Unfolding of D-amino acid oxidase

Unfolding intermediate in the peroxisomal flavoprotein

D-amino acid oxidase

Laura Caldinelli¹, Stefania Iametti², Alberto Barbiroli², Francesco Bonomi², Luciano Piubelli¹, Pasquale Ferranti³, Gianluca Picariello³, Mirella S. Pilone¹, and Loredano Pollegioni¹

¹Department of Structural and Functional Biology, University of Insubria, via J.H. Dunant 3, 21100 Varese, Italy, ²DISMA, University of Milan, via Celoria 2, 20133 Milan, Italy, and ³DSA, University of Naples “Federico II”, Parco Gussone, 80055 Portici, Italy

Correspondence to: Loredano Pollegioni

Dipartimento di Biologia Strutturale e Funzionale
Università degli Studi dell’Insubria
via J.H. Dunant 3, 21100 Varese (Italy)
Tel. +332-421506; Fax. +332-421500
E-mail: loredano.pollegioni@uninsubria.it

Running title: Unfolding of D-amino acid oxidase
The flavoenzyme D-amino acid oxidase (DAAO) from *Rhodotorula gracilis* is a peroxisomal enzyme and a prototypical member of the glutathione-reductase family of flavoproteins. DAAO is a stable homodimer with a FAD molecule tightly bound to each 40 kDa subunit. In this work the urea-induced unfolding of dimeric DAAO was compared with that of a monomeric form of the same protein, a deleted dimerization loop mutant. By using circular dichroism spectroscopy, protein and flavin fluorescence, 1-8anilinonaphtalene sulfonic acid (ANS) binding and activity assays, we demonstrated that the urea-induced unfolding of DAAO is a three state process, yielding an intermediate, and that this process is reversible. 

The intermediate specie lacks the catalytic activity and the characteristic tertiary structure of native DAAO, but has significant secondary structure and retains flavin binding. Unfolding of DAAO proceeds through formation of an expanded, partially unfolded inactive intermediate, characterized by low solubility, by increased exposure of hydrophobic surfaces, and by increased sensitivity to trypsin of the β-strand F5 belonging to the FAD-binding domain. The oligomeric state does not modify the inferred folding process. The strand F5 is in contact with the C-terminal α-helix containing the Ser-Lys-Leu sequence corresponding to the type 1 peroxisomal targeting signal, and this structural element interacts with the N-terminal βαβ flavin binding motif (Rossmann fold). The expanded conformation of the folding intermediate (and in particular the higher disorder of the mentioned secondary structure elements) could match the structure of the inactive holoenzyme required for *in vivo* trafficking of DAAO through the peroxisomal membrane.

*Abbreviations:* DAAO, D-amino acid oxidase (EC 1.4.3.3); Cm, concentration of urea to give half-unfolded protein; ANS, 1-8anilinonaphtalene sulfonic acid; CD, circular dicroism
Protein folding/unfolding is a highly cooperative process. It has been shown that the folding/unfolding of small globular proteins occurs via a two-state process, whereas the folding/unfolding of larger proteins (> 100 amino acids) is complex and often involves the formation of intermediate(s) (1-3). The most thorough investigations of protein folding and stability have been done with unusually small proteins, which are folded into single domains and display simple two-state unfolding processes. It is of interest, however, to extend studies to larger, more complex, and therefore more typical proteins. D-amino acid oxidase (EC 1.4.3.3, DAAO) has attracted our attention as a suitably more complex subject because it is considered the paradigm of the dehydrogenase/oxidase class of flavoproteins (4), and in particular of those in which the flavin is non-covalently bound. In fact, many proteins in nature require the non-covalent binding of cofactors to perform their biological activity, and these molecules fold in a cellular environment where their cognate cofactors are present. However, the manner in which cofactors affect the folding pathway remains poorly understood, because kinetic folding studies are frequently conducted in the absence of potentially complicating ligands. Furthermore, flavoproteins are often multi-subunit proteins constituted either by identical or by different polypeptide chains. Up to now, deep and complete investigations have been restricted to small flavoproteins, such as flavodoxin (5).

To have insights on the relationships between cofactor uptake, the stable interaction between identical subunits (and on its significance), folding, and intracellular trafficking, we undertook a study of the stability of structural elements in the peroxisomal flavoenzyme DAAO from the yeast *Rhodotorula gracilis*. Proteins destined for the peroxisome are synthesised on free ribosomes of the cytoplasm and transported into peroxisomes post-translationally. There are two types of peroxisomal targeting signals (PTS): the first one (PTS1) is the SKL C-terminal sequence (or some conservative variants of it) (6,7). Proteins belonging to this group are made at their mature size, do not undergo cleavage of the targeting
Unfolding of D-amino acid oxidase

4

sequence upon transport into peroxisome, and have cytosolic receptors which mediate their association with the peroxisomal import machinery (6,7). It has also demonstrated that stably folded proteins are substrates for peroxisomal import (8).

DAAO catalyses the dehydrogenation of D-isomers of amino acids, to give the corresponding α-keto acids, ammonia and hydrogen peroxide. During the years DAAOs have been the object of extensive investigation (9, 10). In solution, DAAO from *R. gracilis* is a stable 80 kDa homodimer, with a molecule of FAD tightly \(K_d = 2 \times 10^{-8} \text{ M}\), but non-covalently bound to each 40 kDa subunit. The 3D structure of DAAO has been resolved at very high resolution allowing to find the rationale of its high catalytic efficiency (11, 12). In the “side to tail” model of monomer-monomer interaction (with a high buried surface area, 3049 Å²) (12), a large contribution to the interaction between monomers is given by a long (21 amino acids) loop connecting β-strands F5 and F6, that is unique to yeast DAAO. Recently, by rational design and site directed mutagenesis, a stable monomeric holoenzyme form of DAAO has been obtained by partial elimination of this loop (from Ser\(^{308}\) to Lys\(^{321}\): SPLSLGRGSARAAK) (13). The Δloop mutant DAAO shows slightly altered spectral and kinetic properties, a lower temperature stability and a 5-fold increase in the \(K_d\) for FAD binding compared to the wild-type enzyme. We also demonstrated the possibility of obtaining a monomeric form of yeast DAAO by treatment with 0.5 M NH₄SCN, without deletion of the βF5-βF6 loop (14), as well as by removal of the coenzyme to yield the corresponding apoprotein (10). This latter result suggests a structural relationship between the FAD-harboring domain and the regions involved in dimerization. Recently, temperature ramp experiments following different probes allowed the identification of a clear sequence of events in the course of thermal unfolding of wild-type and Δloop protein forms (14). Apparently, a first, low-temperature energetic domain relates to the unfolding of tertiary
structure regions, whereas a second energetic domain relates to the loss of secondary structure elements and to the release of the cofactor at higher temperatures.

In this paper, we expand the results obtained until now, and report a novel, reversible step in the urea-induced unfolding pathway of \textit{R. gracilis} DAAO, that we believe may be relevant to intracellular trafficking of DAAO. Furthermore, to provide a basis for understanding the structure-function relationships in flavoproteins and the determinants of their stability, we have attempted to compare the chemical unfolding of dimeric DAAO with that of a monomeric form obtained by site directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

\textit{Materials} - Recombinant wild-type and \Delta \text{loop} mutant DAAO were expressed and purified from \textit{E. coli} cells as described previously (13, 15). Starting from a 10-liters fermentation broth, 180 mg and 80 mg of pure enzyme with a specific activity of 110 and 86 U/mg protein were obtained for wild-type and \Delta \text{loop} DAAO, respectively. The enzyme concentration was determined by using extinction coefficients at 455 nm of 12.6 mM$^{-1}$cm$^{-1}$ for wild-type and 11.3 mM$^{-1}$cm$^{-1}$ for \Delta \text{loop} DAAOs (10, 13). Urea was from Pierce Chemical Co., and the other reagents were of analytical grade.

\textit{DAAO activity assay} - DAAO activity was assayed with an oxygen electrode at pH 8.5, air saturation, and 25 °C, using 28 mM D-alanine as substrate in the presence of 0.2 mM FAD (10). The effect of urea concentration on enzyme activity of DAAO was determined using the oxygen-electrode assay on protein samples previously incubated at 15 °C for 40 min in the presence of different concentrations of urea.
**Spectroscopy** - All fluorescence measurements were performed by using a 1-ml cell in a Jasco FP-750 instrument equipped with a thermostated cell holder. Tryptophan emission spectra were taken from 300 to 400 nm using excitation wavelengths of 280 nm and 298 nm. Flavin emission spectra were recorded from 475 to 600 nm using an excitation wavelength of 450 nm; 10 and 20 nm bandwidths were used for excitation and emission, respectively. Steady-state fluorescence measurements were performed at 15 °C, and at 0.02 mg/ml protein concentration. All spectra were corrected by subtracting the emission of the buffer.

ANS binding experiments were carried out at 15 °C and at 2.5 µM (0.1 mg/ml) protein concentration. Protein samples were incubated for 40 min in buffer containing different concentrations of urea, and ANS was added to a final concentration of 0.1 mM. Fluorescence emission spectra were recorded in the 450-600 nm range using an excitation wavelength of 370 nm.

CD spectra were recorded on a J-810 Jasco spectropolarimeter and analyzed by means of Jasco software. The cell path was 1 cm for measurements above 250 nm, and 0.1 cm for measurements in the 190-250 nm region. Proteins were in 50 mM potassium phosphate buffer, pH 7.5, containing 10% (v/v) glycerol, and 2 mM EDTA.

**Equilibrium unfolding experiments** - The unfolding equilibrium of DAAO was determined by following the changes in flavin and protein fluorescence as detailed above. To establish the time required to reach the equilibrium, the fluorescence intensity was measured as a function of time until no further changes are observed (40 min at 15 °C). Each point of urea denaturation curves was determined on individual samples, prepared by mixing appropriate volumes of protein, 8 M urea in buffer, and plain buffer (50 mM potassium phosphate, pH 7.5, containing 5% (v/v) glycerol, and 2 mM EDTA).
**Refolding of DAAO** - Wild-type and Δloop DAAOs were incubated at various fixed concentrations of urea for 40 min at 15 °C. The enzymes were refolded by the dilution method (10 times dilution at 15 °C) in 50 mM potassium phosphate, pH 7.5, 5% glycerol, and 2 mM EDTA. The refolding yield was measured by monitoring protein and flavin fluorescence, and the recovery of enzymatic activity using the values for the native and fully denatured enzymes as reference.

**Data analysis** - Unfolding curves were usually analyzed using a two-state mechanism. Unfolding curves for the N↔D transition were normalized to the apparent fraction of the unfolding form, $F_D$, using the following equation (16):

$$F_D = \frac{Y - Y_N}{Y_U - Y_N}$$  \hspace{1cm} (1)

where $Y$ is the observed variable parameter, and $Y_N$ and $Y_U$ are the values characteristic of the native and fully unfolded conformations, respectively. The difference in free energy between the folded and the unfolded state, $\Delta G$, was calculated by the following equation:

$$\Delta G = -RT \ln K = -RT \ln \left[ \frac{F_U}{1 - F_U} \right]$$  \hspace{1cm} (2)

where $K$ is the equilibrium constant, $R$ is the gas constant, and $T$ is the absolute temperature. The data were analyzed assuming the free energy of unfolding or refolding, $\Delta G$, to be linearly dependent on the urea concentration (denoted here by $C$), as described in detail previously (17):

$$\Delta G = \Delta G_w - mC = m(C_m - C)$$  \hspace{1cm} (3)

in which $\Delta G_w$ and $\Delta G$ represent the free energy of unfolding or refolding in the absence and presence of urea, respectively; $C_m$ is the midpoint concentration of urea required for unfolding or refolding; and $m$ stands for the slope of the unfolding or refolding curve at $C_m$. A least-squares curve fitting analysis was used to calculate the values of $\Delta G_w$, $m$, and $C_m$ by a
software routine. The same equation accounts for the free energy of formation of partly unfolded folding intermediates from native molecules.

The urea-unfolding curves corresponding to a two-state model were analysed using Eq. (4), derived by (18), that incorporates Eq. (1-3):

\[
Y_N + Y_U e^{\left(\frac{\Delta G_w - mC}{RT}\right)}
\]

\[
Y = \frac{1}{1 + e^{\left(\frac{\Delta G_w - mC}{RT}\right)}}
\]

The analysis of the equilibrium unfolding transition using the flavin fluorescence data was performed according to a three-state denaturation pathway (N ↔ I ↔ U) according (19)

\[
F_{N,I} \frac{\text{[urea]}^{n_1}}{C_{m1} + \text{[urea]}^{n_1}} + F_{I,U} \frac{\text{[urea]}^{n_2}}{C_{m2} + \text{[urea]}^{n_2}}
\]

in which \(C_{m1}\) and \(C_{m2}\) are the midpoint concentration of urea for the N ↔ I and I ↔ U transitions, respectively; \(F_{N,I}\) and \(F_{I,U}\) represent the percentage change of flavin fluorescence associated to the N ↔ I and I ↔ U transitions, respectively; exponents \(n_1\) and \(n_2\) reflect the steepness of the transition between states as a function of urea concentration.

**LC/MS analysis after limited tryptic digestion** – Following incubation of 1 mg/ml wild-type DAAO with 0.1% trypsin at 15 °C for 30 min in the absence and in the presence of 2 M urea, the reaction mixture (50 µg) was fractionated by HPLC using an HP 1100 modular system (Agilent Technology, Palo Alto, CA, USA). Samples were loaded onto a Vydac 218TP52, 5 µm reversed-phase C18, 250 x 2.1 column. Solvent A was water containing 0.1 % trifluoroacetic acid (TFA) and solvent B was acetonitrile containing 0.1 % TFA. A linear gradient from 5 % to 70% solvent B was applied over 90 min, after 5 min of isocratic elution at 5 % solvent B, at a constant flow rate of 0.2 ml/min. UV detection was carried out at 220
nm. Effluents were directly injected, through a 100 µm i.d. fused silica capillary, into the Electrospray source of a Platform single quadrupole mass spectrometer (Waters, Manchester, UK). The ESI mass spectra were scanned, in the positive ion mode, from 2000 to 800 u at a scan cycle of 4.9 s/scan and 0.1 s inter-scan delay. The source temperature was 200 °C and the capillary and orifice voltages were 3.6 kV and 40 V, respectively. Mass scale calibration was performed using the multiple charged ions from a separate injection of horse heart myoglobin (20). Mass spectra were elaborated using the software MassLynx 2.0, furnished with the spectrometer. Mass values were reported as average masses.

RESULTS

Spectral properties of wild-type and Δloop DAAO - Wild-type and Δloop DAAOs are purified as holoenzymes showing little difference in the visible absorbance spectra (10, 13). On the contrary, spectral properties depending on the protein folding (flavin and protein fluorescence, and UV CD spectra) profoundly distinguish the monomeric and dimeric DAAO forms. Tryptophan emission fluorescence at 345 nm (following excitation at 280 nm), a marker of protein conformation, is 2-3 fold higher for native Δloop than for wild-type DAAO, indicating a lower relevance of quenching interactions between tryptophan side chains (in particular that of Trp-243 at the monomer-monomer interface) and nearby side chains in the monomeric form of yeast DAAO with respect to the dimeric form (14). The emission fluorescence of the FAD cofactor at 520 nm, following excitation at 450 nm, is higher for Δloop than for wild-type DAAOs, pointing to a different microenvironment surrounding the FAD coenzyme in the two proteins (14). On the other hand, the protein and flavin emission fluorescence determined for the urea-unfolded forms of wild-type and Δloop DAAOs are
quite similar at 8 M urea (see Table I). For both proteins, unfolding by urea is accompanied by an increase in both flavin and tryptophan fluorescence.

Far-UV CD spectra of monomeric and dimeric DAAOs did not reveal any major difference in the features related to the secondary structure of the two proteins while the near-UV CD spectra of the wild-type and ∆loop mutant DAAO were different. The differences have been ascribed to a different contribution from aromatic amino acid residues, which are responsible for most transitions in the near-UV spectral region. Since the deleted portion does not contain aromatic residues, the different spectroscopic features of the two proteins have been explained in terms of an altered mutual relationships between nearby structural elements (14). Following the addition of ≥ 6 M urea, the Far- and Near-UV spectra of both wild-type and ∆loop DAAOs were superimposable.

**Equilibrium unfolding studies** - The stability of wild-type and ∆loop DAAO forms was studied at first by equilibrium unfolding measurements. In the experiments reported in the following sub-sections, different spectroscopic signals, catalytic activity, and the associative behaviour of both proteins were monitored after equilibration in the presence of increasing urea concentration.

**Protein fluorescence** - Tryptophan emission fluorescence can be considered a sensitive marker of protein conformation, and in particular of changes in hydrophobic regions of the structure. The intrinsic fluorescence of tryptophan residues was used as probe of the unfolding of DAAO. Because there are eight tryptophans in yeast DAAO (11), the overall changes in fluorescence reflect global changes in protein structure, and only the average microenvironments of tryptophans can be assessed. As stated above, tryptophan emission at ≈ 345 nm (following excitation at 280 nm or 298 nm) is significantly higher for ∆loop DAAO than for wild-type. At increasing urea concentration, the two DAAO forms show a different
increase in the intensity of the protein emission that is anyway complete at 2-3 M urea (Fig. 1). Treatment with urea also causes the emission maximum of tryptophan fluorescence in both DAAO forms to shift from 337 nm for wild-type and 339 nm for Δloop DAAO to 352-353 nm at 5-6 M urea, a change that does not parallel the change in fluorescence intensity (Fig. 1). The fluorescence red shift stems from transfer of tryptophan side chains to a more polar environment upon protein unfolding (21).

Plots of changes in the intensity protein fluorescence at equilibrium as a function of the urea concentration apparently suggest a simple two-state transition (Fig. 1). The free energy of unfolding, ΔG, was calculated according to Eq. 2. The free energy of unfolding in the absence of the denaturant (ΔGw) can be obtained by extrapolation of ΔG to zero denaturant concentration by using Eq. 3. The energy value determined for the monomeric Δloop enzyme is lower than the value for wild-type DAAO (Table I). The different dependence of the intensity of protein fluorescence and of the wavelength of emission maximum on the urea concentration indicates that the overall urea-induced denaturation of DAAO is a more complex process, pointing to the presence of a detectable intermediate.

*Flavin fluorescence* – As shown in Fig. 2, urea-dependent changes in flavin fluorescence are indicative of a two-step, three-state process for both proteins. An intermediate is formed at ≈ 3 M urea, and apparently it retains a bound cofactor, since its flavin fluorescence does not correspond to the much higher one typical of free flavin (10). Considering the transition occurring at ≤ 3 M urea, increasing urea concentrations have a different effect on the two proteins. Flavin fluorescence of the Δloop mutant starts to increase at lower urea concentration than that of wild-type DAAO. At ≥ 7 M urea, the fluorescence intensity of wild-type DAAO attains values similar to those of the Δloop mutant, and corresponds to that of the free flavin. Equilibrium values determined from changes in flavin fluorescence, analyzed by using Eq. 5 according to a three-state mechanism, are reported in Table I.
The increase in protein and flavin fluorescence at increasing concentration of urea is different for the two DAAO forms (as confirmed by the $C_m$ values in Table I), and changes in tryptophan fluorescence in both proteins appears to anticipate release of flavin cofactor (Figs. 1, 2).

**Secondary and tertiary structure: CD studies** – To test whether the unfolding transition monitored by fluorescence measurements reflects a disruption of the overall protein structure or just indicative of local unfolding, and to have a better knowledge of the structural properties of the intermediate detected at $\approx 3$ M urea, we analyzed urea-induced denaturation of both DAAO forms by CD spectroscopy. When urea-induced loss of secondary structure elements was monitored by following the changes in ellipticity at 223 nm (Fig. 3A), a different increase in the CD signal is observed for the two DAAO forms. The increase of the 223 nm-signal (corresponding to the unfolding of the secondary structure elements) starts at 1 M urea for the monomeric $\Delta$loop DAAO, but only at $\geq 2.0$ M urea for the dimeric wild-type enzyme. In both cases, a two-state transition is observed, with $C_m$ values significantly higher than those observed in protein fluorescence studies and for the first transition seen in flavin fluorescence studies and leading to formation of the intermediate (Table I). Thus, the observed transition may correspond to loss of the secondary structure of the intermediate (which accumulates at [urea] $\approx 2.5$ M, see above), and to conversion of the intermediate into an unfolded state.

Analysis of the CD spectra recorded on either protein at 3 M urea allowed a calculation of the residual content of secondary structures. Alpha helix content decreased from 30.4% in the native wild-type protein to 25.2% (from 33.3 to 22.4% in $\Delta$loop mutant DAAO), with a concomitant increase in random coiled structures (from 35.2 to 39.2% in wild-type DAAO, and from 33.2 to 41.7% in $\Delta$loop mutant DAAO).
Addition of urea also has different effects on the tertiary structure of both wild-type and Δloop mutant DAAO (as showed by the change in CD signals at 253 nm, Fig. 3B). A two-state transition is observed at significantly lower denaturant concentration for the monomeric Δloop mutant than for wild-type DAAO. This suggests that dimer dissociation sensibly altered the tertiary structure in each monomer, and confirms a much higher sensitivity to urea for the Δloop DAAO mutant with respect to the wild-type protein. For both proteins, changes in tertiary structure are complete at urea concentrations in which the intermediate hypothesized above is likely predominant.

However, near-UV CD transitions for wild-type and Δloop DAAO show no apparent intermediate state(s). The $\Delta G_w$, $C_m$ and $m$ values calculated using a two-state mechanism are listed in Table I. The $\Delta G_w$ estimated from near-UV CD studies is significantly higher than that based on fluorescence spectroscopy (e.g., 5.0 kcal/mole vs. 3.4 kcal/mole, in the case of wild-type DAAO, see Table I).

Enzymatic activity – Protein activity can be regarded as the most sensitive probe for studying the changes in protein conformation during denaturation, as it reflects even the subtlest readjustments at the active site. The effect of urea on the activity of DAAO was investigated by measuring the enzyme activity – in the standard assay mixture - on DAAO samples previously incubated for 40 min at 15 °C in the presence of different urea concentrations, by using the standard assay mixture. As reported in Fig. 4, the enzyme activity of wild-type DAAO measured in these conditions starts to decline at $> 1$ M urea and is lost at 2.5 M urea, concomitantly to formation of the intermediate discussed above (see also Figs. 1-3). Activity of the Δloop mutant protein is also completely lost after pre-incubation with 2.5 M urea. The mutant is more sensible to an increase in denaturant concentration than dimeric wild-type DAAO (as shown in Fig. 4, 50% of the initial activity is lost at 1.9 and 1.45 M urea.
concentration for wild-type and Δloop DAAOs, respectively). These $C_m$ values correspond to the first transition observed in flavin fluorescence studies (Table I), and demonstrate that the loss of catalytic activity occurs under conditions where the conversion of the native to the intermediate state also occurs (compare Figs. 1-4 and Table I).

**Associative behavior** – The effects of urea on the oligomerization state and/or the molecular size of wild-type and Δloop DAAOs were monitored by size-exclusion chromatography (Fig. 5). In the absence of denaturant, the two enzymes were, respectively, fully monomeric (Δloop, $V_e = 14.8$ ml) and fully dimeric (wild-type, $V_e = 13.4$ ml). However, upon pre-incubation with urea, both DAAO forms eluted as multiple peaks at significant lower retention volumes, indicating an increase in molecular dimensions (higher aggregation state or increased hydrodynamic radius) under these conditions. The eluted peaks had retention volumes corresponding to DAAO aggregates comprising 2, 3, 5, 6, 7, 8 and 10 subunits, as determined using a calibration curve obtained with standard proteins. The presence of multiple peaks indicates an equilibrium among protein species under the chromatographic conditions used here.

As also evident from Fig. 5, the total peak area in the chromatograms decreased significantly (to about 20% of that measured for the untreated enzyme) at urea concentration of 2 and 3 M urea for Δloop and wild-type DAAO, respectively. This result clearly indicates that the solubility of both proteins is minimal at the urea concentrations required for formation of the unfolding intermediate, which in both proteins is characterized by a strong tendency to aggregation. Addition of non-ionic detergents to the chromatographic buffer did not affect the aggregation phenomena (not shown).

When the urea concentration is increased further, a large fraction of the insoluble aggregates formed by the unfolding intermediate were apparently converted into soluble
polymeric forms, as made evident by the increase in the total area of the eluted peaks (up to 60% and 80% of the value determined for the untreated wild-type and Δloop DAAO, respectively).

Consistent with our flavin fluorescence studies, the peak at very high elution volume (> 18.0 ml) contained the released flavin, and its area increases up to ~ 2 and ~ 3 M urea for Δloop and wild-type DAAOs, respectively (Fig. 5). At higher concentration of urea the area of this peak remained quite constant, suggesting that the flavin cofactor was dissociated during the gel-permeation chromatography from the enzyme at denaturant concentrations corresponding to those required for formation of the intermediate (see above). The conformation of such an intermediate likely loses rigidity in the FAD binding domain, resulting in a significant increase in $K_d$ for flavin binding.

**Probing the exposition of hydrophobic regions** - The effect of urea-induced changes in DAAO on the exposure of buried hydrophobic clusters was studied by using the hydrophobic fluorescent probe ANS. Binding of this probe to solvent-accessible clusters of non-polar side chains in proteins results in a marked increase in its fluorescence accompanied by a blue-shift in its emission fluorescence spectrum. The steady-state fluorescence intensity of ANS significantly increased in the presence of both DAAO forms. This effect was much greater in Δloop mutant than in wild-type DAAO (fluorescence increase was 57 and 14 arbitrary units, respectively, at 2.5 μM protein), likely because larger hydrophobic patches are exposed in the monomeric enzyme.

Binding of ANS was used to study the partial exposition of hydrophobic patches upon loosening of the protein tertiary structure at increasing concentration of urea. At first, both DAAO forms were titrated with ANS in the presence of various urea concentrations (22). An empiric parameter such as the ratio between the change in fluorescence emission at 500 nm at
saturating ANS and the \( K_d \) for ANS binding (23) was highly dependent on the urea concentration. Values of 0.84, 1.92, 0.58 and 0.44 arbitrary units/\( \mu \)M for wild-type DAAO were obtained at 0, 2, 4, and 8 M urea, respectively. This value indicates that new and ANS-accessible hydrophobic regions are exposed following the treatment of DAAO with 2 M urea.

In separate studies, the intensity of ANS fluorescence at 500 nm in solutions containing 0.1 mM ANS and increasing denaturant concentration showed two transitions for both DAAO forms. An increase in ANS fluorescence up to 2 M urea (paralleled by a change in the ANS emission wavelength maximum from 516 nm to 498 and 490 nm for wild-type and \( \Delta \)loop DAAOs, respectively) was followed by a quenching of ANS fluorescence (along with a shift in the emission wavelength maximum form 498 to \( \approx \) 520 nm) at higher urea concentrations (Fig. 6). The first transition coincides with the urea-induced unfolding transition observed in flavin, protein fluorescence, and enzymatic activity studies, and resulting in the formation of the unfolding intermediate. Thus, while new hydrophobic ANS-binding surfaces were exposed in the course of the first transition, the surfaces progressively disappeared as urea concentrations were increased further.

Reversibility of the unfolding process - The refolding efficiency of wild-type and \( \Delta \)loop DAAO holoenzymes was studied kinetically by monitoring the time-courses of changes in tryptophan and flavin fluorescence, and in enzymatic activity, following a 10-fold dilution of urea-treated proteins (1.5-8 M urea initial concentration). Tryptophan fluorescence was followed taking measurements at time intervals, in order to avoid complications arising from photobleaching.

For both DAAO forms, a large part of the changes in tryptophan and flavin fluorescence were observed in the dead-time of the experiment (~20 s), even when starting from 8 M urea. The subsequent smaller changes in tryptophan fluorescence were very slow.
In fact, return to tryptophan fluorescence values appropriate for the urea concentration present after dilution took about 24 hours for both DAAO forms (see Fig. 7A for wild-type DAAO).

Changes in flavin fluorescence showed a different behaviour. Still the largest part of the decrease in fluorescence was still observed during the experiment dead-time, but the final absolute values (at ≥ 24 hours) were higher than those observed during protein unfolding experiments at the urea concentration present after dilution. This indicates that cofactor uptake during the refolding process was somewhat impaired (see Fig. 7B).

All together, these results show that the refolding of urea-treated wild-type and Δloop DAAOs is reversible considering the tertiary protein structure, although some alterations are evident between the native and refolded DAAO in the microenvironment surrounding the flavin cofactor.

Following the unfolding of wild-type DAAO with 2 M urea (at which ~ 80% of the initial activity is lost, see Fig. 4), and its refolding at 0.2 M urea for 10 min at 15 °C, the refolded enzyme showed ≥ 90% of the initial specific activity. Under comparable conditions, the recovery of enzymatic activity was lower for the Δloop mutant (Fig. 4, inset). Starting from fully denatured (8 M urea) wild-type and Δloop DAAOs, about 30% and 15% of the initial activity was recovered, respectively, after 10 min of refolding at 0.8 M urea. Activity recovery figures increased significantly when refolding was allowed to occur for longer times, and reached 60 and 35 % for wild-type and Δloop DAAOs, respectively, at 4 hours after denaturant dilution. These figures did not improve further at 24 hours, and are in good agreement with the fraction of folded enzyme determined in flavin fluorescence recovery studies (≈ 70% for wild-type DAAO).

Probing the conformation of DAAO by limited proteolysis - Limited proteolysis experiments have been extensively used to have insights on the structural properties of yeast
DAAO (13, 24-26). These experiments demonstrated that three peptide bonds are highly susceptible to trypsin in the native protein, namely: R\textsuperscript{305}–T\textsuperscript{306}, R\textsuperscript{318}–A\textsuperscript{319}, and R\textsuperscript{364}–E\textsuperscript{365}. The main proteolytic product was a nicked and truncated monomeric holoenzyme of 38.3 kDa, still catalytically active, in which both the peptide T\textsuperscript{306}–R\textsuperscript{318} and the four C-terminal peptide were cleaved off by trypsin. Residues at the C-terminal are also not visible in the electron density (11), and contain the SKL tripeptide corresponding to the peroxisomal targeting signal PTS1. Structural flexibility in this region is of fundamental importance for interaction with the receptor (6). The loop region between β-strands F5 and F6 (from P\textsuperscript{302} to D\textsuperscript{322}) is instead involved in the dimerization of the enzyme (11-14).

Proteolysis of wild-type DAAO in the presence of 2 M urea is very fast at 10%, 1% and 0.1% (w/w) trypsin, and does not allow the identification of any (partially) stable proteolytic product. Mass spectrometry analysis of the reaction mixture after 30 min of incubation at 0.1% (w/w) trypsin and 10 °C shows the presence of the -5ARIRLMHS.. protein (40690 Da), of the 1MHS.. protein (40182 Da), and of a number of short polypeptides. Among these latter, the 5469.4 Da (A\textsuperscript{319}–L\textsuperscript{368}) and the 5841 Da (G\textsuperscript{315}–L\textsuperscript{368}) polypeptides are largely produced even during limited proteolysis of wild-type DAAO even in the absence of urea, while shorter polypeptides originating from cleavage at level of arginines 288 and 289 (G\textsuperscript{290}–T\textsuperscript{326} 2871 Da, R\textsuperscript{289}–T\textsuperscript{326} 2871 Da, and G\textsuperscript{290}–A\textsuperscript{330} 3087 Da) are specifically produced only when starting from a partially unfolded DAAO. An inspection of DAAO structure shows that the sites sensitive to trypsinolysis in the presence of 2 M urea belong to the loop preceding the long β-strand F5, which is a structural element of the flavin binding domain (11, 12) and is not in direct contact with the flavin cofactor or with the monomer-monomer interaction surface (see Fig. 8). This site is not accessible to attack by trypsin under native conditions, and becomes exposed (and therefore sensitive to proteolysis) in the partially unfolded (intermediate) state.
DISCUSSION

Urea-induced unfolding of both wild-type and Δloop DAAOs is a two step (three-state) process yielding detectable intermediate(s). The presence of intermediates in the unfolding process is demonstrated by the different urea sensitivity of the various protein features used to monitor the folding status of DAAO. Urea-dependent changes in flavin fluorescence are the most indicative of a three-state process, and show that the flavin cofactor is still bound to an unfolding intermediate in which the intensity of the flavin fluorescence is between that of protein-bound flavin (low) and that of the free flavin (high). According to tryptophan fluorescence and near-UV CD data, the change in flavin fluorescence is due to two different events: alteration of the tertiary structure specifically consequent to the addition of urea at concentrations ≤ 2.5 M, and release of the cofactor at higher urea concentrations. The deviation from a classical two-state behavior does not arise from an urea effect on a dimer-monomer transition of DAAO, since similar unfolding profiles have been observed using the dimeric wild-type and the monomeric Δloop DAAOs.

Chemical denaturation experiments show that most of the characteristic tertiary structure is destroyed at 3 M urea, where most of the secondary structure is almost retained. Therefore, at 2-3 M urea, a stable equilibrium intermediates are formed. These protein forms still binds the flavin cofactor, although with a significant increase in dissociation constant, but their catalytic activity is totally lost. A model for the equilibrium unfolding of DAAO based on a three-state model is shown in Fig. 9. Folding intermediates with these characteristics, often referred to as molten globule-like species, have been reported for a few proteins (27). Our ANS binding studies confirm that the unfolding intermediate observed at 2-3 M urea has exposed hydrophobic patches on structural sites that are accessible to ANS in both the wild-
type and the Δloop mutant DAAO. This feature, together with the increased affinity for the probe and the fluorescence shift in the probe emission evident at ≈ 2.5 M urea, are considered to be trademark of a molten-globule like status.

At urea concentrations corresponding to the formation of the unfolding intermediate, and to the loss of the enzymatic activity, the solubility of DAAO is significantly decreased. This may be consequent to the exposure of “sticky” surface hydrophobic sites/patches, as detected by ANS binding studies, that favor hydrophobic interaction between surfaces belonging to distinct proteins (23). At increasing urea concentration, the chaotropic effect of this molecule disrupts the solvating water structure of DAAO and leads to stabilization of an expanded, fully unfolded, and soluble multimeric apoprotein aggregate (see Fig. 9). Size-exclusion chromatography shows that both wild-type and Δloop DAAOs at > 3 M urea are in a multimeric conformation, thus the intermediate should correspond to aggregated molten globules. An expansion of the protein before full denaturation has been reported for several multimeric enzymes (see (28), and references therein). In spite of the formation of aggregates, unfolding of both DAAO forms may be considered as reversible. Only slight alterations in the flavin binding regions are indicative of differences between the native and the refolded proteins. The reversibility of DAAO unfolding is a prerequisite to enable the future application of hydrogen exchange pulse labeling methods in combination with NMR spectroscopy or mass spectroscopy, and real time NMR experiments to study DAAO folding in atomic detail.

A further conclusion of the present study is that the dimerization increases the structural stability of DAAO. All our equilibrium spectroscopic measurements show that the urea concentration required for the unfolding of monomeric Δloop mutant is lower than that of dimeric wild-type DAAO, and that different urea concentrations are required for complete unfolding of each protein (see Table I). It should be noted that the region susceptible to
trypsin cleavage in the unfolding intermediate is far away from the monomer-monomer interaction area. Previous studies indicated a significant lower thermal stability of the Δloop mutant with respect to wild-type DAAO (13). Thermal unfolding of both DAAO forms was characterized by two distinct steps (14): a first energetic domain unfolded with a $T_m$ of 45.1 °C, whereas a more thermostable domain unfolded with a $T_m$ of 47.8 °C (and likely involved a larger portion of the protein structure). The loss of tertiary structure elements involving hydrophobic regions of the protein occurs at lower temperatures, followed in order of increasing temperature by the loss of secondary structure elements and finally by FAD release. In the case of urea-induced unfolding the overall sequence of events seems to reproduce what observed in thermal stability studies. Production of the intermediate (which was not observed in thermal denaturation studies) is accompanied by significant changes in tertiary structure, while complete unfolding is connected to change in secondary structure and flavin release. It is thus possible to envisage that the unfolding intermediate detected in chemical unfolding studies corresponds to modifications in the first energetic domain made evident in thermal denaturation studies.

The factors that cause the stabilization of the DAAO folding intermediate need additional attention, and will require further experimental work. The fact that the unfolding intermediate accumulates in substantial amounts under equilibrium conditions should make it possible to characterize its structure and dynamics by techniques like NMR or MS. The evidence presented in this study about the accessibility to trypsinolysis of specific sequences in the unfolding intermediate indicates that the β-strand F5 of the flavin binding domain is the region specifically more expanded in such protein form, although the binding with FAD and the overall protein conformation is largely maintained. Strand F5 is connected through the C-terminal α-helix to the βαβ N-terminal motif, corresponding to the Rossmann fold known as the dinucleotide binding motif (29). The C-terminal α-helix is highly susceptible to
proteolysis (24, 25) and contains the SKL sequence corresponding to the PTS1 signal for peroxisomal import (see Fig. 8). Recently, it has been demonstrated that at least 12 C-terminal residues of a given substrate protein are implicated in PTS1 signal recognition: 1) the C-terminal tripeptide, 2) four residues upstream interacting with the surface of Pex5 receptor, and 3) a polar, solvent-accessible and unstructured region (five residues) with linker function (30). The expanded structure of the C-terminal sequence in the DAAO folding intermediate could represent the optimal conformation for interaction of DAAO with its cognate PTS receptor during in vivo trafficking to peroxisome.

The investigations of the folding process of DAAO should give also a clue as for the rules that govern folding of other flavoproteins, an delivery of peroxisomal proteins to the peroxisome in an “inactive and partially folded state”. Recent experimental evidence demonstrated that proteins which are incapable of assuming their native conformation are also substrates for peroxisomal import (31). When combined with previous reports demonstrating the import of stably folded proteins (8), these results were used to support the model in which tertiary structure was immaterial as for protein import into the peroxisomal matrix (31).

Such a conclusion may be not true in the case of “dangerous” proteins such as DAAO. The inactive, FAD-bound DAAO intermediate should represent a suitable form to target this “dangerous” enzyme (it efficiently produces hydrogen peroxide) from cytoplasm to the peroxisomal matrix, in a partially folded state and thus in agreement to that reported for other peroxisomal proteins (8).

In conclusion, the relevance of our results may extend beyond the single case of DAAO, since from a structural standpoint it is a component of the large glutathione reductase family of flavoproteins (DAAOs belong to the GR2 sub-group). Based on both structural and sequence homologies (12), all members of this family adopt the Rossmann fold (29) and they are characterized by sequence similarity in the N-terminal region that represents the
Unfolding of D-amino acid oxidase

dinucleotide-binding motif. Cofactor binding, which involves a large portion of the protein, may be common to all members of this class and the expanded conformation of the folding intermediate detected here for the first time may resemble the structure of the inactive holoenzyme required in vivo for trafficking through the peroxisomal membrane.

Acknowledgements - This work was supported by a grant from FAR 2002 to L. Pollegioni and from Fondo Progetti di Eccellenza 2001 - University of Insubria, to M. S. Pilone and L. Pollegioni. Individual FIRST grants (MiUR, Rome, Italy) supported the other principal investigators (S. Iametti, F. Bonomi).
REFERENCES

1. Dobson, C. M., and Karplus, M. (1999) *Curr. Opin. Struct. Biol.* 9, 92-101

2. Englander, S. W. (2000) *Annu. Rev. Biophys. Biomol. Struct.* 29, 213-38

3. Samuel, D., Kumar, T. K., Balamurugan, K., Lin, W. Y., Chin, D. H., and Yu, C. (2001) *J. Biol. Chem.* 276, 4134-4141

4. Massey, V., and Hemmerich, P. (1980) *Biochem. Soc. Trans.* 8, 246-255

5. van Mierlo, C. P., van Dongen, W. M., Vergeldt, F., van Berkel, W. J., and Steensma, E. (1998) *Protein Sci.* 7, 2331-2344

6. Subramani, S. (1993) *Annual Rev. Cell. Biol.* 9, 445-478

7. Terlecky, S. R., and Fransen, M. (2000) *Traffic* 1, 465-473

8. Walton, P. A., Hill, P. E., and Subramani, S. (1995) *Mol. Biol. Cell.* 6, 675-683

9. Curti, B., Ronchi, S., and Pilone Simonetta, M. (1992) *Chemistry and Biochemistry of Flavoenzymes* (ed. F. Müller), vol. III, pp. 69-94, CRC Press, Boca Raton, FL

10. Pilone, M. S. (2000) *Cell. Mol. Life Sci.* 57, 1732-1747

11. Umhau, S., Pollegioni, L., Molla, G., Diederichs, K., Welte, W., Pilone, M. S., and Ghisla, S. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 12463-12468

12. Pollegioni, L., Diederichs, K., Molla, G., Umhau, S., Welte, W., Ghisla, S., and Pilone, M. S. (2002) *J. Mol. Biol.* 324, 535-546

13. Piubelli, L., Caldinelli, L., Molla, G., Pilone, M. S., and Pollegioni, L. (2002) *FEBS Lett.* 526, 43-48

14. Pollegioni, L., Iametti, S., Fessas, D., Caldinelli, L., Piubelli, L., Barbiroli, A., Pilone, M. S., and Bonomi, F. (2003) *Protein Sci.* 12, 1018-1029

15. Molla, G., Vegezzi, C., Pilone, M. S., and Pollegioni, L. (1998) *Prot. Expr. Purif.* 14, 289-294
16. Tanford, C. (1968) Adv. Protein Chem. 23, 121-282
17. Pace, C. N. (1990) Trends Biotechnol. 8, 93-98
18. Santoro, M. M., and Bolen, D. W. (1988) Biochemistry 27, 8063-8068
19. Ayed, A., and Duckworth, H. W. (1999) Protein Sci. 8, 1116-1126
20. Iametti, S., Rasmussen, P., Frøkiær, H., Ferranti, P., Addeo, F., and Bonomi, F. (2002) 
   Eur. J. Biochem. 269, 1362-1372
21. Burstein, E. A., Vedenkina, N. S., and Ivkova, M. N. (1973) Photochem. Photobiol. 18, 
   263-279
22. Cairoli, S., Iametti, S., and Bonomi, F. (1994) J. Protein Chem. 13, 347-354
23. Iametti, S., De Gregori, B., Vecchio, G., and Bonomi, F. (1996) Eur. J. Biochem. 237, 106-
   112
24. Pollegioni, L., Ceciliani, F., Curti, B., Ronchi, S., and Pilone, M. S. (1995) Biochem. J. 
   310, 577-583
25. Campaner, S., Pollegioni, L., Ross, B. D., and Pilone, M. S. (1998) Biochem. J. 330, 615-
   621
26. Piubelli, L., Molla, G., Caldinelli, L., Pilone, M. S., and Pollegioni, L. (2003) Protein 
   Eng. 16, 1063-1069
27. Ptistyn, O. B. (1992) Protein folding (ed. Creighton T.E.), pp. 243-300, W.H. Freeman & 
   Co, NY
28. Prakash, K., Prajapati, S., Ahmad, A., Jain, S. K., and Bhakuni, V. (2002) Protein Sci. 11, 
   46-57
29. Rossmann, M. G., Moras, D., and Olsen, K. W. (1974) Nature 250, 194-199
30. Neuberger, G., Maurer-Stroh, S., Eisenhaber, B., Hartig, A., and Eisenhaber, F. (2003) J. 
   Mol. Biol. 328, 567-579
31. Brocard, C. B., Jedieszko, C., Song, H. C., Terlecky, S. R., and Walton, P. A. (2003) *Traffic* **4**, 74-82
TABLE I

Comparison of thermodynamic parameters for urea-induced unfolding of native wild-type and Δloop DAAOs at 15 °C, monitored by measurement of protein and flavin fluorescence (excitation at 280 nm and 450 nm, respectively) and CD.

|          | Wild-type |          | Δloop  |
|----------|-----------|----------|--------|
|          | exc 450 nm | CD       | exc 450 nm | CD       |
| exc 280 nm | F→I       | I→U<sup>b</sup> | Near | Far | F→I       | I→U<sup>b</sup> | Near | Far |
| ΔG<sub>w</sub> (kcal/mole) | 3.4 (1.2) | 2.8 | 5.0 | 3.6 | 1.9 (1.4) | 1.8 | 4.1 | 2.8 |
| m        | 2.4 (0.7) | 1.6 | 6.2 | 1.8 | 1.1 | 1.8 (0.7) | 1.4 | 5.6 | 2.1 | 0.8 |
| Y<sub>N</sub> | 93 | 5 | 169 | 16.4 |
| Y<sub>L</sub> | 137 | 144 | 129 | 130 |
| Y<sub>U</sub> | 476 | 210 | 472 | 230 |
| ΔY       | 383 | 132 | 303 | 112 |
| C<sub>m</sub> (M) | 1.4 (1.8) | 1.8 | 2.8 | 3.7 | 1.0 (2.0) | 1.3 | 1.9 | 3.0 |
DAAO samples were at a 0.02 mg protein/ml concentration for fluorescence measurements and at a 0.5 mg protein/ml concentration for CD measurements.

\(^{a}\)Values similar to those reported were also obtained using the protein fluorescence values determined following the excitation at 298 nm. In parenthesis are reported the values determined following the shift of protein fluorescence emission maximum (see Fig. 1).

\(^{b}\)These values have been estimated using a three-state model according to (19).
FIG. 1. Equilibrium denaturation curves of wild-type (A) and Δloop (B) DAAOs detected by means of tryptophan fluorescence (emission spectra were recorded from 300 to 400 nm with excitation at 280 nm). Fraction of unfolded DAAO determined from the fluorescence intensity at ≈ 340 nm (circle) and emission peak maximum (square) as function of urea concentration. Samples of DAAO (0.02 mg/ml) were equilibrated at 15 °C in the presence of increasing urea concentrations in 50 mM potassium phosphate, pH 7.5, 5% (v/v) glycerol, and 2 mM EDTA with varying concentrations of urea and the fraction unfolded was determined by means of fluorescence change. The reported values have been corrected for the emission of the solution prior to protein addition. Solid lines represent the best fit obtained using a two-state denaturation model (Eq. 4).

FIG. 2. Equilibrium denaturation curves of wild-type (circle) and Δloop (square) DAAOs detected by means of flavin fluorescence. DAAO samples were incubated for 40 min at 15 °C and the flavin emission spectra were recorded from 475 to 600 nm with excitation at 450 nm. See legend of Figure 1 for details. Solid lines represent the best fit obtained using a three-state denaturation model (eq. 5).

FIG. 3. Equilibrium denaturation curves of wild-type (A) and Δloop (B) DAAOs detected by far-UV at 223 nm (circle) and near-UV at 253 nm (square) CD spectra. Proteins were 0.5 mg/ml in 50 mM potassium phosphate, pH 7.5, containing 10% glycerol and 2 mM EDTA. Measurements were performed at 15 °C. Solid lines represent the best fit obtained using a two-state denaturation model (Eq. 4)

FIG. 4. Effect of urea concentration on enzyme activity of wild-type (circle) and Δloop (square) DAAOs. Unfolding reaction: the activity was determined using the oxygen-
Unfolding of D-amino acid oxidase

30
electrode assay on protein samples incubated at 15 °C for 40 min in the presence of different concentrations of urea. Data were fitted to Eq. 4. Inset. Refolding reaction: the activity was determined on protein samples unfolded as above and refolded by 10-fold dilution in 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 5% glycerol, for 10 min (open symbols) or for 24 hours (filled symbols) at 15 °C. The urea concentration reported is that in the refolding solution, after dilution. The point at 3 M urea was obtained by a 10-fold dilution of a protein sample at 8 M urea with a 2.75 M urea containing refolding buffer.

**Fig. 5. Effect of urea on the molecular dimension of wild-type (A) and Δloop (B) DAAOs.** Size exclusion chromatographic profile of DAAO samples (0.2 ml, 1 mg/ml, incubated for 40 min at 15 °C in the presence of different urea concentration) on a Superdex 200 column in 50 mM potassium phosphate buffer, pH 7.5, 5% (v/v) glycerol, and 2 mM EDTA, containing the same urea concentration used in the pre-incubation and at 25 °C.

**Fig. 6. ANS binding.** ANS fluorescence in the presence of wild-type (A) and Δloop (B) DAAOs as a function of urea concentration: fluorescence intensity at 500 nm (circle) and emission maximum (square). Samples of DAAO (2.5 µM = 0.1 mg/ml) were equilibrated for 40 min at 15 °C in the presence of increasing urea concentrations in 50 mM potassium phosphate, pH 7.5, 5% (v/v) glycerol, and 2 mM EDTA with varying concentrations of urea. Fluorescence spectra were recorded from 450 to 600 nm with excitation at 370 nm after the addition of 100 µM ANS. The reported values have been corrected for the emission of the solution prior to protein addition.

**Fig. 7. Refolding of wild-type DAAO following the fluorescence signals.** Comparison of (A) protein and (B) flavin fluorescence of native (thick continuous line) and 1 M urea-treated
(- - -) enzyme with that of the same protein denatured with 8 M urea (—) and then refolded by 10-fold dilution in the absence of urea (see legend of Fig. 4 inset for further details) after 24 hours (● ● ● ●) of incubation at 15 °C.

**Fig. 8.** Detail of DAAO structure highlighting the interaction in the flavin binding domain (11) between the C-terminal α-helix (containing the SKL sequence corresponding to the PTS1 signal for peroxisomal targeting, blue), the N-terminal βαβ motif (the Rossmann fold, pink), and the βF5 strand (green) containing at its N-terminus R<sup>289</sup> (the specific site of trypsinolysis in the unfolding intermediate, red). The flavin cofactor is shown in yellow.

**Fig. 9.** A schematic representation of the structural changes ensuing from increasing concentrations of urea of dimeric wild-type and monomeric Δloop DAAOs. PF: protein fluorescence; FF, flavin fluorescence.
0 M UREA → 2.5 M UREA → 7 M UREA

FOLDED, HOLoenzyme
LOW FF - LOW PF
DIMERIC
FULLY SOLUBLE
FULLY ACTIVE

INTERMEDIATE, HOLoenzyme
MEDIUM FF - HIGH PF
HIGH HYDRODYNAMIC RADIUS
LOW SOLUBILITY
INACTIVE

UNFOLDED, APOProtein
HIGH FF (free flavin) - HIGH PF
HIGH HYDRODYNAMIC RADIUS (multimeric)
GOOD SOLUBILITY
INACTIVE

change in tertiary structure
(susceptibility to trypsin of R289)
loss of activity
decrease in flavin binding

change in secondary structure
loss of coenzyme
Unfolding intermediate in the peroxisomal flavoprotein D-amino acid oxidase
Laura Caldinelli, Stefania Iametti, Alberto Barbiroli, Francesco Bonomi, Luciano
Piubelli, Pasquale Ferranti, Gianluca Picariello, Mirella S. Pilone and Loredano
Pollegioni

J. Biol. Chem. published online April 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403489200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts