Studies on the structural and functional aspects of *Rhodotorula gracilis* D-amino acid oxidase by limited trypsinolysis

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The structure–function relationships of purified *Rhodotorula gracilis* D-amino acid oxidase (in its holo-, apo- and holo-enzyme–benzoate complex forms) was analysed by digestion with trypsin. In all cases trypsin cleaves this 80 kDa dimeric enzyme at the C-terminal region, since the peptide bonds sensitive to proteinase attack are clustered in this region. Digestion of native enzyme with trypsin produced a nicked and truncated form of 38.3 kDa containing two polypeptides of 34 and 5 kDa starting from Met^{1} and Ala^{319} respectively, and with detachment of the Thr^{306}-Arg^{318} and Glu^{365}-Leu^{368} peptides. Our results show that this 'core', folded into a compact structure, is catalytically competent. The acquisition of this nicked form was marked by a shift from a dimeric to a monomeric active enzyme, a result never previously obtained. The deleted sequences, Thr^{306}-Arg^{318} and Glu^{365}-Leu^{368}, are essential for the monomer–monomer interaction, and, in particular, the region encompassing Thr^{306}–Arg^{318} should play an essential role in the dimerization process. Interestingly, the Ser^{308}–Lys^{321} sequence present in the lost peptide corresponds to a sequence not present in other known D-amino acid oxidases [Faotto, Pollegioni, Ceciliani, Ronchi and Pilone (1995) Biotechnol. Lett. **17**, 193–198]. A role of the cleaved-off region for the thermostabilization of the enzyme is also discussed.

INTRODUCTION

D-Amino acid oxidase (DAAO, EC 1.4.3.3) from the yeast *Rhodotorula gracilis* is a member of the class of flavin dehydrogenase/oxidases [1,2]. The enzyme catalyses the oxidative deamination of D-amino acids to give the corresponding 2-oxo acids, H_2O_2 and NH_3. DAAO from *R. gracilis* in its native form is a 80 kDa dimer of identical subunits, each subunit containing one FAD moiety; this aggregation state is not dependent on a polymerization equilibrium [3]. The corresponding apoprotein is quantitatively present as a 40 kDa monomer. No active DAAO monomer has been so far isolated, owing to the rapid shift to the dimeric state when the apoprotein is reconstituted with coenzyme FAD [4].

The amino acid sequence of the enzyme has now been determined. The enzyme consists of 368 amino acids [5]; however, the three-dimensional structure of DAAO has not yet been solved. Kinetically and mechanistically, DAAO from *Rhodotorula* has been extensively characterized [6,7]. The rate-determining step in the kinetic mechanism corresponds to the reductive half-reaction, in contrast with the mammalian kidney enzyme, where this step is represented by the product release from oxidized enzyme [8]. The active-site topography of the yeast enzyme was depicted by the use of specific chemical modifiers and artificial flavins [9–11]. Furthermore, the enzyme presents other properties that distinguish it from other characterized DAAOs: elevated turnover [6,7], a tight binding with the coenzyme FAD, a stable dimeric aggregation state [4] and substantial differences in the primary sequence [5]. All these properties are also relevant to the exploitation of this flavoenzyme reaction in biotechnological and industrial work [12].

The goal of using limited proteolysis focused on obtaining valuable information on the function–structure relationships of this protein, considering, as mentioned above, the lack of information that exists on the three-dimensional structure of DAAO. In the case of the well-known enzyme from pig kidney, limited proteolysis provided data on protein conformation and topology [13]. In the present paper we particularly report the use of limited trypsin proteolysis, performed on *R. gracilis* apo- and holo-DAAO, to provide information on the location of the polypeptide chain segment involved in enzyme dimerization, on the structural requirements for catalytic activity and binding of the coenzyme FAD, and on the conformational stability of proteolysed forms.

MATERIALS AND METHODS

Materials

The Superose 12 HR 10/30 column was from Pharmacia LKB. Phenylmethanesulphonyl fluoride was from Sigma; sequencing-grade trypsin and tosylphenylalanylchloromethane (Tos-Phe-CH_2Cl; 'TPCK') were from Boehringer Mannheim; other reagents were of analytical grade.

DAAO was purified as described in [14] from *R. gracilis* cells (A.T.C.C. 26217) grown to late-exponential phase in a synthetic medium at pH 5.6 containing 30 mM D-alanine [15]. The pure enzyme had an A_{274}/A_{455} ratio of 8.2 and a specific activity, with D-alanine as substrate, of 180 units/mg of protein at 37 °C; the enzyme concentration was determined by its flavin content, an ϵ_{455} value of 12600 $M^{-1} \cdot cm^{-1}$ [3].

Enzyme-activity assay

Enzyme activity was assayed polarographically by measuring the oxygen consumption at 30 or 37 °C with 28 mM D-alanine as substrate [3] or, alternatively, spectrophotometrically, by monitoring the increase in A_{243} at 25 °C with 25 mM D-phenylglycine as substrate [16].

Steady-state kinetic measurements were carried out polarographically in 75 mM sodium pyrophosphate buffer, pH 8.5, at 30 °C, as described in [6]. Coenzyme and substrate solutions were

Abbreviation used: DAAO, D-amino acid oxidase.

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prepared daily. The effect of inhibitors was tested by differential spectroscopy, monitoring the spectral changes in the enzyme after each addition of inhibitor to sample and reference cuvettes. K_d values were determined as described in [17]. Data analysis was performed on a IBM PS2/386 computer using Enzfitter (Elsevier–Biosoft) program.

Limited proteolysis

Purified holo- or apo-DAAO (28 μM) was incubated at 25 °C in 10 mM sodium pyrophosphate buffer, pH 8.3, in the presence of 10% (w/w) trypsin. For the proteolysis of the holoenzyme–benzoate complex, 35 mM sodium benzoate (final concentration) was added to the incubation mixture. At different times aliquots (2 μg of DAAO) were withdrawn for activity measurements: proteolysis was stopped by diluting the sample in 100 mM sodium pyrophosphate, pH 8.5, containing 0.2 mM Tos-Phe-CH_2Cl. For electrophoretic analysis, samples (8–10 μg of DAAO) were diluted in SDS sample buffer and then heated at 100 °C for 3 min. The kinetics of inactivation, of loss and formation of individual fragments were best fitted with a single- or double-exponential decay equations as described in [18] using Grafit (Erythacus Software) and GraphPad (ISI Software) data-analysis programs.

Isolation of proteolysis product

Preparative experiments were carried out by incubating 20–50 μM DAAO–benzoate complex with 3% (w/w) trypsin as described above for 120 min at 25 °C. The proteolysis product was separated by gel filtration on Superose 12 column in an FPLC system, equilibrated in 20 mM potassium phosphate (pH 7.5)/250 mM NaCl/0.3 mM EDTA/5 mM 2-mercaptoethanol/10% (v/v) glycerol.

Electrophoretic methods

Discontinuous PAGE was performed as described in [19], and analytical SDS/PAGE was carried out using either 10% or linear-gradient gel slabs as described in [20]. Gels were stained for protein content with Coomassie Blue R-250 or for DAAO activity [3]. Densitometric measurements of the stained gels were obtained with a LKB 2202 Ultroscan laser densitometer at 630 nm interfaced with an Apple personal computer. Analytical isoelectrofocusing was carried out in polyacrylamide-gel slabs in 1% (v/v) Pharmalyte ampholines (Pharmacia) over the pH range 3.5–10.0 at 10 °C [21].

Amino acid analysis, N-terminal sequencing and electrospray MS

Gas-phase hydrolysis of protein was carried out *in vacuo* at 105 °C for 24 h in 6 M HCl/1% (v/v) phenol. Amino acid analysis was performed after pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [22], using Jasco HPLC equipment with a 820-FP detector. N-terminal sequences were determined both on trichloroacetic acid-precipitated protein and on poly(vinylidene difluoride) electro-transferred samples [23] using an automated protein sequencer (Applied Biosystems, model 477A). Complete tryptic digestion was carried out in 0.1% (w/v) NH_4HCO_3, pH 8.5, at 37 °C for 4 h using a proteinase/DAAO ratio of 1:25 (w/w). Peptide purification was achieved by reverse-phase HPLC chromatography as described in [5]. Electrospray ms was performed on a VG PLATFORM mass spectrometer (Fisons Instruments) using a voltage of 3.5 kV and a nebulizer-gas (N_2) flow of 13 litres/h.

Apoprotein preparation and reconstitution with FAD and 8-chloro-FAD

The apoprotein of *R. gracilis* DAAO was prepared by dialysis against 250 mM potassium phosphate, pH 7.5, containing 2 M KBr, 20% (v/v) glycerol, 0.3 mM EDTA and 5 mM 2-mercaptoethanol as described in [4].

Flavin and protein-fluorescence measurements were carried out in a Jasco FP-777 spectrofluorimeter [4]. The dissociation constant for the apoprotein–FAD complex was estimated as described in [24].

Thermostability analysis

The time-dependence of thermal inactivation of native and proteolysed form of DAAO was determined by heating enzyme solutions (3.2 μM) in sealed tubes over the temperature range 20–50 °C, in 10 mM potassium phosphate (pH 7.5)/10% (v/v) glycerol/2 mM EDTA/5 mM 2-mercaptoethanol. At different times, aliquots were taken and assayed for DAAO activity as described above. The inactivation rate constants were obtained from the slopes of the first-order inactivation plots and were used to calculate the activation free energies of inactivation ($\Delta G\ddagger$) at each temperature [25]. The activation energy (E_a) for the thermal inactivation of the enzyme was obtained from the slope of the Arrhenius plot, and from these data the activation enthalpy ($\Delta H\ddagger$) and entropy ($\Delta S\ddagger$) at each temperature was then calculated.

RESULTS

Kinetics of inactivation

Holoenzyme

Incubation of *R. gracilis* DAAO holoenzyme with 10% (w/w) trypsin at 25 °C resulted in a biphasic loss of the catalytic activity (Figure 1); the first phase had a rate constant of 0.152 min^{-1} and the second phase had a rate constant of $3.3 \times 10^{-3} \cdot min^{-1}$ (Table 1). The time course of limited trypsin digestion, followed by

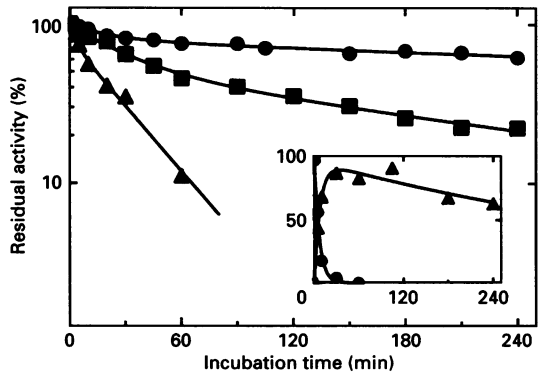


Figure 1 Proteolytic inactivation by 10% trypsin (w/w) at 25 °C of *R. gracilis* DAAO

The data were fitted to a double-exponential process ($y = A \cdot e^{-k_1 \cdot x} + B \cdot e^{-k_2 \cdot x}$) using the rate constants and parameters indicated in parenthesis. ■, Holoenzyme ($A = 27.0$, $k_1 = 0.151\ min^{-1}$, $B = 71.8$, $k_2 = 3.66 \times 10^{-3}\ min^{-1}$); ●, holoenzyme–benzoate complex ($A = 20.6$, $k_1 = 0.147\ min^{-1}$, $B = 78.9$, $k_2 = 1.15 \times 10^{-3}\ min^{-1}$); ▲, apoprotein ($A = 24.5$, $k_1 = 0.206\ min^{-1}$, $B = 77.0$, $k_2 = 31.2 \times 10^{-3}\ min^{-1}$). The inset shows a linear plot of the time course of proteolytic cleavage of the holoenzyme–benzoate complex monitored by densitometric analysis of SDS/PAGE slabs. ●, 40 kDa ($A = 100$, $k_1 = 0.141\ min^{-1}$, B and $k_2 = 0$); ○, 34 kDa ($A = -99.4$, $k_1 = 0.156\ min^{-1}$, $B = 98.3$, $k_2 = 2.72 \times 10^{-3}\ min^{-1}$).

Table 1 Inactivation and proteolysis rates of *R. gracilis* DAAO by 10% trypsin, at 25 °C

	Inactivation ($min^{-1} \cdot 10^{-3}$)		40 kDa	34 kDa	
	First phase	Second phase	Degradation ($min^{-1} \cdot 10^{-3}$)	Formation ($min^{-1} \cdot 10^{-3}$)	Degradation ($min^{-1} \cdot 10^{-3}$)
Holoenzyme	152±32	3.32±1.33	180±73	214±88	3.55±0.96
Holoenzyme–benzoate complex	139±89	1.22±0.17	145±71	126±22	1.71±0.47
Apoenzyme	n.d.	31.6±4.3	1309±115	1510±303	32.7±3.8

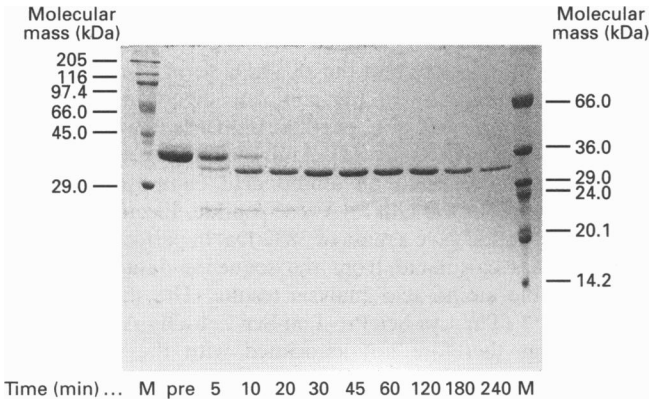


Figure 2 SDS/PAGE of the time course for the limited proteolysis of DAAO

Holo-DAAO was incubated at 25 °C with 10% (w/w) trypsin for the indicated time periods. Each well contained 10 μg of DAAO and 1 μg of trypsin (seen as a band of 23.8 kDa). M, markers.

denaturing SDS/PAGE analysis, showed the progressive digestion of the 40 kDa band (corresponding to the intact enzyme monomer) and the concomitant appearance of a protein band with higher mobility corresponding to a molecular mass of 34.0±0.55 kDa (Figure 2). Densitometric analysis of the gels indicated that the 40 kDa band was totally converted into the 34 kDa fragment in about 20 min. At this time about 80% residual activity was still present (see Figure 1), providing evidence that a catalytically competent form of the enzyme had been produced. At longer incubation times, the 34 kDa fragment was degraded without the appearance of detectable transient bands, with a rate similar to that of the second phase of inactivation ($k_{obs.} = 3.6 \times 10^{-3} \cdot min^{-1}$). The degradation of the 40 kDa form, calculated by densitometric analysis, was a single-exponential process with a rate of 0.180 min^{-1}, a value similar to that estimated for the formation of the 34 kDa band (0.214 min^{-1}), suggesting a direct conversion of the 40 kDa into the 34 kDa form. These rates match that of the first phase of inactivation (0.152 min^{-1}), corresponding to an extrapolated activity loss of 15%.

Holoenzyme–benzoate complex

Incubation of *R. gracilis* DAAO holoenzyme in the presence of 35 mM benzoate with 10% (w/w) trypsin at 25 °C resulted, as for the holoenzyme, in a biphasic loss of catalytic activity (Figure 1) characterized by a first phase ($k_{obs.} = 0.139\ min^{-1}$) which led to an 18% inactivation, and a second phase with a rate constant of $1.2 \times 10^{-3} \cdot min^{-1}$ (with a residual activity of 65% after 240 min). SDS/PAGE analysis of the time course of digestion showed the progressive degradation of the 40 kDa band, in a single-exponential process, and the concomitant appearance of the 34 kDa fragment. The densitometric analysis showed that the rate of formation of the 34 kDa fragment matched that of the disappearance of the 40 kDa species (inset to Figure 1; Table 1). The 34 kDa fragment obtained from the holoenzyme–benzoate complex was more stable to further trypsin digestion than was the free holoenzyme, with a degradation rate constant of $1.7 \times 10^{-3} \cdot min^{-1}$, a value close to that of the second phase of the inactivation process ($1.2 \times 10^{-3} \cdot min^{-1}$).

Apoenzyme

The apoprotein of *R. gracilis* DAAO was rapidly inactivated in presence of 10% (w/w) trypsin at 25 °C (Figure 1) in a biphasic process (Table 1). Densitometric analysis of the SDS/PAGE gels revealed the progressive conversion of the 40 kDa band into the 34 kDa fragment and a complete degradation of the latter in 90 min, with a rate which paralleled the inactivation process ($k_{obs.} = 3.3 \times 10^{-2} \cdot min^{-1}$). Benzoate had no effect on the pattern of apoprotein inactivation.

Isolation, molecular-mass determination and aggregation state of the proteolytic fragments

In order to obtain high amounts of proteolysed enzyme, the holoenzyme–benzoate complex was incubated with 3% (w/w) trypsin at 25 °C for 120 min, as described in the Materials and methods section. The tryptic fragment, largely produced under these controlled conditions, was isolated by Superose 12 FPLC (Figure 3). Under these conditions the proteolytic product was eluted with an apparent molecular mass of 38.3±0.4 kDa (peak B in Figure 3), slightly different from that of monomeric apoprotein (40 kDa), but markedly different from the value of 80 kDa typical of the native dimeric enzyme (peak A in Figure 3). In the molecular-mass range of the SDS/PAGE system used, the analysis of the peak B showed only a protein band of 34 kDa. The apparent discrepancy between the molecular-mass values determined by gel-filtration and SDS/PAGE analyses is explained on the basis of the structural analysis of the proteolytic products (see below).

Characterization of limited-proteolysis products

After SDS/PAGE separation of proteolytic products, the protein band with a molecular mass of about 34 kDa was transferred directly to a poly(vinylidene difluoride) membrane and sequenced. Its N-terminal sequence (15 residues were determined) corresponds to that of the native protein [5], indicating that the lost peptide(s) is (are) derived from the C-terminal region of the

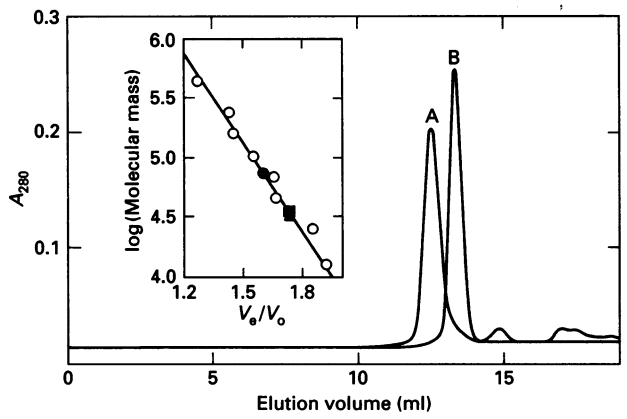
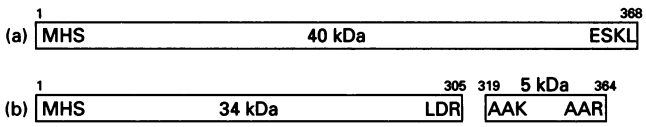


Figure 3 FPLC of native and proteolysed DAAO

Chromatography of native DAAO (A) and chromatography of enzyme after limited digestion with 3% trypsin (w/w) at 25 °C for 120 min (B). Loaded samples contained 0.16 mg of protein.



Scheme 1 Structural models of native (a) and trypsin proteolytic product (b) of *R. gracilis* DAAO

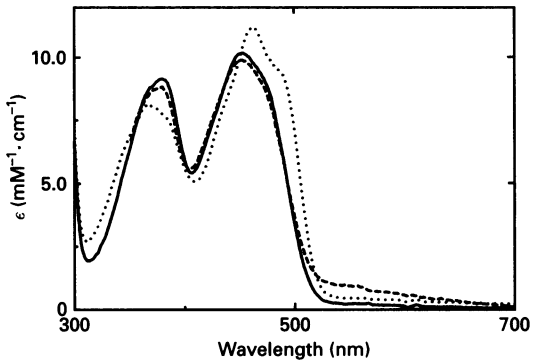


Figure 4 Spectral effects induced by benzoate and anthranilate on binding to the 38.3 kDa proteolysed form of *R. gracilis* DAAO

——, 7.8 μM 38.3 kDa DAAO in 20 mM potassium phosphate buffer (pH 7.5)/250 mM NaCl/10 % (v/v) glycerol/0.3 mM EDTA/5 mM 2-mercaptoethanol; · · · · · ·, after addition of 100 mM sodium benzoate; – – – –, same sample as in —— after addition of 5 mM sodium anthranilate. The temperature was 15 °C.

enzyme. N-terminal-sequence analysis of proteolytic products, isolated by gel filtration on Superose 12 (peak B of Figure 3), showed the presence of two peptides in equal amounts. One peptide corresponded to the N-terminal portion of the protein, whereas the second one started from Ala^{319} [5]. This result indicates the presence in the proteolysed product of a 'nicked' protein. The mass spectrum of this product showed the presence of two peaks, with a molecular mass in agreement with those of sequences Met^{1}–Arg^{305} and Ala^{319}–Glu^{365} respectively (see Scheme 1).

Table 2 Apparent kinetic parameters of native and 38.3 kDa form of DAAO

Measurements were performed by a polarographic method at 30 °C and pH 8.5.

	K_m (mM)		$V_{max.}$ (μmol of O_2/min per mg of enzyme)	
Substrate	Native	38.3 kDa form	Native	38.3 kDa form
D-Alanine	0.83	1.29	140.0	125.4
D-Proline	21.5	15.5	116.0	104.7

The formation of a nicked protein during the limited-proteolysis process was confirmed by experiments involving exaustive trypsin digestion of the 38.3 kDa form. Reverse-phase HPLC chromatography of the complete digest indicated the presence of a peptide (R_t = 62 min) the sequence of which started at Glu^{324} (15 residues analysed). Amino-acid-sequence analysis of this peptide indicated an amino acid composition corresponding to that of the Glu^{324}–Arg^{358} peptide. Electrospray MS of the same peptide gave a mass of 3832 Da, in perfect agreement with the mass calculated from the sequence data (3832 Da), confirming the amino-acid-analysis results. The short peptide Thr^{306}–Arg^{318} (Thr-Lys-Ser-Pro-Leu-Ser-Leu-Gly-Arg-Gly-Ser-Ala-Arg) was therefore not associated with the proteolysed enzyme forms.

These results indicate that at least three peptide bonds susceptible to trypsin, i.e. 305–306, 318–319 and 364–365, are present in the C-terminal portion of *R. gracilis* DAAO, the cleavage of which resulted in the release of the C-terminal tetrapeptide Glu-Ser-Lys-Leu and in a nicked and truncated enzyme form (see Scheme 1).

Spectral and kinetic characterization of the proteolytic fragment

The purified 38.3 kDa enzyme form showed a spectrum typical of an FAD-containing protein, with absorbance maxima at 455, 378 and 274 nm (Figure 4); the A_{274}/A_{455} ratio was about 9.8, a value slightly higher than that determined for the native enzyme [3]. The absorption coefficient of the truncated form was determined on the free coenzyme obtained after heat-denaturation, using absorption coefficient for FAD of 11300 $M^{-1} \cdot cm^{-1}$: a value of 10140 $M^{-1} \cdot cm^{-1}$ was calculated. On the basis of this value and from the amino-acid-analysis data, an $A^{0.1\%}_{274}$ of 2.74 was calculated.

The proteolysed enzyme showed a specific activity on D-alanine and D-proline as substrates corresponding to 85–90 % of the values determined for the native enzyme [6] (Table 2), with no significant changes in K_m values. The proteolysed enzyme did not require exogenous FAD in the assay mixture for maximal activity, thus indicating that the slightly higher spectral ratio reported above was not dependent on loss of coenzyme. The activity of the truncated form of DAAO on different D-amino acids was tested and compared with the activities of native enzyme form (Table 3): D-norvaline, D-methionine and D-phenylalanine were the best substrates, whereas D-valine (the highest activity for native enzyme) and D-tryptophan showed a lower activity. Interestingly, with basic amino acids as substrate the proteolysed protein presented a 2-fold higher activity with respect to the native enzyme, whereas acid substrates were still not oxidized.

Benzoate binding perturbed the visible spectrum of native and truncated forms of *R. gracilis* DAAO, causing in both cases an increase of the shoulder at 480 nm, a red shift of the absorption

Table 3 Substrate specificity of native and 38.3 kDa forms of yeast DAAO

Measurements were performed by a polarographic method at 30 °C and pH 8.5 with saturating substrate concentrations. The activity with D-alanine as substrate was taken as 100.

Substrate	Relative activity (%)	
	Native	38.3 kDa form
D-Alanine	100	100
D-Proline	83	84
D-Methionine	82	118
D-Norvaline	103	119
D-Valine	127	67
D-Phenylalanine	99	113
D-Tryptophan	103	76
D-Lysine	7	15
D-Arginine	6	15
Glycine	0*	0*
D-Aspartate	0	0
D-Glutamate	0	0

* Reduction of the enzyme under anaerobic conditions was observed.

maximum from 455 at 460 nm and a decrease in the absorbance below 400 nm (Figure 4). The K_d value for benzoate binding to proteolysed enzyme, at 15 °C and pH 7.5, as determined by absorption-difference spectroscopy, had a value similar to those previously determined for the native enzyme (0.54 mM as against 0.245 mM) [3]. The absorption spectrum of proteolysed enzyme in the presence of anthranilate showed a broad band at long wavelengths centred at about 565 nm, which is typical of a π–π charge-transfer complex [26] and similar to those obtained with native enzyme (Figure 4) [6]; a K_d value of 1.3 mM at 15 °C and pH 7.5 was calculated.

Coenzyme binding

The apoprotein form of proteolysed enzyme, as for the native enzyme, was obtained by the procedure described in the Materials and methods section, with a yield, in terms of protein recovery, of about 60%. The proteolysed apoprotein was completely inactive if assayed in the absence of exogenous FAD; it regained over 90% of its specific activity, with respect to the native holoenzyme form, when assayed in presence of excess FAD. When proteolysed apoprotein was titrated with increasing amounts of FAD, the extrapolation of the initial slope of the titration curve intercepted the maximum observed activity at a stoichiometry of 1 mol of FAD/mol of apoprotein. The reconstituted holoenzyme showed an absorption spectrum identical with that of proteolysed holo-DAAO.

The equilibrium binding of FAD to apoprotein was measured by FAD and protein fluorescence. The points that lay off the two linear parts of the curve were used to calculate the dissociation constant; a K_d of 1.2×10^{-8} M was determined, a value similar to that determined for the native enzyme (2.0×10^{-8} M) [4].

Binding of 8-mercapto-FAD to the proteolysed apoprotein was accompanied by a red absorption shift and an enhanced resolution of the visible absorption band of 8-mercaptoflavin, with stabilization of the paraquinoid (canonical) form ([9]; results not shown). 8-Thio-FAD-proteolysed enzyme did not react when incubated with 85 mM iodoacetamide, since the spectrum typical of 8-thioalkylated flavin did not appear [27a].

Table 4 Thermodynamic activation parameters for thermal inactivation of holo-, apo-, holo-38.3 kDa form and apo-38.3 kDa form of *R. gracilis* DAAO, over the 20–45 °C temperature range

$\Delta G_m‡$ is the medium value over the temperature range tested (20–45 °C).

Parameter	Holoenzyme	Apoprotein	38.3 kDa form	
			Holoenzyme	Apoprotein
$E_{act.}$ (kJ·mol^{-1})	239.3 ± 16.6	221.8 ± 28.3	227.8 ± 0.9	207.9 ± 19.9
$\Delta G_m‡$ (kJ·mol^{-1})	87.2 ± 3.6	85.4 ± 5.3	86.4 ± 3.9	83.1 ± 3.8
$\Delta H‡$ (kJ·mol^{-1})	236.7 ± 0.1	219.2 ± 0.1	225.3 ± 0.1	205.4 ± 0.1
$\Delta S‡$ (kJ·mol^{-1}·K^{-1})	0.483 ± 0.01	0.430 ± 0.02	0.456 ± 0.01	0.407 ± 0.02

Thermal stability of native and proteolysed DAAO

The thermal inactivation of the native and the proteolysed enzyme followed first-order kinetics at all temperatures. The proteolysed protein was markedly less stable than the native one (an inactivation rate constant of 1.68 min^{-1} as against 6.30 min^{-1} at 30 °C). Arrhenius plots for both enzyme forms were thus drawn using the first-order rate constant for the inactivation process at different temperatures. Their slopes corresponded to an activation energy for the thermoinactivation process of 227.84 ± 0.86 kJ/mol for the proteolysed enzyme as compared with 239.32 ± 16.66 kJ/mol determined for the native one. Over the 20–45 °C temperature range, neither $\Delta H‡$ nor $\Delta S‡$ for both enzyme forms was significantly affected by temperature, whereas $\Delta G‡$ decreased linearly with temperature (from 89.3 kJ/mol at 25 °C to 82.4 kJ/mol at 40 °C in the proteolysed enzyme). The activation energy values and the thermodynamic parameters for the thermoinactivation of the 38.3 kDa and native DAAO (and their corresponding apoproteins) are reported in Table 4. As expected, the apoprotein forms in both cases showed a lower thermostability as compared with the corresponding holoenzyme (with a decrease of the inactivation energy of about 18–20 kJ/mol and of about 0.05 kJ/mol·K in $\Delta S‡$ values).

DISCUSSION

Information on the tertiary structure of proteins can be inferred by the use of limited proteolysis of native enzymes. Cleavage by proteinases has been reported to occur frequently in the 'hinge' flexible region, linking the structural domains of the enzymes [27b]. In DAAO from *R. gracilis*, the primary sites of proteolytic attack are located at the C-terminal region. This result indicates the existence of a large region folded into a more compact structure not accessible to trypsin; transient degradation bands were not detected, in contrast with what was reported for limited trypsin proteolysis of mammalian DAAO [13], where the slow degradation of holoenzyme to a stable 37 kDa fragment was paralleled by direct degradation to smaller fragments. Also the DAAO from *Trigonopsis variabilis* gave a different proteolytic pattern, since during the purification procedure it was largely proteolysed, leaving two peptides of 27 and 12 kDa, which were still bound and active [28].

In the case of native globular proteins, limited proteolysis often occurs at the level of one or very few peptide bonds in topologically distinct sites, producing nicked protein species which can then be degraded [29]. This is the case for the proteolytic cleavage at the C-terminal region of *R. gracilis* DAAO, which yields a nicked and truncated enzyme (Scheme 1) that is fully active and still retains its ability to tightly bind the coenzyme. The proteolysed enzyme has an apparent M_r of about

38300, containing two polypeptides of 34 and 5 kDa starting from the Met^{1} and Ala^{319} residues respectively, tightly associated under non-denaturing conditions, even in the absence of the coenzyme. This result indicates that FAD is not involved in the interaction between two polypeptides. Trypsin cleavages occurred with detachment of the Thr^{306}–Arg^{318} and Glu^{365}–Leu^{368} peptides, yielding a nicked enzyme. The acquisition of this nicked form resulted in a shift from a dimeric to a monomeric form of the enzyme.

Our results demonstrate that the proteolytic enzyme is structured as a catalytically competent 'core' less susceptible to trypsin attack. Until now all attempts to demonstrate the activity of the protein monomer was unsuccessful, owing to the stability of the homodimeric native enzyme [3]. The missing portions, Thr^{306}–Arg^{318} and Glu^{365}–Leu^{368}, are essential for the monomer–monomer interaction and dimerization of the yeast enzyme. A role has been already postulated for the C-terminal tripeptide: the typical signal sequence Ser-His/Lys-Leu has been reported to be a recognition site for the import of proteins into the peroxisomes [30]. The region encompassing Thr^{306}–Arg^{318} should play an essential role in the dimerization process; this result is analogous to what has been observed in the three-dimensional structure of flavocytochrome b_2, where the residues 486–511 of the C-terminal tail are in contact with each of the remaining subunits [31], and as the truncated form (21 C-terminal amino acids missing) of the protein product of gene 2.5 of bacteriophage T_7 shifts to a monomeric state [32]. It should be also noted that the Ser^{308}–Lys^{321} sequence, a positively charged region present in the lost polypeptide, corresponds to one that is not present in other DAAOs [5]. On a speculative basis, the removal of such a charged region could play an important role on the dimerization process.

The different sensitivity to tryptic cleavage of the apo-, holoenzyme and holoenzyme–benzoate complex (Figure 1) points to the presence of different conformational states of yeast DAAO, as previously reported in the case of pig kidney enzyme [13]. For the yeast enzyme, in all cases a cleavage by trypsin occurred at position 305↓306; a faster formation and degradation rate of this 'core' product was observed with the apoprotein form, confirming its loose conformation [4]. Benzoate partially protected the proteolytic product from further inactivation and degradation by trypsin (the second phase of inactivation kinetics), probably stabilizing the conformation of the active site, whereas no effect is exerted on the dimer degradation (the first phase of inactivation kinetics). In this context it is conceivable that benzoate binding occurs at a region topologically distinct from the site(s) of proteinase attack. At present time it is still unclear where benzoate binds to native DAAO, even though evidence has been presented that its binding destroys, directly or indirectly, the inductive effect of the positively charged group interacting with the N^{1}–C^{2}=O locus [33].

Proteolysed *R. gracilis* DAAO maintains its coenzyme- and benzoate-binding properties, confirming that the N-terminus of the protein plays an essential role in FAD binding, similarly to other flavoproteins [34] and retains the spectral properties of native enzyme [3,9]. These conclusions are confirmed by the apoprotein-reconstitution experiments with FAD and 8-mercapto-FAD: the reconstituted monomeric holoenzyme shows the same spectrum, solvent flavin accessibility at position 8 and tight coenzyme binding of the native dimeric enzyme [9].

The 38.3 kDa proteolysed enzyme is still active (specific activity on D-alanine and D-proline was about 90 % of the native DAAO), but a different substrate specificity was observed. Of interest was the decrease in relative activity with D-valine and, in particular, the increase with basic amino acids as substrate (Tables 2 and 3) [6]. At present, two different hypotheses can be speculatively drawn for this observation: (a) proteolysis, removing positively charged groups, weakens the electrostatic repulsion counteracting the accessibility of basic substrates to active site; (b) the accessibility of basic substrates to enzyme active site is easier in the monomeric proteolysed enzyme than in the dimeric intact protein, owing to a conformational effect (this point requires further investigation). The purified proteolysed enzyme conserved all the residues considered important for the active site and particularly, in the 5 kDa polypeptide, a histidine residue homologous with His^{307} of pig kidney DAAO; this residue has been proposed, by site-directed mutagenesis and D-propargylglycine chemical modification, to be involved in the kinetic mechanism of this flavo-oxidase [35–37].

The monomeric proteolysed enzyme form showed a lower thermostability than the native dimeric DAAO (Table 4). The difference in the activation energy for the thermoinactivation process corresponds to a free-energy value lower than the energy of one hydrogen bond, confirming that, in this case also, weak forces contribute significantly to the stabilization or destabilization of a globular molecule [38]. From these results a stabilizing function of the dimerization process can be speculatively proposed for the yeast DAAO, since neither the catalytic activity nor the coenzyme binding was affected by the dissociation.

In addition, FAD binding appears to play a stabilization role in protein conformation, as has already been reported [4].

The results of trypsin limited digestion obtained with *R. gracilis* DAAO differ significantly from those reported for the enzyme purified from pig kidney [13] and *T. variabilis* DAAO [28], which both are more susceptible to proteolytic degradation/inactivation. A more compactly folded conformation can be thus inferred for the native *R. gracilis* enzyme, with a limited presence of exposed and flexible loops. The reported differences in the primary structure (see above) in this context may exert an important role. A precise knowledge of the three-dimensional structure of this model flavo-oxidase must await the X-ray crystallographic analysis.

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