Conversion of the dimeric D-amino acid oxidase from *Rhodotorula gracilis* to a monomeric form. A rational mutagenesis approach

Luciano Piubelli*, Laura Caldinelli, Gianluca Molla, Mirella S. Pilone, Loredano Pollegioni

Department of Structural and Functional Biology, University of Insubria, Via J.H. Dunant 3, 21100 Varese, Italy

Received 21 May 2002; revised 8 July 2002; accepted 15 July 2002

First published online 30 July 2002

Edited by Hans Eklund

Abstract The relevance of the dimeric state for the structure/function relationships of *Rhodotorula gracilis* D-amino acid oxidase (RgDAAO) holoenzyme has been investigated by rational mutagenesis. Deletion of 14 amino acids in a surface loop (connecting β-strands 12 and 13) transforms RgDAAO from a dimeric protein into a stable monomer. The mutant enzyme is still catalytically competent and retains its binding with the FAD coenzyme. Dimerization has been used by this flavoenzyme in evolution to achieve maximal activity, a tighter interaction between the protein moiety and the coenzyme, and higher thermal stability.

Key words: Flavoprotein; Oligomerization state; Rational design; Functional evolution

1. Introduction

Many proteins in their native condition are constituted by more than a single polypeptide chain. We investigated the role of monomer–monomer interaction by using the enzyme D-amino acid oxidase (DAAO, EC 1.4.3.3) as a model tool. The enzyme catalyzes the dehydrogenation of D-amino acids to the corresponding imino acids, which spontaneously hydrolyze to α-keto acids and NH_4^+. The reduced FAD cofactor is then reoxidized by molecular oxygen. As a result of the wealth of structural and kinetic information obtained about this enzyme (for recent reviews see [1,2]), DAAO has been considered the prototype for the flavin oxidase class. It contains as a coenzyme a non-covalently bound molecule of FAD per 40-kDa protein monomer. The enzyme purified from the yeast *Rhodotorula gracilis* (RgDAAO) shows properties different from those of the pig kidney DAAO (pkDAAO), such as a tight binding of the coenzyme FAD, a high catalytic activity, and a stable dimeric state in solution (whereas the mammalian enzyme is present in solution in an oligomerization state depending on the protein concentration) [2]. Crystals of both pkDAAO and RgDAAO have been obtained, allowing the resolution of their 3D structure [3,4]. pkDAAO is present in the crystallographic unit as a dimer [3]; for it a 'head-to-head' interaction of the subunits, resulting in an elongated dimer of cylindrical shape, was reported [3]. For RgDAAO a mode of dimerization different from that of pkDAAO has been proposed in which a 'head-to-tail' interaction between the two monomers yields a spherical dimer (Fig. 1). The buried surface area in RgDAAO is much wider than that observed in the 'head-to-head' dimer of pkDAAO (3049 $Å^2$ vs. 1512 $Å^2$ in yeast and mammalian, respectively) [5]. This wider interaction area between the two monomers of RgDAAO has been proposed to be mainly due to the presence of a long loop (21 residues, from Pro^{302} to Glu^{322}) connecting β-strands 12 and 13, near the C-terminus (β12–β13 loop). It has been theoretically calculated that eliminating this loop in the proposed model halves the interaction area between the two monomers [5]. This sequence is peculiar to RgDAAO, since it is not conserved in any of the other known DAAOs (Figs. 2 and 3A,C). The proposed model is supported by previous limited proteolysis studies: this loop can be easily cleaved off yielding a monomeric RgDAAO form [6]. The conversion to a monomeric form can also be achieved by removing the coenzyme to yield the corresponding apoprotein form [7].

In order to determine whether the β12–β13 loop plays a role in the dimerization process and affects the dimer stability in RgDAAO, a mutant enzyme was designed, produced, and characterized in which the major part of the β12–β13 loop (from Ser^{308} to, and including, Lys^{321}) was deleted.

2. Materials and methods

2.1. Mutagenesis, enzyme expression and purification, and spectral characterization

The RgDAAO gene fragment containing the desired mutation was generated by mutagenic PCR, performed using *Taq* DNA polymerase and the cDNA of the RgDAAO wild-type as template. The DNA was manipulated as described in [8]. A 205-bp fragment of the RgDAAO wild-type cDNA was amplified using the *Bam*HI oligonucleotide (GGCAGAACGGATCGTCCTGCCTCTCGACCGGACAAAGGAGAAGGAGGTCACGCTTGTGCATGCGTATGGCGC, 72-mer), which carries the desired deletion as an upstream primer, and the *Sph*I oligonucleotide (CGGTCCAGCGGATCCTCAAGCACTGCTTGCG, 31-mer) as a downstream primer. To insert the digested PCR product into the expression vector (pT7.7), the *Bam*HI restriction site of the plasmid was eliminated. The DAAO wild-type cDNA was inserted into the resulting plasmid using the *Eco*RI restriction site, to obtain pT7ΔBam/Hind-DAAOwt. The PCR product was digested with *Bam*HI and *Sph*I and then cloned into the pT7ΔBam/Hind-DAAO vector digested with the same enzymes, obtaining the pT7-ΔLOOP plasmid. The presence of the desired deletion was con-

*Corresponding author. Fax: +39-0332-421-500.
E-mail address: luciano.piubelli@uninsubria.it (L. Piubelli).

Abbreviations: DAAO, D-amino acid oxidase (EC 1.4.3.3); RgDAAO, *Rhodotorula gracilis* D-amino acid oxidase; pkDAAO, pig kidney D-amino acid oxidase

0014-5793/02/$22.00
PII: S0014-5793(02)03111-3

firmed by restriction analysis and DNA sequencing. The plasmid pT7-ΔLOOP was used to transform the *Escherichia coli* BL21(DE3)pLysS used as an expression strain. The recombinant enzyme was purified by a procedure similar to that reported in [9], but replacing hydrophobic interaction chromatography on the phenyl Sepharose CL-4B column with cationic exchange chromatography on a Source 15S column in an ÄKTA system (Amersham Pharmacia Biotech). After chromatography on DEAE–Sepharose, precipitation with ammonium sulfate, and dialysis, the sample was loaded on Source 15S, which was equilibrated with 50 mM MES, pH 6.3, 10% (v/v) glycerol, and 2 mM EDTA. The bound enzyme was then eluted using the same buffer in the presence of 1 M NaCl and the purified enzyme concentrated and desalted using a Sephadex G25 column equilibrated in 50 mM potassium phosphate, pH 7.5, 10% (v/v) glycerol, and 2 mM EDTA. Absorption spectra of the free enzyme and titration experiments with various ligands were carried out in the same buffer at 15°C [9]. The extinction coefficient for the oxidized mutant DAAO was determined at 15°C in the same buffer without EDTA by measuring the change in absorbance after release of the flavin by heat denaturation (an extinction coefficient of 11300 M^{-1} cm^{-1} at 450 nm for free FAD was used). The apoprotein form of the DAAO-ΔLOOP mutant was obtained by dialysis in the presence of a chaotrophic agent, as described in [7].

2.2. Determination of oligomerization state and of isoelectric point

Gel permeation chromatography was carried out using a Superose 12 HR column in an ÄKTA system. The column was equilibrated with 20 mM potassium phosphate, pH 7.5, 0.25 M NaCl, 10% (v/v) glycerol, 0.3 mM EDTA, and 5 mM 2-mercaptoethanol. To determine the molecular mass, a calibration curve was set up using dextran blue (M_r = 2 000 000) and the following standard proteins: cytochrome *c* (M_r = 12 400), carbonic anhydrase (M_r = 29 000), bovine serum albumin (M_r = 66 000), alcohol dehydrogenase (M_r = 150 000), β-amylase (M_r = 200 000). Isoelectrofocusing was carried out in a flat bed apparatus FBE-3000 (Amersham Pharmacia Biotech), using a 6% (w/v) PAA gel slab containing 2.5% (v/v) of both Pharmalyte® 5-8 and Pharmalyte® 3-10 (Amersham Pharmacia Biotech). As anodic solution 1 M H_3PO_4 was used and as catodic solution 1 M NaOH. The gel was stained with Coomassie blue R-250.

2.3. Activity assay, thermal and pH stability and pH activity profiles

DAAO activity was assayed with an oxygen electrode at 25°C as described in [10,11] using 28 mM D-alanine as substrate. To investigate the thermal stability of the DAAO-ΔLOOP, samples of enzyme (1.4 μM) were incubated in 50 mM potassium phosphate, pH 7.5, 10% (v/v) glycerol, and 2 mM EDTA at different temperatures and the residual activity was determined at intervals with the polarographic method as described above. For pH stability, 0.6 μM of mutant DAAO was incubated at 15°C in 15 mM Tris–HCl, 15 mM Na_2CO_3, 15 mM H_3PO_4, and 250 mM KCl, adjusted at the desired pH value, and the residual activity was assayed as reported above. Activity at different pH values was tested in the same buffer as above adjusted at different pH values.

2.4. Limited proteolysis experiments

DAAO-ΔLOOP (0.33 mg/ml) was incubated at 25°C in 100 mM sodium pyrophosphate, pH 8, with 10% (w/w) trypsin, and in the absence or in the presence of 0.2 mM exogenous FAD. For electrophoretic analysis, protein samples (7 μg of DAAO) taken at different times after the addition of trypsin were diluted in the sample buffer for SDS–PAGE and boiled for 3 min. Gels were stained with Coomassie blue R-250 and the intensity of the protein bands were determined by densitometric analysis using Image Master 1D (Amersham Pharmacia Biotech). Kinetic activity was assayed by the same polarographic method described above, using about 0.3 μg of DAAO. Kinetic data of inactivation and of protein fragment formation and degradation were fitted to either a single or a double exponential decay equation using KaleidaGraph™ (Sinergy Software).

3. Results

3.1. Design and production of DAAO-ΔLOOP deletion mutant

The deletion mutant was designed in order to remove a substantial part of the β12–β13 loop (14 amino acid residues, from Ser^{308} to Lys^{321}, Fig. 2). This mutation converts the 21-residue-long loop into a shorter, 7-residue-long loop (from Pro^{302} to Glu^{308}, new numeration, Fig. 2). This mutation also alters the charge distribution in the interaction area because of the loss of three positively charged residues (Arg^{314}, Arg^{318} and Lys^{321}). A model of the 3D structure of the mutant, named DAAO-ΔLOOP, was built with the SWISS MODEL automated protein modeling server [12] on the basis of the amino acid sequence of the designed mutant and the 3D coordinates of the RgDAAO wild-type (PDB code: 1c0p) [4]. The conformation of the altered loop was refined using the software package Swiss-PDBViewer, both by minimizing the energy as computed by partial implementation of the GROMOS force field and by scanning a database of experimentally determined loop structures ([13], URL: http://www.expasy.ch/spdbc/). This analysis showed that the designed loop is still able to connect the residue Lys^{307} with Glu^{322} (about 4 Å apart) without substantially altering the overall 3D structure of the protein (Fig. 3B).

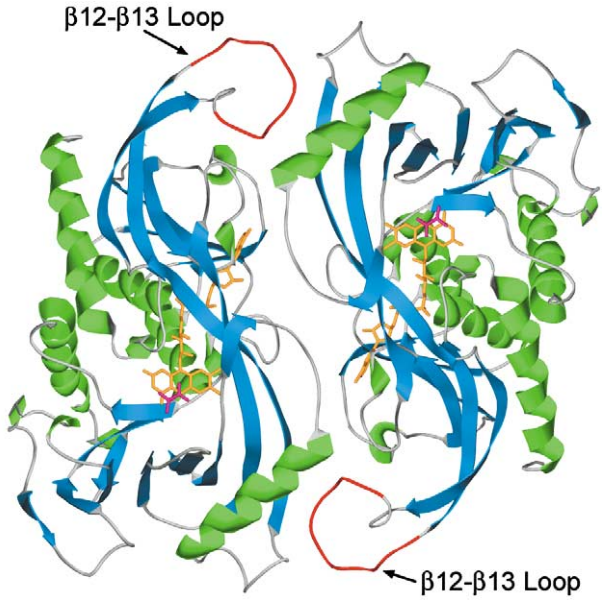


Fig. 1. 'Head-to-tail' mode of monomer–monomer interaction of RgDAAO. The deleted portion of the β12–β13 loop is depicted in red, the flavin cofactor is in yellow and the substrate D-alanine is in magenta.

For both wild-type and ΔLOOP DAAOs, the cloning procedure results in a fusion protein since six additional residues (Met^{-6}-Ala-Arg-Ile-Arg-Leu^{-1}) are added at the N-terminus of the protein before the original starting methionine [9]. In the 3D structure of RgDAAO only two (Arg^{-2} and Leu^{-1}) out of the six residues of this segment can be modelled into the electron density map, the remaining four being in a flexible conformation [4,5]. The presence of this short peptide does not alter the overall properties of the wild-type RgDAAO [9]. The DAAO-ΔLOOP mutant was expressed using the strain BL21(DE3)pLysS *E. coli* as host and purified as reported in [9]; however, the hydrophobic interaction chromatography step was replaced by cationic exchange chromatography. This modification of the original procedure was necessary because, in preliminary experiments, a loss of the FAD coenzyme from the mutant DAAO-ΔLOOP was observed during the hydrophobic interaction chromatography

Rg wt	298	RIVLPLDRTK**SPLSLGRGSARAAK**EKEVTLVHA	330
RgΔLOOP	298	RIVLPLDRTK----------EKEVTLVHA	316
Fs	316	KL----------DDETWIVHN	326
Tv	313	KI----------PGVGFVVHN	323
pk	295	QLRFGSSNTE----------VIHN	308
Hum	295	QLRTGPSNTE----------VIHN	308
Rab	295	QLSAGPSKTE----------VIHN	308
Mou	293	WLRFGSSSAE----------VIHN	306

Fig. 2. Comparison of amino acid sequences of different DAAOs. Rg wt, *R. gracilis* wild-type; Rg ΔLOOP, *R. gracilis* ΔLOOP; Fs, *Fusarium solani*; Tv, *Trigonopsis variabilis*; pk, pig kidney; Hum, man; Rab, rabbit; Mou, mouse. The sequence corresponding to the β12–β13 loop (residues Pro^{302}–Glu^{322}) of the RgDAAO is underlined. The sequence deleted in the mutant DAAO-ΔLOOP is in bold.

on phenyl Sepharose. The overall yield of the purification was of 40–45%, similar to the yield obtained with other RgDAAO forms.

The expressed protein possesses a electrophoretic mobility in SDS–PAGE higher than that of the wild-type RgDAAO, corresponding to an apparent molecular mass of 40.0 ± 1.1 kDa (the theoretical value calculated on the basis of the amino acid sequence is 39 334 Da). The isoelectric point of the mutant was determined under native conditions by isoelectrofocusing and was compared to the value calculated theoretically under denaturing conditions by appropriate software available on the http://www.expasy.ch web site. In both cases, and as expected for the deletion of an exposed and charged loop, the *p*I of the mutant protein was more acidic than that of the wild-type enzyme (8.8 and 7.6 for wild-type and 8.2 and 6.8 for DAAO-ΔLOOP under denaturing and native conditions, respectively). This result suggests that the β12–β13 loop contributes significantly to the overall surface charge in the native DAAO.

3.2. Oligomerization state

The oligomerization state of the ΔLOOP mutant was investigated by gel-filtration chromatography. The elution volume of DAAO-ΔLOOP is significantly different from that of the wild-type (14.0 vs. 12.6 ml, respectively, see Fig. 4). From the elution volume and the calibration curve reported in the inset of Fig. 4, a molecular mass of 37.2 ± 2.2 kDa for the ΔLOOP mutant was estimated, to be in good correspondence with the value determined under denaturing conditions by means of SDS–PAGE. Deletion of the β12–β13 loop clearly produces a monomeric holoenzyme. Analogously to wild-type DAAO [7], the oligomerization state of DAAO-ΔLOOP mutant is not dependent on protein concentration, as demonstrated by performing gel-filtration chromatography at different protein concentrations (0.24, 2.4, and 24 mg/ml).

3.3. FAD and ligands binding, and steady-state kinetics

The purified DAAO-ΔLOOP mutant retains the binding of the FAD coenzyme, as confirmed by the absorption spectrum in the visible region, which is typical of a FAD-containing protein. The extinction coefficient of the mutant at 455 nm is lower than that of the wild-type: 11 300 M^{-1} cm^{-1} and 12 600 M^{-1} cm^{-1} [14], respectively. Perturbations in the visible absorption spectrum indicate changes in the microenvironment surrounding the isoalloxazine moiety of the FAD molecule. The binding constant for the coenzyme was determined

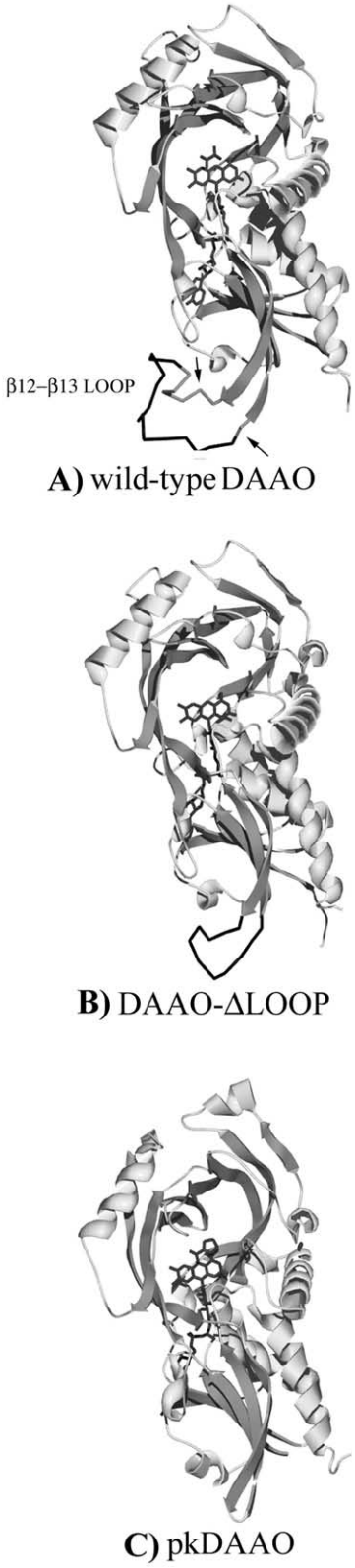


Fig. 3. Comparison of the structure of the protomer of wild-type RgDAAO (A) [4], with the model obtained for the DAAO-ΔLOOP mutant (B), and with the protomer of wild-type pkDAAO (C) [3]. Arrows indicate the peptide bonds sensible to proteolytic attack [6].

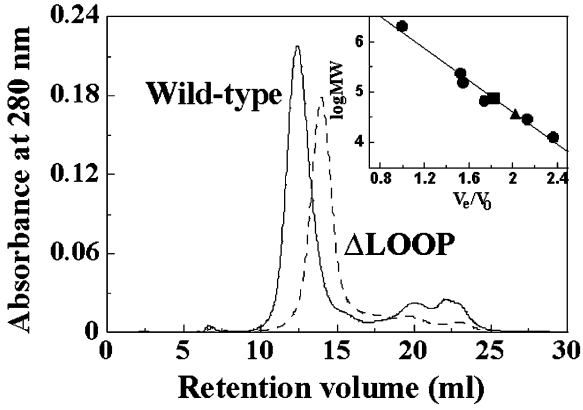


Fig. 4. Gel-permeation chromatography on Superose 12 column of about 0.4 mg of RgDAAO wild-type (solid line) and of DAAO-ΔLOOP (dashed line). Inset: calibration curve of Superose 12 column; circles: proteins used as gel-filtration molecular mass markers; square: RgDAAO wild-type; triangle: DAAO-ΔLOOP.

by titrating the apoprotein with increasing amounts of FAD and by monitoring the reconstitution following the quenching of the FAD fluorescence at 342 nm [7]: a K_d value of the apoprotein–FAD complex was found to be five-fold higher than that of the wild-type RgDAAO (Table 1).

The binding of typical DAAO competitive inhibitors (such as benzoate, anthranilate, and crotonate) causes changes in the visible region of the absorption spectrum of the enzyme [14,15]. The titration of DAAO-ΔLOOP with these inhibitors resulted, in all cases, in spectral perturbations similar to those observed with wild-type DAAO. Dissociation constants were only slightly different from those determined for the wild-type enzyme, indicative of a small effect of the mutation on the binding between RgDAAO and its active site ligands (Table 1). The ΔLOOP mutant reacts with sulfite, producing a covalent adduct: a K_d similar to that observed for wild-type DAAO was estimated (Table 1), indicating that the deletion does not affect the overall redox properties of the enzyme.

The steady-state kinetic parameters were determined using D-alanine (the most frequently used DAAO substrate) and D-tryptophan (in order to evaluate whether the mutation affects the ability of the mutant to accommodate a substrate with a bulky side-chain) as substrates at a fixed (21%) oxygen concentration. With both substrates, only a slight modification of the apparent kinetic parameters is evident. The most significant change is a 1.8-fold decrease in the V_{max} value determined with D-Trp as substrate, and a 1.9-fold increase in K_m for D-Ala (Table 1).

3.4. pH stability and activity and thermal inactivation

Stability and activity profiles of DAAO-ΔLOOP as a function of pH are similar to those of the wild-type RgDAAO: both enzymes are stable between pH 6 and 9 and show maximum activity at pH 8.5 (data not shown). More pronounced effects were evident when the thermal stability of the two DAAO forms was compared. Up to 35°C, the stability of the ΔLOOP mutant holoenzyme lies between that of native holoenzyme and apoprotein DAAOs [16], whereas at higher temperatures the mutant enzyme is much less stable: at 40°C the ΔLOOP DAAO holoenzyme retains less then 5% of the initial activity after 30 min of incubation, as compared to 60% and 20% observed with wild-type holoenzyme and apoprotein, respectively.

3.5. Limited proteolysis

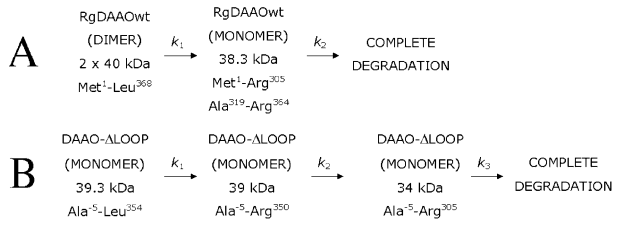
Experiments carried out with DAAO purified from *R. gracilis* cells demonstrated that three peptide bonds were susceptible to digestion with trypsin: Arg^{305}–Thr^{306}, Arg^{318}–Ala^{319}, and Arg^{364}–Glu^{365} [6]. Analysis of the proteolytic products revealed the formation of a nicked and truncated form of 38.3 kDa, still catalytically competent, in which both the peptide Thr^{306}–Arg^{318} and the four C-terminal amino acids (Glu-Ser-Lys-Leu) are cleaved off by trypsin (Scheme 1A). The activity loss was a biphasic process: the rate of the first phase matched the rate of conversion of the intact 40-kDa enzyme into the 38.3-kDa form, whereas the rate of the second phase matched the rate of degradation of the 38.3-kDa intermediate [6].

SDS–PAGE analysis of the time course of trypsinolysis of DAAO-ΔLOOP under the same experimental conditions used for wild-type RgDAAO showed that the deleted mutant is rapidly converted (more than 95% in less than 1 min) to a form of 39.0 ± 1.8 kDa (Fig. 5). Since its N-terminal sequence is unchanged, appearance of this form is probably due to the cleavage of the four C-terminal amino acids (Glu-Ser-Lys-Leu), resulting in a polypeptide with a theoretical molecular mass of 38 876 Da. At longer incubation times, a band corresponding to a molecular mass of 34.0 ± 2.1 kDa appeared. Densitometric analysis of the gel showed that the rate of degradation of the 39-kDa form corresponds to the rate of formation of the 34-kDa form (Table 2). Since the N-terminal sequence of the 34-kDa form is unchanged, it probably arises from the cleavage of the Arg^{305}–Ala^{306} bond, that generates a polypeptide with a theoretical molecular mass of 33 921 Da. This digestion pattern is quite different from that observed with the wild-type enzyme [6]. In the DAAO-ΔLOOP the peptide bond Arg^{305}–Thr^{306}, whose cleavage produces the 34-kDa form (Met^{1}–Arg^{305}) in the RgDAAO wild-type [6], is much

Table 1
Comparison of dissociation constants determined for various ligands and for FAD and of kinetic parameters determined on D-alanine and D-tryptophan as substrates for wild-type and ΔLOOP RgDAAO

Enzyme form	Binding (K_d)					D-alanine		D-tryptophan	
	FAD (μM)	Benzoate (mM)	Anthranilate (mM)	Crotonate (mM)	Sulfite (mM)	V_{max} (μmol O_2/min mg_{enzyme})	K_m (mM)	V_{max} (μmol O_2/min mg_{enzyme})	K_m (mM)
Wild-type	0.02[a]	0.9[b]	1.9[b]	0.4[b]	0.12[b]	110[c]	0.8[c]	120[c]	0.3[c]
ΔLOOP	0.1	3.2 ± 0.7	2.8 ± 0.3	0.2 ± 0.02	0.08 ± 0.006	86 ± 2	1.5 ± 0.2	68 ± 2	0.4 ± 0.05

[a]From [7].
[b]From [15].
[c]From [9].



Scheme 1. Models for trypsinolysis of wild-type (A) and of ΔLOOP (B) RgDAAO. Data for wild-type RgDAAO are from [6]. Numbering is referred to that reported in Fig. 1. Note that the purified ΔLOOP mutant contains five out of the six amino acids of the N-terminal presequence (Ala-Arg-Ile-Arg-Leu) numbered from −5 to −1.

less susceptible to trypsinolysis. This observation has also been confirmed by a comparison of the rate constants observed for the degradation of the enzyme's native form: for wild-type RgDAAO, this value is about 30-fold higher than the corresponding rate constant determined for the DAAO-ΔLOOP (cf. Table 1 in [6] and Table 2). On the other hand, the rate constant following complete degradation of the 34-kDa form is similar for the two enzyme forms. The activity loss of ΔLOOP is essentially monophasic, and shows a rate constant comparable to that of formation of the 34-kDa form and also to that of its degradation (Table 2). A comparison of the results obtained by digestion with trypsin on wild-type and ΔLOOP DAAOs are summarized in Scheme 1.

However, since the binding between FAD and apoprotein is weaker in the mutant than in the wild-type RgDAAO, the loss of activity and the full degradation could be partially ascribed to the formation of the corresponding apoprotein form. We previously reported that, in the intact DAAO, the apoprotein is significantly more sensitive than the holoenzyme form to proteolytic attack and to the inactivation because of the increased exposure of the β12–β13 loop to the solvent [6]. As shown in Table 2, incubation of DAAO-ΔLOOP with 10% trypsin in the presence of exogenous FAD (0.2 mM) showed that the rate of activity loss is lower than that determined in the absence of exogenous FAD. Although the rate of conversion of the 39-kDa polypeptide into the 34-kDa form was increased, further degradation of this latter polypeptide, which is responsible for the complete loss of activity, is decreased. This latter result highlights a protecting effect exerted by the addition of exogenous FAD on the 34-kDa proteolysed form, due to the maintenance of the mutant DAAO in the holoenzyme form.

4. Discussion

Limited proteolysis and crystallographic studies suggest that the long loop connecting β-strands 12 and 13 is involved in the dimerization of the enzyme [4–6]. A mutant form of RgDAAO was designed by rational mutagenesis: the region removed ranges from residues Ser^{308} to Lys^{321}. The DAAO-

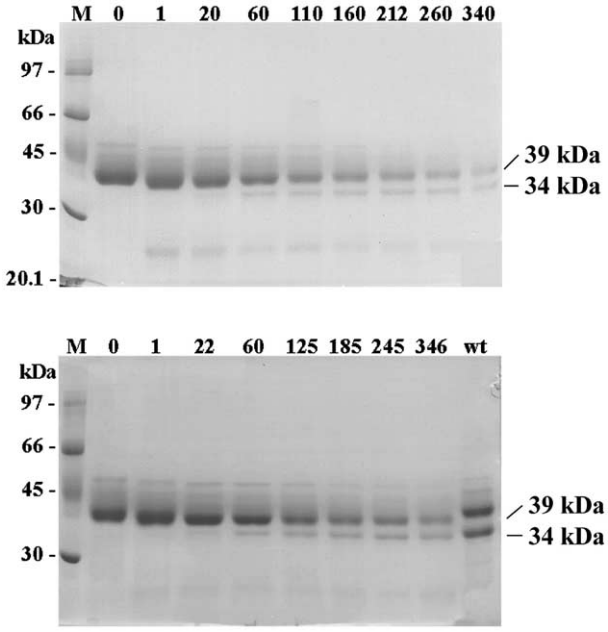


Fig. 5. SDS–PAGE showing the time course for the tryptic digestion of DAAO-ΔLOOP in the absence (upper panel) and in the presence (lower panel) of 0.2 mM exogenous FAD. Each lane contained 7 μg of DAAO-ΔLOOP and 0.7 μg of trypsin (visible as a band at about 24 kDa). M: molecular mass markers; wt: wild-type RgDAAO after 2 min of incubation with 10% trypsin in the presence of 0.2 mM exogenous FAD. The number above each lane corresponds to the incubation time expressed in minutes.

ΔLOOP mutant was purified to homogeneity as a monomeric and active holoenzyme, confirming the original hypothesis that the β12–β13 loop is essential for the dimerization of the RgDAAO. The monomeric oligomerization state of DAAO-ΔLOOP is not dependent on the protein concentration. The mutant enzyme retains the binding with the FAD coenzyme, but the interaction between the protein moiety and the coenzyme is slightly modified, as indicated by the comparison of the absorption spectrum of the two enzyme forms and by the increase in the K_d value for the apoprotein–FAD binding. The ability of the monomeric form of DAAO to bind the FAD coenzyme confirms the observation that in RgDAAO dimerization follows FAD binding [17]. In addition to the alteration of FAD binding, even the limited proteolysis experiments point to some conformational changes following the deletion of the β12–β13 loop, e.g. the Arg^{305}–Thr^{306} peptide bond becomes significantly less sensitive to the trypsin attack. This result indicates significant and different conformational changes in this region following conversion to the monomeric state in the holo- and apoprotein form. In the dimeric DAAO, the β12–β13 loop is only partially exposed, since the main part of it is involved in the monomer–monomer interaction. In the monomeric apoprotein the higher exposure of the β12–β13 loop results in an increase in the rate of

Table 2
Rates of inactivation and proteolysis of 0.33 mg/ml DAAO-ΔLOOP by 10% (w/w) trypsin, at 25°C and pH 8.0

Exogenous FAD	Inactivation ($min^{-1} \times 10^{-3}$)	39-kDa form	34-kDa form	
		Degradation ($min^{-1} \times 10^{-3}$)	Formation ($min^{-1} \times 10^{-3}$)	Degradation ($min^{-1} \times 10^{-3}$)
NO	7.8 ± 0.9	4.8 ± 0.3	6.8 ± 0.5	6.9 ± 0.8
YES	5.8 ± 0.5	17.2 ± 5.1	16.7 ± 3.0	n.d.

proteolysis with respect to that observed for the dimeric wild-type holoenzyme [6].

None of the residues involved in the monomer–monomer interaction is close to the active-site cleft. In fact, DAAO-ΔLOOP retains the major part of the catalytic activity of the wild-type enzyme, as well as its pH stability and activity optima. The substrate specificity of DAAO-ΔLOOP is essentially unchanged, as well as its ability to bind classical DAAO inhibitors. These observations indicate that oligomerization is of no significance for enzyme function. However, the partial elimination of the loop affects both the thermal stability of the enzyme and its maximal activity.

These results provide further support for the role of higher oligomerization state in thermal stabilization, as recently showed by studies on triosephosphate isomerase [18]. Interestingly, in the case of RgDAAO, the conversion from a dimeric to a monomeric oligomerization state does not affect the catalytic competence. This result is different from that observed for other oligomeric proteins, e.g. for multimeric dihydroneopterin aldolase, where a single amino acid substitution that disrupts tetramer formation also yields enzyme forms totally inactive [19]. In conclusion, our results show that it is possible to design a stable monomeric holoenzyme form of RgDAAO acting on the length of the β12–β13 loop. The dimerization is not required to accomplish catalytic activity and FAD binding, whereas it is a tool to achieve a better stability and activity of this flavoprotein.

Acknowledgements: This work was supported by grants from Italian MURST to M.S.P. (*PRIN MM05C73482*). We thank Dr. Stefania Iametti and Dr. Fabio Forlani (University of Milano) for the densitometric analyses of SDS–PAGE.

References

[1] Mattevi, A., Vanoni, M.A. and Curti, B. (1997) Curr. Opin. Struct. Biol. 7, 804–810.
[2] Pilone, M.S. (2000) Cell. Mol. Life Sci. 57, 1732–1747.
[3] Mattevi, A., Vanoni, M.A., Todone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M. and Curti, B. (1996) Proc. Natl. Acad. Sci. USA 93, 7496–7501.
[4] Umhau, S., Pollegioni, L., Molla, G., Diederichs, K., Welte, W., Pilone, M.S. and Ghisla, S. (2000) Proc. Natl. Acad. Sci. USA 97, 12463–12468.
[5] Umhau, S., Diederichs, K., Welte, W., Ghisla, S., Pollegioni, L., Molla, G., Porrini, D. and Pilone, M.S. (1999) in: Flavins and Flavoproteins 1999 (Ghisla, S., Kroneck, P., Macheroux, P. and Sund, H., Eds.), pp. 567–570, Weber, Berlin.
[6] Pollegioni, L., Ceciliani, F., Curti, B., Ronchi, S. and Pilone, M.S. (1995) Biochem. J. 310, 577–583.
[7] Casalin, P., Pollegioni, L., Curti, B. and Pilone Simonetta, M. (1991) Eur. J. Biochem. 197, 513–517.
[8] Sambrook, J., Fritsch, E.P. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[9] Molla, G., Vegezzi, C., Pilone, M.S. and Pollegioni, L. (1998) Protein Expr. Purif. 14, 289–294.
[10] Pilone Simonetta, M., Vanoni, M.A. and Casalin, P. (1987) Biochim. Biophys. Acta 914, 136–142.
[11] Pollegioni, L., Falbo, A. and Pilone, M.S. (1992) Biochim. Biophys. Acta 1120, 11–16.
[12] Peitsch, M.C. (1996) Biochem. Soc. Trans. 24, 274–279.
[13] Guex, N. and Peitsch, M.C. (1997) Electrophoresis 18, 2714–2723.
[14] Pilone Simonetta, M., Pollegioni, L., Casalin, P., Curti, C. and Ronchi, S. (1989) Eur. J. Biochem. 180, 199–204.
[15] Harris, C.M., Molla, G., Pilone, M.S. and Pollegioni, L. (1999) J. Biol. Chem. 274, 36233–36240.
[16] Pilone, M.S., Pollegioni, L. and Butò, S. (1993) in: Stability and Stabilization of Enzymes (van den Tweel, W.J.J., Harder, A. and Buitelaar, R.M., Eds.), pp. 415–420, Elsevier Science Publisher.
[17] Pollegioni, L. and Pilone, M.S. (1996) Arch. Biochem. Biophys. 332, 58–62.
[18] Walden, H., Bell, G.S., Russell, R.J.M., Siebers, B., Hensel, R. and Taylor, G.L. (2001) J. Mol. Biol. 306, 745–757.
[19] Thomas, M.C., Ballantine, S.P., Bethell, S.S., Bains, S., Kellam, P. and Delves, C.J. (1998) Biochemistry 37, 11629–11636.