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Chemical modification of tryptophan residues of D-amino acid oxidase from *Rhodotorula gracilis*

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Abstract

D-Amino acid oxidase was inactivated by *N*-bromosuccinimide (NBS) at 30°C and pH 8. The reaction followed pseudo-first order kinetics with second-order rate constants of 69.8 mM^{-1} min^{-1} for the apoenzyme and 0.63 mM^{-1} min^{-1} for the holoenzyme. The presence of substrates or benzoate protected the enzyme against inactivation. Difference absorption spectra at 280 nm, low consumption of NBS per mole of enzyme, the decrease in the fluorescence emission at 335 nm, integrity of the protein backbone and the absence of cysteine oxidation pointed to the modification of tryptophan residues. The statistical analysis of the residual fractional activity vs. the number of modified tryptophan residues led to the conclusion that one tryptophan residue is essential for the enzyme activity. This tryptophan residue was not involved in binding of FAD or dimerization of the enzyme. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: D-Amino acid oxidase; Enzyme modification; *Rhodotorula gracilis*; Tryptophan; Essential residues

1. Introduction

D-Amino acid oxidase (EC 1.4.3.3. DAAO) is an FAD-oxygen oxidoreductase that catalyzes the oxidative deamination of D-amino acids to yield the corresponding α-keto acid and ammonia [1]. In addition to the well-studied protein from mammalian kidney, related DAAOs have been described from several yeast [2,3]. DAAO isolated from *Rhodotorula gracilis* showed a very high catalytic efficiency [4] as compared with that purified from pig kidney [1]. This characteristic, together with a low dissociation constant for FAD render this DAAO an interesting enzyme for biotechnological work in the production of semisynthetic cephalosporins [5–7].

In spite of the number of biochemical, spectroscopic and kinetic studies [3,4,8,9], the mechanism of DAAO from *R. gracilis* has remained controversial.

Although 3D-structure of the pig kidney enzyme has been resolved [10], the crystal structure of DAAO from *R. gracilis* could not be obtained yet. Active site studies have shown the relevance of several amino acid residues in the reaction catalyzed by the yeast enzyme. To date, a histidine residue have been shown to be essen-

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tial for the reaction catalyzed for the enzyme [11], this histidine has been involved in the catalytic event of the reaction and not in binding of the substrate [12]. The critical role of His^{329} (307 in pig kidney enzyme) was demonstrated because replacement of His^{329} for Leu, by site-directed mutagenesis, caused complete loss of enzyme activity (unpublished data). Arg^{285} has been involved in binding to the carboxylate group of the substrate or in the interaction with the flavin N(1)–C(2)=O locus [13] and Cys^{208} in FAD binding [14]. Lysine residues have also shown as essential for the oxidation of D-amino acids catalyzed by the enzyme from *R. gracilis* [15].

The present paper reports, for the first time, the possible role of tryptophan residues in the active site of *Rhodotorula* enzyme, using the chemical modification approach by the use of *N*-bromosuccinimide (NBS). The results suggest that in this enzyme at least one tryptophan residue is essential for catalysis.

2. Experimental

2.1. Chemicals

D-Alanine, NBS, dithiobisnitrobenzoic acid (DTNB), FAD and 2,4-dinitrophenylhydrazine were from Sigma (St. Louis, MO, USA). Cephalosporin C was a gift from Antibióticos (León, Spain). All other reagents and solvents were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Enzyme purification

D-Amino acid oxidase was isolated and purified from *R. gracilis* (American Type Culture Collection, strain number 26217) cultures as previously described [11]. Apoenzyme was prepared by dialysis against potassium bromide, as described by Casalin et al. [16], using a strategy originally proposed for preparation of mammalian apo-D-amino acid oxidase [17].

2.3. Enzyme assay

The activity of D-amino acid oxidase was routinously assayed in incubation mixtures containing 7.6 nM enzyme, 10 mM D-alanine in 50 mM potassium phosphate buffer, pH 8, saturated with air, at 30°C for 10 min. The release of pyruvic acid was determined by reacting with 2,4-dinitrophenylhydrazine and the corresponding hydrazone was monitored at 450 nm. The product formation proceeded linearly with time for this period.

Reconstitution of D-amino acid oxidase from apoenzyme was carried out by incubation of protein (2.37 μM) with FAD (16.6 μM) in 50 mM potassium phosphate buffer, pH 7.5, during 5 min at 4°C. Activity of reconstituted holoenzyme was assayed in the same conditions as native DAAO.

2.4. Enzyme modification with NBS

A 0.152-μM D-amino acid oxidase (holo- or apoenzyme) was preincubated with different concentrations of NBS in 50 mM potassium phosphate buffer, pH 8.0, at 30°C. Aliquots were withdrawn at different times of preincubation and the reaction was stopped by adding a solution containing 60 mM L-tryptophan and 0.18 M β-mercaptoethanol in the same buffer. Then, the enzyme activity was assayed after adding 10 mM D-alanine, under standard assay conditions. NBS-treated apoenzyme was reconstituted with FAD and the remaining activity was assayed under standard conditions. Controls of activity were carried out in the presence of either tryptophan (60 mM) or β-mercaptoethanol (0.18 mM) or in the presence of both of them.

Protection experiments were carried out by preincubation of the enzyme (holo- or apoenzyme) with 10 mM D-alanine, 20 mM cephalosporin C or 10 mM sodium benzoate in 50 mM potassium phosphate buffer, pH 8.0, at 30°C for 10 min. Then, NBS solution was added to the mixture at the indicated concentrations in

the corresponding figures. The inactivation conditions were as above.

The number of tryptophan residues that react with NBS was calculated by the decrease in absorbance at 280 nm as described by Spande and Witkop [18]: 95 μl enzymatic solution (7.2 μM) were mixed with 5 μl of 0.5 mM NBS freshly prepared water solution during 1 min and the spectrum was recorded between 230 and 330 nm. Then, spectra were successively recorded after withdrawn an aliquot of 2.5 μl and added another aliquot of 2.5 μl 0.5 mM NBS solution. In each case, the number of oxidized tryptophan residues (n_{wox}) were estimated according to the following equation [18]:

$$n_{wox} = \Delta A_{280} 1.31/4500[E_m] \quad (1)$$

were ΔA_{280} is the difference absorption change of the modified enzyme at 280 nm, 4500 is the molar extinction coefficient for tryptophan in proteins at 280 nm [19] and $[E_m]$ is the protein concentration after m cycles of modification.

In order to characterize the extension of modification, upon excitation at 280 nm, the fluorescence spectra of the enzyme were recorded between 250 and 450 nm, in the same conditions as above using a Perkin-Elmer MPF-44E spectrofluorimeter and a quartz cuvette, light path 1 cm, at room temperature.

2.5. Titration of cysteine residues

Cysteine residues were determined in both apo- and holo-D-amino acid oxidase before and after treatment with NBS according to the method described by Ellman [20].

Native or NBS-treated DAAO (3 mol NBS/mol holo- or apoenzyme) were incubated with 10 mM 5,5′-dithiobis(2-nitrobenzoic) acid in 50 mM potassium phosphate buffer, pH 8. After 5 min at room temperature, the absorbance was recorded at 412 nm.

2.6. Determination of methionine residues

Methionine residues were determined in both apo- and holo-D-amino acid oxidase before and after treatment with NBS, using ^{14}C-labelled iodoacetic acid, according to the method of Caldwell et al. [21].

Native or NBS-treated DAAO (3 mol NBS/mol holo- or apoenzyme) were incubated in a solution containing 2.32 mM (7.57 Ci/mole) iodo[^{14}C]acetic acid adjusted with HCl to pH 3.0. Alkylation was driven at 40°C for 24 h in the dark and solutions were then filtered through Millipore filters with an exclusion limit of 5000 Da. Filters were then washed three times with water and once with ethanol. After drying, the filters were placed in vials containing 10 ml of Ready Safe scintillation cocktail and counted in a Tricarb (Packard) liquid scintillation counter.

2.7. Gel filtration studies

Native D-amino acid oxidase (holo- and apoenzyme), NBS-modified D-amino acid oxidase (holo- and apoenzyme) or NBS-treated and reconstituted apoenzyme with FAD were applied onto a Biosep-Sec 3000 HPLC column by using 50 mM potassium phosphate buffer, pH 8.0 containing 2 mM EDTA as mobile phase.

2.8. Electrophoretic methods

Analytical SDS-PAGE was carried out as described by Laemmli [22], in 12.5% slab gels. Native electrophoresis was performed in 5% polyacrylamide slabs gels in 25 mM Tris–HCl buffer, pH 8.3, 129 mM glycine. Proteins were stained with Coomassie Blue R-250 and activity was obtained by incubating gels with D-alanine according to Pilone Simonetta et al. [3].

3. Results and discussion

NBS rapidly and completely inactivates both the apo and holo forms of DAAO from *R. gracilis*.

Chemical modification of proteins with NBS has been used for state discrimination of tryptophan residues and for the exploration of their role in the catalytic function of enzymes. Native D-amino acid oxidase from *R. gracilis* is a dimer of identical subunits with a molecular mass of 79 kDa that contains one molecule of tightly non-covalently bound FAD per monomer. By contrast, the apoenzyme is entirely present as a monomeric protein [3].

The sequence of the 368 amino acid residues in the enzyme from *R. gracilis*, that has been recently determined, showed eight tryptophans per monomer [9].

D-Amino acid oxidase from *R. gracilis* quickly lost all its activity when treated with NBS. Fig. 1 shows the time-dependent inactivation of both DAAO forms, apo- and holoenzyme. As it can be observed all the tryptophans that are susceptible of modification are oxidized within the first minute of the reaction. The plots were linear during the first minute of reaction indicating that the inactivation follows, in both cases, pseudo-first-order kinetics. The second-order rate constant (k) was calculated from the replot of log k_{app} vs. log[NBS]:

$$\log k_{app} = \log k + \log[\mathrm{NBS}] \quad (2)$$

where k_{app} is the pseudo-first-order rate constant at a particular NBS concentration. From the intercepts of such replots (Fig. 1A and B, inset), values of $k_{apo} = 69.8\ \mathrm{mM}^{-1}\ \mathrm{min}^{-1}$ and $k_{holo} = 0.63\ \mathrm{mM}^{-1}\ \mathrm{min}^{-1}$ were obtained. The slopes of the replots give reaction orders of about 1 for the apoenzyme and 2 for the holoenzyme, indicating that 1 mole of NBS reacts with

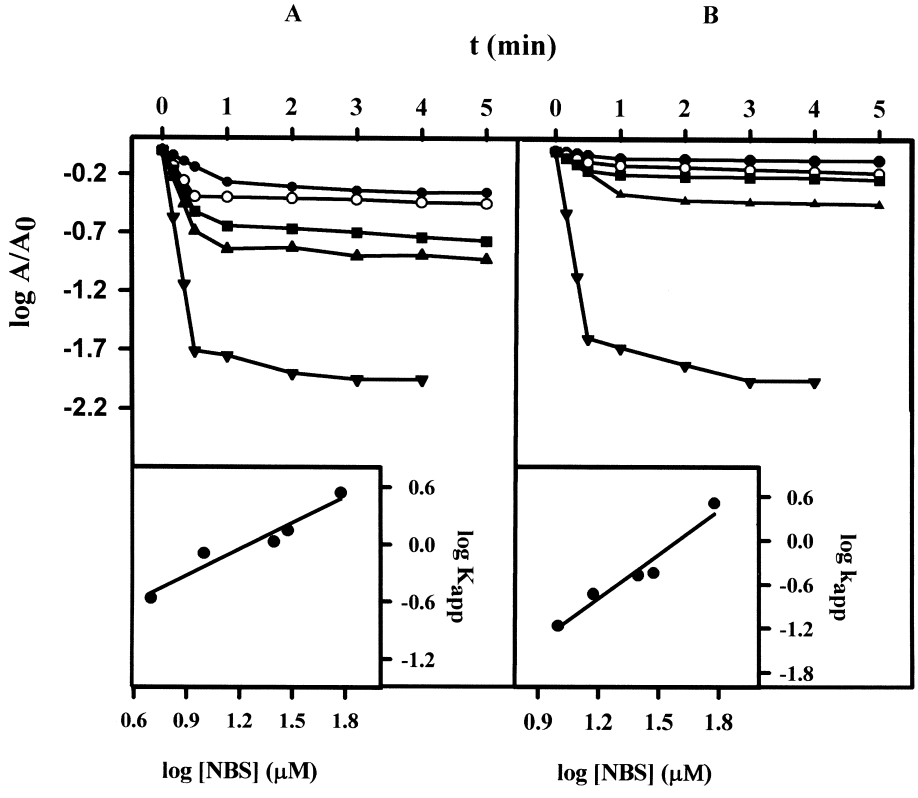

Fig. 1. Kinetics of inactivation of DAAO-apoenzyme (A) and DAAO-holoenzyme (B) by NBS. 0.152-μM enzyme in 50 mM potassium phosphate buffer, pH 8 was incubated with NBS: (-●-) 5 μM, (-○-) 10 μM, (-■-) 25 μM, (-▲-) 30 μM, (-▼-) 60 μM. Enzyme activities were assayed at the indicated times after addition of 10 mM D-alanine. Inset: Replot of log of the pseudo-first order rate constants against log NBS concentration.

a mole of apoenzyme and 2 moles of NBS react with a mole of holoenzyme.

The reaction of tryptophan residues with NBS can be followed by difference spectroscopy; when oxindole is formed, a decrease in absorbance at 280 nm is observed [18]. Modification of holo- and apoenzyme with NBS produced a difference absorption spectra having a minimum at 280 nm and a peak at 250 nm that indicate the formation of oxindolalanine [18]. The spectra were recorded within 1 min after the beginning of the reaction. An isosbestic point at 263 nm could be observed in the absorbance spectra of both forms of DAAO which indicates that tyrosine residues were not oxidized to dienone structure(s).

Fig. 2 shows the correlation of DAAO activity with the extent of tryptophan oxidation. The

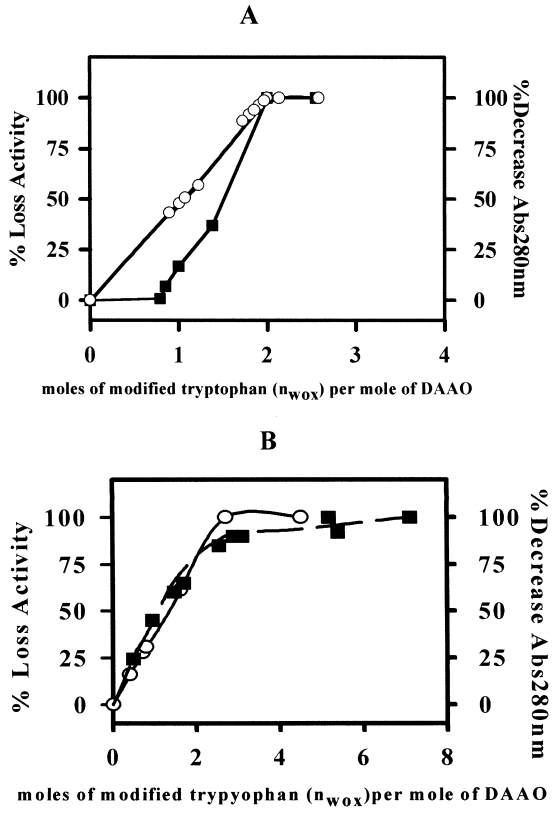

Fig. 2. Correlation of DAAO activity with the extent of tryptophan oxidation. (A) Holoenzyme and (B) apoenzyme. (-■-■-) percent loss activity, (-○-○-) percent absorbance decrease at 280 nm.

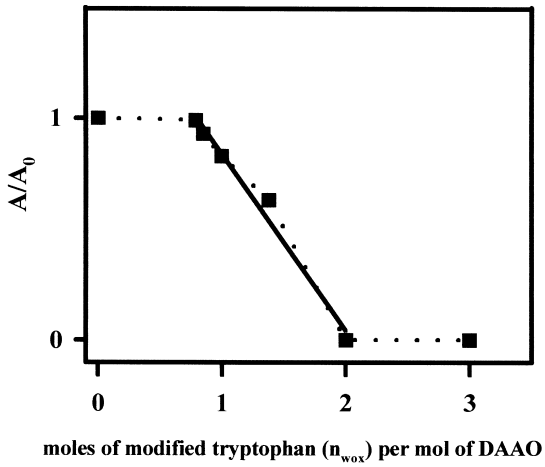

Fig. 3. Plot of residual activity of DAAO-holoenzyme against the number of tryptophan residues modified. The fitting to the straight line was carried out by the method of Tsou, using Eq. (3) with $s = 1$, $p = 1$ and $i = 1$.

decrease in absorbance paralleled both the loss in activity and the extent of oxidation of the enzyme. The destruction of two residues of tryptophan which requires 3 moles of NBS per mole of enzyme monomer (apo- or holoenzyme) led to loss of all the activity and the ΔA_{280} absorbance.

The consumption of NBS did not exceed 1.5 mole reagent/mole oxidized tryptophan. This value indicates selective modification of tryptophan residues, since the consumption of reagent during oxidation of other residues as tyrosine is much higher [18,19].

Fig. 2A revealed that about one residue of tryptophan can be readily oxidized without impairment of activity in the holoenzyme. However, subsequent modification of the enzyme was accompanied by a rapid and total loss of activity.

The number of essential tryptophans can be obtained by the statistical method of Tsou [23], which includes for the holoenzyme the fitting of the following equation:

$$a^{1/i} = \frac{p + s - m}{p} \qquad (3)$$

where s represents the kind of tryptophans in the protein that react most rapidly and are non-

essential. A second kind of tryptophans is p, among which are i essential, and react appreciably only after the reaction with the s groups has almost reached completion, m are the number of tryptophans modified per molecule.

A plot of $a^{1/i}$ against m will give a straight line with a slope of $-1/p$, and s can also be obtained from the intercept at $a^{1/i}=1$. As observed in Fig. 3, the best fit to a straight line is obtained with $s=1$, $p=1$ and $i=1$.

The number of tryptophan residues necessary for complete inactivation, obtained for extrapolation to zero activity was also 2 for the apoenzyme (Fig. 4). But, in this case, the number of essential tryptophans obtained by the statistical method of Tsou [23] included the fitting to the following equation:

$$\log\left[\left(nx/(A/A_0)^{1/i}\right)-p\right] = \log(n-p)+\left[(\alpha-1)/i\right]\log(A/A_0) \quad (4)$$

where n is the total number of modifiable residues of which p react at a defined rate (k_1). These include i essential residues. The remaining ones $(n-p)$ react at different rate ($k_2=\alpha k_1$) and x is the total fraction of non-modified residues remaining at a given moment of the modification. A plot of $\log[(nx/(A/A_0)^{1/i})-p]$ vs. $\log(A/A_0)$ must be a straight line for positive integral values of n, p and i. As observed in Fig. 4B, a satisfactory straight line is obtained with $n=4$, $p=1$, and $i=1$ ($r=0.97$). The value of α was 0.179 indicating that the essential tryptophan react 5.58 times faster than the ''slow'' reacting group.

These results indicate that only one tryptophan residue is essential for activity in both holo- and apoenzyme forms of DAAO. Moreover, the kinetic observation that the reaction orders with respect to NBS are close to 1 in the apoenzyme and 2 in the holoenzyme, together with the observation that the inactivation of both forms of the enzyme is pseudo-first order, suggest that the modification of a single tryptophan residue per active site is sufficient for inactivation.

Protection against inactivation by NBS was achieved by preincubating the enzyme with benzoate, a competitive inhibitor of D-amino acid oxidase [1] or with substrates as D-alanine or cephalosporin C (Fig. 5). All these compounds induced full protection of the enzyme against inactivation, indicating that the essential tryptophan is at or near the active site of the enzyme.

NBS-treated apoenzyme binds FAD with a $K_d=8.5\times10^{-9}$ M almost identical to that for the native enzyme ($K_d=9.2\times10^{-9}$ M). Native-PAGE and gel filtration studies showed that NBS-treated apoenzyme reconstituted with FAD

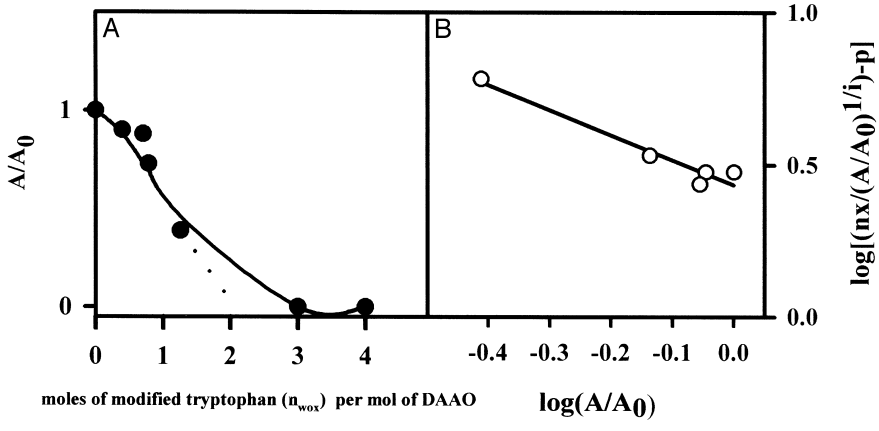

Fig. 4. (A) Plot of residual activity of DAAO-apoenzyme against the number of tryptophan residues modified. (B) Tsou's plot of the data from (A). The points in the figure are experimental values, while the line was calculated using Eq. (4) with $n=4$, $p=1$ and $i=1$.

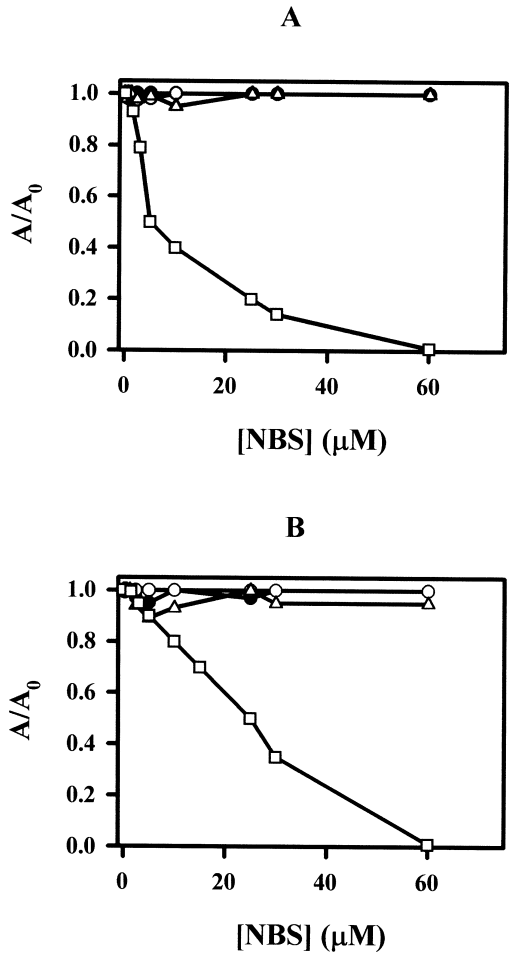

Fig. 5. Protection by benzoate, D-alanine and cephalosporin C against inactivation of DAAO by NBS. (A) Apo-DAAO modified with NBS (-□-□-). Enzyme preincubated during 5 min with 10 mM alanine (-●-●-), 20 mM cephalosporin C (-○-○-) or 10 mM benzoate (-△-△-). (B) Holo-DAAO, conditions and symbols as for (A). Controls of enzyme activity were performed under the same conditions but in the absence of NBS.

dimerize as did native apoenzyme [16]. These results indicate that the essential tryptophan was not involved in binding of FAD or dimerization.

Fluorescence spectrophotometry for the reaction of the enzyme with NBS was carried out (Fig. 6). There was a marked decrease in the fluorescence emission as the tryptophan residues of apo- (Fig. 6A) and holoenzyme (Fig. 6B) were modified. On excitation at 280 nm, 90% and 75% losses in fluorescence emission were found at 335 nm when four and two tryptophan residues per monomer of enzyme were modified respectively in apo- and holoenzyme. The conformational change that occurs when the enzyme binds FAD and dimerize [24] could be responsible for the burying of some tryptophans, preventing their contact with the modifier in the holoenzyme.

The fluorescence emission maximum at 335 nm is typical of tryptophan emission in proteins, indicating that the fluorescence is solely due to tryptophan residues exposed on the protein surface [25]. On the other hand, the fluorescence intensity changes were in good accord with the absorption changes (data not shown), indicating that both changes are linked and are two facets of the same reaction [19].

NBS is a potent oxidizing agent that is capable of eliciting a variety of effects in proteins. Under certain conditions, scission of peptide bonds is possible [26]. Although at pH 4 in aqueous acetate buffer, the cleavage of tryptophyl peptides is optimal, while above pH 5.5 it becomes negligible, we have assess the integrity of the protein backbone of the oxidized enzyme by SDS-PAGE and gel filtration studies. NBS-treated enzyme (both apo and holo forms) migrated as a single band and its apparent molecu-

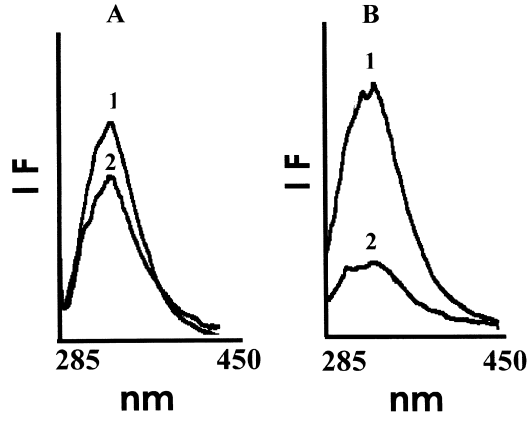

Fig. 6. Decrease in the relative fluorescence intensity of the tryptophan emission with the modification of tryptophan residues by NBS. (A) Native apo-DAAO (1), gain 3, NBS-treated apo-DAAO (NBS/apoenzyme = 3) (2), gain 10. (B) Native holo-DAAO (1), gain 10; NBS-treated holo-DAAO (NBS/enzyme = 3) (2), gain 10.

lar weight was identical with unmodified enzyme. A single symmetrical protein peak was observed by gel filtration chromatography (data not shown).

The presence of free –SH groups in proteins may interfere with the titration of tryptophan residues by NBS [27,28] but, if this occurs, several moles of NBS (higher than 5) would be consumed before any decrease in absorbance at 280 nm were detected. As stated above, full inactivation of DAAO was achieved with 1.5 moles of NBS per mole of enzyme. Nevertheless, as DAAO from *R. gracilis* has six cysteines per monomer [9], the possibility that the loss of enzyme activity was caused by oxidation of cysteines was further investigated. Titration of NBS-treated DAAO (3 mol NBS/mol holo- or apoenzyme) with Ellman reagent [20] yielded six cysteines per monomer, indicating that the sulfhydryl groups of DAAO remain intact during the course of the reaction.

The possibility that methionine residues in the protein undergo modification with NBS could be discarded after treatment of native and NBS-treated DAAO (holo- or apoenzyme) with iodo[^{14}C]acetate, which alkylates the methionine residues but not the oxidized ones. After treatment of D-amino acid oxidase with NBS, none of the methionine residues in holo- or apoenzyme were modified

Taken together, the present data strongly indicate that the inactivation of DAAO by NBS, in the conditions stated in the present work is due to the modification of tryptophan residues. This conclusion is supported by the following facts. (a) The decrease in absorbance at 280 nm paralleled both the loss in activity and the extent of oxidation of DAAO. (b) The absence of absorbance changes at 263 nm ruled out the modification of tyrosine residues. Moreover, modification of tyrosine residues with tetranitromethane and *N*-acetylimidazole did not cause inactivation of both apo- and holoenzyme (data not shown). This result could indicate that, at difference of D-amino acid oxidase from pig kidney [29], there are no essential tyrosines in DAAO from *R. gracilis*. (c) The consumption of NBS did not exceed 1.5 moles reagent per mole of oxidized tryptophan. This value is the same that for the oxidation of *N*-acetyl-L-tryptophan ethyl ester that has been employed as model compound [19] and one of the lower than described for different proteins [30]. Consumption of reagent during oxidation of other residues is much higher [18,19]. (d) The decrease in the fluorescence emission at 335 nm was in accordance with the absorbance changes at 280 nm. (e) NBS-treated DAAO retained primary structure integrity. (f) No oxidation of cysteines was detected. (g) No oxidation of methionine residues was detected. Involvement of tryptophan residues in activity of D-amino acid oxidase from *R. gracilis* has not hitherto been reported.

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