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Characterization of a Fully Active N-terminal 37-kDa Polypeptide Obtained by Limited Tryptic Cleavage of Pig Kidney D-Amino Acid Oxidase*

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In order to obtain further information on the structure of D-amino acid oxidase (EC 1.4.3.3), limited proteolysis experiments have been carried out on its apo-, holo-, and holoenzyme-benzoate forms. The enzyme is unsensitive to 10% (w/w) chymotrypsin, while incubation with 10% (w/w) trypsin, under nondenaturating conditions, produces inactivation and proteolysis patterns which are different for the three forms of enzyme analyzed.

These results confirm the previously reported conformational changes which occur upon binding of coenzyme to the apoprotein, and of benzoate to holoenzyme.

The stable 37.0-kDa polypeptide, obtained from the apo- and holoenzyme-benzoate complex upon cleavage of a C-terminal 2.0-kDa fragment, retains full catalytic activity with unaltered kinetic parameters, and the coenzyme binding properties of the native enzyme. These results are in agreement with the tentative localization of the FAD-binding domain in the N-terminal region of the enzyme, and with the hypothesis that the function of the C-terminal region of D-amino acid oxidase could be related to the import of the enzyme into the peroxisomes, as suggested by Gould *et al.* (Gould, S. J., Keller, G. A., and Subramani, S. (1988) *J. Cell. Biol.* 107, 897–905).

A large number of kinetic and structural studies have been carried out on pig kidney D-amino acid oxidase (EC 1.4.3.3), a flavoprotein which catalyzes the oxidative deamination of D-amino acids to yield the corresponding α-keto acid, ammonia, and H_2O_2 (1, 2). Unfortunately, in spite of earlier attempts (3), no crystals of D-amino acid oxidase suitable for X-ray studies have been obtained yet. Thus, due to the lack of a three-dimensional structure of the macromolecule, a considerable amount of experimental work on the kinetic and chemical mechanism of the enzyme and on its active site cannot be fully exploited.

Limited proteolysis has been proven to be an extremely helpful tool for the investigation of specific aspects of the protein structure, such as the localization of hydrophilic regions, the existence of flexible portions on the molecule surface, or the presence of functional domains (4–6).

In D-amino acid oxidase, three different forms of the enzyme, *i.e.* apoprotein, holoenzyme, and holoenzyme-benzoate complex, have been characterized. Spectroscopic and kinetic experiments (7, 8) have shown that different conformational states of the protein are associated with the three forms. Thus, it was interesting to investigate whether limited proteolysis of the protein could distinguish among the three enzyme forms at the structural level. Our data, while confirming the existence of different conformational states of the protein, show that the integrity of the first half of the sequence is required for activity. The results also indicate that the C-terminal 2.0-kDa portion of the protein is not essential for catalysis and coenzyme binding, but could play a role in the recognition and import of D-amino acid oxidase into the peroxisomes (9).

EXPERIMENTAL PROCEDURES

Enzymes—D-Amino acid oxidase was purified from pig kidney as described in Ref. 10. Apoenzyme was prepared as described in Ref. 11. Holoenzyme and holoenzyme-benzoate complex were obtained from apoenzyme upon incubation for 20 min in ice with 40-fold excess FAD, and 40-fold excess FAD and 500-fold excess sodium benzoate, respectively. TPCK[1]-treated trypsin and TLCK-treated chymotrypsin were from Sigma.

Materials—8-Cl-FAD was a generous gift of Prof. Sandro Ghisla (University of Konstanz, Konstanz, Federal Republic of Germany). All other reagents were of analytical grade.

Limited Proteolysis—Apo-D-amino acid oxidase was incubated in 50 mM Hepes/K^+ buffer, pH 8.0, in the presence of 10% (w/w) trypsin, unless otherwise specified. During treatment of holo- and holoenzyme-benzoate forms of D-amino acid oxidase, 1 mM FAD, and 1 mM FAD and 12.5 mM sodium benzoate, respectively, were included in the incubation mixture. At different times aliquots (3 μg) were withdrawn for activity measurements, and diluted in the standard assay mixture; for electrophoretic analyses, at the same times, aliquots (8–10 μg) were diluted 2-fold in 125 mM Tris/Cl^-, pH 6.8, 4% (w/v) sodium dodecyl sulfate, 1.42 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride. Soybean trypsin inhibitor (10/1, w/w with respect to trypsin) or TLCK (5 mM) were also included. Samples were immediately incubated at 100 °C for 5–10 min and subjected to SDS-PAGE. Identical experiments were carried out by replacing trypsin with chymotrypsin. In this case TPCK was substituted for TLCK.

Enzyme Activity Assay—D-Amino acid oxidase activity was assayed spectrophotometrically (12) at 243 nm and 25 °C by monitoring the increase of absorbance of the reaction mixture containing 100 mM sodium pyrophosphate, pH 8.5, 12.5 mM D-α-phenylglycine, and 10 μM FAD.

Protein Concentration Measurements—D-Amino acid oxidase con-

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[1] The abbreviations used are: TPCK, N^α-*p*-tosyl-L-phenylalanine chloromethyl ketone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; TLCK, N^α-*p*-tosyl-L-lysine chloromethyl ketone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 8-Cl-FAD, 8-nor-8-chloro-FAD; 8-SH-FAD, 8-nor-8-mercapto-FAD.

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centration was determined spectrophotometrically (10) or through amino acid analysis.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate—SDS-PAGE was carried out as described in Ref. 13. After electrophoresis, protein bands were stained either by diffusion with Coomassie Blue or by using the silver staining procedure (14).

Densitometric Measurements—Densitometric measurements on the Coomassie Blue-stained polyacrylamide gels were performed at 630 nm, using a LKB laser densitometer interfaced with an Apple personal computer.

Isolation of Proteolysis Products—Preparative experiments were carried out by incubating 25 μM apo-, holo-, and holoenzyme-benzoate complex of D-amino acid oxidase with 10% (w/w) trypsin as described above. At different times, aliquots (400 pmol) were withdrawn. The proteolysis products were separated by SDS-PAGE and electrotransferred on a polyvinylidene difluoride membrane (Immobilon, Millipore) as in Ref. 15 for subsequent analysis. Alternatively, 5 mM TLCK or 0.15 mg of soybean trypsin inhibitor were added to 150 μl of the incubation mixture, and the proteolytic fragments were separated by fast protein liquid chromatography on a Superose 12 gel filtration column equilibrated with 25 mM Hepes/K^+, pH 8.0, containing 0.1 M KCl. When the D-amino acid oxidase-benzoate complex was analyzed, the column equilibration buffer also contained 1 μM FAD and 0.2 mM sodium benzoate. Aliquots of each fraction were analyzed for D-amino acid oxidase activity spectrophotometrically, and for peptide composition through SDS-PAGE.

Amino Acid Analysis—Amino acid analyses were performed by the *o*-phthalaldehyde post-column derivatization procedure, after gas-phase hydrolysis of proteins in 6 M HCl, 1% (v/v) phenol, at 110 °C for 24, 48, or 72 h (16), using a Jasco amino acid analyzer.

N-terminal Protein Sequencing—Sequence analyses were performed using an automated Protein Sequencer (Applied Biosystems Model 477/A) equipped with an on-line HPLC (Applied Biosystems Model 120/A) for phenylthiohydantoin-derivative identification (17).

Steady-state Kinetic Measurements—Apparent values of K_M for D-α-phenylglycine, and for FAD were obtained by measuring the initial velocity of reaction mixtures at 25 °C and 243 nm, in 0.1 M pyrophosphate buffer, pH 8.5. To determine the K_M for D-α-phenylglycine, FAD concentration was held constant at 10 μM, and D-α-phenylglycine was varied between 1.7 and 12.5 mM. For the determination of the apparent K_M for FAD, the concentration of the coenzyme was varied between 0.7 and 10 μM in the presence of 12.5 mM D-α-phenylglycine. To determine the K_I for benzoate, its concentration was varied between 1.3 and 18 μM in the presence of 7.5, 5, 3.33, 2.5 mM D-α-phenylglycine and 10 μM FAD. In all cases the reactions were started by adding 0.4–0.8 μg of protein to the reaction mixture equilibrated at 25 °C.

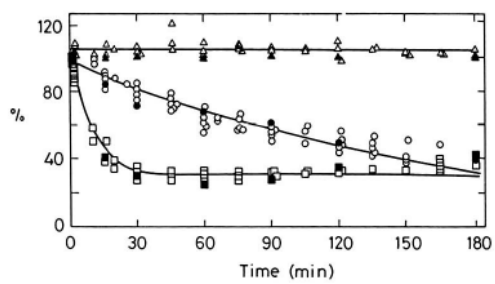

FIG. 1. **Proteolytic inactivation of D-amino acid oxidase.** Apo-, holo-, and holoenzyme-benzoate complex of D-amino acid oxidase were incubated with 10% trypsin (w/w) at 25 °C (see "Experimental Procedures"). At different times, aliquots of the incubation mixtures (3 μg) were assayed spectrophotometrically for activity. Aliquots (8 μg) were also subjected to SDS-PAGE, followed by densitometric analysis of the Coomassie Blue-stained protein bands. The percentages of residual activity (*open symbols*) and of the density corresponding to the sum of the 39- and 37-kDa species *versus* the total protein density (*closed symbols*) are presented in the figure for apo- (○, ●), holo- (□, ■), and the holoenzyme-benzoate complex (△, ▲) of D-amino acid oxidase.

Reconstitution of the 37-kDa Proteolysis Fragment with 8-Cl-FAD and 8-SH-FAD—10 nmol (400 μg) of D-amino acid oxidase-benzoate complex was incubated with trypsin (10%, w/w) for 170 min. An 8.5-nmol aliquot was withdrawn and trypsin was inactivated by addition of 5 mM TLCK. The 37-kDa polypeptide was separated by fast protein liquid chromatography on the Superose 12 column as described above. The polypeptide containing fractions were concentrated with a Centricon 10 (Amicon) microconcentrator, and incubated with 210 nmol of 8-Cl-FAD, in ice, overnight. The unbound 8-Cl-FAD was removed by gel filtration on a Sephadex G-25 column equilibrated in 25 mM Hepes/K^+, pH 8.0. Protein-bound 8-Cl-FAD was converted to 8-SH-FAD by addition of 5 μmol of Na_2S, at 17 °C (18). The reaction was monitored spectrophotometrically.

Data Analysis—Analysis was accomplished using the Enzfitter program (Elsevier Biosoft).

RESULTS

Kinetics of Inactivation—25 μM apo-, holo-, and holoenzyme-benzoate forms of D-amino acid oxidase were incubated with 10% (w/w) trypsin. At different times, aliquots were withdrawn for activity assays and electrophoretic analysis. The Coomassie Blue-stained gels were submitted to densitometric analysis to compare the rate of enzyme inactivation with the rate of degradation and of formation of the various polypeptide bands.

Incubation of the holoenzyme-benzoate complex with trypsin resulted in no loss of catalytic activity, while SDS-PAGE revealed the progressive digestion of the 39-kDa native form of the enzyme, and the concomitant appearance of a stable 37-kDa fragment (Figs. 1 and 2*C*). When apoenzyme was submitted to tryptic digestion, the activity loss was well described by a single exponential process, which proceeded with a rate constant of 0.006 min^{-1} (Figs. 1 and 4). SDS-PAGE (Fig. 2*A*) showed the cleavage of the native enzyme to yield a 37-kDa polypeptide, which was further degraded to smaller peptides of 25, 20, and 13.4 kDa. Finally, in the case of tryptic digestion of holoenzyme, the rate of inactivation was faster than that measured for the apoenzyme, but it leveled off at 30–40% of the initial value; a rate constant for activity loss of 0.106 min^{-1} was calculated (Figs. 1 and 4). SDS-PAGE showed that the holoenzyme undergoes two parallel proteolytic processes: slow degradation to the stable 37-kDa fragment, and direct degradation to smaller fragments of 25 and 14.5 kDa, the latter being further degraded to a 13.4-kDa peptide (Fig. 2*B*). A digestion of the holoenzyme in the presence of 2% (w/w) trypsin, which was performed to investigate the early proteolytic events, confirmed the proteolytic

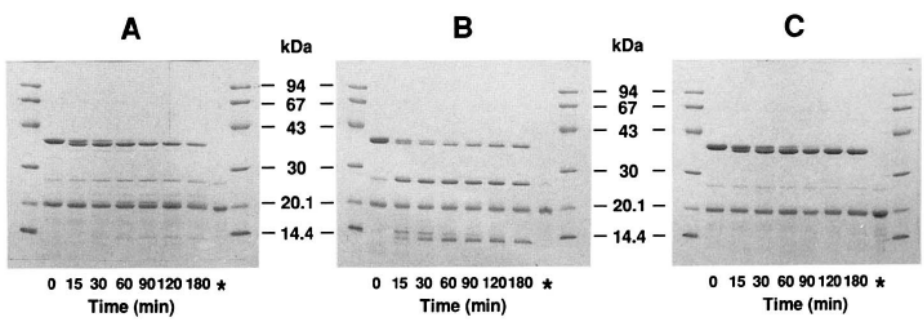

FIG. 2. **Proteolysis products of D-amino acid oxidase.** 1 mg/ml apo- (*panel A*), holo- (*panel B*), and holoenzyme-benzoate forms (*panel C*) of D-amino acid oxidase were incubated with 10% (w/w) trypsin at 25 °C (see "Experimental Procedures"). At different times, aliquots (8 μg) were subjected to SDS-PAGE. In the three gels shown the outer lanes correspond to the protein standards, while the lane marked with a *star* shows the migration of trypsin (23.8 kDa) and trypsin inhibitor (20.1 kDa).

sequence described above (data not shown). Densitometric analyses of gels after SDS-PAGE confirmed the qualitative results reported above. These analyses also allowed us the calculation of the rates of degradation of the native 39-kDa protein, and of formation of the 37.0-kDa polypeptide obtained by tryptic cleavage of the three forms of D-amino acid oxidase (Figs. 3, *A–C*, and 4). The degradation of the 39-kDa native form in apoprotein and the holoenzyme-benzoate complex (Fig. 3, *panels A* and *C*) was a single exponential process, whose calculated rates were 0.036 min^{-1} and 0.024 min^{-1}, respectively. The degradation of the 39-kDa species in holoenzyme was well described by the sum of two exponential processes, where the rates of the slow phase (which involves 20–30% of the total protein) and of the fast phase (which accounts for the remaining 70–80% of the protein present) were 0.01 min^{-1} and 0.103 min^{-1}, respectively (Figs. 3*B*, and 4). The biphasic inactivation and degradation pattern of the holoenzyme was independent from protein concentration (data not shown). In the holoenzyme-benzoate complex, the rate of formation of the 37-kDa species matched the rate of degradation of the native protein, as expected (Fig. 3*C*). In the case of apoenzyme (Figs. 3, *panel A*, and 4), the 37-kDa fragment was formed at a rate of 0.037 min^{-1}, and it was degraded at about 0.004 min^{-1}. Finally, in holoenzyme, the rate of formation of the stable 37-kDa species matched that of the slow phase of loss of the native protein band ($k = 0.007$ min^{-1}). In all cases, the rate of activity loss was equal to the rate of loss of the sum of the 39- and 37-kDa species (Fig. 1). This indicates that the 37-kDa polypeptide has identical catalytic properties as the native enzyme, while all other proteolytic products are completely inactive.

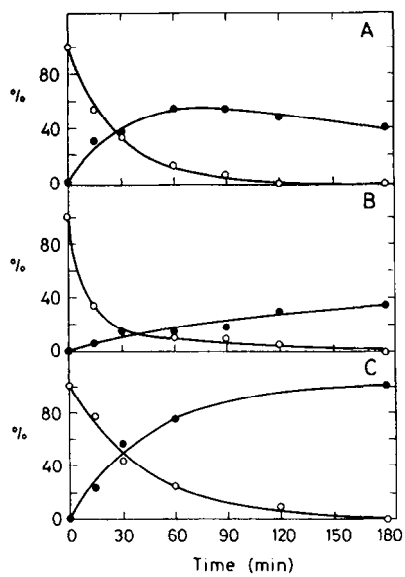

FIG. 3. **Kinetics of proteolytic cleavage of native D-amino acid oxidase and of formation of the stable 37-kDa polypeptide.** Densitometric analysis of the electrophoretic patterns shown in Fig. 2 allowed the calculation of the rates of degradation of the native 39-kDa protein (○), and appearance of the stable 37-kDa polypeptide (●) in the apo- (*panel A*), holo- (*panel B*), and holoenzyme-benzoate complex (*panel C*) of D-amino acid oxidase. For clarity, the percentages of other proteolytic products over the total protein present in the sample are not shown.

Characterization of the Products of Limited Proteolysis—The major proteolytic products obtained upon tryptic digestion of the three forms of D-amino acid oxidase were isolated by either fast protein liquid chromatography gel filtration or SDS-PAGE, and subjected to N-terminal sequencing and amino acid analysis to locate the sites of tryptic cleavage within the enzyme primary structure (19; Fig. 5).

In all cases, the 37-, 25-, and 20-kDa fragments showed the same N-terminal sequence as the native enzyme. Due to the low amount of the 20-kDa peptide present at any time in our samples, this was not further analyzed. Amino acid analysis indicated that the 37-kDa polypeptide originated from the tryptic cleavage at Lys-328 of the native enzyme, while the 25-kDa species derived from cleavage at Arg-221 of the native form of D-amino acid oxidase, in holoenzyme, or of the 37-kDa polypeptide, in apoprotein (Figs. 4 and 5). The N-terminal sequences of the 14.5- and 13.4-kDa fragments indicated that they were the result of a different tryptic attack. In the case of holo-D-amino acid oxidase, the 14.5-kDa fragment originated from cleavage at Arg-221 of the native enzyme; a further cleavage at the C-terminal, presumably at Lys-328, yielded the 13.4-kDa fragment. In apoprotein, the 13.4-kDa peptide derived directly from cleavage at Arg-221 of the 37-kDa species (Figs. 4 and 5).

Kinetic Characterization of the 37-kDa Polypeptide—From the results of the tryptic digestion of the three enzyme forms, it appeared that the 37-kDa polypeptide is fully active (Fig. 1). In order to further characterize the 37-kDa polypeptide, this was isolated by gel filtration chromatography of samples (400 pmol) of apo- or holoenzyme-benzoate complex forms incubated with 10% (w/w) trypsin for 135 and 170 min, respectively. Interestingly, under these experimental conditions (1 mg/ml protein), the 37-kDa fragment originated from the apoenzyme digestion eluted as a monomer, while the one derived from the holoenzyme-benzoate complex eluted as a dimer, in agreement with the behavior of the corresponding native forms of D-amino acid oxidase (20). The apparent K_M values for FAD and D-α-phenylglycine, and the K_I for ben-

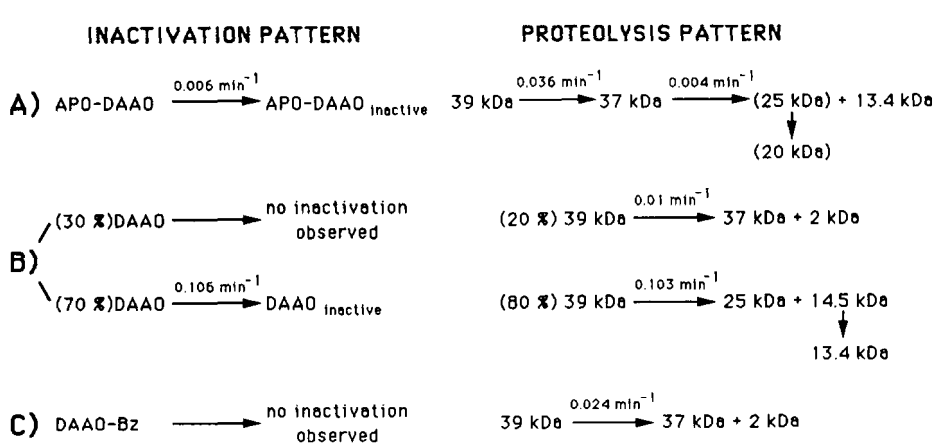

FIG. 4. **Comparison of the inactivation and proteolysis patterns upon limited tryptic digestion of D-amino acid oxidase.** The pattern and the rates calculated for the tryptic inactivation of apo- (*panel A*), holo- (*panel B*), and holoenzyme-benzoate complex (*panel C*) of D-amino acid oxidase (see Fig. 1), and those deduced for the proteolytic degradation of the protein (see Figs. 2 and 3) are compared. The species in parentheses do not accumulate, although they are detectable in SDS-PAGE. *APO-DAAO*; apo-D-amino acid oxidase; *DAAO*; holo-D-amino acid oxidase; *DAAO-Bz*; holoenzyme-benzoate complex of D-amino acid oxidase.

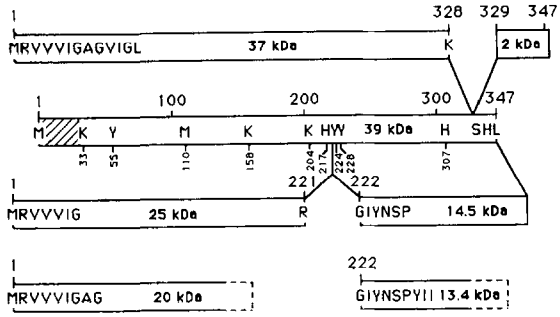

FIG. 5. **Characterization of limited proteolysis products of D-amino acid oxidase.** The experimentally determined N-terminal sequences of the isolated proteolytic fragments of D-amino acid oxidase are shown in the figure. For clarity, the relative sizes of the polypeptides have not been taken into account. In the schematic representation of the native protein (39-kDa species) the *shaded area* indicates the region of D-amino acid oxidase that is homologous to glutathione reductase and *p*-hydroxybenzoate hydroxylase (19) and amino acid residues that have been chemically modified are indicated (2, 23, 19). Due to the low amount of 20-kDa fragment present in our samples at any time, it has not been possible to determine its amino acid composition, thus its C-terminal region is uncertain.

zoate of the isolated 37-kDa polypeptide were determined ($K_{M(FAD)}$ = 2.03 μM, $K_{M(D\text{-}\alpha\text{-phenylglycine})}$ = 9.1 mM, and $K_{I(benzoate)}$ = 13.7 μM), and they agreed well with the corresponding values obtained for control samples of native D-amino acid oxidase ($K_{M(FAD)}$ = 1.24 μM, $K_{M(D\text{-}\alpha\text{-phenylglycine})}$ = 7.14 mM, and $K_{I(benzoate)}$ = 12.7 μM). Furthermore, the specific activity of native enzyme and of 37-kDa fragment were also calculated. Aliquots of both enzyme forms used for the determination of maximal velocity in the presence of 10 μM FAD, and at different concentrations of D-α-phenylglycine, were subjected to amino acid analysis in order to determine the protein concentration. Values of 2.78 and of 2.71 ΔA_{243} min^{-1} $nmol^{-1}$ were obtained for the native and the cleaved 37-kDa enzyme species, respectively.

Finally, to test whether the coenzyme binding site of the 37-kDa polypeptide retained the same properties as in native enzyme, 8-Cl-FAD and 8-SH-FAD were used as flavin-binding probes (18). The 37-kDa polypeptide binds 8-Cl-FAD. As in the case of native D-amino acid oxidase, the coenzyme exchange could be accomplished by incubation of the FAD-bound 37-kDa fragment with excess 8-Cl-FAD. This confirms that the relative affinity for the two flavins is similar in native D-amino acid oxidase, and in the 37-kDa fragment. Furthermore, treatment of the 8-Cl-FAD/37-kDa polypeptide with Na_2S gave rise to the spectral changes and protein stabilization of the 8-SH-FAD benzoquinoid form reported for the native enzyme (18, 21).

DISCUSSION

The existence of different conformational states of D-amino acid oxidase has been confirmed by the present limited proteolysis experiments. In fact, apo-, holo-, and holoenzyme-benzoate complex showed different sensitivity to tryptic cleavage, which can be interpreted as due to a different accessibility, in the three species, of the protein surface to trypsin digestion. Interestingly, chymotrypsin, up to 10% (w/w), was in all cases without effect (data not shown). Fig. 5 summarizes the sites of tryptic cleavage of the enzyme that resulted from our limited proteolysis experiments. Lys-328 is always exposed to the tryptic attack, while in the case of holo- and apoenzyme, the second major site of cleavage is Arg-221. Interestingly, this residue belongs to a sequence (residues 217–228) containing at least 3 amino acid residues essential for catalysis (2). Remarkably, despite that Arg-221 is within a highly hydrophilic region, according to the hydropathy plot of D-amino acid oxidase (22), this residue is not available for tryptic cleavage in the holoenzyme-benzoate complex of D-amino acid oxidase. This result suggests that the binding to the holoenzyme of the substrate analog benzoate induces a dramatic change in the environment of the active site peptide. In apoprotein, Lys-328 is the primary site of tryptic attack; cleavage at Arg-221 of the 37-kDa initial tryptic product follows. In holoenzyme, two different conformational states of the enzyme can be detected. 20–30% of the enzyme is present in a conformation where only Lys-328 is exposed to trypsin. However, cleavage at this position to yield the stable 37-kDa polypeptide is slower than in the case of apo- or holoenzyme-benzoate forms. In the remaining 70–80% of the holoenzyme, Arg-221 is the primary site of tryptic cleavage to give the unstable and inactive 25- and 14.5-kDa peptides. While the former is rapidly degraded to lower molecular mass forms, the 14.5-kDa species is further cleaved (presumably at Lys-328) to yield the 13.4-kDa fragment. These data are consistent with the heterogeneous behavior of D-amino acid oxidase holoenzyme observed during chemical modification studies (11).

The densitometric analysis of the digestion products of the three enzyme's forms treated with 10% trypsin allowed us to calculate the rates of formation and degradation of the various peptide fragments, and to compare these data with the rate and extent of disappearance of catalytic activity for the same enzyme species (Fig. 4). This analysis is in agreement with the proteolytic sequence discussed above, and strongly indicates that both the native enzyme (39 kDa) and the 37-kDa fragment are the catalytically active species present, while the lower molecular mass products are fully inactive.

The 37-kDa polypeptide obtained by tryptic digestion of apo- or holoenzyme-benzoate forms a D-amino acid oxidase shows the same kinetic parameters, coenzyme binding properties, and chromatographic behavior of the corresponding native forms of D-amino acid oxidase. These data are consistent with those reported by several authors on D-amino acid oxidase (2). In fact, on the basis of studies on the similarities among the amino acid sequences of several flavoproteins, part of the coenzyme binding domain has been tentatively assigned to the N-terminal region of D-amino acid oxidase (19). Unfortunately, our attempts to isolate the 25- and 20-kDa inactive fragments chromatographically, or electrophoretically, under nondenaturing conditions were unsuccessful, thus preventing us from testing the coenzyme-binding properties of these two peptides which contain the N-terminal region of D-amino acid oxidase. Furthermore, active site studies of D-amino acid oxidase (2, 19, 23) have indicated that most of the amino acid residues involved in the catalytic mechanism of the enzyme are included in the 204–307 region of the primary structure (see Fig. 5). We observed that cleavage within this region causes loss of activity and produces fragments greatly susceptible to further tryptic degradation. This finding suggests that the integrity of the upstream region of the protein (residues 1–328), which is preserved upon binding of benzoate to D-amino acid oxidase, is sufficient not only for catalysis, but also to maintain the correct folding of the protein.

Our data also indicate that the C-terminal portion of D-amino acid oxidase (residues 329–347) is not essential for catalytic activity, coenzyme binding, or monomer-monomer interaction. Interestingly, Gould *et al.* (9) recently showed by gene fusion experiments that in several peroxisomal enzymes a common C-terminal sequence (Ser-Lys/His-Leu) functions as a target for the recognition and import of proteins into the peroxisomes. The triplet Ser-His-Leu (345–347) is in fact

present in the C terminus of D-amino acid oxidase. Our results and those of Gould *et al.* (9) even if not necessarily correlated, may support the role of the C-terminal region of the enzyme in the translocation process of the protein into the peroxisomes. At this stage it cannot be ruled out that other residues in the C-terminal peptide of D-amino acid oxidase (residues 329–347) may play a role in the translocation process.

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