Research Article

Inhibition of Recombinant D-Amino Acid Oxidase from *Trigonopsis variabilis* by Salts

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Inhibition of recombinant D-amino acid oxidase from *Trigonopsis variabilis* (*Tv*DAAO) activity in the presence of different sodium salts and potassium chloride is reported. A competitive inhibition pattern by sodium chloride was observed, and an inhibition constant value of $K_i = 85$ mM was calculated. Direct connection of NaCl inhibition with FAD cofactor dissociation was confirmed by measuring the fluorescence of tryptophanyl residues of the holoenzyme.

1. Introduction

D-amino acid oxidase (EC 1.4.3.3, DAAO) is a flavoprotein that catalyzes the enantioselective oxidative deamination of D-amino acids to yield the corresponding α-imino acids, which are spontaneously hydrolyzed to α-oxoacids and ammonia. Reoxidation of the reduced FAD by molecular oxygen is accompanied by the release of hydrogen peroxide [1, 2]. DAAO is almost ubiquitous in eukaryotic organisms and fulfills different physiological functions: from a catabolic role in yeasts, which allows the use of D-amino acids as carbon and energy sources, to a regulatory role in the human brain, where it controls the levels of the neuromodulator D-serine [3, 4].

In recent years, a major biotechnological application of DAAOs has emerged for the industrial production of 7-aminocephalosporanic acid (7-ACA), a key starting material for the preparation of semisynthetic cephalosporin antibiotics [5]. D-amino acid oxidases can catalyze the conversion of cephalosporin C to glutaryl-7-aminocephalosporanic acid (glutaryl-7-ACA), the first intermediate in the two step route that leads to 7-ACA. In the second step of the process, glutaryl-7-ACA is hydrolyzed to 7-ACA by a glutaryl-7-ACA acylase, an enzyme from the group of penicillin amidohydrolases (EC 3.5.1.14) [6]. DAAOs from several microbial sources have been demonstrated to work efficiently in the oxidative deamination of cephalosporin C [7–10] whereas DAAO from pig kidney (*pk*DAAO) has been considered a poor catalyst in the same reaction [10]. In fact, immobilized *Trigonopsis variabilis* cells with D-amino acid oxidase activity are actually the most employed biocatalyst for 7-ACA production in industry [11]. Cloning of the DAAO gene of *T. variabilis* in different hosts, such as *Escherichia coli* [12–16], *Saccharomyces cerevisiae*, *Kluyveromyces lactis* [17], *Schizosaccharomyces pombe* [18], and *Pichia pastoris* [19, 20], has allowed obtaining the recombinant enzyme for biotechnological applications. In many cases, overproduction of hexahistidine-tagged *Tv*DAAO has been successfully achieved in order to obtain an engineered form of the protein that could facilitate its downstream processing in *E. coli* [12–16] and *P. pastoris* [20]. As reported previously, a chimeric *Tv*DAAO containing a hexahistidine tag at the N-terminal end was largely expressed as apoenzyme by a recombinant *E. coli* strain [12], and the pure apoprotein could be purified in a single step by using metal-chelate affinity chromatography. The holoenzyme could be reconstituted from the recombinant apoenzyme by addition of exogenous FAD to yield a fully active enzyme. The dissociation constant (K_d) for FAD of this *Tv*DAAO, which was quite similar of that reported for *pk*DAAO, is 10-fold larger than that for

Rhodotorula gracilis DAAO [21]. Thus, the weak noncovalent binding of FAD to the recombinant enzyme might explain the observation that the flavin cofactor is lost upon dilution or prolonged dialysis [22]. In the present paper, we report experiments demonstrating that recombinant *Tv*DAAO activity was inhibited by different salts and that the direct inhibition by NaCl is due to FAD dissociation as confirmed by measuring the fluorescence of tryptophanyl residues of the holoenzyme.

2. Materials and Methods

2.1. Chemicals. D-alanine, 2,4-dinitrophenyl-hydrazine (DNPH), EDTA, FAD, imidazolem, and 2-mercaptoethanol were supplied by Sigma. Potassium phosphate, potassium hydroxide, and glycerol were purchased from Scharlab (Barcelona, Spain).

2.2. Enzyme Purification. His-tagged D-amino acid oxidase from *Trigonopsis variabilis* (*Tv*DAAO) was produced and purified as previously described in [12]. Recombinant *Tv*DAAO was largely expressed as apoenzyme (about 90%), and further enzyme purification and dyalisis against the appropriate buffer lead to a 100% pure apoenzyme preparation. This apoenzyme solution was further dialyzed against 20 mM potassium phosphate buffer pH 8.0, 20% glycerol, 5 mM EDTA, and 5 mM β-mercaptoethanol at 4°C and, then, centrifuged at $11,600 \times g$ for 5 minutes. An excess of exogenous cofactor FAD (5-fold enzyme concentration) was added to apoenzyme solution to obtain the holoenzyme solution.

2.3. Protein Determination. Apoenzyme concentration was determined spectrophotrically using a molar absorption coefficient at 276 nm of $\varepsilon_{276} = 70.5 \, mM^{-1} \cdot cm^{-1}$ [21].

2.4. Enzyme Activity Assay. The standard method measured the release of α-ketoacid from D-alanine during the reaction by the formation of the corresponding hydrazone from DNPH, which could be monitored at 450 nm [23]. The reaction mixture contained 80 μl of 100 μM FAD dissolved in distilled water and 100 μl of 100 mM D-alanine dissolved in 100 mM potassium phosphate buffer pH 8.0. The mixture was preincubated for 5 minutes at 30°C, and the reaction was started by adding 20 μl of enzyme solution. After 10 minutes, the reaction was stopped with 20 μl of a saturated solution (approximately 10 mM) of DNPH in 1N HCl. After 15 minutes of incubation with DNPH, the colour was developed by the addition of 180 μl of 2N NaOH. Pyruvic acid formation proceeded linearly during the 10-minute reaction period. The activity was calculated by spectrophotometric absorption measurement at 450 nm, using a pyruvic acid calibration curve. All samples were measured three times and the standard error was always below 5%. One activity unit (U) was defined as the amount of enzyme producing 1 μmol of pyruvic acid per minute under the conditions mentioned.
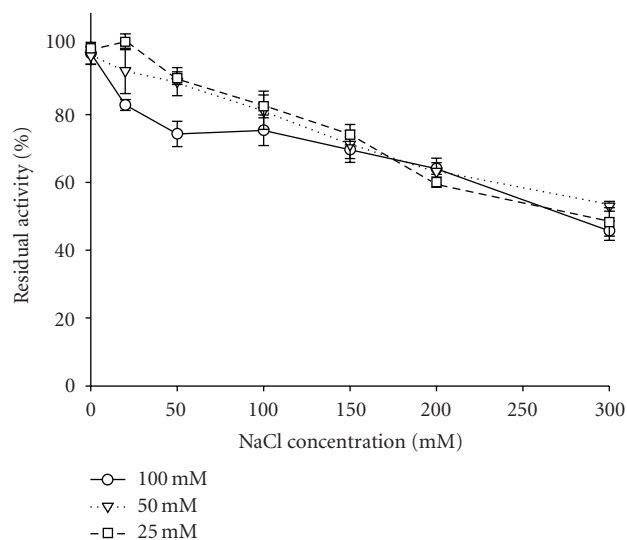

Figure 1: Effect of sodium chloride concentration on recombinant His-tagged *Tv*DAAO activity. Reactions were performed at 25 mM (□), 50 mM (∇), and 100 mM (○) potassium phosphate buffer pH 8.0 at 30°C under the standard assay conditions (see Materials and methods).

2.5. Effect of Inhibitors. Enzyme inhibition by different salts such as NaCl and KCl was tested. The activity was assayed at different concentrations of the inhibitor. The activity assay was performed under standard enzyme assay conditions as mentioned above, at a buffer concentration of 50 mM.

2.6. Intrinsic Fluorescence Measurements. Intrinsic fluorescence measurements were performed in an SLM Aminco 8000C spectrofluorimeter (Spectronic Instruments, USA) equipped with a thermostated cell holder using a 2 mL cell at 25°C. Emission spectra were recorded using an excitation wavelength of 295 nm (tryptophan emission). Excitation and emission bandwidths were set at 5 nm and 6 nm, respectively. Scattering was minimized by crossed Glan-Thompson polarizers.

3. Results and Discussion

3.1. Inhibition of Enzyme Activity by Salts. We studied the influence of the presence of different salts on recombinant *Tv*DAAO activity. As shown in Figure 1, we observed a significant decrease of enzyme activity when NaCl concentration was increased in the reaction medium. At a concentration of 300 mM NaCl, the enzyme only displayed 50% of the original activity. The influence of buffer concentration on enzyme inhibition was ignored as no differences in activity were observed. The phenomenon of flavoenzyme inhibition by salts has been described in the literature [24–26]. As reported, enzyme inhibition by high salt concentrations often occurs due to the abundance of negatively charged ions, which lead to conformational perturbations preventing holoprotein formation. In fact, flavoproteins that bind their cofactor rather weakly can be deflavinated using bromide
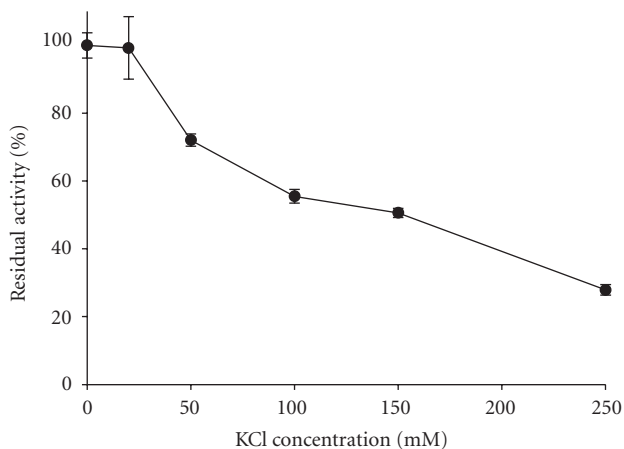
FIGURE 2: Effect of potassium chloride concentration on recombinant His-tagged *Tv*DAAO activity. Reactions were performed at different salt concentrations in 50 mM potassium phosphate buffer pH 8.0 at 30°C.
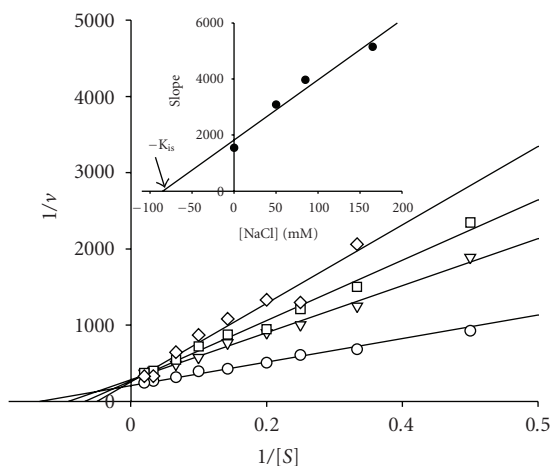

FIGURE 3: Competitive inhibition of recombinant His-tagged *Tv*DAAO by sodium chloride. Reactions were performed in 100 mM potassium phosphate buffer pH 8.0 at 30°C. Sodium chloride concentrations: Nil (○), 50 mM (▽), 85 mM (□), and 165 mM (◇).
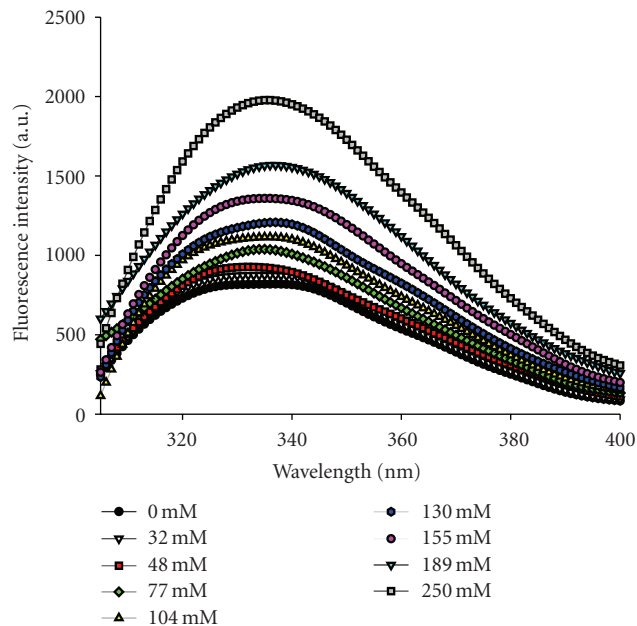

FIGURE 4: Fluorescence emission spectra of recombinant His-tagged *Tv*DAAO at different sodium chloride concentrations. Intrinsic fluorescence spectra of holoenzyme were recorded at 25°C after excitation at 295 nm.

ions at high concentration [27–32]. Chloride has been reported to be less chaotropic and therefore less effective in removal of flavin [33]. In our case, reconstituted *Tv*DAAO may be deflavinated in the presence of increasing concentrations of NaCl. As mentioned above, chloride negative charges could interfere with the weak interactions between cofactor and enzyme, favouring FAD dissociation. The same deactivation was observed when KCl concentration was increased in the reaction medium (Figure 2). Apart from halide ions, also other negative charged groups were described to induce FAD dissociation, such as cyanate or cyanide [24].

3.2. Inhibition by Sodium Chloride.
An extensive study of the inhibition effect of recombinant *Tv*DAAO activity by NaCl

was carried out in order to know the inhibition type and the inhibition constant. The enzyme activity was measured with different concentrations of D-alanine in the absence and presence of several concentrations of sodium chloride under the same standard assay conditions, at 50 mM potassium phosphate buffer pH 8.0. As shown in Figure 3, the inhibition exerted by NaCl was competitive, indicating that the chloride anion diminishes the affinity of the enzyme for the substrate, probably due to conformational perturbation of the active site, which could be related to the binding of the negative ion to some residues. Nevertheless, such hypothesis should be checked when the *Tv*DAAO three-dimensional structure will be available. The replot of the slope versus inhibitor concentration fitted to a straight line whose intercept in the x-axis indicated an inhibition constant value of $K_i = 85$ mM.

3.3. Effect of Chloride Anion on Tryptophanyl Fluorescence of Recombinant TvDAAO.
In order to check possible FAD dissociation from the holoenzyme due to NaCl, fluorescence emission spectra of reconstituted *Tv*DAAO in the presence of different NaCl concentrations were measured with excitation at 295 nm (Figure 4). Excitation at this wavelength selectively allows measurements of the contribution of tryptophan residues to the emission spectra of the protein. Fluorescence intensity was increased up to threefold when increasing concentrations of NaCl were added to the enzyme solution. In addition, a small red shift from 338 nm to 340 nm was observed, indicating that the tryptophan residues were in a slightly more hydrophilic environment. Such an effect may be related to the loss of cofactor FAD and the corresponding exposure of tryptophan residues, which were quenched by

the cofactor prior to NaCl addition. As a matter of fact, *Tv*DAAO apoenzyme, which contains no FAD, shows an emission maximum at 340 nm with intensity 3-fold higher than that of the holoenzyme, whose maximum is at 338 nm [21]. The decreased fluorescence intensity of holoenzyme can be explained by a conformational transition upon FAD binding or subunit association that would affect the local environment surrounding the indole ring of tryptophan residues. FAD dissociation from the holoenzyme in the presence of halide anions such as iodide, bromide, or chloride has also been reported for *pk*DAAO [28], in which tryptophanyl fluorescence intensity was stronger than that of apoenzyme [27]. In our case, a significant increase of the intrinsic fluorescence intensity can be observed at salt concentrations higher than 77 mM. Above this concentration the red shift was also clearly observed. This result is quite similar to the inhibition constant obtained in the kinetic experiments, strongly indicating that competitive inhibition by NaCl could be related to FAD dissociation. As flavin cofactor binding is essential for catalytic activity, FAD dissociation induced by chloride anions would lead to enzyme inactivation. The same effect has been described for other flavoproteins [24].

4. Conclusions

Recombinant *Tv*DAAO activity is inhibited by the presence of high salt concentrations in the reaction media. Sodium chloride behaved as a competitive inhibitor, leading to conformational perturbations in the active site that might induce FAD dissociation.

Acknowledgments

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