Limited proteolysis and site-directed mutagenesis reveal the origin of microheterogeneity in *Rhodotorula gracilis* D-amino acid oxidase

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When analysed by isoelectric focusing, D-amino acid oxidase from the yeast *Rhodotorula gracilis* normally consists of three molecular isoforms (pI 7.8, 7.4 and 7.2, respectively) all with the same N-terminal sequence. However, only a single band of pI 7.8 is detected with the recombinant wild-type protein expressed in *E. coli*. To determine whether the molecular basis of this heterogeneity is due to proteolysed forms of the protein, we treated *R. gracilis* D-amino acid oxidase with various proteases. Limited proteolysis by chymotrypsin and thermolysin produced truncated and nicked monomeric holoenzymes containing two polypeptides of ≈ 34 kDa (Met¹-Leu³¹²) and one of ≈ 5 kDa (Ala³¹⁹-Arg³⁶⁴ with chymotrypsin or Ala³¹⁹-Ala³⁶² with thermolysin). On the other hand, treatment with endoproteinase Glu-C gave a dimeric holoenzyme lacking the C-terminal SKL tri-

peptide. This cleavage of Glu³⁶⁵-Ser³⁶⁶ peptide bond caused the disappearance of the three isoelectric bands and a single homogeneous band (pI 7.2) appeared. To study this protein form, we used site-directed mutagenesis to produce a mutant form of *R. gracilis* D-amino acid oxidase lacking the SKL C-terminal tripeptide (which is the targeting sequence PTS1 for peroxisomal proteins). As expected, the SKL-deleted mutant gave a single band (pI 7.2) in isoelectric focusing. The three-band pattern of native yeast enzyme was generated by *in vitro* experiments using an equimolar mixture of the wild-type (pI 7.8) and the SKL-deleted recombinant (pI 7.2) DAAOs. The microheterogeneity of yeast DAAO thus stems from the association of two polypeptide chains differing in the C-terminal tripeptide, giving three different holoenzyme dimers.

INTRODUCTION

D-Amino acid oxidase (DAAO, EC 1.4.3.3) catalyses the oxidative deamination of D-amino acids to give the corresponding 2-oxo-acids, hydrogen peroxide and ammonia. DAAO from the red yeast *Rhodotorula gracilis* (RgDAAO) in its native form is a stable 80 kDa dimer of identical subunits, each subunit containing one FAD molecule. The corresponding apoprotein is entirely present as a 40 kDa monomer. This aggregation state is not dependent on a polymerization equilibrium [1]. The yeast DAAO differs considerably from the pig kidney enzyme, in that it has very high catalytic efficiency, tightly binds FAD and exists as a stable dimer (for a review see [2]).

RgDAAO is a peroxisomal flavoenzyme whose presence in yeast cells can be induced by growth on media containing D-amino acids as carbon and/or nitrogen source [3]. Induction of DAAO synthesis is associated with a 241% increase of peroxisome volume density and a 31% increase of peroxisome size, relative to cells grown on basal medium [4]. Peroxisomal proteins contain signals that are required for targeting to peroxisome and for interacting with an import machinery. For many soluble matrix peroxisomal proteins the signal is the Ser-Lys-Leu carboxy-terminal tripeptide motif (PTS1) but a subset of proteins utilizes a cleavable amino-terminal cleavable sequence (PTS2) (for a review see McNew and Goodman [5]). All known DAAO sequences employ a PTS1 signal for peroxisome import (SKL or its variants) [6]. Recent evidence suggests that proteins may be translocated into peroxisomal matrix in a folded or oligomeric state and that a subunit lacking the PTS1 sequence can be targeted to peroxisome by a carrier subunit or protein having the PTS1 sequence [5].

Isoelectric-focusing (IEF) studies on DAAO proteins revealed in pig kidney DAAO the presence of two bands at pI 7.0 and 7.2, the latter pI in good agreement with the theoretical value calculated from amino acid sequence [7]. In *Trigonopsis variabilis* DAAO a single active band at pI 5.1 was instead detected [8]. RgDAAO gives three bands on IEF (pI 7.8, 7.4 and 7.2) all with the same N-terminal sequence.

We report here our analysis of the products of limited proteolysis of RgDAAO with three different proteases, and the results of site-directed mutagenesis experiments with a recombinant form of DAAO lacking the C-terminal SKL tripeptide. Our aim was to account for the isoelectric heterogeneity of RgDAAO. Chymotrypsin and thermolysin proteolysis gave truncated and nicked monomeric holoenzymes, while only treatment with endoproteinase Glu-C afforded a single isoform from which the C-terminus SKL tripeptide had been cleaved. Recombination experiments with recombinant wild-type (which has a single isoform) and SKL-deleted DAAOs revealed that RgDAAO dimers are built up by two subunits differing by the C-terminal three residues. Free association of these two subunits yields three different dimers, thus explaining RgDAAO microheterogeneity.

MATERIALS AND METHODS

Materials

Sequencing-grade chymotrypsin A4, *Bacillus thermoproteolyticus* thermolysin, *Staphylococcus aureus* V8 endoproteinase Glu-C and restriction enzymes were from Boehringer Mannheim (Germany). Superose 12 HR 10/30 column was from Pharmacia

Abbreviations used: DAAO, D-amino acid oxidase; RgDAAO, *Rhodotorula gracilis* D-amino acid oxidase; Holo-Bz, DAAO:benzoate complex; Δ SKL, SKL-deleted DAAO chain; IEF, isoelectric focusing; PTS1, peroxisomal targeting signal type 1; TB, terrific broth; IPTG, isopropyl β -D-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight spectrometry.

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LKB (Sweden). Pro-blot membranes were from Applied Biosystems. LMW and HMW standard proteins were from Sigma Chemical Co. The pKK223-3 plasmid was from Pharmacia; pAlter plasmid was from Promega Co. Other reagents were of analytical grade.

D-Amino acid oxidase was purified according to Pollegioni and Pilone [9] from *R. gracilis* cells (ATCC 26217) grown to late logarithmic phase in synthetic medium at pH 5.6 containing 30 mM D-alanine [3]. The pure enzyme had a A_{274}/A_{455} ratio of 8.2 and a specific activity on D-alanine as substrate of 120 U/mg protein at 25 °C; enzyme concentration was determined as flavin content using $\epsilon_{455} = 12600 \text{ M}^{-1} \text{ cm}^{-1}$ [1]. The apoprotein of RgDAAO was prepared by dialysis against 250 mM potassium phosphate buffer, pH 7.5, containing 2 M KBr, 20% (v/v) glycerol, 0.3 mM EDTA and 5 mM 2-mercaptoethanol, according to the procedure described in [10].

Enzyme activity assay

Enzyme activity was assayed polarographically measuring oxygen consumption with 28 mM D-alanine as substrate [11] or, alternatively, spectrophotometrically following the increase in absorbance at 243 nm with 25 mM D-phenylglycine as substrate, both at 25 °C [12]. Steady-state kinetic measurements were carried out polarographically in 75 mM sodium pyrophosphate buffer, pH 8.5, at 25 °C, according to [13].

Limited proteolysis and isolation of proteolysis products

6.5 μM Holo- or apo-DAAO was incubated at 25 °C in 10 mM sodium pyrophosphate buffer, pH 8.3, in the presence of 10% (w/w) chymotrypsin, or in 50 mM Hepes/KOH buffer, pH 7.5, and 2 mM calcium acetate with thermolysin, or in 50 mM potassium phosphate and 50 mM ammonium bicarbonate buffer pH 7.8, with endoproteinase Glu-C. For the proteolysis of 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. For electrophoretic analysis samples (8–10 μg of DAAO) were diluted in SDS-sample buffer and then heated at 100 °C for 3 min. Kinetic data on inactivation, loss and formation of individual fragment were best fitted with a single or double exponential decay equations using Grafit (Erythacus Software) data analysis program.

The proteolysis products were purified by FPLC chromatography on a Superose 12 column equilibrated in 20 mM potassium phosphate buffer, pH 7.5, containing 250 mM NaCl, 0.3 mM EDTA, 5 mM 2-mercaptoethanol and 10% (v/v) glycerol.

Structural studies

Analytical SDS/PAGE was carried out using either 10% or linear gradient-pore gel slabs described by O'Farrell [14]. Gels were stained for protein content with Coomassie Blue R-250 and silver-binding methods or for DAAO activity [11]. Densitometric measurements of the stained gels were obtained with an LKB 2202 Ultrosan laser densitometer at 630 nm interfaced with an Apple personal computer. Non-denaturing isoelectric focusing was carried out in polyacrylamide gel slabs in 1% (v/v) Ampholytes (Pharmalyte, Pharmacia) over a pH range of 3.5–10.0 at 10 °C [15]. Denaturing IEF was performed in 5% polyacrylamide gel containing 8 M urea, 2.5% (w/v) Ampholytes pH 5–8 and 1% (v/v) Triton X-100 [16]. Theoretical isoelectric points were

calculated with the Isoelectric program included in the GCG software package (Genetics Computer Groups, Inc.).

N-terminal sequences were determined both on aqueous protein sample and on Pro-blot electrotransferred samples [17] using an automated Protein Sequencer (Applied Biosystems Model 492 Procise). Matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry was performed using a Voyager Biospectrometry Workstation (Perspective Biosystems, Vestec Mass Spectrometry Products).

Glycosylation analysis of RgDAAO samples was performed using the DIG glycan ELISA kit (Boehringer Mannheim); protein concentration ranged from 50 to 2500 ng/well. The glycoprotein fetuin and recombinant creatinase from *E. coli* were used as positive and negative controls, respectively.

Thermal inactivation

DAAO samples (3.2 μM) in 50 mM potassium phosphate buffer, pH 7.5, containing 10% (v/v) glycerol, 1 mM EDTA and 5 mM 2-mercaptoethanol were heated over a temperature range of 20–45 °C. Aliquots were withdrawn at different times and assayed for DAAO activity. Rate constants, determined from the slope of first-order inactivation plot, were used to calculate activation free energy of inactivation ($\Delta G^\ddagger$). The activation energy (E_{act}) for thermal inactivation was obtained from the slope of the Arrhenius plot and used to calculate enthalpy ($\Delta H^\ddagger$) and entropy ($\Delta S^\ddagger$) values at each temperature [18].

Site-directed mutagenesis

Site-directed mutagenesis was performed by subcloning the *EcoRI* fragment of DAAO cDNA from pKKDAAO plasmid ($\approx 1.2 \text{ kb}$) [19] into the *EcoRI* site of the multiple cloning site of pAlter-Ex2 vector, a plasmid containing tetracycline resistance and inactivated chloramphenicol resistance genes. A mutant form of DAAO lacking the C-terminal SKL-tripeptide was constructed by replacing the codon for Ser³⁶⁶ (TCG) with an amber stop codon (TAG) using the Altered Sites II kit (Promega) and the 5'-GCGGGAGTAGAAGTTGTA-3' mutagenic oligonucleotide. Plasmid purified from the repair-defective strain was transformed into JM109 cells and the mutation was confirmed by automated nucleotide sequencing. DNA preparation and manipulation was performed as detailed in [20].

Expression in *E. coli* and purification of recombinant SKL-deleted mutant DAAO

A pKKDAAO(ΔSKL) plasmid encoding SKL-deleted DAAO was obtained by recovering the mutant cDNA from pAlter plasmid using *EcoRI* restriction sites. The SKL-deleted DAAO cDNA was expressed by insertion into the *EcoRI* site of the multiple cloning site of pKK223-3 plasmid under the p_{tac} promoter, similarly to the wild-type cDNA [19]. The direction of insertion was checked by restriction analysis. The recombinant wild-type and SKL-deleted DAAOs were purified from transformed JM109 *E. coli* cells grown at 37 °C on TB medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin and induced with 1 mM IPTG, using the procedure of Pollegioni et al. [19].

RESULTS

Electrophoretic analysis of RgDAAO

DAAO purified from *R. gracilis* cells had a single homogeneous N-terminal sequence, and in SDS/PAGE gave a single band of 40 kDa, as reported previously [11]. No degradation pattern was ever observed. In IEF analysis the enzyme gave a three-band

Figure 1 IEF analysis of different DAAO forms. (A) Recombination of wild-type and SKL-deleted recombinant DAAO investigated by non-denaturing IEF; (B) denaturing IEF

Lanes 1 and 5: wild-type recombinant DAAO (6 µg); lanes 2 and 7: SKL-deleted DAAO (6 µg); lane 3: mixture of wild-type and SKL-deleted DAAO; lanes 4 and 6: DAAO from *R. gracilis* (6 µg). (A) DAAO activity staining; (B) Coomassie Blue staining for total proteins.

pattern (pI 7.8, 7.4 and 7.2) (Figure 1A). Since all three bands stained for activity, with or without exogenous FAD (data not shown), the three isoforms are functional holoenzymes. This molecular heterogeneity is not due to glycosylation, as no carbohydrate content was detected by the DIG glycan ELISA

kit. Interestingly, wild-type recombinant DAAO from *E. coli* gave a single band of pI 7.8 in IEF (Figure 1A).

Limited proteolysis of RgDAAO

As a first approach, protease digestion was employed to reveal a possible degradation pathway producing the three-band pattern in isoelectric focusing. The aim was to distinguish between post-translational amino acid modification and proteolytic processing.

Chymotrypsin and thermolysin

The time course of limited chymotrypsin or thermolysin digestion of holo-, apo- and holo-benzoate complex of RgDAAO at 25 °C resulted in a biphasic loss of catalytic activity (Table 1). The first phase led to a 18% inactivation. Proteolysis was followed by SDS/PAGE which in all cases showed progressive conversion of the band at 40 kDa (corresponding to the intact DAAO monomer) to a band of electrophoretic mobility corresponding to a molecular mass of ≈ 34 kDa plus an additional peptide of a molecular mass ≈ 5 kDa. Gels stained with Coomassie Blue and silver-staining methods showed identical electrophoretic patterns. The rates of degradation and accumulation were calculated from densitometric analyses of SDS/PAGE gels: degradation of the 40 kDa form (a single exponential process) correlated with the formation of the 34 kDa band; the second phase of inactivation corresponded to the degradation rate of the 34 kDa band, with the formation of a transient band at 31 kDa, thus indicating the catalytic competence of the first proteolysis product (Table 1 and Scheme 1). N-terminal sequence analysis confirmed the amount of the different protein forms during proteolysis as isolated by SDS/PAGE (see also below).

Digestion of the holoenzyme–benzoate complex with thermolysin or chymotrypsin resulted in the complete disappearance of the 40 kDa band corresponding to intact DAAO and the concomitant formation of a 34 kDa band. The stabilizing effect of benzoate on the 34 kDa polypeptide is explained by an increase in the k_1/k_2 ratio due to a slowing down of the second phase of inactivation. On the other hand, DAAO apoprotein was completely degraded by chymotrypsin and thermolysin and rapidly proteolysed, resulting in the complete disappearance of the 40 kDa band in ≈ 10 min with a transient appearance of a faint band of ≈ 34 kDa and the complete loss of enzyme activity (Table 1).

The proteolytic products after treatment of the holoenzyme–

Table 1 Kinetics of inactivation and proteolysis of *R. gracilis* DAAO with various proteases at 25 °C

Holo, RgDAAO holoenzyme; HoloBz, RgDAAO:benzoate complex; Apo, RgDAAO apoprotein. N.D., not determined. All values are expressed as $\text{min}^{-1} \times 10^{-3}$.

Proteolytic enzyme	Inactivation		40 kDa	34 kDa	
	k_1	k_2	Degradation (k_1)	Formation (k_1)	Degradation (k_2)
Chymotrypsin (10%)					
Holo	28.3 ± 7.3	10.6 ± 1.3	27.0 ± 3.9	30.7 ± 2.7	10.0 ± 1.6
HoloBz	N.D.	3.1 ± 0.2	33.1 ± 5.0	28.2 ± 13.2	3.4 ± 1.2
Apo	739 ± 171	39.0 ± 3.3	455 ± 120	599 ± 216	63.9 ± 12.1
Thermolysin (3%)					
Holo	14.7 ± 3.4	1.0 ± 0.8	17.0 ± 3.1	18 ± 2.2	0.7 ± 0.5
HoloBz	19.1 ± 9.3	0.58 ± 0.26	12 ± 7	17 ± 5	N.D.
Apo	120 ± 37	35 ± 6.1	N.D.	N.D.	N.D.
Endoproteinase Glu-C (3%)					
Holo	Rapid loss of ≈ 10 –20% of the initial activity in the dead time of the experiment				
HoloBz	Rapid loss of ≈ 10 –20% of the initial activity in the dead time of the experiment

Scheme 1 Diagram illustrating degradation pattern of *R. gracilis* DAAO with chymotrypsin, thermolysin and endoproteinase Glu-C

This figure is based on the kinetics of degradation and formation of polypeptides as determined by migration in SDS/PAGE and N-terminal sequence analysis. In the absence of sufficient information, the sites of cleavage were tentatively localized from the estimated mass of the polypeptides (square brackets). The polypeptides with star were found associated under non-denaturing conditions (i.e. gel filtration chromatography analysis).

Table 2 Peptides obtained by limited proteolysis of *R. gracilis* DAAO at 25 °C

The NH₂-terminal residue numbers were assigned by comparison to the total protein sequence [6]. GF, FPLC gel filtration chromatography on Superose 12; MS, MALDI-TOF mass spectrometry.

Proteolytic enzyme	Apparent mass (kDa)			NH ₂ -terminal sequence	NH ₂ -terminal residue (no.)	Possible fragment (theoretical mass, kDa)
	GF (aggregation state)	SDS/PAGE	MS			
Chymotrypsin	37.1 ± 2.3 (monomeric)	33.5 ± 0.3 ≈ 5	≈ 34.0 5.051 ± 0.010	MHSQKR... 1	1	Met ¹ -Leu ³¹² (34.037)
Thermolysin	37.1 ± 2.4 (monomeric)	33.75 ± 0.2 ≈ 5		AAKEKEV... 319	319	Ala ³¹⁹ -Arg ³⁶⁴ (5.015)
				MHSQKR... 1	1	Met ¹ -Leu ³¹² (34.037)
Endoproteinase Glu-C	78.0 ± 1.0 (dimeric)	39.5 ± 0.3	39.745 ± 0.007	MHSQKR... 1	1	Ala ³¹⁹ -Ala ³⁶² (4.787)
				MHSQKR... 1	1	Met ¹ -Glu ³⁶⁵ (39.748)
	+ SDS during boiling	35 ± 0.4		MHSQKR... 1	1	Met ¹ -Glu ³²² (35.021)
		32.5 ± 0.5 18 ± 1.0		MHSQKR... 1	1	Met ¹ -Asp ²⁹⁷ (32.348)
Native RgDAAO	80.0 ± 1.2 (dimeric)	40.0 ± 0.1	40.058 ± 0.011	MHSQKR... 1	1	Met ¹ -Asp ¹⁵⁸ (17.632) or Met ¹ -Asp ¹⁶⁶ (18.575)
				MHSQKR... 1	1	Met ¹ -Leu ³⁶⁸ (40.076)
Recombinant SKL-deleted DAAO	79.0 ± 0.4 (dimeric)	40.0 ± 0.2	39.763 ± 0.017	MHSQKR... 1	1	Met ¹ -Glu ³⁶⁵ (39.745)

benzoate complex with 10 % (w/w) chymotrypsin for 240 min, or with 3 % (w/w) thermolysin for 180 min, both at 25 °C, were purified by gel filtration chromatography. In both cases DAAO was largely present in a peak corresponding to a molecular mass of 37.1 ± 2.3 kDa (≈ 50 % protein recovery), i.e. in a monomeric aggregation state. SDS/PAGE of this product on an 8–22 % polyacrylamide gradient revealed the presence of the ≈ 34.0 kDa

band plus a band of low molecular weight (*M_r* < 6000), indicating the production of a nicked proteolysed protein under non-denaturing conditions. This finding was confirmed by N-terminal sequence analysis of soluble proteolysed DAAOs after gel filtration, where two equimolar sequences were detected simultaneously starting respectively from the native N-terminal methionine and Ala³¹⁹ (Table 2). Hence, the N-terminal sequence

Figure 2 SDS/PAGE of the proteolytic products obtained by limited proteolysis of *R. gracilis* D-amino acid oxidase with chymotrypsin (lane 4), endoproteinase Glu-C (lane 5) and thermolysin (lane 6)

Each well contained 3 μ g of protein purified by Superose 12 gel filtration chromatography after protease treatments of RgDAAO. Lanes 1 and 7: HMW and LMW standard proteins; lanes 2 and 3: 7 μ g of RgDAAO.

Table 3 Spectral and kinetic properties on D-alanine as substrate of native, proteolysed and recombinant wild-type and SKL-deleted DAAOs (at 25 °C and pH 8.5)

	$V_{\max}$ (μ mol O ₂ /min mg E)	K_m Ala (mM ⁻¹)	A_{278}/A_{455} ratio
<i>R. gracilis</i> DAAO*	125 $\pm$ 2.9	0.83 $\pm$ 0.14	8.2
Wild-type DAAO†	126 $\pm$ 2.4	0.97 $\pm$ 0.18	8.2
Glu C-treated RgDAAO	105 $\pm$ 8.5	0.76 $\pm$ 0.21	8.7
SKL-deleted DAAO	103 $\pm$ 3.1	0.70 $\pm$ 0.05	8.4
Chymotrypsin or thermolysin-treated DAAO	120 $\pm$ 4.9	0.86 $\pm$ 0.12	9.6

* [12].

† [19].

of the 34 kDa fragment, separated by SDS/PAGE (Figure 2) and electroblotted on a Pro-blot membrane, matched the native N-terminus (Table 2 and Scheme 1). MALDI-TOF mass spectrometry of the proteolysis product after chymotrypsin digestion showed two polypeptides of $\approx$ 34.0 and 5.051 kDa; values corresponding to the molecular weights of the Met¹-Leu³¹² and Ala³¹⁹-Arg³⁶⁴ sequences, respectively. These results indicate the production of a monomeric nicked and truncated holoenzyme of about 39 kDa, as reported also for the proteolytic product obtained under similar experimental conditions with trypsin [21].

The nicked and truncated DAAO forms possessed spectral and kinetic properties similar to native RgDAAO (Table 3). The proteolysed enzyme obtained with thermolysin showed a doubled reactivity towards basic substrates, slight changes for substrates with apolar side chain (with D-methionine as the 'best' substrate) and confirmed the lack of reactivity towards acid substrates (data not shown). In all cases, the proteolysed DAAOs did not require exogenous FAD in the assay mixture for maximal activity, showing that the slightly higher spectral ratio (see Table 3) was not dependent on loss of coenzyme.

Endoproteinase Glu-C

Treatment of RgDAAO holoenzyme with 3, 8 and 15% (w/w) endoproteinase Glu-C in phosphate or bicarbonate buffer, pH 7.8, caused in all cases $\approx$ 20% loss of activity. The rate of this limited deactivation could not be determined as it was completed in 1–2 min at 25, 15 and 4 °C (Table 1). SDS/PAGE analysis of the time course of the reaction showed the conversion of the 40 kDa band to a single fragment of slightly higher mobility when SDS was added to the reaction mixture after boiling, and production of molecular species with lower molecular weight (35, 32.5 and 18.5 kDa) when SDS was added to the reaction mixture before boiling. All these proteolytic products had the same N-terminus (starting from Met¹) (Scheme 1 and Table 2). Changes in reaction conditions (protease concentration, presence of benzoate, use of carbonate buffer) did not affect the kinetics of inactivation or the electrophoretic pattern of the products.

DAAO digested with 8% (w/w) endoproteinase Glu-C for 180 min at 25 °C was applied to a Superose 12 column. The protein eluted in a single peak of a molecular mass of $\approx$ 78.0 $\pm$ 1.0 kDa which gave a single band by SDS/PAGE having a slightly lower molecular mass than the native DAAO ($\approx$ 39.5 kDa) (Figure 2). This sample, recovered after chromatography with 80% yield, had an N-terminal sequence identical to that of the native DAAO and in MALDI-TOF mass spectrometry gave a mass of 39745 $\pm$ 7 Da, corresponding to a polypeptide lacking the SKL C-terminal tripeptide (Table 3). This sample had spectral properties closely resembling the native enzyme and possessed a specific activity of 107 U/mg with D-alanine as substrate, at 25 °C (Table 3); it did not require exogenous FAD for maximal activity. When this Glu-C treated DAAO was analysed in non-denaturing IEF it focused as a single active band of pI 7.2 (Figure 1A). Therefore, treatment of native RgDAAO with endoproteinase Glu-C eliminates iso-electrofocusing microheterogeneity.

Expression and biochemical characterization of an SKL-deleted mutant DAAO

In order to study the effect of the C-terminal tripeptide SKL on the heterogeneity of DAAO, we planned to use for recombination experiments a mutant DAAO form lacking the PTS1 tripeptide. The mutant DAAO was obtained by replacing the TCG codon for Ser³⁶⁶ with a TAG stop codon. The expression system and the purification procedure previously used for the wild-type enzyme was used to obtain the mutant DAAO [19]. The SKL-deleted DAAO was expressed in a completely soluble form and was purified to homogeneity in $\approx$ 30% yield (corresponding to $\approx$ 0.25 mg of pure DAAO per gram of *E. coli* cells).

The spectrum of the recombinant SKL-deleted DAAO in the oxidized form had absorbance maxima at 455, 378 and 278 nm and a A_{278}/A_{455} ratio of 8.4. The kinetic properties of wild-type and SKL-deleted recombinant enzymes on D-alanine as substrate are shown in Table 3 in comparison to DAAO purified from *R. gracilis*. In gel filtration chromatography, both recombinant enzymes eluted in a single symmetric peak corresponding to a molecular mass of $\approx$ 80 kDa, i.e. in a dimeric aggregation state; whereas mass spectrometry revealed a 295 Da difference in mass, corresponding to the mass of the missing SKL C-terminal tripeptide (Table 2).

Thermal inactivation of wild-type and SKL-deleted DAAOs followed first-order kinetics at all temperatures tested (20–45 °C range) (data not shown). The activation energy values and other thermodynamic parameters for the thermoinactivation of

both recombinant proteins were similar ($E_{act} = 235.5$ and 239.3 kJ/mol, $\Delta S^\ddagger = 0.453$ and 0.483 kJ/mol·K, $\Delta H^\ddagger = 232.7$ and 236.7 kJ/mol for SKL-deleted and wild-type DAAOs, respectively). This result indicates that SKL-deletion does not change the thermostability.

Recombination studies

Treatment of RgDAAO with endoproteinase Glu-C converted the three band isoelectric pattern of the native RgDAAO (pI 7.8, 7.4 and 7.2) (Figure 1A) into a proteolytic product with a single band at pI of 7.2 (see above). This result, according to the structural studies, supports the hypothesis that the heterogeneity of RgDAAO could be ascribed to sequence differences in its C-terminal portion. Recombinant wild-type and SKL-deleted mutant DAAOs also gave single active bands in IEF under non-denaturing conditions, at pI 7.8 and 7.2, respectively (lanes 1 and 2, Figure 1A). These bands correspond to the two extreme bands observed for the native RgDAAO (lane 4, Figure 1A). The three-band pattern of the latter enzyme form was restored by mixing equimolar amounts (0.15 nmol) of wild-type and SKL-deleted recombinant DAAOs, for 16 h at 4 °C (lane 3, Figure 1A).

Under denaturing conditions, isoelectric focusing of RgDAAO produced two bands (pI 7.9 and 8.1), while the recombinant enzymes gave single bands (pI of 8.1 for the wild type and 7.9 for the SKL-deleted enzyme) (Figure 1B). This difference in isoelectric point (≈ 0.2 pH unit) agrees with the theoretically calculated difference resulting from removal of the C-terminal SKL tripeptide. From these results, we conclude that molecular microheterogeneity of RgDAAO is due to the formation of three dimeric holoenzymes from two polypeptide chains differing only in the presence of the three C-terminal residues.

DISCUSSION

In the present study a combination of limited proteolysis experiments using various proteases (namely chymotrypsin, thermolysin and endoproteinase Glu-C) and site-directed mutagenesis has been used to investigate the molecular heterogeneity of RgDAAO. Limited proteolysis experiments demonstrate that this enzyme is sensitive to proteolysis only in the C-terminal portion of the protein, indicating that most of the rest of the protein is folded into a compact structure not accessible to proteolytic enzymes. Cleavage most consistently occurred at sites located in the proximity of residue(s) Leu³¹² and Arg³⁶⁴. This is a major difference compared to mammalian DAAO, where transient degradation bands were reported during limited trypsinolysis [22] and to *T. variabilis* DAAO which during the purification is extensively proteolysed to two peptides of 27 and 12 kDa, which remain bound to each other and active [23]. RgDAAO is less susceptible to proteolysis in the central part of the protein and this only occurs during the second phase of inactivation with chymotrypsin and thermolysin (probably at Val²⁷⁷ yielding a 31 kDa polypeptide) when it is accompanied by the total cleavage and inactivation of the enzyme. Limited chymotrypsin and thermolysin proteolysis gave two larger fragments that remain associated, yielding a nicked and truncated enzyme form (Scheme 1) together with detachment of the Gly³¹³-Arg³¹⁸ and Glu³⁶⁵-Leu³⁶⁸ peptides. This treatment resulted in a shift from the dimeric to monomeric form. The missing Gly³¹³-Arg³¹⁸ fragment is therefore essential for monomer-monomer interaction and the dimerization of the yeast enzyme. It is noteworthy that the Ser³⁰⁸-Lys³²¹ sequence, a positively charged region containing the lost fragment, corresponds to a sequence that is not present in other DAAOs [6].

The carboxy-terminal peptide (E)SKL was cleaved off by all the proteases tested. It contains the PTS1 recognition sequence for importing protein into peroxisomes [24]. Interestingly, also in the 3D-structure of the mammalian enzyme the carboxy-terminal tail of pig kidney DAAO is not solved, indicating that it is highly mobile [25,26].

The ≈ 39 kDa holoenzyme purified by gel filtration chromatography following chymotrypsin and thermolysin proteolysis retained the spectral properties of native enzyme [1] and was normally active on D-alanine but there was a two-fold increase in catalytic efficiency with basic amino acids as substrate, as also reported for the product obtained after limited trypsinolysis [21]. At the present, two different hypotheses can be speculatively drawn for this observation: (a) by removing positively charged groups, proteolysis weakens the electrostatic repulsion that inhibits the accessibility of basic substrates to the active site; (b) basic substrates may access the enzyme active site more easily in the monomeric proteolysed enzyme than in the dimeric intact protein due to a conformational effect. Clearly, this intriguing point requires further investigation.

Benzoate induces a conformational change which partly protected the nicked and truncated ≈ 39 kDa product from further proteolytic inactivation and degradation as indicated by the slowing down of the second phase of inactivation and degradation of the 34 kDa fragment in the presence of the competitive inhibitor (Table 1). The access to the inhibitor-binding site in the 3D-structure of pig kidney DAAO [25] is blocked by a lid formed by the loop between β strands β I5 and β I6, highly susceptible to trypsinolysis in the absence of benzoate. Benzoate presence fully protected pig kidney DAAO (no cleavage at site Arg²²¹) [22], while in RgDAAO benzoate does not protect totally against proteolysis. This indicates a major difference between the structure of the yeast and mammalian DAAOs, as also indicated by differences in the rate-limiting step in the catalytic pathway. The limiting step is in the reductive half-reaction for the yeast DAAO, whereas for the mammalian enzyme it is represented by product release, probably reflecting lid movement [27,28]. From these considerations we can conclude that the native RgDAAO has a more compactly folded conformation than the *T. variabilis* and pig kidney enzymes, with limited presence of exposed and flexible loops.

An important finding of this paper is the different proteolysis pattern obtained using endoproteinase Glu-G, which resulted in a product missing the SKL C-terminal, characterized by a dimeric aggregation state and a single active band on isoelectric focusing (Scheme 1 and Table 2). A DAAO form lacking the Ser-Lys-Leu C-terminal tripeptide has been also obtained by site-directed mutagenesis of the recently cloned RgDAAO cDNA [19]. The mutant enzyme was also dimeric, fully active, stable and had a tightly bound FAD, allowing exclusion of the possibilities that the SKL sequence is involved in catalysis, coenzyme binding or thermostabilization. Furthermore, expression of the SKL-deleted DAAO in *E. coli* as a dimeric holoenzyme allowed us to exclude definitively the involvement of the PTS1 sequence in dimerization, thus pointing to a crucial role in this process of the Gly³¹³-Arg³¹⁸ region.

The IEF heterogeneity of RgDAAO was restored by mixing the homogeneous wild-type enzyme with the SKL-deleted recombinant DAAO (Figure 1). This result indicates that heterogeneity is not due to post-translational covalent modification of amino acid side chains, but indicates post-translational protease processing. In fact, DAAO purified from *R. gracilis* appears to be a mixture of two homodimers (i.e. w/w and Δ SKL- Δ SKL, having identical chains of 368 or 365 amino acids, respectively) and one heterodimer (w/ Δ SKL). The three forms result from an equi-

librium between the two different DAAO polypeptide chains differing for the PTS1 sequence. The SKL C-terminal is solvent exposed, as it is easily removed by proteases; we also conclude that it is on the protein surface because it contributes significantly to the overall surface charge of the enzyme.

Further investigations are in progress by *in vivo* expression experiments using yeast expression vectors for wild-type, SKL-deleted and a monomeric mutant of RgDAAO. The aim is to identify the fate of the different forms of this flavoenzyme in yeast cells and to determine whether RgDAAO, as well as other multimeric complexes, can be imported into peroxisome even if one of the constituents lacks the peroxisomal targeting signal.

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