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Microbial oxidases of acidic D-amino acids

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Abstract

Two microbial oxidases of acidic D-amino acids have been purified to homogeneity. One is a D-aspartate oxidase of the yeast *Cryptococcus humicolus* UJ1 that was induced markedly with D-aspartate and is far more active toward D-aspartate than D-glutamate. The other is a D-glutamate oxidase of *Candida boidinii* 2201 that preferred D-glutamate to D-aspartate as a substrate in terms of k_{cat}/K_m, but was not induced very effectively by D-glutamate. The most potent competitive inhibitor of the *C. humicolus* D-aspartate oxidase was malonate, and that of the *C. boidinii* D-glutamate oxidase was D-malate. The former enzyme was a homotetramer of 160 kDa consisting of subunits of 40 kDa, each of which contained 1 mol of FAD, while the latter was a monomer of 45 kDa. The N-terminal sequences of both enzymes were similar to those of other FAD enzymes and contained a consensus sequence common to most enzymes binding ADP-containing nucleotides. Peroxisomal localization of the *C. humicolus* D-aspartate oxidase was shown by subcellular fractionation and morphological analysis via electron microscopy of *C. humicolus* cells, where induction of the enzyme was accompanied by induction of catalase and development of peroxisomes. The apo-form of *C. humicolus* D-aspartate oxidase, prepared by removal of FAD was a monomeric protein of 40 kDa, and its binding with FAD proceeded in two stages. The K_d for the apoprotein-FAD complex was very low (8.2×10^{-12} M) consistent with the observed tight binding. The *C. humicolus* D-aspartate oxidase was essentially similar to other flavoprotein oxidases of acidic and neutral D-amino acids with respect to its spectral properties and sensitivity to specific modifying reagents for arginyl and histidyl residues.

Keywords: D-Aspartate oxidase; D-Glutamate oxidase; Acidic D-amino acid oxidase; Yeast (*Cryptococcus humicolus* and *Candida boidinii*); Flavoprotein

1. Introduction

The D-amino acid oxidase that was initially characterized in early 1930s was mammalian D-amino acid oxidase [1]. The enzyme from pig kidney has been most extensively studied and an enormous amount of information has been obtained on this enzyme, including the early finding that acidic D-amino acids such as D-aspartate and D-glutamate are not substrates. Later, in 1949, oxidizing activity which catalyzes, for acidic D-amino acids, the reaction shown in Fig. 1 was first identified in rabbit kidney and liver by Still et al. [2], who showed that the enzyme was different from D-amino acid oxidase. In 1987, purification of D-aspartate oxidase from bovine kidney was reported [3].

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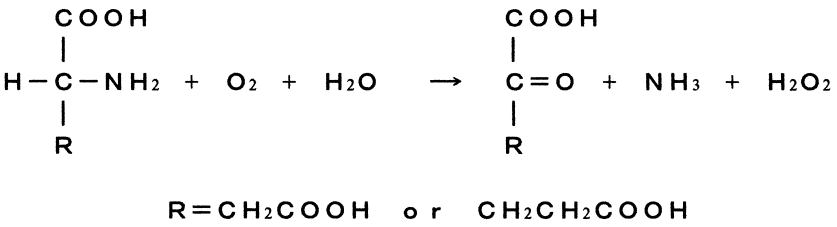

Fig. 1. Reaction catalyzed by oxidases for acidic D-amino acids.

As regards, organisms other than mammals, cephalopods [4], crustaceans [5], amphibians [6], fishes [7], avians [8] and microorganisms [9,10] were found to posses oxidase activity for acidic D-amino acids, and D-aspartate oxidase was purified from octopi (*Octopus vulgaris*) [11].

The first microbial enzyme of this kind that was purified to homogeneity was the D-aspartate oxidase of the yeast *Cryptococcus humicolus* UJ1 [12], which we isolated from soil. In addition to this enzyme, which is highly specific for D-aspartate, we purified another enzyme from the yeast *Candida boidinii* [13]. This enzyme has been designated as D-glutamate oxidase, since it prefers D-glutamate to D-aspartate as a substrate. The present review describes various aspects of these enzymes in comparison to D-aspartate oxidases and D-amino acid oxidases from other sources.

2. Isolation of *C.humicolus* UJ1 and induction of D-aspartate oxidase

Microoganisms that grew on D-aspartate as a nitrogen source in the presence of chloramphenicol were selected from soils collected in Nagaoka [12]. Of the 20 strains, thus, obtained the strain UJ1 had the highest D-aspartate oxidase activity. This strain was identified as the yeast *Cryptococcus humicolus* by Dr. T. Nakase at the Institute of Physical and Chemical Research, Wako, Japan. It is deposited as JCM 9575 in the Japan Collection of Microorganisms.

This yeast produced a remarkable D-aspartate oxidase activity, but only when it was grown on D-aspartate as a nitrogen source. Other amino acids, including D-glutamate, were as ineffective in stimulating enzyme activity as ammonium chloride. When *C. humicolus*, grown with ammonium chloride as a nitrogen source, was transferred to D-aspartate-containing medium, the enzyme activity increased time-dependently. This increase was completely inhibited by the addition of 400 μM cycloheximide, suggesting that the induced *de novo* synthesis of the enzyme was occurring. Interestingly, the effect of DL-aspartate on induction was remarkably smaller than that expected from the amount of D-aspartate present. This was probably because L-aspartate somehow inhibited the induction, for example, through competition with D-aspartate for entry into the yeast cells.

3. Purification of *C.humicolus* D-aspartate oxidase and its substrate- and inhibitor-specificity

C. humicolus cells were grown in a medium containing 1% D-aspartate [14], and ammonium sulfate fractionation was performed on the cell-free extract and FPLC on Butyl-Sepharose 4FF and Superdex 200 (twice) was conducted to produce a protein which appeared homogeneous on a SDS-polyacrylamide gel. The purified preparation first obtained had a specific activity of 76.1 μmol/min mg protein. The specific activity increased about twice as the purification procedure was later improved as reported elsewhere.

The enzyme was active only toward acidic D-amino acids, as expected, and followed Michaelis–Menten kinetics in the range of 0.1–20 mM substrate, for the three substrates shown in Table 1. The behavior of this enzyme is distinct from that of the bovine or octopod D-aspartate oxidases, which are subject to substrate activation. Comparison of the k_{cat}/K_m values given in Table 1 indicate that the *C. humicolus* enzyme is far more active toward D-aspartate than D-glutamate, and that *N*-methyl-D-aspartate is a better

Table 1
Comparison of acidic D-amino acid oxidases in kinetic parameters for three substrates

Enzyme source	K_m (mM) for			k_{cat} (s^{-1}) for			k_{cat}/K_m ($s^{-1}\,M^{-1}$) for		
	D-Aspartate	D-Glutamate	*N*-methyl-D-aspartate	D-Aspartate	D-Glutamate	*N*-methyl-D-aspartate	D-Aspartate	D-Glutamte	*N*-methyl-D-aspartate
C. humicolus UJ1[b]	3.65	152	28.0	54.2	2.04	106	14800	13.4	378
C. boidinii 2201[c]	12.9	20.1	182	19.2	60.0	21.0	1490	2980	115
Bovine kidney[d]	2.7	8.8	0.2	4.7	2.2	16.3	1741	250	81500
Human brain[e]	2.7	ND[a]	6.8	52.5	ND[a]	37.7	19400	ND[a]	5540
O. vulgaris[d]	4.3	9.7	47.7	6.8	11.0	2.4	1581	1134	50

[a] ND: not determined.
[b] Data taken from [12].
[c] Data taken from [13].
[d] Data taken from [15] are expressed in a modified manner to be suited for comparison.
[e] Data taken from [16].

Table 2
Comparison of the most potent competitive inhibitors for two microbial acidic D-amino acid oxidases

D-Aaspartate oxidase from *C. humicolus*[a]		D-Glutamate oxidase from *C. boidinii*[b]	
Inhibitor	K_i (mM)	Inhibitor	K_i (mM)
Malonate	5.4	D-Malate	9.0
D-Malate	13.3	Glutarate	11.2
meso-Tartrate	61.0	*meso*-Tartrate	15.8

[a] Data taken from [12].
[b] Data taken from [13].

substrate than D-glutamate, but still markedly poorer than D-aspartate. This demonstrates the high specificity of the enzyme for D-aspartate, and suggests that the enzyme could be a useful tool for determination, identification and removal of D-aspartate.

As shown in Table 2, malonate was the most effective competitive inhibitor of the compounds tested, followed by D-malate. *meso*-tartrate, which was found to be the strongest inhibitor of pig kidney D-aspartate oxidase [19], was not a very effective inhibitor. Nor was benzoate, which is known to inhibit D-amino acid oxidase.

4. Structure and components of *C.humicolus* D-aspartate oxidase

4.1. Subunit, coenzyme and apoprotein

The molecular mass of the native enzyme, as determined from its behavior on gel filtration, was 160 kDa, while that of the monomer subunit, as estimated via SDS-polyacrylamide gel electrophoresis, was 40 kDa, suggesting that the enzyme is a homotetramer [12]. This makes an interesting contrast to the monomeric structure of acidic D-amino acid oxidases from other sources, i.e. bovine kidney [3], human brain [16], octopus hepatopancreas [15] and the yeast *C. boidinii* [13].

The native enzyme showed an absorption spectrum typical of flavoproteins and addition of D-aspartate to the enzyme solution resulted in a spectrum similar to that of reduced flavoproteins. Addition of malonate to the enzyme induced some spectral changes, including the appearance of a shoulder at around 495 nm and a red shift of the maximum at 453 nm, as shown in Fig. 2, representing the perturbation of the spectrum by the binding of malonate [17]. By plotting the reciprocal of the increase in A_{497} versus the reciprocal of malonate concentration, the K_d for the binding of malonate to the enzyme was estimated to be 154 μM, which was much lower than the K_i for malonate given in Table 2. K_d values of 26 and 58 μM were reported for the binding of L-tartrate to the bovine and octopod D-aspartate oxidases [3,4].

A common feature in flavoprotein oxidases is reactivity with sulfite to form an N(5)-adduct with spectral properties resembling those of reduced flavin. Fig. 3 shows that *C. humicolus* D-aspartate oxidase shares this feature. Analysis of the relation between the decrease in A_{449} and sulfite concentration gave a K_d of 213 μM [17]. This value was higher than the K_d values for the adduct of bovine D-aspartate oxidase (7.4 μM) [3] and octopod D-aspartate oxidase (6.3 μM) [14], but

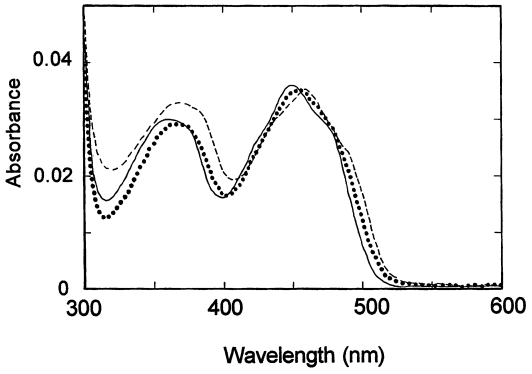

Fig. 2. Malonate titration of *C. humicolus* D-aspartate oxidase. Sodium malonate stock solutions were added to D-aspartate oxidase in 20 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA to give the final concentration indicated. Final protein concentration was 0.121 mg/ml. Only selected spectra are shown. Malonate concentration was (—) 0 mM, (···) 0.15 mM and (- - -) 10 mM.

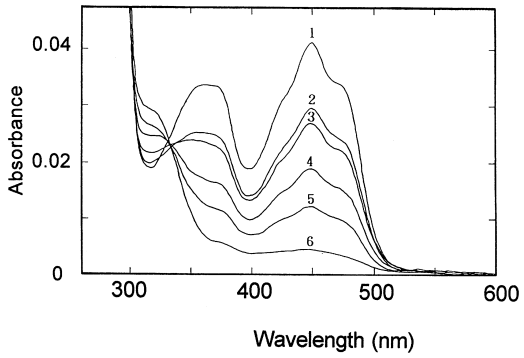

Fig. 3. Sulfite titration of *C. humicolus* D-aspartate oxidase. Sodium sulfite stock solutions were added to D-aspartate oxidase in 20 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA to give a final concentration of (1) 0 mM, (2) 0.0625 mM, (3) 0.125 mM, (4) 0.3125 mM, (5) 0.625 mM and (6) 10 mM. Final protein concentration was 0.185 mg/ml.

was rather similar to that of *R. gracilis* D-amino acid oxidase (110 μM) [18].

That the flavin moiety was tightly bound to the protein was indicated by the fact that the absence of FAD in the standard assay mixture or the addition of FMN in place of FAD did not affect enzyme activity. The flavin moiety was released from the enzyme by heating, indicating that it was not covalently bound, and was identified with FAD by thin layer chromatography.

The apoprotein of the enzyme was obtained at pH 7.0 by dialyzing the holoenzyme for three days at 4°C against 3 M KBr in 250 mM potassium phosphate, 0.3 mM EDTA and 5 mM 2-mercaptoethanol [20]. Although this procedure did not completely remove FAD and allowed 5–10% of the original holoenzyme to remain, the apoprotein was readily purified by gel filtration on Superdex 200. This was because the apoprotein was entirely present as a monomer of 40 kDa, while the holoenzyme was a tetramer of 160 kDa. It was also confirmed that holoenzyme reconstituted from the apoprotein and FAD behaved as a tetrameric protein.

The apoprotein was much less stable than the holoenzyme. It completely lost activity after 10 min incubation at 45°C, while the native enzyme retained >90% activity after the same treatment. In addition, the presence of 2-mercaptoethanol was essential for stabilization, as indicated by observed rapid protein precipitation when the thiol was omitted.

Activity titration of the apoprotein with FAD indicated that full activity was attained when their molar ratio was 1:1.

When excited at 285 nm, the apoprotein showed a fluorescence spectrum having a peak at 338 nm with a markedly increased intensity (3.5 times) in comparison to that of the native enzyme (which has a peak at 335 nm). When excited at 450 nm, the apoprotein showed no flavin fluorescence, while the native enzyme and the reconstituted holoenzyme showed flavin fluorescence with a peak at 522 nm whose intensity was about 10% of that of free FAD.

The equilibrium binding of FAD to apoprotein was determined from the quenching of flavin fluorescence, and a very small K_d value (8.2×10^{-12} M) was calculated, consistent with the tight binding described above. This value is markedly lower than that determined for the bovine D-aspartate oxidase (5.0×10^{-8} M) [3], *R. gracilis* D-amino acid oxidase (2.0×10^{-8} M) [21] and pig D-amino acid oxidase (2.2×10^{-7} M) [22]. Considering that the dissociation constant for most flavoproteins is of the order of 10^{-7}–10^{-10} M [23], the binding in the present enzyme may be classified as amongst the tightest.

The kinetics of formation of the apoprotein-FAD complex were studied via the quenching of protein and flavin fluorescence and through activity measurements. The reaction apparently proceeded in two stages: a rapid first phase, followed by a slower second phase. The rapid phase was observed in the change in protein fluorescence and initial rapid phase of decrease in FAD fluorescence, representing at least initial binding of FAD to monomeric apoprotein. The slower phase correlated with a second phase of FAD fluorescence quenching and the appearance of catalytic activity. This stage may be associated with some intramolecular changes which are essential for activity.

4.2. Amino acid composition

The amino acid composition of this enzyme was determined [17] and is compared with those of other acidic D-amino acid oxidases and D-amino acid oxidase in Table 3. The most marked differences are found in the contents of alanyl and lysyl residues: the number of the former is greater and that of the latter is smaller in the D-aspartate oxidase from *C. humicolus* than in the other enzymes. With respect to the prevalence

Table 3
Comparison of the amino acid composition of D-aspartate oxidase from *C. humicolus* UJ-1 with those of D-aspartate oxidases and D-amino acid oxidases from several sources

Amino acid	Mol residue/monomer					
	Ch-DASPO[a]	O-DASPO[b]	B-DASPO[c]	H-DASPO[d]	P-DAAO[e]	Rg-DAAO[f]
Asx	25.7	36.4	22	22	32	25
Thr	21.9	20.0	21	24	22	22
Ser	12.2	23.8	22	23	13	28
Glx	20.6	35.1	30	30	36	35
Pro	21.8	ND	22	21	22	23
Gly	37.6	24.5	30	31	32	35
Ala	50.4	19.9	22	25	17	35
Cys	7.7	1.1	7	9	5	6
Val	26.4	10.6	30	28	26	27
Met	4.8	5.9	6	3	5	4
Ile	10.2	15.9	20	18	20	16
Leu	32.8	31.1	31	32	36	33
Tyr	8.3	8.9	7	7	14	11
Phe	7.2	14.6	12	13	15	8
Lys	3.0	21.8	19	13	12	17
His	9.9	9.9	9	13	9	9
Arg	27.5	7.5	20	21	21	26
Trp	ND	ND	8	8	10	8

[a] Ch-DASPO: D-aspartate oxidase from *C. humicolus* UJ1 [17]. Each value except that for Cys and Trp represents the average of four determinations obtained after 24 h of hydrolysis at 110°C in 6 N HCl. Amino acids were analyzed with a Hitachi amino acid analysis system consisting of a 655A-52 column unit, an L-6300 intelligent pump, an L-6000 pump, an L-4250 UV–VIS detector, an L-500 reaction unit and an AS-2000 auto sampler, according to a standard programmed procedure. Cys was determined as *S*-carboxymethylcysteine by derivatization before hydrolysis of protein. Reliable data were not obtained for Trp, although hydrolysis was conducted with mercaptoethanesulfonic acid. Calculation was based on the molecular mass of the monomer subunit (40 kDa).
[b] O-DASPO: D-aspartate oxidase from *O. vulgaris*, determined by amino acid analysis [15].
[c] B-DASPO: D-aspartate oxidase from beef kidney, deduced from the sequence [24].
[d] H-DASPO: D-aspartate oxidase from human brain, deduced from cDNA [16].
[e] P-DAAO: D-amino acid oxidase from pig kidney, deduced from the sequence [25].
[f] Rg-DAAO: D-amino acid oxidase from *R. gracilis*, deduced from the sequense [26].

of other residues, the present enzyme is more or less similar to the other enzymes.

4.3. N-terminal sequence

The N-terminal sequence of the enzyme is shown in Fig. 4 in comparison with various D-amino acid oxidases, and an FAD enzyme, phytoene dehydrogenase from *Myxococcus xanthus* [27]. The sequence is obviously similar to those of all the enzymes compared, especially at positions 6–19, where the consensus sequence common to most enzymes binding ADP-containing nucleotides [28] is conserved. Thus, the comparison suggests that the N-terminal region is involved in the binding of FAD and, thus, has no relation to substrate specificity.

4.4. Chemical modification of arginine and histidine residues

Recent studies on enzymes similar to the *C. humicolus* D-aspartate oxidase have identified some amino acid residues essential for the enzyme activity. Negri et al. pointed out the importance of Tyr-228 and His-307 (the numbers refer to pig D-amino acid oxidase) by comparing the primary structure of bovine D-aspartate oxidase with those of D-amino acid oxidases from various sources [24].

Gadda et al. identified Arg-285 (corresponding to Arg-283 of pig D-amino acid oxidase) in *R. gracilis* D-amino acid oxidase as (probably) the basic residue interacting with the substrate carboxylate anion [29]. The presence of this residue at the active site of pig

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                  1         5         10        15        20        25        30
Ch-DASPO:         M P S D P I I V L G A G V I G L T T A
Cb-DGLUO:         A Q T K E I V I V G A G V I G L S X A Y L L V L S G Y K V T

O-DASPO:                V K I A V I G A G V V G L S T A L Q V K Q N F P I X S
B-DASPO:          M D T V R I A V V G A G V M G L S T A V C I S K M V P G C S
H-DASPO:          M D T A R I A V V G A G V V G L S T A V C I S K L V P R C S

Rg-DAAO:        M H S Q K R V V V L G S G V I G L S S A L I L A R K G Y S V H
P-DAAO:                 M R V V V I G A G V I G L S T A L C I H E R Y H S V L

M-PHYDH: M S A S T Q G R R I V V V G A G V G G L A A A A R L A H Q G F D V Q

Consensus:                k I X v X G X G X X G X X X a
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Fig. 4. Comparison of N-terminal amino acid sequences of D-aspartate oxidase from *C. humicolus* and D-glutamate oxidase from *C. boidinii* with those of D-aspartate oxidases and D-amino acid oxidases, and a phytoene dehydrogenase. Underlined letters indicate tentatively identified residues. Ch-DASPO, *C. humicolus* D-aspartate oxidase [13] Cb-DGLUO, *C. boidinii* D-glutamate oxidase [13]; O-DASPO, octopus D-aspartate oxidase [15]; B-DASPO, bovine D-aspartate oxidase [24]; H-DASPO, human D-aspartate oxidase [16]; Rg-DAAO, *Rhodotorula gracilis* D-amino acid oxidase [26]; P-DAAO, pig D-amino acid oxidase [25]; M-PHYDH, *Myxococcus xanthus* phytoene dehydrogenase [27]. Most of the data for comparison were obtained using the FASTA program from the DNA Data Bank of Japan, national Institute of Genetics. The consensus sequence indicated is common to most enzymes binding ADP-containing nucleotides [28]. Upper case letters refer to the G residues which need to be strictly identical, while lower case letters indicate one of various conserved amino acids which can be found at a certain position in accordance with the consensus sequence detailed in [28].

D-amino acid oxidase was confirmed by the crystallographic structure of the pig enzyme [30]. In addition, bovine D-aspartate oxidase was shown to lose specificity to acidic D-amino acids and become active toward neutral D-amino acids after chemical modification of some of its arginine residues, probably due to loss of the basic residue interacting with the β-carboxyl group of D-aspartate [31].

We have examined the effect of some specific chemical modifiers on *C. humicolus* D-aspartate oxidase for comparison with similar enzymes [17], and are also trying to determine the primary structure of the enzyme.

4.4.1. Modification of arginyl residues with phenylglyoxal

Treatment of *C. humicolus* D-aspartate oxidase with an arginine-specific reagent, phenylglyoxal, caused a time dependent loss of activity in a biphasic manner (Fig. 5). The first phase was too rapid to analyze kinetically and did not show any clear dependence on reagent concentration. The slow second phase followed pseudo-first-order kinetics. Analysis of the relation between the apparent first-order rate of the slow phase (k_{obs}) and phenylglyoxal concentration suggested that the reaction order was 1 with respect to the reagent concentration and a reversible complex was formed between the reagent and the enzyme prior to the covalent modification. The modification was determined to be irreversible under our experimental

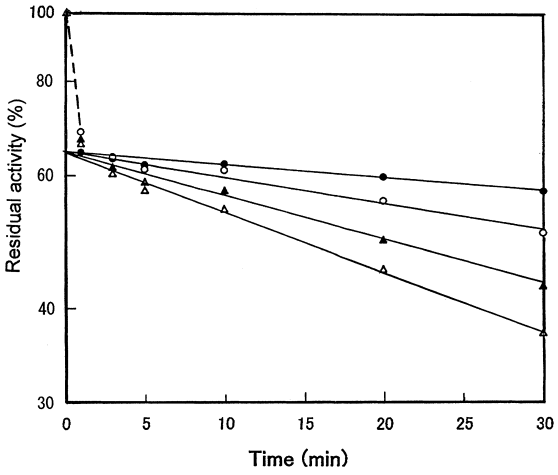

Fig. 5. Time dependent inactivation of D-aspartate oxidase from *C. humicolus* with phenylglyoxal. The enzyme (51.9 μg/ml) was incubated in 50 mM potassium phosphate buffer (pH 7.0) at 20°C with 10 mM (●), 20 mM (○), 30 mM (▲) and 40 mM (△) phenylglyoxal. At the indicated times the reaction was stopped by the addition of 50 volumes of the same buffer containing 5 mM L-arginine and 0.5 mg/ml bovine serum albumin at 0°C, and the diluted solutions were dialyzed before assay for enzyme activity.

conditions since no recovery of enzyme activity was observed after removing excess reagent by dialysis and stopping the inactivation with the addition of excess L-arginine.

The effect of malonate, which is the most potent competitive inhibitor as shown in Table 2, on inactivation by phenylglyoxal was examined. Malonate showed no effect on the first rapid phase, but partially protected the enzyme from the second slow phase of inactivation. The malonate concentration which showed a half-maximal protection was about 5 mM, which is in good agreement with the K_i of this compound, suggesting that malonate and glyoxal compete for the substrate binding site of the protein. This would mean that phenylglyoxal modifies some arginyl residue at or near the substrate binding site. Addition of FAD to the reaction mixture yielded no protection from fast or slow phase of inactivation.

A partially inactivated enzyme preparation (remaining activity, about 25%) was prepared by incubating the enzyme with 40 mM phenylglyoxal for 40 min under the conditions described in Fig. 5. The enzyme was isolated from the reaction mixture by stopping the inactivation through the addition of L-arginine, followed by dialysis and gel filtration. This preparation was likely to contain little intact enzyme, since the employed reaction period was long enough for the enzyme to be modified by at least the first rapid-phase reaction. The V_{max} value of the preparation was much lower than that of the native enzyme, whereas the K_m value was little affected as shown in Table 4. The substrate specificity of this preparation was, by and large, the same as that of the native enzyme: D-aspartate was a much better substrate than D-glutamate and none of neutral D-amino acids behaved as a substrate. Thus, no change in substrate specificity similar to that described above for the bovine D-aspartate oxidase [31] was observed. This may mean that the basic residue interacting with the β-carboxylate anion is not arginine, or that it is an arginine residue that is not accessible. On gel filtration, the modified enzyme preparation appeared to have a mass of 160 kDa, indicating that the tetrameric structure was unaffected, and its absorption spectrum suggested that its FAD content was not changed. However, its FAD fluorescence was about 3.5-fold higher than that of the native enzyme, suggesting a change in the environment surrounding the bound FAD.

The apoprotein of *C. humicolus* D-aspartate oxidase lost its functional activity after phenylglyoxal treatment much more rapidly than the holoenzyme, and malonate did not confer protection from this process. Addition of the thus-modified apoprotein to FAD did not cause any quenching of FAD fluorescence unlike the native apoprotein described above, suggesting that the protein did not bind FAD. This would suggest that there is an arginyl residue at the FAD binding site.

All the findings taken collectively, the first rapid phase of inactivation may represent modification of arginyl residues distant from active site, which does not affect the binding of substrate. The second slow phase, which ultimately causes complete inactivation, may be due to modification of the residue that corresponds to Arg-283 of pig D-amino acid oxidase. The unaffected K_m shown in Table 4 is probably due to

Table 4
Comparison of native and phenylglyoxal-modified D-aspartate oxidase in substrate specificity and kinetic parameters

Substrate	Native enzyme			Modified enzyme[b]		
	Activity (μmol/min mg)[a]	V_{max} (μmol/min mg)	K_m (mM)	Activity (μmol/min. mg)[a]	V_{max} (μmol/min. mg)	K_m (mM)
D-Aspartate	175.6	221.3	2.89	43.8	45.6	2.86
D-Glutamate	2.73	–	–	0.67	–	–
D-Proline	0	–	–	0	–	–
D-Alanine	0	–	–	0	–	–
D-Valine	0	–	–	0	–	–
D-Serine	0	–	–	0	–	–
Glycine	0	–	–	0	–	–

[a] Activity was measured with each substrate at 20 mM concentration.
[b] Modified enzme was prepared by incubation of the enzyme with 40 mM phenylglyoxal for 40 min at 20°C.

the presence of enzyme species in which this residue remained unmodified.

4.4.2. Modification of histidyl residues with diethyl pyrocarbonate

Diethyl pyrocarbonate is known to be relatively specific to histidyl residues. On treatment with this reagent at pH 7.0, *C. humicolus* D-aspartate oxidase was inactivated in a biphasic manner as shown in Fig. 6. The first phase was very rapid and was completed in less than 1 min. Its extent was apparently dependent on the reagent concentration and was not influenced by the presence of malonate or D-aspartate. The slow second phase followed pseudo-first-order kinetics and the presence of malonate or D-aspartate partially protected the enzyme from this phase of inactivation, suggesting that this phase involves the modification of some histidyl residue near or at the substrate binding site.

As reported by Ramón et al. [32], absorbance at 242 nm increased as inactivation progressed reflecting the modification of histidyl residues (Fig. 7). A plot of relative activity against the number of modified histidyl residues as estimated by increase in A_{242} was analyzed according to the method of the authors described above.

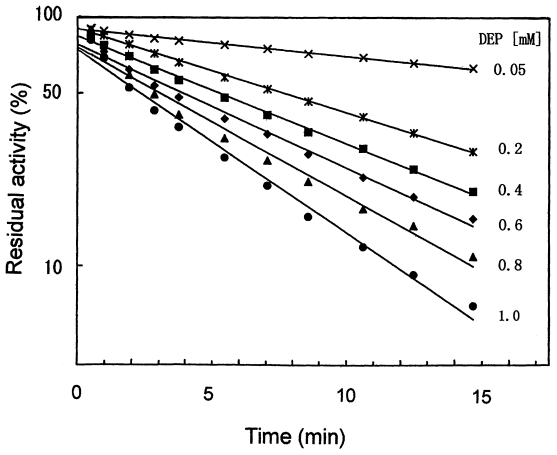

Fig. 6. Time dependent inactivation of D-aspartate oxidase from *C. humicolus* with diethyl pyrocarbonate. The enzyme (25 μg/ml) was incubated in 50 mM potassium phosphate buffer (pH 7.0) at 20°C with diethyl pyrocarbonate at the indicated concentrations. Enzyme activity was assayed after stopping the reaction, by the addition of 4 volumes of the same buffer containing 5.5 mM L-histidine and 0.5 mg/ml bovine serum albumin at 0°C.

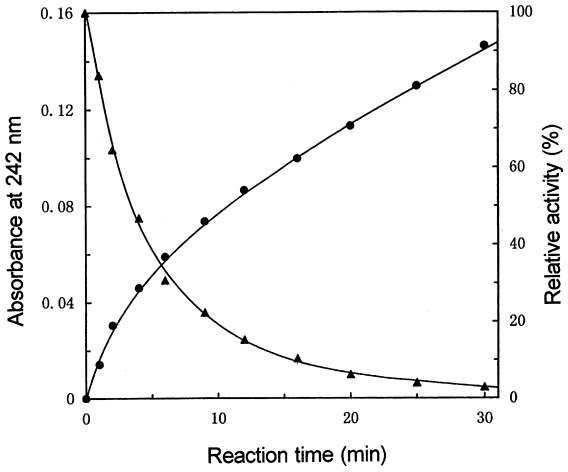

Fig. 7. Correlation of inactivation of D-aspartate oxidase from *C. humicolus* by diethylpyrocarbonate (▲) with the increase in absorbance at 242 nm (●). The enzyme (200 μg/ml) was incubated in 50 mM potassium phosphate buffer (pH 7.0) at 20°C with 1 mM diethyl pyrocarbonate. At the indicated times the absorbance was measured and the enzyme activity was assayed as described for Fig. 7.

The result suggested that the modification of five histidyl residues was necessary for complete inactivation. Further analysis according to the statistical method of Tsou [33] suggested that one residue was essential for enzyme activity. These results suggest the presence of an essential histidyl residue at or near the substrate-binding site. It is probable that this residue corresponds to His-307 of pig D-amino acid oxidase.

5. Peroxisomal localization and development of peroxisomes

The subcellular localization of oxidase specific for acidic D-amino acids in organisms other than mammals was unknown, while the D-amino acid oxidase of mammals [34] and yeasts [35,36], and the D-aspartate oxidase of mammals were found to be localized in peroxisomes [37–39]. We, therefore, examined whether or not the D-aspartate oxidase of *C. humicolus* was subcellularly localized in peroxisomes [14]. Cells of the yeast grown on D-aspartate as a nitrogen source were treated with Novozyme 234 to prepare protoplasts, which were then homogenized gently

and subjected to differential centrifugation. Enzyme activity was found mainly in a particulate fraction, which also contained catalase and urate oxidase, marker enzymes for peroxisomes, and cytochrome C oxidase, a marker enzyme for mitochondria. This particulate fraction was further subfractionated by a discontinuous sucrose density gradient. D-Aspartate oxidase was mainly in the heavy particle fraction as were catalase and urate oxidase, whereas cytochrome C was detected in the light particulate fractions. These results clearly suggest D-aspartate oxidase is in fact localized in peroxisomes.

When cells grown on D-aspartate as a nitrogen source were compared with those grown on L-aspartate, the extract from the former contained 500-fold and three-fold higher activities of D-aspartate oxidase and catalase, respectively, as shown in Table 5. Moreover, morphometric analysis of the peroxisomes with electron microscopy showed that the former cells contained more and larger peroxisomes than the latter cells.

This morphological development of peroxisomes together with the simultaneous induction of D-aspartate oxidase and catalase confirms the peroxisomal localization of D-aspartate oxidase suggested by the subfractionation. The degree of peroxisome development observed is comparable to that reported in *Rhodotorula gracilis* where D-alanine induces D-amino acid oxidase [36].

6. Purification and properties of D-glutamate oxidase from *C. boidinii* 2201

Those oxidases specific for acidic D-amino acids so far purified tend to prefer D-aspartate to D-glutamate as a substrate; D-aspartate is a much better substrate than D-glutamate for the bovine kidney enzyme [15] and the *C. humicolus* enzyme [12]. This seems to be also the case for the human brain enzyme, since the reported Lineweaver–Burk plots [16] suggest that the k_{cat}/K_m value for D-glutamate is much smaller than that for D-aspartate, although the K_m and k_{cat} for the former could not be determined. With respect to the enzyme from octopus, the k_{cat}/K_m value for D-aspartate is a little higher than that for D-glutamate, suggesting that the former is an effective or, at least, an almost equivalently effective substrate.

We, therefore, searched for an acidic D-amino acid oxidase with higher activity toward D-glutamate and found that the yeast *C. boidinii* 2201 was a candidate to produce this type of enzyme, which is appropriately called D-glutamate oxidase: extracts from *C. boidinii* 2201 cells showed oxidase activity that was about twice as high toward D-glutamate as toward D-aspartate at 20 mM [13]. This finding led us to employ the former as the substrate in the standard assay mixture.

After replacement of ammonium chloride with D-glutamate as a nitrogen source in the standard medium for cell growth, the specific activity of D-glutamate oxidase increased only about two-fold. Since the effect was not marked enough to justify using this expensive amino acid, this modification of the medium was not adopted for cell growth on a large scale. The effect of methanol added to the medium in place of glucose, the carbon source in the standard medium, was also tested, since *C. boidinii* 2201 is known to show induction of peroxisomes when it utilizes methanol as a carbon source. This replacement was found, surprisingly, to lower the specific activity of the oxidase.

The enzyme was purified from the crude extract 2285-fold to homogeneity as indicated by SDS-polyacrylamide gel electrophoresis. The molecular mass determined by the electrophoresis was 45 kDa, while gel filtration of the native enzyme showed a molecular mass of 43 kDa, indicating the native enzyme to be a monomer. The monomeric structure is similar to that of mammalian and octopod D-aspartate oxidases and contrasts with the homotetrameric structure of *C. humicolus* D-aspartate oxidase as described above. This enzyme required FAD for activity and FMN could not replace FAD. The purified preparation had a specific activity of 41.4 μmol/min mg protein with 20 mM D-glutamate as a substrate. The optimum pH and temperature were 7.0 and 37°C. The K_m for D-glutamate was higher than that for D-aspartate, while the k_{cat} was more markedly higher for the former than for the latter, resulting in a higher k_{cat}/K_m value for D-glutamate (Table 1). This indicates that D-glutamate is the better substrate and supports the designation of the enzyme as D-glutamate oxidase. *N*-methyl-D-aspartate was a far poorer substrate than these two compounds. Table 1 further shows that no other oxidases specific

Table 5
Effects of D-aspartate and L-aspartate as a nitrogen source for growth of *C. humicolus* on peroxisomal enzyme activities and morphology of peroxisomes[a]

Nitrogen source	D-Aspartate oxidase (nmol/min. mg)	Catalase (μmol/min. mg)	D-Amino acid oxidase (nmol/min. mg)	Urate oxidase (nmol/min. mg)	Number of peroxisomes observed in one in one cell section	Total area of peroxisomes ($\mu M \times 10^3$)
D-Aspartate	567 ± 305[b]	90 ± 45[c]	4.4 ± 1.7	11.4 ± 6.4[b]	2.40 ± 0.82	172 ± 55.2
L-Aspartate	1.0 ± 0.3	28 ± 13	3.0 ± 0.5	5.7 ± 6.2	1.50 ± 0.71	53.2 ± 29.6

[a] Data are taken from [14]; values for enzyme activities are means±S.D. for 6–7 determinations; values for peroxisomes are means±S.D. for examination of 25 and 18 electron micrographs of cells grown on D-aspartate and L-aspartate, respectively.
[b] Significant difference between the nitrogen sources, $P < 0.001$.
[c] Significant difference between the nitrogen sources, $P < 0.05$.

for acidic D-amino acids so far purified are more active toward D-glutamate than toward other substrates as is this enzyme. It may be added that partially purified preparations of the D-glutamate oxidase of the crayfish showed a higher V_{max} for D-glutamate than for D-aspartate [5], but the V_{max}/K_m was higher for D-aspartate. As shown in Table 2, D-malate was the most potent competitive inhibitor, followed by glutarate and *meso*-tartrate. Interestingly, malonate, the strongest inhibitor for the D-aspartate oxidase of *C. humicolus* [12], was a relatively weak inhibitor. The substrate and inhibitor specificities thus compared suggest that the enzyme of *C. boidinii* has more room for the binding of substrates than that of *C. humicolus*. The N-terminal sequence of the enzyme shown in Fig. 4 was similar to those of other FAD enzymes, including D-aspartate and D-amino acid oxidases from various sources. The consensus sequence common to most enzymes binding ADP-containing nucleotides [28]was, as expected, conserved in this N-terminal sequence.

7. Discussion

This review has summarized various aspects of two oxidases specific for acidic D-amino acids purified from two strains of yeast, in comparison with those of corresponding oxidases and D-amino acid oxidases from other sources.

Of the findings described, the homotetrameric structure revealed for the *C. humicolus* D-aspartate oxidase contrasts with the monomeric structure of the other oxidases for acidic D-amino acids. This feature cannot be considered common to microbial enzymes, since the D-glutamate oxidase of *C. boidinii* is a monomer. Thus, this feature might be rather considered unique to this enzyme, linked to its function. The functional features of this enzyme may be represented by high specificity to D-aspartate and high affinity to FAD. It seems likely that these functional features are supported by the unique quarternary structure.

It is doubtless that this enzyme plays an essential role in *C. humicolus* growing in media containing D-aspartate as a nitrogen source. Taking into account that D-aspartate is the specific substrate and the specific inducer of the enzyme, it may be safely postulated that genes for this enzyme were developed in the past and kept to utilize D-aspartate whenever required. In this context, the tetrameric structure of the enzyme may be one of the most developed forms among similar enzyme species.

The D-glutamate oxidase of *C. boidinii* is the only acidic D-amino acid oxidase that has been shown to be more active toward D-glutamate than D-aspartate in terms of k_{cat}/K_m.

The difference in substrate and inhibitor specificity between this enzyme and the *C. humicolus* D-aspartate oxidase, as well as other similar enzymes, presents an intriguing question about the difference in the structure of the active site. To answer this question and provide further information on these enzymes, comparable to that available for more extensively studied similar enzymes, we are studying the primary structure and the gene structure of these enzymes.

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