower) phase of flavin reoxidation observed in the presence of iminoacid and corresponding to reoxidation of the free reduced enzyme form ($k_6$ in Scheme 1c) is shown in parentheses. $^c$ The values for the reductive half-reaction of Q144R-DAAO are assumed to be very close to those for wt-DAAO, as selected kinetic traces for both species were superimposable under the same conditions.
From this, we deduce that the first, fast phase corresponds to the (re)oxidation of E-Flred\textsuperscript{-}P present at equilibrium, and the second one to the reoxidation of uncomplexed E-Flred [21]. The $k_{\text{obs}}$ values obtained by this method are reported in Fig. 4B as a function of O₂ concentration. This dependence does not show indications of saturation with O₂ concentration; it can thus be assumed to reflect a second-order process.

Table 2 reports the various rate constants for the oxidative half-reactions for wt-DAAO and mutant DAAO. It is noteworthy that whereas the rate of flavin reduction ($k_2$) is not significantly altered in m-DAAO (Fig. 3B) as compared with wt-DAAO, that of the bimolecular reaction with O₂ suggests a slight increase. The effect (Fig. 4B and Table 2), although of small magnitude (~1.2-fold), was observed consistently in different sets of experiments and with different enzyme preparations.

The overall kinetic mechanism

In order to identify the rate constant(s) that might have been altered by the substitutions introduced in m-DAAO (Fig. 3B) as compared with wt-DAAO, that of the bimolecular reaction with O₂ suggests a slight increase.

For the kinetic set-up of Scheme 1a,b, the steady state is described by Eqns (1,2), by analogy to that derived completed in the dead-time of the stopped-flow instrument ($k_{\text{obs}} > 250$ s\textsuperscript{-1} at 15 °C). It is noteworthy that a good correspondence is also evident between the steady-state $\Phi_O$ Dalziel coefficient and the reciprocal of $k_3$, the second-order rate constant for reoxidation of E-Flred\textsuperscript{-}P (compare the values in Tables 1 and 2).

\[
e_t/v = \frac{\Phi_0 + \Phi_{\text{D-Ala}}/[\text{D-Ala} + \Phi_{O_2}/[O_2] + \Phi_{\text{D-Ala}O_2}/[\text{D-Ala}]*[O_2]}{\Phi_{\text{D-Ala}O_2}/[\text{D-Ala}]*[O_2]} \tag{1}
\]

\[
e_t/v = \frac{[k_2 + k_4]/(k_2 \cdot k_4)}{\Phi_0 + \Phi_{\text{D-Ala}}/[\text{D-Ala} + \Phi_{O_2}/[O_2] + \Phi_{\text{D-Ala}O_2}/[\text{D-Ala}]*[O_2]} + \frac{[k_1 + k_2]/[k_1 \cdot k_2[O_2]] + [(k_1 + k_2)/[k_1 \cdot k_2[O_2]]} + \frac{[(k_1 + k_2)/[k_1 \cdot k_2[O_2]]} \tag{2}
\]

where $k_{\text{cat}} = 1/\Phi_0$; $K_{m,\text{D-Ala}} = \Phi_{\text{D-Ala}}/\Phi_0$; and $K_{m,O_2} = \Phi_{O_2}/\Phi_0$.

$\Phi$ values are the steady-state kinetic coefficients: $\Phi_0$ is the reciprocal of the maximum rate, $\Phi_{\text{D-Ala}}$ and $\Phi_{O_2}$ are the monomolecular terms of dependence on D-Ala and O₂ concentration, respectively, and $\Phi_{\text{D-Ala}O_2}$ is the bimolecular term showing the dependence for both substrates concentration.

An sample comparison between an experimental trace at 455 nm and the simulation is shown in Fig. 2A. Therein, a good reproduction of the experimental traces for wt-DAOO and m-DAAO is obtained by using the rate constants listed in the legend of Fig. 2 and in Table 2: the main difference between the two enzymes is for the rate constant of product dissociation from the reoxidized enzyme form ($k_4$). For m-DAAO, a 10-fold lower rate is required for good simulation as compared with wt-DAAO. The simulations suggest that for wt-DAAO, the simplification $k_4 > k_2$ (Scheme 1) is valid. This yields $k_{\text{cat}} = [k_2 \cdot k_4]/(k_2 + k_4) \sim k_2$ [21] (Table 1). The expression $K_{m,O_2} = [k_4 \cdot (k_2 + k_4)]/[k_3 \cdot (k_2 + k_4)]$ can also be simplified to $k_2/k_3$, and $K_{m,\text{D-Ala}} = [k_4 \cdot (k_1 + k_2)]/[k_1 \cdot (k_2 + k_4)]$ simplifies to $(k_1 + k_2)/k_4$.

The validity of these assumptions can be assessed by comparing the estimated values of $k_{\text{cat}}$, $K_{m,O_2}$, and $K_{m,\text{D-Ala}}$ with those derived from steady-state turnover data. From Table 1, it is apparent that the correspondence is very good, the discrepancy between the two values being ≤1.6-fold. This simplification does not apply for m-DAAO, since the results from simulations indicate that $k_4 \sim k_2$. This leads to a situation where $k_{\text{cat}} \sim k_2/2$ and thus the flavin reduction step is no longer fully rate-limiting in catalysis. A good correlation between the experimental values (obtained using the full equations described in [21] and without the simplification $k_4 > k_2$ and those from simulations is thus found also for m-DAAO (Table 1).

A measurement of the rate of product release from the (re)oxidized enzyme as previously performed [23] with pig kidney DAAO is not feasible with yeast DAAO, because with the latter the process is completed in the dead-time of the stopped-flow instrument ($k_{\text{obs}} > 250$ s\textsuperscript{-1} at 15 °C). The DAAO application in cell cultures

The m-DAAO mutant showed significantly higher activity at low O₂ and D-Ala concentrations (30 μM and 100 μM, respectively) than wt-DAAO (Fig. 5A). The ability of the different DAAO forms to produce H₂O₂ in vivo was assessed with a cytotoxicity assay performed on mouse tumor cell lines. In these studies, D-Ala was used, because it is the optimal substrate of DAAO ($K_m < 1$ mM) [15]. DAAO or D-Ala alone showed no cytotoxicity against tumor cells (not shown). On the other hand, application of the different DAAO forms to N2C tumor cells resulted in a remarkable D-Ala (prodrug substrate) concentration-dependent
cytotoxicity (Fig. 5B). Importantly, m-DAAO generated greater cytotoxicity than wt-DAAO and Q144R-DAAO [in particular at a low (1 mM) d-Ala concentration; Fig. 5B,C], a result resembling the relative activity measured at low substrate concentrations (Fig. 5A). The cytotoxicity was most evident on N2C and glioblastoma U87 tumor cells as compared with COS-7 fibroblasts or HEK293 embryonic control cells, whereas the metastatic 4T1 tumor cell line from mammary glands was insensitive to DAAO treatment (Fig. 5D). This result correlates with the observation that, in control experiments, the 4T1 cells showed >90% survival after treatment with exogenously added H$_2$O$_2$ at 1 mM, a ROS concentration at which all the further tested cell cultures showed full cytotoxicity (not shown). It is noteworthy that DAAO-induced cytotoxicity was previously demonstrated to be apoptotic [1,26–28].

**Discussion**

The reactivity of flavoprotein oxidases to O$_2$ depends on two factors: the intrinsic reactivity of the reduced flavin cofactor to O$_2$, and the ability of the latter to travel through the protein scaffold to the locus, where the primary redox step takes place [14,29]. Although a combination of both factors is assumed to be operative in most cases, detailed insights at the molecular level that might be of help in developing approaches aimed at modifying the O$_2$ reactivity is still elusive. For these reasons, in our effort to optimize the activity of DAAO at low O$_2$ and d-amino acid concentrations, we resorted to the directed evolution approach.

The present data show that evolution of the catalytic efficiency of DAAO towards improved reactivity to O$_2$, and consequently enhanced suitability for cancer treatment, is indeed feasible. On the other hand, the analysis of kinetic data for m-DAAO has produced unexpected results, in that the improved efficiency does not result from an increase in the rate of reaction of reduced enzyme with O$_2$. First, it should be stated that, on the basis of the spectral and kinetic parameters used, it can be deduced that the general folding pattern and the topology of the active center are probably very similar for the mutants and wt-DAAO. In agreement with this, the (limiting) rate of the chemical step in the reductive half-reaction $k_2$ (see Scheme 1) is essentially the same for wt-DAAO and m-DAAO (Fig. 3). The higher rate of enzyme reduction observed with m-DAAO at low substrate concentrations (e.g. [d-Ala] = 40 μM; Fig. 3A) might result from $k_1$, the rate of substrate binding, being ≥2-fold that of wt-DAAO. This will result in a lower $K_d$ and faster formation of E–Fl$_{ox}$S (Michaelis complex) (see Scheme 1). The affinity for O$_2$, as expressed by the $K_{m,O_2}$ parameter, is ~10-fold lower for m-DAAO than for wt-DAAO (Table 1). The effect of the enhanced apparent affinity for O$_2$ is especially evident at low concentrations of the latter (see Fig. 2), and manifests itself in the results of the screening tests. In fact, the $k_{cat}/K_{m,O_2}$ parameter for m-DAAO calculated for low substrate concentrations from the data in Table 1 is approximately 3.6-fold better than that of wt-DAAO. This number correlates very well with the data in Fig. 5 showing an approximately three-fold better effect on tumor cell lines, this arguably resulting from a correspondingly enhanced production of H$_2$O$_2$. 

**Fig. 5.** Activity and cytotoxicity of wt-DAAO and mutants. (A) Comparison of the activity of wt-DAAO and mutants with 0.1 mM d-Ala and at 2.5% O$_2$ as substrates (25 °C, pH 8.5); 100% corresponds to the values determined at 21% O$_2$ for each enzyme; wt-DAAO, 12.9 U mg$^{-1}$ protein; Q144R-DAAO, 10.2 U mg$^{-1}$ protein; m-DAAO, 12.6 U mg$^{-1}$ protein. (B) Comparison of the cytotoxicity of the different DAAO forms on cultured N2C tumor cells, and dependence on the concentration of the substrate d-Ala. The effect was observed after 24 h of incubation using 10 μM of wt-DAAO (white bars), Q144R-DAAO (gray bars), and m-DAAO (black bars). (C) Comparison of cytotoxicity observed using the indicated DAAO forms in the presence of 20 mM d-Ala [conditions as in (B)]. (D) Cytotoxicity of m-DAAO on the indicated tumor cell lines and comparison with the control cell lines (COS-7 and HEK293). Cytotoxicity is the percentage of cell death after 24 h of incubation with 10 μM of enzyme and using the indicated d-Ala concentrations, as estimated using the thiazolyl blue tetrazolium bromide assay (see Experimental procedures). The data are reported as the average of at least three separate determinations, and the error bars indicate the standard deviation.
One important conclusion emerging from comparison of the rate constants estimated singularly from rapid reaction studies with the parameters resulting from steady-state studies is that the mentioned difference in $K_{m;O_2}$ cannot be attributed to the modification of a unique step. On the contrary, it appears that the ‘improvement’ of several steps contributes to generating the observed, overall effect on $K_{m;O_2}$. Such minor factors might act synergistically in optimizing the availability of E-Fl$_{ox}$~P for the reaction with $O_2$ (see Scheme 1).

Specifically, faster substrate binding ($k_1$, $\sim$ 2-fold) and an increase in $k_3$ ($\sim$ 1.2-fold) contribute additively to the observed effect. Further effects that cannot be assessed experimentally, such as the rates of product dissociation from E-Fl$_{ox}$~P ($k_4$), might contribute to increasing the ‘oxygen affinity’ to the observed level. Excellent simulations of the steady-state traces were obtained by lowering the rate of $k_4$ $\sim$ 10-fold (see Fig. 3A). As stated in [30], a properly positioned positive charge (from the protein moiety or from a ligand) can enhance $O_2$ reactivity. We thus cannot exclude the possibility that the presence, for a prolonged period, of a positive charge (due to the charged iminoacid product) in the active site of m-DAAO as compared with the wild-type enzyme also might contribute to increasing the activity at low $O_2$ concentrations, the site whose substitutions alter substrate affinity and kinetic properties.

In conclusion, the evolved m-DAAO mutant, which contains five point substitutions (Fig. 1), shows significantly higher activity at low $O_2$ and d-Ala concentrations than wt-DAAO (Fig. 5A). This results in an ‘improved’ enzyme that induces remarkably increased cytotoxic effects on mouse tumor cells (see Fig. 5): this new DAAO variant is expected to lead to a suitable tool for a cancer treatment that exploits the production of $H_2O_2$.

**Experimental procedures**

**Protein engineering**

The pT7-HisDAAO wild-type and pT7-HisDAAO–Q144R plasmids were used as templates, and the whole cDNA sequence encoding DAAO was chosen as the target of mutagenesis by error–prone PCR [16]. A library of DAAO mutants was then generated in BL21(DE3)pLysS E. coli cells [16]. For the identification of DAAO mutants with increased enzymatic activity at low $O_2$ concentrations, the following screening procedure was implemented. Three hundred microliter volumes of recombinant E. coli cultures were grown, starting from a single colony. Protein expression was induced with 1 mM isopropyl thio-$\beta$-d-galactoside and, after 2 h, the oxidase activity was assayed on crude extracts following cell lysis (100 $\mu$L of lysis buffer: 50 mM sodium pyrophosphate, pH 8.5, 100 mM sodium chloride, 1 mM EDTA, 40 $\mu$g mL$^{-1}$ lysozyme, and 1 $\mu$g mL$^{-1}$ DNase I). The activity was assayed by addition of 100 $\mu$L of 90 mM d-Ala, 0.3 mg mL$^{-1}$ $o$-dianisidine and 1 unit of horseradish peroxidase in 100 mM sodium pyrophosphate (pH 8.5) and 2.5% (30 $\mu$L) $O_2$ using the AtmosBag incubation system (Sigma-Aldrich, Milano, Italy). After 6 h at 25 $^\circ$C, the reaction was stopped by the addition of 100 $\mu$L of 10% trichloroacetic acid, and the absorbance at 440 nm was recorded using a microtiter plate.

**Protein purification**

The pT7-HisDAAO recombinant plasmids coding for yeast DAAO variants selected from the screening procedure were directly transferred to BL21(DE3)pLysS E. coli cells. These were grown overnight at 37 $^\circ$C in LB medium containing 100 $\mu$g mL$^{-1}$ ampicillin and 34 $\mu$g mL$^{-1}$ chloramphenicol and induced at saturation by adding 1 mM isopropyl thio-$\beta$-d-galactoside; the cells were cultivated at 30 $^\circ$C for 5 h and then collected by centrifugation (10 000 g for 10 min). Crude extracts were prepared by French press treatment, and the DAAO mutants were purified as previously reported for wild-type, His-tagged DAAO [32]: $\sim$ 3.2 ± 0.5 mg of pure enzyme per liter of fermentation broth was obtained.
enzymes was determined using the known turnover technique was used to assess steady-state kinetic (BioLogic, Grenoble France) equipped with a J&M diode rated enzyme with an air-saturated solution of (pH 8.5), containing 1% (v/v) sodium pyrophosphate (BioLogic, Grenoble France) and was treated as records of the reaction of O2 (the limiting substrate). These traces were analyzed according to Gibson et al. [22]: the area covered by the experimental curve is proportional to the concentration of O2. The trace is divided into segments along the time axis; for each segment, a velocity is calculated at the corresponding concentration of the remaining limiting substrate, and these values are used to build the e/v versus 1/[O2] Lineweaver–Burk, double-reciprocal plot. The concentration of d-Ala (at least five concentrations were used) was varied over a range so as to obtain sufficient information about $k_m$ and $k_{cat}$ values. Steady-state kinetic parameters were then determined from secondary plots reporting the $x$-intercept and the $y$-intercept from the primary plot versus [d-Ala] or [O2]. For reductive half-reaction experiments, the stopped-flow instrument was made anaerobic by overnight incubation with a sodium dithionite solution followed by rinsing with argon-equilibrated buffer: the oxidized DAAO was reacted with increasing d-Ala concentrations in the absence of O2. For anaerobic experiments, the final solutions contained 100 mM glucose, 0.1 mM glucose oxidase, and 30 mM catalase; anaerobiosis was obtained by repeated cycles of evacuation and flushing with O2-free argon. For the study of the oxidation of reduced enzyme, two different enzyme forms were used: (a) the free reduced DAAO (E-Flred), which was generated by reacting oxidized DAAO with a four-fold excess of d-Ala; and (b) the reduced DAAO~P complex (E-Flred~P), which was generated analogously, but in the presence of 400 mM NH4Cl and 20 mM pyruvate to generate iminopyruvate (see Scheme 1a). These species were then reacted with solutions of appropriate O2 concentration. Reaction rates for both the reductive and the oxidative half-reactions (Scheme 1) were estimated from traces extracted at specific wavelengths where absorbance changes are optimal for data evaluation (e.g. 455 nm and 530 nm) and by fitting using the application BIKINE32 (BioLogic) and one to three exponential terms (for example, for a biexponential fit: $y = A \cdot e^{-kt} + B \cdot e^{-kt} + C$, where $A$ and $B$ are amplitudes, and $C$ is an initial value). Fits of the reductive half-reaction traces obtained using three exponents did, in some instances, yield marginally better results, in that the step corresponding to flavin reduction ($k_2$ in Scheme 1) is not strictly monophasic. Such a bias for a biphasic behavior of $k_2$ has been observed and discussed previously by others [24,33] for DAAOs from different sources and also for sarcosine oxidase [34]. As the different modes of analysis would not affect kinetic conclusions pertinent to the present case, they are not discussed here. The global analysis of the absorption spectra obtained for the reductive half-reaction was carried out using the application SPECTIT/32 (Spectrum Software Associates, Chapel Hill, NC, USA). This allows the estimation of the spectra of intermediates, of rate constants, and of the concentration of intermediates as a function of time. The same program was used to simulate kinetic processes [35]. Of relevance for the present case, the estimation of the lower limits of the rates of steps $k_1$ and $k_{-1}$ was performed in two steps. First, the values of $k_1$ and $k_{-1}$ were assumed to be large in comparison with those of all subsequent steps (see Scheme 1, below), and the simulation was optimized by variation of the latter. Then, these steps were held fixed, and the values of $k_1$ and $k_{-1}$ were lowered in successive increments. The minimal values are taken as the rates of $k_1$ and $k_{-1}$ at the point where they just do not lower the quality of the simulation.

In vitro cytotoxicity assay

The cytotoxicity of DAAO was assessed by the thiazolyl blue tetrazolium bromide assay [36] on mouse CT26 (colon carcinoma), 4T1 (mammary gland), N2C (mammary gland) and TSA (mammary adenocarcinoma) and on human U87 (glioblastoma) cancer cell lines, as well as on monkey COS-7 (kidney) fibroblasts and human embryonic HEK293 (kidney) cells as control. Cells plated in 96-well culture plates at a density of 3000 cells per well were cultured overnight at 37 °C in a 5% CO2 incubator in DMEM (Euroclone, Pero, Italy) supplemented with 10% fetal bovine serum, 4.5 g L−1 glucose, 1 mM L-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin, and then exposed to increasing concentrations of DAAO and d-Ala for 24 h. Following the removal of the growth medium,
100 μL of 0.5 mg·mL⁻¹ thiazolyl blue tetrazolium bromide was added; after 4 h at 37 °C, the liquid was removed, 100 μL of dimethylsulfoxide was added, and the absorbance at 600 nm was recorded. The value measured for the control (i.e. cells incubated similarly but without DAAO and/or d-Ala addition) was taken as 100% of survival. Toxicity was quantified as the fraction of surviving cells relative to the untreated cells as control.

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