Reactivity of histidyl residues in D-amino acid oxidase from *Rhodotorula gracilis*

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Abstract Incubation of D-amino acid oxidase from the yeast *Rhodotorula gracilis* with excess dansyl chloride at pH 6.6 and 18°C caused an irreversible inactivation of D-amino acid oxidase. Benzoate, a competitive inhibitor of the enzyme, completely protected the enzyme from inactivation. The dansylated-enzyme, isolated by gel-filtration, was in part still active while the substrate specificity was altered substantially. It was completely reduced by D-alanine in anaerobiosic conditions and did stabilize the red anion semiquinone upon photochemical reduction with EDTA. The results provide evidence for the presence of essential histidyl residue(s) in the active center of the yeast enzyme.

Key words: D-Amino acid oxidase; Histidine; Covalent modification; Dansyl chloride; Methyl-*p*-nitrobenzenesulfonate; *Rhodotorula gracilis*

1. Introduction

D-Amino acid oxidase (EC 1.4.3.3) is a flavoprotein that catalyses the oxidative deamination of D-amino acids giving the corresponding α-keto acids, ammonia and hydrogen peroxide. In recent years DAAO from the yeast *Rhodotorula gracilis*, the first DAAO to be purified from a microorganism, has been extensively characterised in its kinetic and functional aspects [1–5], and also the complete amino acid sequence of the enzyme has been elucidated ([6]; Faotto, L., Pollegioni, L., Ceciliani, F., Ronchi, S. and Pilone, M.S., manuscript submitted). The yeast enzyme shows relevant characteristics (e.g. a quite high turnover [4], a rate-limiting step in the reductive half-reaction [5], and a stable dimeric aggregation state [2]), which render it an enzyme of utmost interest in basic research and biotechnology [7].

Since the three-dimensional structure of the enzyme is not available, investigation of the active site topography has been performed using specific chemical modifiers. Lately, arginine and lysine residues have been identified at or near the active site of the enzyme. Reaction with phenylglyoxal results in the modification of arginine residues; that number is decreased by one if the reaction takes place in the presence of the competitive inhibitor benzoate [8]. This residue has been identified in the primary structure of the yeast enzyme as a highly conserved arginine (Arg-285) which could act either as the positively charged residue exerting an inductive effect on the N(1)–C(2) = O flavin locus [9] or as a basic residue involved in binding the carboxylate moiety of the substrate. The presence of one or more essential lysyl residue(s) has been inferred by the use of trinitrobenzensulfonic acid [10]. From a comparison of the amino acid sequence of the *R. gracilis* enzyme with that of known DAAOs ([11]; Faotto et al., manuscript submitted), only Tyr-223 and His-329 (corresponding to Tyr-228 and His-307 of pig kidney DAAO, respectively) besides Arg-285 are conserved out of the residues mapped in the mammalian enzyme by the chemical modification approach [12,13].

We report here on the presence of essential histidine residue(s) in the active center of *R. gracilis* DAAO as demonstrated by the data obtained with the dansyl-chloride modification.

2. Materials and methods

MNBS and DNS-Cl were purchased from Sigma. DAAO was purified following the method described in [14] from *R. gracilis* cells (ATCC 26217) grown in a synthetic medium at pH 5.6 containing 30 mM D-alanine [15]. The ratio A_{274}/A_{455} for the pure enzyme was 8.2, and the specific activity on D-alanine as substrate was 180 μmol $O_2 \times min^{-1} \times mg\ protein^{-1}$ at 37°C; the enzyme concentration was determined spectrophotometrically from the flavin content using A_{455} = 12,600 $M^{-1} \cdot cm^{-1}$ [2].

Fresh stock solutions of MNBS in 100% acetone were prepared for daily experiments. DAAO (3.1 μM) was incubated with MNBS (1.4–5.9 mM) in 0.1 M phosphate buffer, pH 7.4, 10% glycerol at 25°C in the dark. At different times, aliquots were withdrawn and assayed spectrophotometrically for residual enzymatic activity using D-phenylglycine as substrate at 25°C.

Fresh stock solutions of DNS-Cl in 100% acetone were prepared for daily experiments. The reagent concentration was determined in acetone by absorption at 369 nm using an extinction coefficient of 3,690 $M^{-1} \cdot cm^{-1}$ [16]. DAAO (3.0–15.0 μM) was incubated with 1.5 mM DNS-Cl in 50 mM phosphate buffer, pH 6.6, 10% glycerol at 25°C or 18°C in the dark. The final acetone concentration in the incubation mixture was 3% (v/v) and did not affect enzyme activity and stability. At different times, aliquots were withdrawn and assayed spectrophotometrically as above. To assess the effect of benzoate on the inactivation, the enzyme was incubated for 5 min with the compound before the inactivation reaction was started. Reversibility of the inactivation by DNS-Cl was studied by Sephadex G-25 chromatography (PD-10 column; Pharmacia).

The dansylated enzyme was prepared, removing excess reagent by gel-filtration on a Sephadex G-25 column. Stability of the dansylated enzyme was studied by incubating the modified enzyme in 50 mM phosphate buffer, pH 6.6, and 10% glycerol at different temperatures. The reversibility of inactivation in the presence of 5 mM 2-mercaptoethanol or 5.5 mM hydroxylamine was performed under the same conditions. Determination of kinetic parameters of the dansylated enzyme was performed polarographically at 30°C using different D-amino acids as substrates [1]. The extent of dansyl incorporation was determined by difference spectrophotometry, assuming a molar extinction coefficient equal to that of DNS-Cl free in solution. Absorption spectra were obtained at 10°C. For experiments involving reduction of the dansylated-modified enzyme, after gel-filtration the sample was placed in a 1-ml cell equipped with side arms and was made anaerobic by several cycles of evacuation and flushing with purified N_2. Photoreduction was performed in the presence of 5 mM EDTA.

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Abbreviations: DAAO, D-amino acid oxidase; DNS-Cl, dansyl chloride; MNBS, methyl-*p*-nitrobenzenesulfonate; FAD, flavin adenine dinucleotide.

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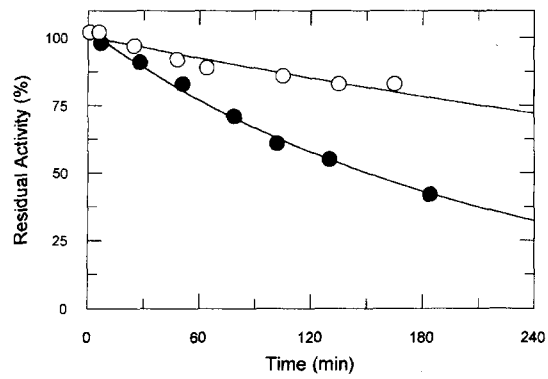

Fig. 1. Time-dependent inactivation of DAAO with DNS-Cl in the presence and absence of benzoate. 4.9 μM DAAO was incubated in the dark with 1.5 mM DNS-Cl in 50 mM phosphate buffer, pH 6.6, 10% glycerol at 25°C. (●) No benzoate; (○) 20 mM benzoate.

3. Results

MNBS is a strong methylating reagent previously used by Swenson and co-workers to study the active center of pig kidney DAAO, where His-217 was identified as an essential residue [17]. Treatment of *R. gracilis* DAAO with MNBS in phosphate buffer, pH 7.4, at 25°C did not lead to loss of enzyme activity after 2 h of incubation (data not shown). The same results were obtained under different experimental conditions (0.1 M phosphate buffer, pH 6.6, or 0.15 M borate buffer, pH 8.0, in the presence of 2 mM EDTA and 5 mM 2-mercaptoethanol).

Reaction of DAAO from *R. gracilis* with DNS-Cl at pH 6.6 is slow and leads to an irreversible inactivation of the enzyme. As shown in Fig. 1, the process is time-dependent and follows pseudo-first-order kinetics for at least two half-lives. Thereafter a non-linearity ensued, which was observed after 2 half-lives and was due to hydrolysis of DNS-Cl in water [16]; thus further addition of reagent restored the inactivation process. In the presence of 1.5 mM DNS-Cl at 25°C the rate of inactivation was 0.00475 min^{-1}. The second-order rate constant of inactivation was not determined, as the actual concentration of reagent in solution was unknown due to the low solubility of DNS-Cl in buffer solutions. DNS-Cl modification of DAAO was irreversible since activity was not restored after removing the excess reagent by gel-filtration. Benzoate fully protected the enzyme from inactivation; in the presence of the inhibitor the rate of activity loss was the same as in control experiments at 25 and 18°C (Fig. 1).

After 24 h of incubation at 18°C the dansylated enzyme was subjected to gel-filtration. Within a few minutes, a substantial fraction (about 30%) of the recovered protein denaturated, irrespectively of the temperature being 0°C. Thus, all experiments were carried out on freshly made preparations of modified enzyme, after removing the denaturated fraction of protein by centrifugation. When DAAO was reacted with DNS-Cl at 25°C, after 7 h of incubation about 50% (six experiments) of the total enzyme was recovered with a specific activity corresponding to 44–60% of that of native DAAO. The same inactivation was obtained after 24 h of incubation at 18°C. It should be noted that incubation times longer than the reported ones resulted in a lower protein recovery, and in no case was a lower specific activity of the reacted enzyme obtained. Control experiments, in which DAAO was kept at 18°C for 24 h in the absence of DNS-Cl, gave a recovery of enzyme of 82%. Thus, the low yield observed in the presence of the reagent was due to extensive dansyl incorporation, probably resulting in denaturation of the enzyme. The dansylated DAAO was stable and soluble when kept at 10°C (100% residual activity after 5 h), whereas a significant loss of activity was observed at 25°C ($t_{1/2}$ = 173 min). The dansylated DAAO retained coenzyme binding capacity since addition of exogenous FAD did not increase the specific activity. The difference spectrum of the DNS-modified enzyme and untreated DAAO showed an absorption maximum at 338 nm (data not shown). Using the molar extinction coefficient of free DNS-Cl [16], 1.3–2.9 mol dansyl incorporated per mol DAAO was calculated when incubation was carried out at 18°C, whereas a slightly higher incorporation was obtained at 25°C (3.2–4.1 mol dansyl per mol DAAO). On the basis of these results, all subsequent preparations were obtained at 18°C.

In order to verify the specificity of DNS-Cl for the histidine residue, the modified enzyme (60% specific activity) was incubated in the presence of 5 mM 2-mercaptoethanol in 50 mM phosphate buffer, pH 6.6, 10% glycerol at 10°C. Under these conditions, no time-dependent changes of the specific activity were observed, ruling out cysteine residues as a target of modification. The dansylated DAAO was also incubated at 10°C with hydroxylamine, which is known to displace weak nucleophilic dansylated residues [18]. Control experiments on the native enzyme showed that the yeast DAAO was extremely sensitive to this reagent: after 5 min at 37°C and neutral pH the enzymatic activity was halved in the presence of 0.6 M reagent. Activity loss as a consequence of incubation with hydroxylamine has been previously observed in the case of L-amino acid oxidase from *C. reinhardi* [19] and of *G. flabelliformis* [20]. Treatment of the dansylated DAAO (56% specific activity) with 5.5 mM hydroxylamine in 50 mM phosphate buffer, pH 6.6,

Table 1
Apparent kinetic parameters of dansylated DAAO with different substrates

Substrate	K_m DAAO[a] (mM)	V_{max} DAAO[a] (U/mgE)	K_m DNS-E[b] (mM)	V_{max} DNS-E[b] (U/mgE)	Specific activity[c] (%)
D-Alanine	0.83	140	0.80	10.3	48
D-Leucine	0.50	120	0.51	38.2	58
D-Tryptophan	0.30	150	0.23	38.3	56
D-Phenylalanine	0.29	144	n.d.	51.6	48

Measurements were performed by the polarographic assay method at 30°C and pH 8.5. [a]From [23]. [b]DNS-E = dansylated-enzyme. [c]Determined with D-phenylglycine as substrate by the spectrophotometric method at 25°C and pH 8.5.

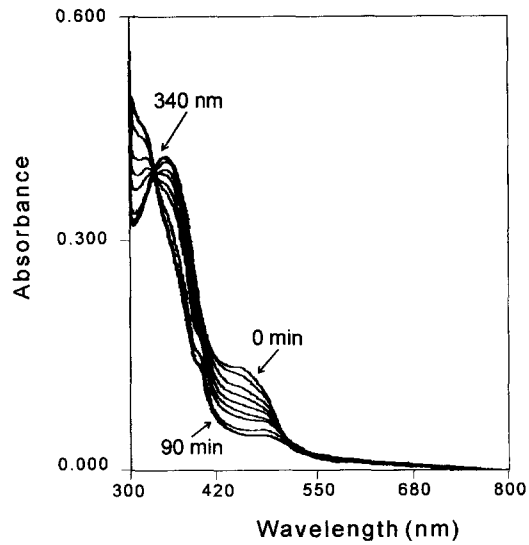

Fig. 2. Photoreduction of dansylated DAAO in the presence of EDTA. 9.44 μM DAAO was incubated in the presence of 5 mM EDTA in 0.1 M phosphate buffer, pH 6.6, 10% glycerol at 10°C. Selected spectra are shown: 0, 1, 3, 6, 12, 20, 30, 60 and 90 min. of irradiation.

10% glycerol at 15°C caused a time-dependent loss of enzymatic activity ($t_{1/2}$ = 275 min). In the same experimental conditions as reported above for the native enzyme, the rate of inactivation was three fold higher compared to that of native enzyme, suggesting that dansylation enhanced the reactivity of the enzyme with this reagent. However, the results do not allow the identification of the modified residues (a weak nucleophile such as histidine or tyrosine).

Isoelectrofocusing of a sample of dansylated enzyme (60% of specific activity) showed the presence of two active bands at pH 7.4 and 7.1, whereas the native enzyme showed the presence of only one active band at pH 7.4. Steady-state measurements of the apparent kinetic parameters showed no substantial changes of K_m for D-alanine, D-leucine and D-tryptophan as substrates (Table 1), whereas different V_{max} were obtained. It appears that substrate specificity is altered by the modification as was also the case upon modification of pig kidney DAAO with propargylglycine [12]. In particular, the highest activity was found with D-phenylalanine (36% that of the native DAAO), whereas D-alanine was a poor substrate (7% compared with native DAAO). Nevertheless, DNS-modified DAAO having 60% of the specific activity of native DAAO for D-alanine is completely reduced by the same substrate in the absence of oxygen and stabilizes the red anion semiquinone upon photochemical reduction in the presence of EDTA (Fig. 2). These observations suggest a functional integrity of the dansylated enzyme. The interaction with sulfite could not be studied because of interfering denaturation of DNS-DAAO. Benzoate binds and induces spectral perturbations qualitatively similar to those observed with native DAAO (Fig. 3) [2]. However, the extent of the perturbations appears to be minor and to correspond to ≅ 30% of those observed with native DAAO, suggesting either impaired binding or an altered $\Delta\varepsilon$.

4. Discussion

In previous studies with pig kidney DAAO, His-217 was tagged using MNBS as well as DNS-Cl [17,18,21] and His-307 using propargylglycine [13,22]. Lack of reactivity of the yeast DAAO vs. MNBS indicates a different topology of the active site of *R. gracilis* DAAO compared to pig kidney DAAO in accordance with previous deductions based on the use of modified flavins [23]. It is noteworthy that, by comparing the amino acid sequences of the two enzymes, His-217, which is tagged by MNBS and DNS-Cl in pig kidney DAAO, is not conserved in *R. gracilis* DAAO (Faotto et al., manuscript submitted). In the present case the complete protection against modification with DNS-Cl induced by benzoate binding suggests that the reaction occurs at or near the active site of DAAO. The high instability of the modified enzyme towards hydroxylamine hampers the conclusion that a histidine is the target of the reagent. Nevertheless, the result obtained with 2-mercaptoethanol speaks against a modification of a cysteine SH, and the low pH at which inactivation occurs argues against a lysil residue [16]. Based on similar reasoning, threonine and serine do not appear to be likely candidates [24]. Unfortunately the low recovery of DNS-modified protein did not allow the direct identification of the modified residue, e.g. by amino acid analysis or sequencing.

As in the case of modification of pig kidney DAAO with propargylglycine [12], modification of *R. gracilis* DAAO with DNS-Cl leads to only partial suppression of the activity, while the substrate specificity is altered substantially. This suggests that the residue in question is important for substrate recognition and specificity, but not essential for catalysis, and that is probably not the residue involved in the abstraction of the substrate α-hydrogen as a proton, the event assumed to initiate enzyme flavin reduction. Although the identification of the modified residue was not attempted in this study, from a comparison of the sequences of yeast and pig kidney DAAOs, His-329 (corresponding to His-307 of mammalian DAAO) appears to be the only one conserved (Faotto et al., manuscript

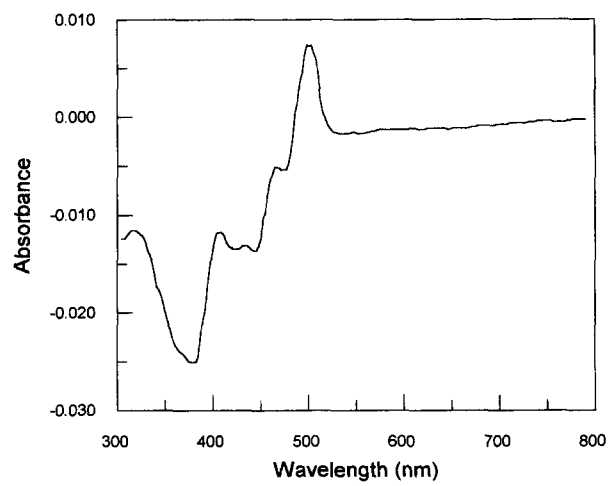

Fig. 3. Difference absorption spectrum between dansylated DAAO–benzoate complex and dansylated DAAO. Modified enzyme (60% specific activity) was prepared as follows. 14.73 nmol DAAO was incubated in the dark with 1.46 mM DNS-Cl in 1 ml of 0.1 M phosphate buffer, pH 6.6, 10% glycerol at 18°C. Reaction was stopped with gel-filtration. Spectra recorded at 10°C.

submitted). Studies are in progress to attempt the identification of the target of DNS-Cl modification.

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