Limited Proteolysis and X-ray Crystallography Reveal the Origin of Substrate Specificity and of the Rate-Limiting Product Release during Oxidation of D-Amino Acids Catalyzed by Mammalian D-Amino Acid Oxidase[†,‡]

Maria A. Vanoni,[*,§] Antonio Cosma,[§] Daniela Mazzeo,[§] Andrea Mattevi,[||] Flavia Todone,[||] and Bruno Curti[§,⊥]

Dipartimento di Fisiologia e Biochimica Generali, Universitá degli Studi di Milano, Via Celoria 26, 20131 Milano, Italy, Dipartimento di Genetica e Microbiologia, Universitá degli Studi di Pavia, Via Abbiategrasso 207, Pavia, Italy, and Centro Interuniversitario per lo Studio delle Macromolecole Informazionali

Received December 10, 1996; Revised Manuscript Received February 12, 1997[⊗]

ABSTRACT: Limited proteolysis of D-amino acid oxidase holoenzyme with trypsin cleaves the protein at Arg 221 and near the C-terminus, producing stable 25, 13.4, and 2 kDa polypeptides [Torri-Tarelli, G., Vanoni, M. A., Negri, A., & Curti, B. (1990) *J. Biol. Chem. 265*, 21242−21246]. The 25 and 13.4 kDa polypeptides remain associated to form a nicked D-amino acid oxidase species. This nicked protein form maintains the ability to bind FAD, but exhibits altered catalytic efficiency toward the oxidation of various D-amino acids when compared to native DAAO. Changes in substrate specificity were first monitored by measuring the activity in the presence of different amino acid substrates at various times during proteolysis. Three amino acid substrates were then selected for further analysis of the properties of the nicked D-amino acid oxidase species produced by limited tryptic proteolysis: D-serine, D-arginine, and D-alanine. The three D-amino acids represented limiting cases of the observed changes of enzyme activity on nicking: loss of activity, increase of activity, and minor activity changes, respectively. D-serine was found to be no longer a substrate of D-amino acid oxidase. D-arginine exhibited a 2.5-fold increased apparent maximum velocity although its K_m value increased 2-fold with the nicked enzyme in comparison to the native species. D-alanine was oxidized 1.5-fold faster by the nicked D-amino acid oxidase at infinite substrate concentration, and its K_m value increased approximately 4-fold. The K_d for benzoate, which was determined kinetically with D-alanine as the enzyme substrate, increased 17-fold in the nicked species. Primary deuterium kinetic isotope effects on V and V/K during the oxidation of D-alanine were also measured. $^DV/K$ increased from 1.4 ± 0.2 to 1.8 ± 0.3 on nicking, while ^DV increased from 1.04 ± 0.1 to 2.53 ± 0.5. All the observed changes of the values of the kinetic parameters and of the observed isotope effects are consistent with the hypothesis that nicking of D-amino acid oxidase at position 221 decreases the strength of binding of both substrates and products to the enzyme active site. The information obtained by limited tryptic proteolysis nicely complements that gathered from the analysis of the three-dimensional structure of D-amino acid oxidase in complex with benzoate, which was recently determined [Mattevi, A., Vanoni, M. A., Todone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M., & Curti, B. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 7496−7501]. Arginine 221 is part of the 216−228 loop that covers the active site and contributes residues to substrate binding and catalysis. The limited proteolysis data support the hypothesis that this loop acts as a lid on the active site and controls both substrate specificity and the rate of turnover of D-amino acid oxidase.

D-Amino acid oxidase (D-amino acid: oxygen oxidoreductase, EC 1.4.3.3, DAAO[1]) is a well characterized FAD-dependent enzyme which catalyzes the oxidative deamination of D-α-amino acids to yield the corresponding α-keto acid and ammonia (Curti et al., 1992).

Among D-amino acid substrates, the enzyme is more efficient toward the oxidation of those containing a neutral side chain, very poorly oxidizes amino acids with a positively charged side chain, and is inactive toward acidic amino acids (Dixon & Kleppe, 1965). D-Aspartate and D-glutamate are oxidatively deaminated by a specific D-aspartate oxidase (Negri et al., 1992). The kinetics of oxidation of amino acids catalyzed by DAAO differ according to the amino acid side chain (as reviewed in Curti et al., 1992). The major distinction is made between neutral and polar amino acids on one side, and basic amino acids on the other. The reaction mechanism with the former substrates is described by a

[†] This work was carried out with funds from Ministero dell' Universitá e la Ricerca Scientifica e Tecnologica and from Consiglio Nazionale delle Ricerche, Rome, Italy.

[‡] The atomic coordinates of D-amino acid oxidase have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973. Filename: 1KIF.

* To whom correspondence should be addressed.

[§] Universitá degli Studi di Milano.

[||] Universitá degli Studi di Pavia.

[⊥] Centro Interuniversitario per lo Studio delle Macromolecole Informazionali.

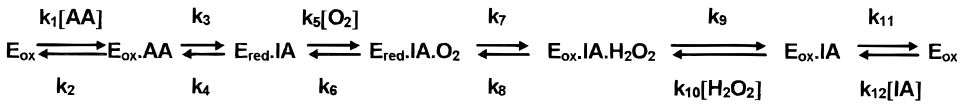
[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: DAAO, D-amino acid oxidase; Apo-DAAO, apoprotein form of DAAO; DAAO-Bz, DAAO-benzoate complex; FPLC, fast protein liquid chromatography; V, apparent maximum velocity; K, apparent Michaelis constant for the substrate indicated in parentheses as the subscript; K_d, dissociation constant for the complex between the enzyme and the compound indicated in parentheses as the subscript; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; SDS, sodium dodecyl sulfate; AA, amino acid; IA, imino acid.

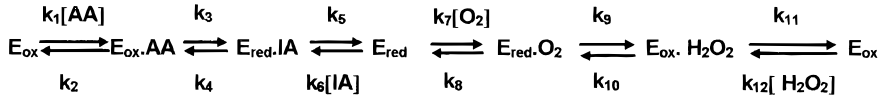
S0006-2960(96)03023-1 CCC: $14.00

Scheme 1: Reaction of DAAO with Neutral and Polar Amino Acids (A) and with Basic Amino Acids (B)

A:



B:



limiting case of an ordered mechanism (Scheme 1A) where reoxidation of the reduced flavin by oxygen takes place prior to release of the imino acid, which is the rate-limiting step in the overall reaction. The oxidation of basic amino acids is well described, instead, by a simple ping-pong mechanism (Scheme 1B; Curti et al., 1992; Yagi et al., 1974; Yagi et al., 1969). Among neutral and polar amino acids, exemplified by D-alanine and D-serine, respectively, functional distinctions have been described (Denu & Fitzpatrick, 1992; Porter et al., 1977). A series of kinetic isotope effect measurements on the reactions of DAAO (Denu & Fitzpatrick, 1992) has shown that D-alanine behaves as a "sticky" substrate ($k_3 \gg k_2$ in Scheme 1A), while D-serine does not.

The lack of a three-dimensional structure of DAAO has prevented in the past a rigorous correlation between the observed functional properties of the enzyme and its structural features. A variety of chemical modification and site-directed mutagenesis studies, along with the comparison of the properties of DAAO from different sources, have been reported (Curti et al., 1992). In our laboratory, we have used limited proteolysis to probe the presence of flexible regions of DAAO exposed to solvent and to compare the susceptibility to limited tryptic proteolysis of the different forms of DAAO that can be prepared, namely: apoprotein, holo-DAAO, and the DAAO−benzoate complex (Torri Tarelli et al., 1990). These studies revealed that in apo-DAAO three sites of the protein are particularly sensitive to trypsin (see Figure 5 of Torri Tarelli et al., 1990). Trypsin first attacks the Lys328 or Arg337 position very rapidly to yield an N-terminal 37 kDa fragment and a C-terminal 2 kDa polypeptide. The N-terminal 37 kDa polypeptide is then cleaved into two fragments resulting from hydrolysis of the 221−222 peptide bond to yield a stable C-terminal 13.4 kDa polypeptide and an N-terminal 25 kDa polypeptide. The latter is unstable and undergoes proteolytic cleavage to yield an N-terminal 20 kDa polypeptide, which is further degraded. The presence of FAD in the holoenzyme protects the N-terminal 25 kDa polypeptide from proteolytic degradation, consistent with the hypothesis that this portion of the protein is involved in FAD binding. Furthermore, in approximately 30% of the holoenzyme the 221−222 peptide bond is also protected from proteolysis, thus leading to the presence of a fraction of DAAO converted to the 37 kDa N-terminal polypeptide. In the DAAO−benzoate complex the only site sensitive to proteolysis is that at the C-terminus of the protein, which leads to the formation of the 37 kDa polypeptide and of the C-terminal 2 kDa fragment. We have also shown that the 37 kDa polypeptide, obtained from the holoenzyme or the DAAO−benzoate complex, exhibits properties indistinguishable from those of the native enzyme, while the protein cleaved at position 221−222 is inactive with respect to D-α-phenylglycine as the substrate. These findings supported the hypothesis that the region surrounding position 221 of DAAO is involved in the formation of the enzyme active site and that extensive conformational changes involving this region do occur on binding of the substrate to DAAO.

In the present work we have extended the analysis of the effects of limited tryptic proteolysis of DAAO holoenzyme. In particular, taking into account the protection exerted by FAD on further degradation of the 25 kDa N-terminal fragment (see above), we aimed to verify if the latter polypeptide maintained the ability to bind FAD and if the loss of activity during proteolysis of the holoenzyme we observed with D-α-phenylglycine as the substrate could be extended to other amino acids. As this work was being completed, the three-dimensional structure of DAAO was solved in our laboratories (Mattevi et al., 1996) and independently by Mizutani and co-workers (Mizutani et al., 1996). This allowed us to compare the results of limited proteolysis experiments with the crystallographic data in order to get a better insight on structure−function relationship of this flavooxidase.

MATERIALS AND METHODS

Enzymes. Preparation of the DAAO−benzoate complex and of the apoprotein were carried out according to established procedures, with modifications reported previously (Curti et al., 1973; Massey & Curti, 1966; Vanoni et al., 1987). The protein concentration was determined using published extinction coefficients (Curti et al., 1973; Massey & Curti, 1966).

Limited Tryptic Proteolysis. Limited tryptic proteolysis on the apo- and holo-DAAO and on the DAAO−benzoate complex was carried out as described in the previous paper (Torri Tarelli et al., 1990). Typically, the form of DAAO under study (1 mg/mL) was incubated with trypsin (0.1 mg/mL) for 3−5 h, at 25 °C, in the dark in 50 mM Hepes/KOH, pH 8.0. Before and at different times from trypsin addition the proteolytic reaction was monitored in different ways. Aliquots (10 μL) were withdrawn and transferred to an Eppendorf tube containing 10 μL of a 20 mM TLCK solution and 20 μL of 2XSDS sample buffer (Laemmli, 1970). Protein was denatured by heating at 100 °C for 5 min. With

this procedure soybean trypsin inhibitor was not necessary to quench trypsin. Proteolytic fragments were resolved by polyacrylamide gel electrophoresis in the presence of SDS on 12% minigels, which were stained with Coomassie blue as described by Torri Tarelli et al. (1990). Aliquots were also withdrawn from the proteolytic reactions and diluted in assay mixtures containing different D-α-amino acids. The activity was determined by various methods as described below. For kinetic analyses of the DAAO form derived from tryptic proteolysis, the enzyme was incubated with trypsin as described above for 3−5 h to allow for maximal conversion of the native protein into the cleaved one. TLCK to a final concentration of 1.54 mM was added to quench trypsin, and the enzyme solution was kept on ice in the dark and used immediately for kinetic analyses. Control experiments showed that 1.54 mM TLCK was sufficient to inactivate trypsin. Furthermore, during most of the experiments a parallel sample was set up, which was treated exactly as the proteolyzed one except for the addition of trypsin. Kinetic determinations were also carried out on such control samples. In these control experiments DAAO was stable and exhibited the same properties of samples that had not been incubated for 3−5 h at 25 °C and were not supplemented with TLCK.

Activity Assays and Steady-State Kinetic Analyses. DAAO activity was measured polarographically using a Clark-type oxygen electrode at 25 °C in 0.1 M pyrophosphate buffer, pH 8.5, in the presence of 10 μM FAD and fixed or varied amounts of the selected D-amino acid. When D-α-phenylglycine was the substrate, its oxidation was monitored at 243 nm (Fonda & Anderson, 1967). Alternatively, the enzyme reaction was measured spectrophotometrically by monitoring the rate of H_2O_2 production, in a coupled assay with horse radish peroxidase (Macheroux et al., 1992). Reaction mixtures (1 mL) contained the given amino acid, 15 μg of horse radish peroxidase, and 0.4 mM *o*-dianisidine, and the increase of absorbance at 436 nm was monitored. An extinction coefficient of 11.6 mM^{-1} cm^{-1} was applied. Reactions were carried out in 50 mM Hepes/KOH buffer, pH 8.0, at 25 °C. Control experiments demonstrated that the different assay methods gave essentially the same results, once the composition of the assay mixture was the same with respect to buffer, pH, and substrate concentrations.

In a series of preliminary experiments, the values of *V* and *K* for the oxidation of the different amino acids were determined by varying amino acid concentrations in air-saturated buffers with the oxygen electrode or the spectrophotometric method, using native DAAO. In order to monitor activity values at different times during the proteolysis, the activity of DAAO was measured in the presence of concentrations of substrate that were at least 10-times the *K* value we determined with native DAAO, unless this was prevented by the low solubility of the substrate and/or the high *K* value exhibited by the reaction.

Inhibition by Benzoate. The inhibition constant exhibited by benzoate with respect to the native and nicked DAAO forms was determined by varying benzoate concentration between 0 and 300 μM in the presence of three different concentrations of D-alanine (8.4, 14, and 28 mM) in the coupled assay with the horse radish peroxidase.

Primary Deuterium Kinetic Isotope Effects on D-Alanine Oxidation. In order to measure the primary kinetic isotope effects on the oxidation of D-alanine exhibited by native and nicked DAAO, the apparent *V* and *V*/*K* values were determined independently using DL-(α-^{1}H)- or DL-(α-^{2}H)-alanine in two series of assays. The coupled assay with horse radish peroxidase was used for this experiment, and DL-[α-^{2}H]-alanine was a kind gift of Dr. P. Fitzpatrick.

Data Analysis. The kinetics of activity loss or gain during proteolysis were well described by single exponential processes (Torri Tarelli et al., 1990), and the data were analyszed by fitting the values of percent residual activity (*A*) at a given time (*t*) to eq 1 or 2, respectively:

$$A = A_1 \exp(-k_1 t) + A_2 \quad (1)$$

$$A = A_1 (1 - \exp(-k_1 t)) + A_2 \quad (2)$$

where: A_1 is the percent activity change associated with activity loss or increase; A_2 in eq 1 is the percent residual activity at infinite reaction times, while A_2 in eq 2 is the percent initial activity.

The steady-state kinetic parameters apparent maximum velocity (*V*) and apparent K_m for the different amino acids (*K*) were obtained by fitting initial velocities (υ) measured at different substrate concentrations (*S*) and constant oxygen concentration (0.253 mM at 25 °C) to the Michelis−Menten equation (eq 3), once visual inspection ensured the linearity of double reciprocal plots (Segel, 1975).

$$\upsilon = (VS)/(K + S) \quad (3)$$

For the determination of the inhibition constant (K_i) for benzoate, the initial velocities (υ) obtained at different inhibitor (*I*) and D-alanine (*S*) concentrations were fitted to eq 4a (Segel, 1975).

$$\upsilon = VS/(K(1 + I/K_i) + S) \quad (4a)$$

For graphical representation, the Dixon plot was chosen (eq 4b; Segel, 1975):

$$1/\upsilon = (K/V)(1/K_i)(1/S)I + ((K/V)(1/S) + (1/V)) \quad (4b)$$

For the determination of the primary deuterium kinetic isotope effects on *V* and *V*/*K*, the primary data obtained with D-[α-^{1}H]- and D-[α-^{2}H]-alanine were fitted to eq 5, where *F* is the fraction of deuterium label in the substrate ($F = 0.0$ and 1.0 for hydrogen- and deuterium-containing substrate, respectively), ^DV is the deuterium isotope effect on *V*, and $^DV/K$ is the deuterium isotope effect on *V*/*K* (Cleland, 1980).

$$\upsilon = VS/(K(1.0 + F(^DV/K - 1)) + S(1.0 + F(^DV - 1))) \quad (5)$$

The Grafit software (Erythacus Software Ltd., UK) run on a Zenith Z-425/SX personal computer was used for data analysis.

RESULTS

Time-Course of Activity Changes of DAAO with Respect to D-Amino Acids during Limited Tryptic Cleavage. The results of previous limited proteolysis experiments (Torri Tarelli et al., 1990), where a different stability of the N-terminal 25 kDa fragment derived from holo- and apo-DAAO was observed, suggested that bound FAD could be the reason of the greater stability of this fragment in the case of the holoenzyme. As a consequence attempts were made

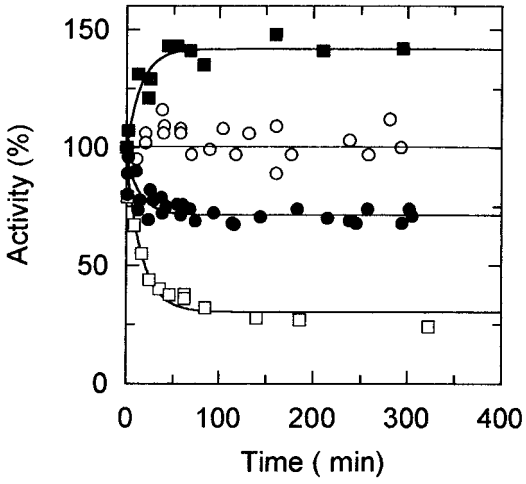


FIGURE 1: Time-course of activity changes during tryptic limited proteolysis of DAAO. DAAO (1 mg/mL) was incubated with trypsin (0.1 mg/mL) at 25 °C as described in Material and Methods. At different times aliquots were withdrawn and the activity of DAAO using the indicated amino acids was measured. The curves shown have been drawn using eq 1 or 2 and the indicated values of A_1, A_2 and k_1 (see Materials and Methods and Table 1): D-Ala (open circles): $A = 100$; D-Val (closed circles): $A = 24e\wedge(-0.067t) + 71$; D-Thr (open squares): $A = 64e\wedge(-0.059t) + 30$; D-Arg (closed squares): $A = 42(1 - e\wedge(-0.066t)) + 100$.

Table 1: Kinetics of Activity Changes during the Course of Limited Tryptic Proteolysis of DAAO with Respect to the Oxidation of Different Amino Acids

		parameters		
amino acid substrate (mM)[a]	equation[b]	A_1	k_1 (min^{-1})	A_2
D-alanine (28)	1	100	0	0
D-isoleucine (11.4)	1	100	0	0
D-methionine (20)	1	100	0	0
D-tyrosine (0.35)	1	21	0.040	87
D-valine (21.34)	1	24	0.044	74
D-proline (43.4)	1	40	0.060	65
D-leucine (6.86)	1	35	0.043	60
D-histidine (100)	1	40	0.052	61
D-α-phenylglycine (12.5)	1	56	0.061	43
D-serine (91)	1	61	0.048	31
D-threonine (150)	1	69	0.064	29
D-arginine (20)	2	37.5	0.087	96
D-lysine (60)	2	19	0.063	102

[a] Activity assays were carried out in the presence of 0.01 mM FAD, 0.4 mM *o*-dianisidine, and 15 μg of peroxidase in 50 mM Hepes/KOH buffer, pH 8.0, at 25 °C. The amino acid substrate concentrations were held constant at the concentration indicated in parentheses. [b] Before and at different times after trypsin addition aliquots of the proteolysis mixture were diluted in activity assay mixtures. The values of residual activity calculated at different proteolysis times were expressed as the percent of the activity measured prior to trypsin addition, corrected for dilution, and were fitted to eqs 1 and 2.

to separate the N-terminal 25 kDa fragment derived from limited tryptic proteolysis of the holoenzyme, from the C-terminal 13.4 kDa polypeptide by several chromatographic techniques. These experiments were unsuccessful, showing that proteolysis of DAAO at position 221−222 resulted in a nicked DAAO species in which the two fragments remained associated. Fluorimetric analysis of samples obtained during these chromatographic separations demonstrated that the nicked DAAO species maintained bound FAD. Thus, such nicked DAAO species is inactive with respect to the oxidation of D-α-phenylglycine (Torri Tarelli et al., 1990), but its ability to maintain bound FAD prompted us to test its activity with respect to other amino acids. In a first series of experiments, DAAO was incubated with trypsin at 25 °C in the dark. At different times aliquots of the proteolysis reaction were diluted in assay mixtures containing either D-α-phenylglycine or another amino acid. In order to determine the amino acid concentration to use in these assays, the *K* values exhibited by each amino acid were determined during separate experiments with the native enzyme. The values we obtained were similar to those reported by Dixon and Kleppe (1965), regardless of the assay method used. Substrate concentration in the assays used to monitor the time course of activity changes during limited proteolysis of DAAO was always at least 10-fold the corresponding *K* value for the native enzyme, except for those amino acids that exhibited high *K* values and low solubility. The results of these experiments are summarized in Figure 1 and in greater detail in Table 1. No time-dependent activity changes were observed using D-alanine, D-isoleucine, and D-methionine; loss of activity comparable to that observed with D-α-phenylglycine was observed with D-serine and D-threonine, while D-valine, D-proline, D-leucine, D-histidine, D-tyrosine, and D-phenylalanine had an intermediate behavior. When D-arginine and D-lysine were the substrates, activity increased.[2] In all cases, activity changes observed with the different amino acids occurred at virtually identical rates, given the experimental error associated with these determinations (compare values in Figure 1 and Table 1) and paralleled the formation of the 25 and 13.5 kDa fragments of DAAO, as revealed by electrophoretic analysis of the proteolysis reaction. Furthermore, it should be kept in mind that the residual activity observed with all D-amino acids behaving like D-α-phenylglycine always correlated with the fraction of 37 kDa polypeptide present at the end of the proteolysis of DAAO. Its quantity was on the average 30% of the total protein. These results confirmed that nicked DAAO maintained the ability to bind FAD and suggested that proteolytic cleavage at position 221−222 altered the conformation of the enzyme active site with an effect on the rates which govern the individual steps of the reaction leading to the altered behavior during the oxidation of different D-amino acids. Similar experiments were performed using the DAAO−benzoate complex which is converted to the 37 kDa N-terminal polypeptide described before (Torri Tarelli et al., 1990). In this case, no activity loss was observed with any of the substrates tested, in agreement with the original finding that the 37 kDa polypeptide exhibits properties similar to those of native DAAO. Furthermore, proteolysis of the apoprotein led to species that are inactive toward all the amino acid tested, in agreement with the observation that the N-terminal 25 kDa fragment produced from apo-protein is unstable and rapidly degraded to low molecular weight peptides. Finally, the enzyme preparations remained inactive with respect to the oxidation of D-glutamate and D-aspartate.

Steady-State Kinetic Analysis of Nicked DAAO. In order to better characterize the properties of the nicked DAAO species formed on proteolysis of DAAO, the enzyme was incubated with trypsin for 3−5 h in order to obtain maximal

[2] The low activity exhibited by DAAO with respect to the oxidation of glycine (Dixon & Kleppe, 1965; Denu & Fitzpatrick, 1992) prevented us from obtaining reliable data with this enzyme substrate.

Table 2: Comparison of Kinetic Parameters Exhibited by Native and Nicked DAAO[a]

	native DAAO			nicked DAAO		
substrate	*K* (mM)	*V* (μmol of H_2O_2/(min·mg)	*V*/*K*	*K* (mM)	*V* (μmol H_2O_2/(min·mg)	*V*/*K*
D-alanine	3.1 ± 0.3	9.6 ± 0.3	3.09	13.0 ± 1.3	13.3 ± 0.7	1.02
D-serine	41.1 ± 5.5	8.7 ± 0.53	0.21	48.3 ± 7.9	3.7 ± 0.3[b]	0.08
D-α-phenylglycine	10.7 ± 3.8	10.8 ± 2.1	1.0	15.5 ± 7.2	2.9 ± 0.8[b]	0.19
D-arginine	26.9 ± 4.8	4.6 ± 0.4	0.17	39.0 ± 4.1	9.4 ± 0.6	0.24

[a] Assays were carried out in the presence of varied concentrations of the amino acid, 0.01 mM FAD, 0.4 mM *o*-dianisidine, 15 μg of peroxidase, 50 mM Hepes/KOH buffer, pH 8.0, and DAAO that had been incubated for 3 h in the absence or presence of 10% (by weight) trypsin. To stop trypsin proteolysis, 1.54 mM TLCK was added, and protein samples were transferred on ice and immediately used for the determination of the steady-state kinetic parameters. [b] The residual activity measured with D-serine and D-α-phenylglycine correlates with the amount of 37 kDa fragment present at the end of the trypsin treatment of DAAO in the experiment (approximately 30%).

conversion into the nicked species. Trypsin was quenched by addition of TLCK, which had no effect on DAAO activity, as shown by control experiments. Enzyme solutions were kept on ice and immediately used for the determination of the apparent kinetic parameters *V* and *K* for D-α-phenylglycine, D-alanine, D-serine, and D-arginine. These amino acids were selected for further analysis because of the wealth of information already available on their reaction with DAAO and because they represented limiting cases: no apparent loss of activity during proteolysis (D-alanine), maximal loss of activity (D-α-phenylglycine and D-serine), maximum activity increase (D-arginine). It should be noted that the proteolysed enzyme preparation exhibited a limited stability (namely, 50% activity loss was observed on storage on ice overnight), preventing more complex and time-consuming experiments than those shown here. Table 2 summarizes the results of several experiments which were carried out with similar results using different assay methods.

It is confirmed that proteolysis of DAAO at position 221−222 leads to a species that is inactive with respect to D-α-phenylglycine (Torri Tarelli et al., 1990). A similar result was obtained with D-serine. The steady-state kinetic analysis of the nicked DAAO form revealed that this protein species oxidized D-alanine with an approximately 30% faster velocity, but the *K* value determined for the amino acid was approximately 4-fold higher than that determined under identical conditions with the native enzyme, leading to a 3-fold decrease of the catalytic efficiency (*V*/*K*) of the nicked DAAO species in this reaction (Figure 2). Finally, *V* increased 2-fold when D-arginine was the nicked DAAO substrate as compared to the native enzyme. Again nicking brought along an increase of the *K* value for arginine (39 mM), from the already high value (27 mM) of the native enzyme.

The nicked DAAO preparations contain approximately 30% of DAAO that had been converted into the 37 kDa polypeptide (Torri Tarelli et al., 1990). This species exhibits the same *V* and *V*/*K* values as native DAAO. By correcting for the contribution of the 37 kDa polypeptide,[3] *V* exhibited by nicked DAAO with D-alanine becomes approximately 15 μmol/(min·mg), (155% that of native DAAO), and that with D-arginine becomes approximately 11.5 μmol/(min·mg), (250% that of native enzyme with the same substrate).

The changes of the steady-state kinetic parameters we observed immediately suggested to us that nicking of DAAO at position 221 led to a "more open" active site. If this were the case, D-serine and D-α-phenylglycine could not bind productively anymore leading to inactivation. D-Alanine and its oxidation product, iminopyruvate, would bind less tightly leading to decreased catalytic efficiency but higher turnover number because product release would be no longer (or less) rate limiting. Finally, we can imagine that in native DAAO the structure of the active site offers steric or electrostatic hindrance to proper binding and positioning of D-arginine, a bulky and charged amino acid. A "more open" active site in the nicked species would allow D-arginine to find a better positioning with respect to catalytic groups and the flavin, thus bringing along the observed increase of *V* and *V*/*K*.

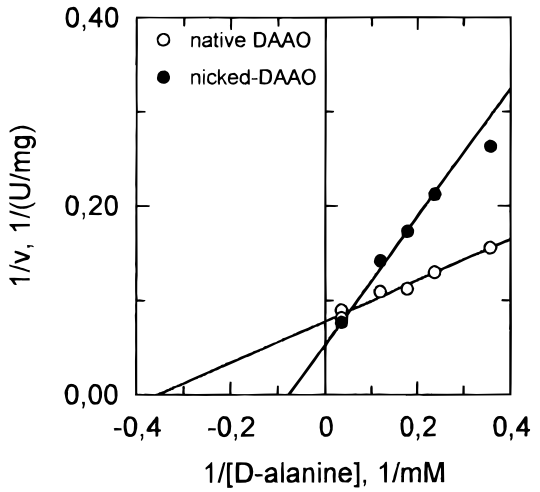


FIGURE 2: Comparison of the steady-state kinetic parameters exhibited by the native and nicked DAAO during D-alanine oxidation. All assays were carried out at 25 °C using the coupled assay with horse radish peroxidase in 50 mM Hepes/KOH buffer, pH 8.0. The reciprocal values of the initial velocities measured in the presence of varying amounts of D-alanine in 50 mM Hepes/KOH, pH 8.0, and 10 μM FAD, at 25 °C, using native DAAO or nicked DAAO are plotted as a function of the reciprocal substrate concentration. The lines are drawn using the values of *V* and *V*/K summarized in Table 2.

Benzoate Binding to Nicked DAAO. If nicking of DAAO at position 221−222 resulted in a looser active site, also the binding of the substrate analog, benzoate, should be weaker than with the native DAAO. The dissociation constant for the DAAO−benzoate complex was determined kinetically. The nicked DAAO exhibited a 17-fold higher K_i for benzoate (271 μM) with respect to native DAAO (16 μM, at pH 8.0 and 25 °C; Figure 3, Table 3).

FAD Binding to Nicked DAAO. Given the fact that proteolysis of DAAO is carried out in the presence of excess FAD in order to prevent formation of the apoprotein, and that the nicked enzyme has a limited stability, we obtained

[3] For the calculation we used the following expression: $V_{obs} = 0.7V_{nickedDAAO} + 0.3*V_{nativeDAAO}$.

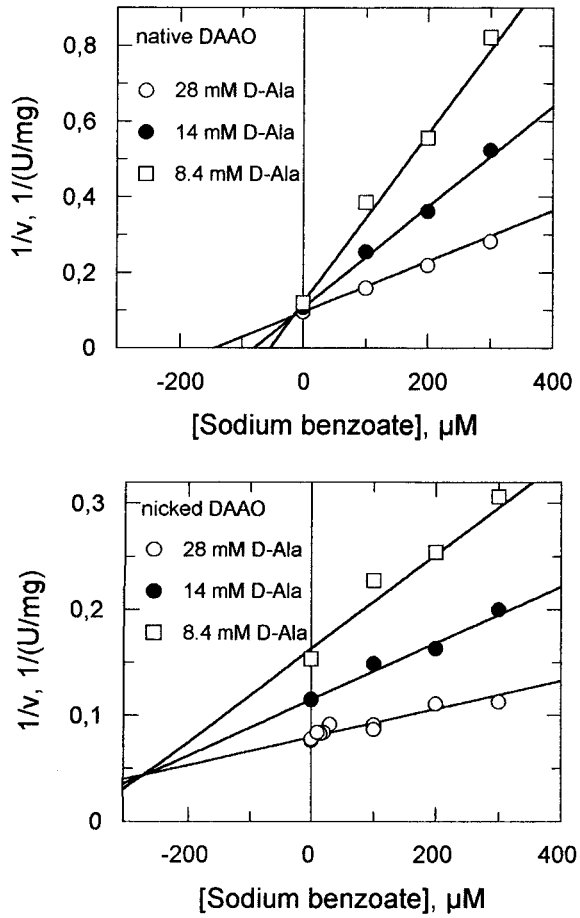


FIGURE 3: Benzoate inhibition of the native and nicked DAAO forms. The K_i values exhibited by the native (top part) and nicked DAAO (lower part) and benzoate have been determined by measuring the initial velocity of reactions containing varying concentrations of benzoate and constant levels of D-alanine in 50 mM Hepes/KOH, pH 8.0, 10 μM FAD, at 25 °C. The Dixon plots were constructed using the parameters summarized in Table 3.

an estimate of the dissociation constant for FAD from a steady-state kinetic experiment. The initial velocity of reactions (1 mL) containing 28 mM D-alanine and amounts of FAD ranging from 0 to 10 μM was measured using the peroxidase-coupled assay. Reactions were started with the addition of 0.5 μL of nicked DAAO solution or 0.5 μL of a parallel sample from which trypsin had been omitted. The FAD concentration was calculated taking into account that the enzyme solutions added to the assays contained 1 mM FAD. The initial velocities measured at the different FAD concentrations were fitted to the Michaelis−Menten equation. Values of $K_{(FAD)}$ of (0.034 ± 0.009) μM and of (0.120 ± 0.022) μM were obtained for the native and nicked DAAO species, respectively (Table 3). This result confirmed that the nicked enzyme maintained FAD bound with an affinity not dramatically different from that of native DAAO. Furthermore, this experiment ensured that both proteolytic treatment of DAAO and all our kinetic analyses had been carried out under saturating concentrations of FAD (1 mM and 10 μM, respectively).

Primary Kinetic Deuterium Isotope Effects on the Oxidation of D-*Alanine by the Nicked DAAO.* A small primary kinetic isotope effect on *V*/*K* and essentially no isotope effect on *V* have been reported for DAAO during the oxidation of D-alanine (Denu & Fitzpatrick, 1992; Porter et al., 1977). The large suppression of the intrinsic deuterium isotope effect on *V*/*K* is due to a large value of the k_3/k_2 ratio (Scheme 1A). On the other hand, the almost full suppression of the intrinsic isotope effect on *V* has been ascribed to the fact that product release is rate limiting during oxidation of D-alanine by DAAO ($k_3/k_{11} \gg 0$). If our hypothesis that the active site of DAAO has become looser following proteolysis at position 221 (with increase of the values of k_2 and k_{11}) is correct, we would expect that the nicked species exhibited greater isotope effects on both *V* and *V*/*K* than the native form. Again, the experimental results were consistent with such hypothesis. A ^{D}V of 1.04 ± 0.1 is observed with the native enzyme, and the $^{D}V/K$ was 1.38 ± 0.25 (Figure 4 and Table 3). This value is similar to that of determined by Denu and Fitzpatrick (1992) under similar pH conditions. With the nicked species, ^{D}V was 2.53 ± 0.47 and $^{D}V/K$ was 1.84 ± 0.33.

DISCUSSION

While the present work was in progress, we solved the three-dimensional structure of DAAO from pig kidney in complex with benzoate (Mattevi et al., 1996). Analysis of the DAAO structure in light of the previous (Torri Tarelli et al., 1990) and present results obtained by limited proteolysis of DAAO allow us both to confirm initial hypotheses on the structure of the enzyme and to elucidate novel functional aspects of the enzyme.

The C-terminal region of DAAO (residues 340−347) is disordered and not visible in the three-dimensional structure of DAAO, thus explaining why a site close or within this region is extremely sensitive to tryptic proteolysis in all DAAO forms. Moreover, from the mass and amino acid composition of the 37 kDa N-terminal fragment of DAAO, we argued that the C-terminal peptide derived from tryptic cleavage at Lys328 without ruling out Arg337 as the proteolysis site. The X-ray structure of DAAO clearly shows that Lys328 is within a α-helix, while Arg337 is located next to the disordered C-terminal region. As a consequence, we suggest Arg337 as the most likely target of proteolysis rather than Lys328.

The active center of DAAO is lined by hydrophobic residues, and its size and shape is, as predicted by the early work of Dixon and Kleppe (1965), optimized for accommodating small hydrophobic amino acids whose side chain is shorter than four carbons. On the basis of the binding mode of benzoate, a model of DAAO in complex with D-alanine was built (Mattevi et al., 1996). More important in relation to a critical evaluation of proteolysis results is the finding that in the three-dimensional structure of the DAAO−benzoate complex positions 221−222 of DAAO belong to a loop extending from position 216 to position 228: it is on the protein surface and limits the active site of DAAO preventing contact with solvent (Figure 5). In the DAAO−benzoate complex it is ordered and contributes to benzoate binding with the side chain of Tyr 224 which lays parallel to the benzoate phenyl ring. From the analysis of the structure of the DAAO−benzoate complex it could be inferred that the 216−228 active site loop must change its conformation in order to allow the entrance and exit of the substrate and products to and from the active site, respectively. In this respect the limited proteolysis data we presented before (Torri Tarelli et al., 1990) and in this work are in full agreement with the crystallographic data and

Table 3: Comparison of the Dissociation Constants for Benzoate and FAD and of the Primary Kinetic Isotope Effects on D-Alanine Oxidation Exhibited by Native and Nicked DAAO[a]

		native DAAO			nicked DAAO		
Substrate (mM)	Inhibitor (μM)	K (mM)	V (μmol of H_2O_2/(min·mg))	K_i (μM)	K (mM)	V (μmol of H_2O_2/(min·mg))	K_i (μM)
Dissociation Constant for Benzoate[b]							
D-alanine (8.4, 14, 28)	benzoate (0−300)	3.5 ± 0.5	11.7 ± 0.3	16 ± 1.9	23.1 ± 4	23 ± 1.9	271 ± 41

		native DAAO		nicked DAAO	
substrate (mM)	FAD (μM)	$K_{d(FAD)}$ (μM)	V (μmol of H_2O_2/(min·mg))	$K_{d(FAD)}$ (μM)	V (μmol of H_2O_2/(min·mg))
Dissociation Constant for FAD[c]					
D-alanine (28)	0.5−10.5	0.034 ± 0.009	12.9 ± 0.11	0.12 ± 0.022	14.1 ± 0.22

	native DAAO				nicked DAAO			
substrate	K (mM)	V (μmol of H_2O_2/(min·mg))	$^DV/K$	^DV	K (mM)	V (μmol of H_2O_2/(min·mg)	$^DV/K$	^DV
Primary Kinetic Deuterium Isotope Effects[d]								
D-[α-^{1}H]-alanine	3.01 ± 0.54	14.8 ± 0.9			12.0 ± 1.9	22.0 ± 2.0		
D-[α-^{2}H]-alanine	4.0	14.2			8.7	8.7		
			1.4 ± 0.2	1.04 ± 0.1			1.84 ± 0.33	2.53±0.47

[a] Assays were carried out in the presence of 0.4 mM *o*-dianisidine, 15 μg of peroxidase, 50 mM Hepes/KOH buffer, pH 8.0, and DAAO that had been incubated for 3 h in the absence or presence of 10% (by weight) trypsin. To stop trypsin proteolysis, 1.54 mM TLCK was added, and protein samples were transferred on ice and immediately used for the determination of the steady-state kinetic parameters. [b] Assays were carried out in the presence of 10 μM FAD, varying concentrations of benzoate and different fixed levels of D-alanine. The data were fitted to eq 4a which describes competitive inhibition. [c] Assays were carried out in the presence of 28 mM D-alanine and varying concentration of FAD. The data were fitted to the Michaelis−Menten equation. [d] Assays were carried out by varying D-[α-^{1}H]- or D-[α-^{2}H]-alanine concentration in the presence of 10 μM FAD. The data were fitted to eq 5, and the values of V and K for the deuterated substrate were calculated using the estimates for the protio substrate and the values of the isotope effects on V and V/K.

further clarify the role of the 216−228 active site loop of DAAO. In the absence of bound substrate the loop is more flexible ("open") and, presumably, in a disordered state leading to a high susceptibility to tryptic attack of position 221−222. Following benzoate (or amino acid) binding, the prevailing form is that with the loop in the ordered ("closed") position observed in the crystal structure where position 221−222 no longer is accessible to trypsin. Interestingly, limited proteolysis of DAAO holoenzyme always showed the presence of a fraction (≈30%) of protein in which the 221−222 position was not accessible to trypsin, suggesting the presence of a slow conformational equilibrium with the loop in the "open" or "closed" position also in the benzoate-free enzyme. Proteolytic nicking of the loop most likely prevents coordinated movement of such protein portion during closure of the active center on substrate binding. Therefore, the ability of DAAO to oxidize various types of amino acids is modified. Such effects range from loss of activity (as exemplified by D-α-phenylglycine and D-serine) to increased activity, although with a lower catalytic efficiency (with D-alanine or D-arginine). With native DAAO, D-serine is not a sticky substrate of the enzyme (k_3/k_2 is relatively small; Denu & Fitzpatrick, 1992). Nicking by proteolysis may increase k_2, thus preventing binding of D-serine to DAAO. Furthermore, the lack of coordinated movement of the active site loop may alter the position of amino acyl residues within the active site to the point where catalysis is prevented. D-Arginine offers an example opposite to that observed with D-α-phenylglycine and D-serine on the effect of nicking of DAAO. Amino acids with bulky and positively charged side chains cannot be easily accommodated by DAAO active site, probably, because of their size and charge (Dixon & Kleppe, 1965; Yagi et al., 1974; Mattevi et al., 1996). This most likely explains the low affinity exhibited by DAAO toward these substrates, the low rate of oxidation of them, and the rapid release of the corresponding oxidation products, which brings along the switch from the order mechanism of Scheme 1A to the ping-pong mechanism of Scheme 1B. With these substrates, disorder of the loop in nicked DAAO most likely improves catalysis because it may allow better positioning of the basic amino acid in the active site. It may even be suggested that the nicked loop exhibits a greater conformational flexibility than in the native protein and can adapt its conformation to the bulky charged substrate and position catalytic groups to favor its oxidation.

D-Alanine is the most commonly used substrate of DAAO. The variety of data available, which describe the reaction of DAAO with this substrate (in particular see Porter et al., 1977 and Denu & Fitzpatrick, 1992), allowed us to define better the properties of nicked DAAO. With the nicked enzyme, the catalytic efficiency of DAAO toward the oxidation of D-alanine decreased 3-fold, but the turnover number increased 1.5-fold. In order to rationalize our results, we evaluated the expression for V and V/K (eqs 6 and 7, respectively. See also footnote 4) when written using the net rate constants method of Cleland (1975) for the mechanism shown in Scheme 1A:

[4] E, total enzyme concentration; $k_{11}' = k_{11}$; $k_9' = k_9$; $k_7' = (k_7k_9)/(k_8 + k_9)$; $k_5' = (k_5[O_2]k_7')/(k_6 + k_7')$; $k_3' = (k_3k_5')/(k_4 + k_5')$; $k_1' = (k_1[AA]k_3')/(k_2 + k_3')$. The primed rate constants are net rate constants calculated as described by Cleland (1975).

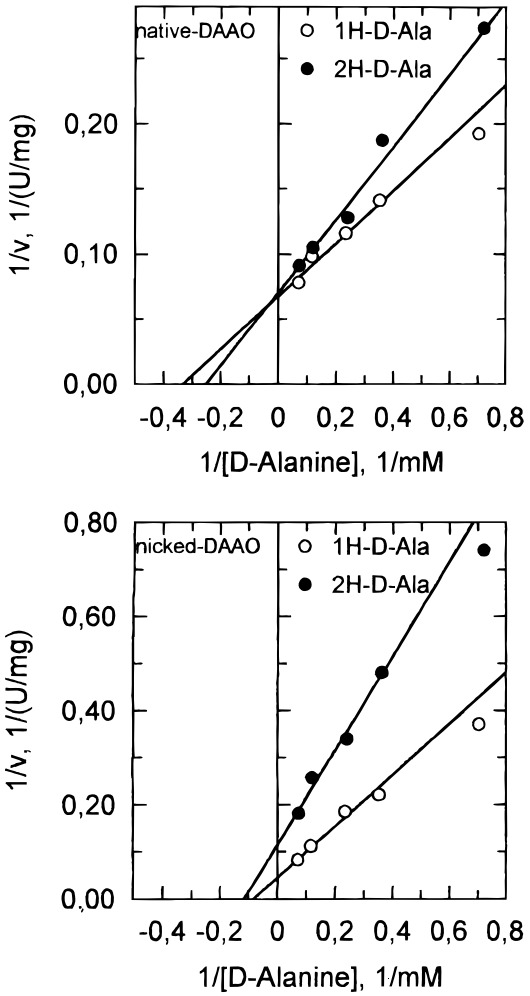


FIGURE 4: Deuterium primary kinetic isotope effects exhibited by native and nicked DAAO. The primary kinetic isotope effects on the oxidation of D-alanine have been determined using native (top part) and nicked DAAO (bottom part) by measuring the initial velocity of reactions containing variable amounts of D-[α-^{1}H]- or D-[α-^{2}H]-alanine in the presence of 10 μM FAD. The lines show the fit to the data in the double reciprocal form, and the calculated parameters *V* and *V*/*K* are summarized in Table 3.

$$E/V = 1/(1/k_3' + 1/k_5' + 1/k_7' + 1/k_9 + 1/k_{11}) \quad (6)$$

$$(V/K)/E = (k_1k_3')/(k_2 + k_3') \quad (7)$$

With D-alanine and the native enzyme, product release (k_{11}) is rate-limiting turnover; k_4 is effectively zero so that $k_3' = k_3$ and $k_3/k_2 \gg 0$ so that $k_1' = k_1$[AA] (Porter et al., 1977; Denu & Fitzpatrick, 1992). Furthermore, reoxidation of DAAO by molecular oxygen is described as a simple bimolecular reaction, so that k_6/k_7 and k_8/k_9 are essentially zero, and k_7 and k_9 are very large. Therefore, eqs 6 and 7 reduce to eqs 8 and 9, respectively.

$$E/V = 1/(1/k_3 + 1/k_5' + 1/k_{11}) \quad (8)$$

$$(V/K)/E = k_1 \quad (9)$$

The changes in *V* and *V*/*K* we observed are consistent with the hypothesis that proteolysis primarily affects k_2 and k_{11}. If k_2 increases following nicking and no longer is $k_3/k_2 \gg 0$, *V*/*K* would be described by eq 7 rather than eq 9 and be smaller than *V*/*K* exhibited by native enzyme by a factor given by $k_3/(k_2 + k_3)$. If the decreased stickiness of the substrate were also accompanied by a faster release of the imino acid product (increase of k_{11} of Scheme 1A), *V* would also increase, as observed. In support of this interpretation is the fact that the K_i for benzoate increases 17-fold on DAAO nicking. In this context, we would like to point out that benzoate is routinely defined as a substrate analog of DAAO. It occurred to us that in light of the low K_i exhibited by benzoate and the native enzyme (in the micromolar range) and its planarity at the Cα position, this compound may act more as a product rather than as a substrate analog.

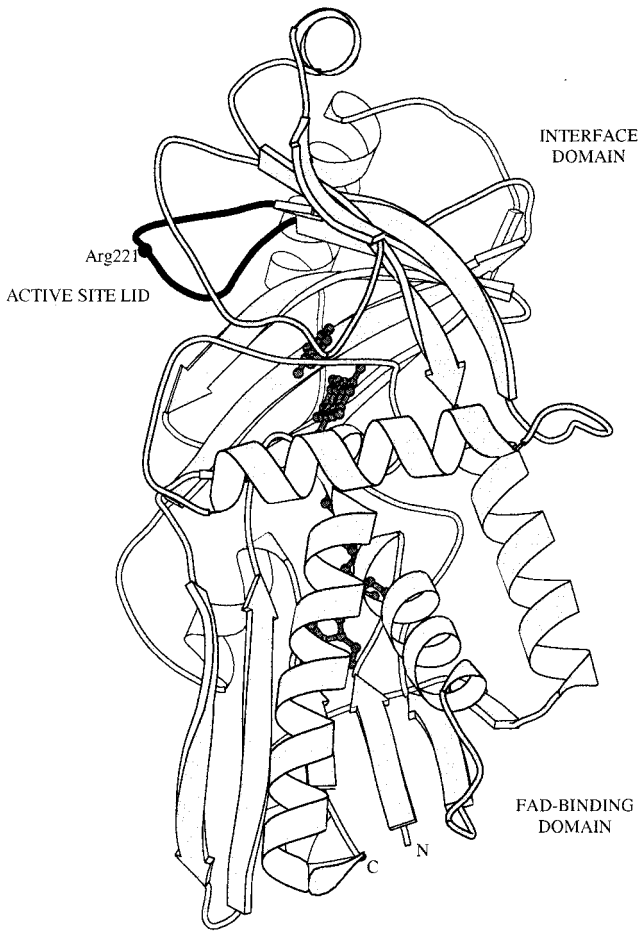


FIGURE 5: Ribbon representation of the two domain structure of pig kidney DAAO subunit. The benzoate inhibitor and the FAD cofactor are shown in ball-and-stick representation. The position of the active site lid and of Arg221 is outlined.

The values of the observed kinetic primary deuterium isotope effects during oxidation of D-alanine also change on nicking of DAAO. Their analysis allows us to further elucidate the effect of nicking of the active site lid of DAAO on its reaction. The expressions for $^DV/K$ and ^DV were derived for the mechanism shown in Scheme 1A (Cleland, 1982), keeping in mind the known properties of DAAO reaction with D-alanine as detailed above:

$$^DV/K = (^Dk_3 + C_f + C_r{}^DK_{eq})/(1 + C_f + C_r) = (^Dk_3 + C_f)/(1 + C_f) = (^Dk_3 + k_3/k_2)/(1 + k_3/k_2) \quad (10)$$

where $^DV/K$ is the observed kinetic isotope effect on *V*/*K*; $^Dk_3$ is the intrinsic isotope effect, and it was estimated to have a value of approximately 5.5 (Denu & Fitzpatrick, 1992). C_f is the forward committment to catalysis; and $C_f = k_3/k_2$; C_r is the reverse committment to catalysis; $k_4/k_5' \approx$

0 because $k_4 \approx 0$. ${}^{D}K_{eq}$ is the primary deuterium kinetic isotope effect on the equilibrium constant:

$$ {}^{D}V = ({}^{D}k_3 + C_{vf} + C_r {}^{D}K_{eq})/(1 + C_{vf} + C_r) = $$
$$ ({}^{D}k_3 + C_{vf})/(1 + C_{vf}) = = ({}^{D}k_3 + k_3/k_5' + k_3/k_{11})/ $$
$$ (1 + k_3/k_5' + k_3/k_{11}) \quad (11) $$

where ${}^{D}V$ is the observed isotope effect on V; C_{vf} is the ratio of catalysis, $k_3/k_5' + k_3/k_{11}$.

${}^{D}V/K$ increases only a little from 1.4 ± 0.2 to 1.8 ± 0.3 on nicking of DAAO, while ${}^{D}V$ increases significantly from 1.04 ± 0.1 to 2.5 ± 0.5, indicating that most of the changes brought along by nicking involve the oxidative half reaction of DAAO. The increase of ${}^{D}V$, together with the 1.5-fold increase of V, indicates that the rate of release of the imino acid (k_{11}) must be approximately 2-fold faster in the nicked species with respect to native enzyme (at 25 °C, O_2 concentration is only 0.25 mM, making k_5' partially rate limiting: 2530 min^{-1} with $k_5 = 1 \times 10^7$ M^{-1} min^{-1} and k_{11} = 600 min^{-1}; Porter et al., 1977). However, a 2-fold increase of k_{11} would not be sufficient to justify the large increase of ${}^{D}V$ we observed. A significant increase of ${}^{D}k_3$ is ruled out by the small change of ${}^{D}V/K$ we observed. Assuming that ${}^{D}k_3$ still has a value of ≈5.5, we can estimate that C_{vf} must decrease at least 10-fold (if we use a value of ${}^{D}V$ with native DAAO of 1.14 and that for the nicked species of 2.0, the maximum and minimum values for ${}^{D}V$ we estimated, respectively). Therefore, we must propose that nicking causes both a faster release of the imino acid from the DAAO active site (increase of k_{11}) and a higher rate of reoxidation of the reduced flavin by oxygen (increase of k_5'). The increase of the K value we calculated for D-alanine on nicking is also consistent with this interpretation. As pointed out by Matthews (1991), an acceleration of the oxidative half reaction of enzymes showing ping-pong mechanisms brings along an increase of the K_m value for the substrate that is oxidized in the enzyme reductive half reaction. A small decrease of k_3 and minor changes of the position of the transition state of the oxidation of the D-amino acid (increase of ${}^{D}k_3$) cannot be ruled out, but would not affect our interpretation of the data. On the contrary we can rule out an increased reversibility of the oxidation of D-alanine in the nicked enzyme. Such an effect would introduce the C_f term in the expression of both ${}^{D}V$ and ${}^{D}V/K$, thus a decrease of their values rather than the observed increase.

The present results lead directly to the proposal that the movement of the active site loop from a "closed" to an "open" position is the step determining the rate of release of the iminopyruvate product from DAAO, thus controlling the rate of turnover. This conclusion is also supported by recent kinetic data of Fitzpatrick and co-workers (1996). Only a very small effect on V and V/K was observed when the viscosity of the reaction medium was increased with sucrose. This finding was interpreted as due to the presence of a conformational change that is limiting the rate of turnover in DAAO. Interestingly, data obtained independently in our laboratory, using glycerol instead of sucrose, are in full agreement with those reported by Fitzpatrick et al. (1996). Furthermore, the present studies show also that the accessibility of oxygen to the reduced flavin seems to be controlled by the 216−228 active site loop of DAAO.

Finally, analysis of the structure of DAAO active site (Mattevi et al., 1996; Todone et al., 1997) and the present experiments do not allow to explain the lack of interaction with D-aspartate or D-glutamate and the native or nicked DAAO forms. In this respect we found interesting the fact that there is a very low similarity between DAAO and D-aspartate oxidase in the loop region (Negri et al., 1992). Also the closely related DAAO from yeast exhibits little similarity with the pig kidney DAAO in the active site loop region (Faotto et al., 1995).

Together these observations support the hypothesis of the major role played by this part of the protein molecule in determining both substrate specificity and the kinetics of the reaction.

ACKNOWLEDGMENT

We are grateful to Dr. P. Fitzpatrick for many helpful discussions during the course of this work.

REFERENCES

Cleland, W. W. (1975) *Biochemistry 14*, 3220−3224.

Cleland, W. W. (1980) *Methods Enzymol. 64*, 104−125.

Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385−428.

Curti, B., Ronchi, S., & Pilone Simonetta, M. (1992) In *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol III, Chapter 3, CRC Press, Boca Raton.

Curti, B., Ronchi, S., Branzoli, U., Ferri, G., & Williams, C. H. (1973) *Biochim. Biophys. Acta 327*, 266−273.

Denu, J. M., & Fitzpatrick, P. F. (1992) *Biochemistry 31*, 8207−8215.

Dixon, M., & Kleppe, K. (1965) *Biochim. Biophys. Acta 96*, 358−382.

Faotto, L., Pollegioni, L., Ceciliani, F., Ronchi, S., Pilone, & M. S. (1995) *Biotechnol. Lett. 17*, 193−198.

Fitzpatrick, P. F., Denu, J. M., & Emanuele, J. J. (1996) In *Flavins and Flavoproteins 1996* (Stevenson, K., Williams, C. H., & Massey, V., Eds.) Calgary University Press, *in press*.

Fonda, M. L, & Anderson, B. M. (1967) *J. Biol. Chem. 242*, 3957−3962.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Macheroux, P., Mulrooney, S. B., Williams, C. H., & Massey, V. (1992) *Biochim. Biophys. Acta 1132*, 11−16.

Massey, V., & Curti, B. (1966) *J. Biol. Chem. 241*, 3417−3423.

Mattevi, A., Vanoni, M. A., Todone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M., & Curti, B. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 7496−7501.

Matthews, R. G. (1991) In *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 593−597, de Gruyter, Berlin.

Mizutani, H., Miyahara, I., Hirotsu, K., Nishima, Y., Shiga, K., Setoyama, C., & Miura, R. (1996) *J. Biochem. 120*, 14−17.

Negri, A., Ceciliani, F., Tedeschi, G., Simonic, T., & Ronchi, S. (1992) *J. Biol. Chem.* 267, 11865−11871.

Porter, D. J. T., Voet, J. G., & Bright, H. J. (1977) *J. Biol. Chem. 13*, 4464−4473.

Segel, E. H. (1975) *Enzyme kinetics*, John Wiley & Sons, New York.

Todone, F., Vanoni, M. A., Mozzarelli, A., Bolognesi, M., Coda, A., Curti, B., & Mattevi, A. (1997) *Biochemistry 36*, 5853−5860.

Torri Tarelli, G., Vanoni, M. A., Negri, A., & Curti, B. (1990) *J. Biol. Chem. 265*, 21242−21246.

Vanoni, M. A., Pilone, S. M., Curti, B., Negri, A., & Ronchi, S. (1987) *Eur. J. Biochem. 167*, 261−267.

Yagi, K., Okamura, K., Naoi, M., Takai, A., & Kotaki, A. (1969) *J. Biochem. (Tokyo) 66*, 581−589.

Yagi, K., Nishikimi, Takai, A., & Ohishi, N. (1974) *Biochim. Biophys. Acta 341*, 256−264.

BI963023S