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* (*TvDAAO*) 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 $K_i = 85 \text{ mM}$ 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 $\alpha$-imino acids, which are spontaneously hydrolyzed to $\alpha$-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 (*pkDAAO*) 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 *TvDAAO* 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 *TvDAAO* 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 *TvDAAO*, which was quite similar of that reported for *pkDAAO*, 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 TvDAAO 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 (TvDAAO) was produced and purified as previously described in [12]. Recombinant TvDAAO was largely expressed as apoenzyme (about 90%), and further enzyme purification and dialysis 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 × 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 spectrophotometrically using a molar absorption coefficient at 276 nm of \( \varepsilon_{276} = 70.5 \text{ M}^{-1}\text{cm}^{-1} \) [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 prepared 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.

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 TvDAAO 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.
3.2. Inhibition by Sodium Chloride. An extensive study of the inhibition effect of recombinant TvDAAO activity by NaCl 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 TvDAAO 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, other negative charged groups were described to induce FAD dissociation, such as cyanate or cyanide [24].

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 TvDAAO 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, TvDAO 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 pkDAO [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 TvDAO 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.

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References

[1] M. S. Pilone, “D-amino acid oxidase: new findings,” Cellular and Molecular Life Sciences, vol. 57, no. 12, pp. 1732–1747, 2000.
[2] V. I. Tishkov and S. V. Khoronenkova, “D-amino acid oxidase: structure, catalytic mechanism, and practical application,” Biochemistry, vol. 70, no. 1, pp. 44–54, 2005.
[3] L. Pollegioni, L. Piubelli, S. Sacchi, M. S. Pilone, and G. Molla, “Physiological functions of D-amino acid oxidases: from yeast to humans,” Cellular and Molecular Life Sciences, vol. 64, no. 11, pp. 1373–1394, 2007.
[4] S. V. Khoronenkova and V. I. Tishkov, “D-amino acid oxidase: physiological role and applications,” Biochemistry, vol. 73, no. 13, pp. 1511–1518, 2008.
[5] M. S. Pilone and L. Pollegioni, “D-amino acid oxidase as an industrial biocatalyst,” Biocatalysis and Biotransformation, vol. 20, no. 3, pp. 145–159, 2002.
[6] H. D. Conlon, I. Baqai, K. Baker et al., “Two-step immobilized enzyme conversion of cephalosporin C to 7-aminocephalosporanic acid,” Biotechnology and Bioengineering, vol. 46, no. 6, pp. 510–513, 1995.
[7] T. Isogai, H. Ono, Y. Ishitani, H. Kojo, Y. Ueda, and M. Kohsaka, “Structure and expression of cDNA for D-amino acid oxidase active against cephalosporin C from Fusarium solani,” Journal of Biochemistry, vol. 108, no. 6, pp. 1063–1069, 1990.
[8] E. Szwajcer-Dey, J. R. Miller, S. Kovacevic, and K. Mosbach, “Characterization of a D-amino acid oxidase with high activity against cephalosporin C from the yeast Trigonopsis variabilis,” Biochemistry International, vol. 20, no. 6, pp. 1169–1178, 1990.
[9] M. S. Pilone, S. Buto, and L. Pollegioni, “A process for biocconversion of cephalosporin C by Rhodotorula gracilis D-amino acid oxidase,” Biotechnology Letters, vol. 17, no. 2, pp. 199–204, 1995.
[10] L. Pollegioni, L. Caldinelli, G. Molla, S. Sacchi, and M. S. Pilone, “Catalytic properties of D-amino acid oxidase in cephalosporin C biocconversion: a comparison between proteins from different sources,” Biotechnology Progress, vol. 20, no. 2, pp. 467–473, 2004.
[11] W. Kiethors and A. Reichert, “An industrial view on enzymes for the cleavage of cephalosporin C,” Chimia, vol. 53, no. 12, pp. 600–607, 1999.
[12] J. Alonso, J. L. Barredo, P. Armisén et al., “Engineering the D-amino acid oxidase from Trigonopsis variabilis to facilitate its overproduction in Escherichia coli and its downstream processing by tailor-made metal chelate supports,” Enzyme and Microbial Technology, vol. 25, no. 1–2, pp. 88–95, 1999.
[13] T. S. Hwang, H. M. Fu, L. L. Lin, and W. H. Hsu, “High-level expression of Trigonopsis variabilis D-amino acid oxidase in Escherichia coli using lactose as inducer,” Biotechnology Letters, vol. 22, no. 8, pp. 655–658, 2000.
[14] L. L. Lin, H. R. Chien, W. C. Wang, T. S. Hwang, H. M. Fu, and W. H. Hsu, “Expression of Trigonopsis variabilis D-amino acid oxidase gene in Escherichia coli and characterization of its inactive mutants,” Enzyme and Microbial Technology, vol. 27, no. 7, pp. 482–491, 2000.
[15] H. Luo, H. Yu, Q. Li, and Z. Shen, “Cloning and co-expression of D-amino acid oxidase and glutaryl-7-aminocephalosporanic acid acylase genes in Escherichia coli,” Enzyme and Microbial Technology, vol. 35, no. 6–7, pp. 514–518, 2004.
[16] X. F. Ma, H. M. Yu, C. Wen, H. Luo, Q. Li, and Z. Y. Shen, “Triple fusion of D-amino acid oxidase from Trigonopsis variabilis with polyhistidine and Vitreoscilla hemoglobin,” World Journal of Microbiology and Biotechnology, vol. 25, no. 8, pp. 1353–1361, 2009.
[17] F. J. González, J. Montes, F. Martín et al., “Molecular cloning of TvDAO1, a gene encoding a D-amino acid oxidase from Trigonopsis variabilis and its expression in Saccharomyces cerevisiae and Kluyveromyces lactis,” Yeast, vol. 13, no. 15, pp. 1399–1408, 1997.
[18] A. Isoai, H. Kimura, A. Reichert et al., “Production of D-amino acid oxidase (DAO) of Trigonopsis variabilis in Schizosaccharomyces pombe and the characterization of bio-catalysts prepared with recombinant cells,” Biotechnology and Bioengineering, vol. 80, no. 1, pp. 22–32, 2002.
[19] J. Yu, D. Y. Li, Y. J. Zhang, S. Yang, R. B. Li, and Z. Y. Yuan, “High expression of Trigonopsis variabilis D-amino acid oxidase in Pichia pastoris,” Journal of Molecular Catalysis B, vol. 18, no. 4–6, pp. 291–297, 2002.
[20] H. Zheng, X. Wang, J. Chen et al., “Expression, purification, and immobilization of His-tagged D-amino acid oxidase of Trigonopsis variabilis in Pichia pastoris,” Applied Microbiology and Biotechnology, vol. 70, no. 6, pp. 683–689, 2006.
[21] M. Arroyo, M. Menéndez, J. L. García et al., “The role of cofactor binding in tryptophan accessibility and conformational stability of His-tagged D-amino acid oxidase from Trigonopsis variabilis,” *Biochimica et Biophysica Acta*, vol. 1774, no. 5, pp. 556–565, 2007.

[22] M. Arroyo, I. de la Mata, M. P. Castillón, C. Acebal, J. L. Garcia, and M. Menéndez, “Inactivation mechanisms of His-tagged D-amino acid oxidase from Trigonopsis variabilis,” in *Modern Multidisciplinary Applied Microbiology*, A. Mendez-Vilas, Ed., pp. 227–231, Wiley-VCH, Weinheim, Germany, 2006.

[23] P. Brodelius, K. Nilsson, and K. Mosbach, “Production of α-keto acids Part I. Immobilized cells of Trigonopsis variabilis containing D-amino acid oxidase,” *Applied Biochemistry and Biotechnology*, vol. 6, no. 4, pp. 293–307, 1981.

[24] H. Y. Neujahr, “Effect of anions, chaotropes, and phenol on the attachment of flavin adenine dinucleotide to phenol hydroxylase,” *Biochemistry*, vol. 22, no. 3, pp. 580–584, 1983.

[25] L. Pollegioni, S. Iametti, D. Fessas et al., “Contribution of the dimeric state to the thermal stability of the flavoprotein D-amino acid oxidase,” *Protein Science*, vol. 12, no. 5, pp. 1018–1029, 2003.

[26] M. H. Hefiti, J. Vervoort, and W. J. H. van Berkel, “Deflavination and reconstitution of flavoproteins: tackling fold and function,” *European Journal of Biochemistry*, vol. 270, no. 21, pp. 4227–4242, 2003.

[27] V. Massey and B. Curti, “A new method of preparation of D-amino acid oxidase apoprotein and a conformational change after its combination with flavin adenine dinucleotide,” *Journal of Biological Chemistry*, vol. 241, no. 14, pp. 3417–3423, 1966.

[28] Y. Nishina, K. Horiike, K. Shiga, Y. Miyake, and T. Yamano, “Effect of halide anions on the binding of FAD to D amino acid oxidase and the tryptophanyl fluorescence of the apoenzyme,” *Journal of Biochemistry*, vol. 81, no. 5, pp. 1455–1463, 1977.

[29] C. C. Ryerson, D. P. Ballou, and C. Walsh, “Mechanistic studies on cyclohexanone oxidase,” *Biochemistry*, vol. 21, no. 11, pp. 2644–2655, 1982.

[30] P. Casalin, L. Pollegioni, B. Curti, and M. Pilone Simonetta, “A study on apoenzyme from Rhodotorula gracilis D-amino acid oxidase,” *European Journal of Biochemistry*, vol. 197, no. 2, pp. 513–517, 1991.

[31] V. Radjendirane, M. A. Bhat, and C. S. Vaidyanathan, “Affinity purification and characterization of 2,4-dichlorophenol hydroxylase from Pseudomonas cepacia,” *Archives of Biochemistry and Biophysics*, vol. 288, no. 1, pp. 169–176, 1991.

[32] L. Pollegioni and M. S. Pilone, “On the holoenzyme reconstitution process in native and truncated Rhodotorula gracilis D-amino acid oxidase,” *Archives of Biochemistry and Biophysics*, vol. 332, no. 1, pp. 58–62, 1996.

[33] S. C. Tu and D. B. McCormick, “Conformation of porcine D-amino acid oxidase as studied by protein fluorescence and optical rotatory dispersion,” *Biochemistry*, vol. 13, no. 5, pp. 893–899, 1974.