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Mutants of Neurospora Deficient in D-Amino Acid Oxidase*

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Although many investigations have been carried out on the D-amino acid oxidase since its discovery in 1935 (2), the metabolic role of this enzyme remains somewhat obscure. It is generally believed, on the basis of indirect evidence, that in animals the enzyme functions in the inversion of exogenous D-amino acids over the pathway D-amino acid → α-keto acid → L-amino acid (3). The first step is assumed to be catalyzed by the D-amino acid oxidase, the second by a transaminase. Other functions that have been proposed for the enzyme include the removal of D-amino acids of endogenous origin (4, 5) and, in some organisms, the synthesis of D-amino acids (6). It has also been suggested that the enzyme has no physiological significance as a D-amino acid oxidase, but that it is a by-product of metabolism, or that it may have some function other than oxidation of D-amino acids (7, 8).

Neurospora crassa is known to possess a D-amino acid oxidase which resembles the mammalian enzyme in substrate specificity (5, 9). The evidence suggests that in this organism, too, the enzyme is required for the inversion of D-amino acids of exogenous origin (5). Operating on this assumption, it has been possible to obtain mutants of Neurospora showing little or no D-amino acid oxidase activity. A description of the mutants and their response to D-amino acids is reported below.

EXPERIMENTAL PROCEDURE

Production of Mutants—In our first series of experiments, mutations were induced with ultraviolet in conidia of methionine-less mutant 38706 (or *me-1*), blocked between homocysteine and methionine (10). The ultraviolet source was a 40-watt sterilizing lamp (American Sterilizer Company, model UV-30); the exposure time was 30 to 45 seconds at 10 cm (50 to 70% mortality). Selection was carried out for mutants unable to grow on D-methionine by use of the filtration enrichment technique of Woodward, De Zeeuw, and Srb (11). (Methionine auxotrophs of Neurospora normally utilize either stereoisomer.) One D-amino acid oxidase-deficient mutant, designated oxD^1, was obtained by this procedure. This mutant is the subject of much of the work reported here.

Later, 12 additional D-amino acid oxidase-deficient mutants were obtained by the "inositolless-death" method of Lester and Gross (12). In this procedure, a double mutant of *me-1* with inositolless 89601 was irradiated with ultraviolet as above, and selection was made for clones unable to grow on D-methionine plus inositol. Of the 12 mutants, four, oxD^8, oxD^9, oxD^{11}, and oxD^{13}, were studied in some detail, as reported below.

Enzyme Assay—Cultures were grown for 5 to 7 days at 25° on appropriately supplemented Fries (10) or Vogel (13) liquid minimal medium. Preparation of extracts and manometric determinations of enzyme activity were as described previously (5). Colorimetric assays, with the use of the method of Friedemann and Haugen (14) for keto acids, were also performed. The latter method was applicable to washed and frozen-and-thawed mycelium, as well as to cell-free extracts. With either method, it was necessary to correct for endogenous oxygen consumption or keto acid production by controls without added substrate.

Growth Tests—Cultures were grown at 25° in 125-ml Erlenmeyer flasks containing 20 ml of liquid medium. The mycelium was harvested after 72 hours of growth, dried at 90°, and weighed.

Compounds—Commercial D-methionine was recrystallized twice from water to remove residual L-methionine. Sodium α-ketomethiolbutyrate was prepared by the procedure of Meister (15); the final product was free of methionine by paper chromatography.

RESULTS

D-Amino Acid Oxidase Activity of oxD Mutants—The D-amino acid oxidase activity of the oxD^1 mutant was found to be not over 5% of that of the strain from which it was derived. Frequently, no activity could be detected in the mutant. In a typical manometric experiment with D-methionine as substrate, an extract of the parental *me-1* strain consumed oxygen at a constant rate of 0.735 μl per minute, while an extract of the double mutant oxD^1 *me-1* took up no oxygen during the 75 minutes of the experiment; an equal mixture of the two extracts consumed 0.351 μl of oxygen per minute, or very close to the rate expected on the basis of simple dilution of the enzyme activity. Addition of FAD (up to 0.1 mM) to the extracts had no effect on oxidase activity.

In tests with mutants oxD^8, oxD^9, oxD^{11}, and oxD^{13}, no D-amino acid oxidase activity at all could be found by methods capable of detecting a few per cent of the wild type activity. Table I shows keto acid production from D-methionine by extracts of wild type and the five *oxD* mutants studied.

Besides D-methionine, the oxD^1 mutation abolished activity toward the following substrates of D-amino acid oxidase of Neurospora: D-alanine, D-valine, D-leucine, D-phenylalanine, D-isoleucine, 3,4-dihydroxy-D-phenylalanine, DL-arginine, DL-α-amino-*n*-butyric acid, DL-norleucine, and DL-tyrosine. The mutant retained the ability to deaminate D-serine, D-threonine, D-homoserine, D-glutamate, and D-aspartate. D-Serine and D-threonine are not substrates of the D-amino acid oxidase of Neurospora (5), but are deaminated by a separate enzyme (16). Since the

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D-serine-threonine deaminase is inactive toward homoserine, the present observations indicate that Neurospora produces a D-homoserine deaminase. D-Glutamate and D-aspartate also are deaminated in Neurospora by an enzyme (or enzymes) separable from the D-amino acid oxidase (16). A specific D-glutamic-aspartic oxidase has been reported in Aspergillus and other molds (17).

Effect of oxD Gene on Utilization of D-Amino Acids for Growth—As already stated, the *oxD*^{1} mutation was induced in methionineless mutant *me-1*. The resulting double mutant, *oxD*^{1} *me-1*, utilizes L-methionine and its α-keto analogue, α-ketomethiolbutyrate, for growth with about the same efficiency as does the original *me-1* strain. Unlike *me-1*, however, the double mutant grows only slightly on D-methionine. Similar results were obtained with *oxD*^{8}, *oxD*^{9}, *oxD*^{11}, and *oxD*^{13} in combination with *me-1* (Fig. 1).

oxD^1 also blocks the utilization of D-arginine and D-leucine by argininelessand leucineless mutants. For these tests, the double mutant *oxD*^{1} *arg-2* and the triple mutant *oxD*^{1} *me-1 leu-1* were constructed. In common with other argininelessmutants, *arg-2* (mutant 33442) grows as well on DL- as on L-arginine (18). *Leu-1* (mutant 33757) grows on DL-leucine at about 0.75 its rate of growth on L-leucine; that is, D-leucine is used only half as effectively as L-leucine (19). When combined with *oxD*^{1}, however, neither of these mutants is able to utilize the D-stereoisomer (Table II).

Effect of oxD^1 *on Sulfate Utilization*—When *oxD*^{1} was freed from its combination with the methionineless gene, it was found, unexpectedly, to be incapable of growing on minimal medium. Further investigation showed that *oxD*^{1} is blocked in the utilization of sulfate—the sole source of sulfur in minimal medium, except for a trace of biotin. The mutant grew normally when supplied with sulfite, thiosulfate, or sulfur-containing amino acids, including L-methionine. D-Methionine could not serve as a sulfur source, although the sulfur atom of this compound is available to wild type and to other mutants blocked in the reduction of sulfate (Table III).

Since this result suggested that the D-amino acid oxidase might have a function in sulfate reduction, genetic experiments were undertaken to determine if the block to sulfate reduction could be dissociated from the D-amino acid oxidase deficiency. Crosses of *oxD*^{1} to closely linked markers were made, and progeny which had experienced crossing over close to the locus of *oxD*^{1} were selected for study. In no case did we find that the crossovers had separated the oxidase deficiency from the requirement for reduced sulfur. Further details are given under "Genetics."

We next attempted to induce a back-mutation of the triple mutant *oxD*^{1} *me-1 leu-1* to the ability to use D-methionine, to learn if the block to sulfate utilization would be removed simultaneously. No back-mutations of the *oxD*^{1} gene were obtained, however.

The problem was finally solved by the isolation of the new series of mutants *oxD*^{8}, *oxD*^{9}, *oxD*^{11}, and *oxD*^{13}. When these were freed from the accompanying *me-1* and *inos* genes and tested on minimal medium, it was found that they grew normally with sulfate as the source of sulfur (Table III). We also found that three independent "sulfiteless" mutants (blocked between sulfate and sulfite) were normal with respect to D-amino acid oxidase activity. The most reasonable interpretation of these findings is that the D-amino acid oxidase does not function in sulfate reduction, but that the *oxD*^{1} mutant involves the simultaneous mutation of two closely linked genes, one controlling D-amino acid oxidase synthesis, the other controlling sulfate reduction.

TABLE I

Keto acid production from D-methionine by extracts of wild type and mutant strains

Incubation mixtures contained 6.7 mM D-methionine, 4 μM FAD (except where indicated), 0.1 M pyrophosphate, pH 8.25, and 0.2 to 1.0 ml of extract in a total volume of 3 ml. The mixtures were shaken in air for 40 minutes at 34°. The reaction was stopped by addition of an equal volume of 10% trichloroacetic acid, followed by heating at 80° for ½ minute and centrifugation. The supernatants were analyzed for keto acids by the method of Friedemann and Haugen (14), with Na-α-ketomethiolbutyrate as the standard.

Strain	Keto acid (as α-ketomethiolbutyrate) per g of fresh mycelium		
	Without D-methionine*	With D-methionine	Difference
	μmoles	*μmoles*	*μmoles*
Wild type	0.44	13.4*	13.0
me-1 inos	1.35	13.4*	12.0
		13.5	12.1
oxD^1	0.89	0.80	−0.09
oxD^8	0.85	0.75	−0.10
oxD^9	0.80	0.67	−0.13
oxD^{11} *me-1 inos*	1.72	1.65	−0.05
oxD^{13}	0.78	0.67	−0.11

* No FAD added.

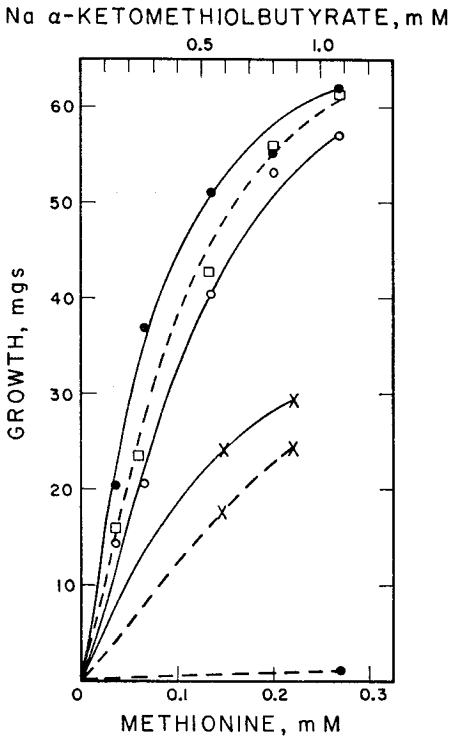

FIG. 1. Growth of the parental strain *me-1 inos* (*solid lines*) and the triple mutant *oxD*^{8} *me-1 inos* (*broken lines*) on L-methionine (○, □), D-methionine (●), and Na-α-ketomethiolbutyrate (×).

Other Effects of oxD Mutations—The foregoing results having shown that loss of D-amino acid oxidase activity does not have a

TABLE II

Effect of oxD¹ mutation on utilization of D-arginine and D-leucine by Neurospora mutants

Values in the table are milligrams (dry weight) of mycelium per flask. All flasks contained 0.67 mM L-methionine.

Supplement	Concentration	Mutants			
		arg-2	*oxD¹ arg-2*	*leu-1*	*oxD¹ leu-1 me-1*
	mg/ml				
L-Arginine-HCl	0.0	2.0	Trace		
	0.0125	15.2	8.4		
	0.025	27.6	18.0		
	0.05	33.8	27.0		
DL-Arginine-HCl	0.025	27.8	9.0		
	0.05	38.4	15.2		
	0.1	45.0	27.0		
L-Leucine	0.0			0.0	0.0
	0.0125			11.4	8.4
	0.025			20.4	18.0
	0.05			38.9	37.6
DL-Leucine	0.025			14.2	
	0.05			31.8	14.2
	0.1				33.4

TABLE III

Utilization of various sulfur sources for growth by wild type and oxD mutants

Values in the table are milligrams (dry weight) of mycelium per flask.

Strain	Sulfur source				
	None	Sulfate, 2 mM	Thiosulfate, 0.2 mM	L-Methionine, 0.335 mM	D-Methionine, 0.335 mM
Wild type	1.8	81.4	69.2	73.4	65.4
oxD¹	Trace	Trace	84.2	78.2	4.7
oxD⁸	3.6	89.2	82.8	67.2	7.8
oxD⁹	2.0	33.4	44.8	52.6	7.8
oxD¹³	3.0	78.4	77.4	60.8	13.8
Mutant 85518*		0.0	64.0	67.4	66.0

* Blocked between sulfate and sulfite.

lethal, or even deleterious, effect on wild type Neurospora, further experiments were carried out in an attempt to detect some natural advantage attaching to possession of the enzyme. It was noted that the mutants behave normally in crosses, as well as vegetatively; *oxD¹*, in particular, showed no impairment of fertility when used either as the male or the female parent.

Attempts to detect accumulation of D-amino acids in the medium or in mycelial extracts, with a commercial preparation of hog kidney D-amino acid oxidase, gave negative results. Such accumulation might have been expected if the role of the enzyme is to remove endogenously synthesized D-amino acids. Dr. Yasushi Watanabe, in unpublished experiments from this laboratory, has found that Neurospora contains little or no D-amino acid in its cell wall.

If no D-amino acids are produced endogenously, then the function of the D-amino acid oxidase, if it has one, must be to deal with D-amino acids of exogenous origin. The latter might be formed in nature by the breakdown of bacterial cell walls, or by racemization of L-amino acids. The D-amino acid oxidase would make these available metabolically. We have tested the ability of wild type Neurospora to grow on D-alanine and D-methionine as the sole source of nitrogen. Growth was very slow at low concentrations of the D-amino acids and was inhibited at high concentrations. The enzyme apparently has insufficient capacity to provide an important fraction of the nitrogen required for growth. It can, however, make sulfur available from D-methionine at a rate sufficient to meet the needs for normal growth. Table III shows that whereas wild type attains a normal growth rate on D-methionine as sole source of sulfur, the *oxD* mutants do not. *oxD¹* is more limited in growth than the other mutants, a fact which suggests that the sulfur atom of D-methionine may be slowly oxidized to sulfate in a reaction that does not involve the D-amino acid oxidase. It is clear, however, that the major pathway of D-methionine metabolism is over the D-amino acid oxidase. The limited capacity of the *oxD* mutants for utilizing D-methionine as a sulfur source is a convenient tag by which they can be identified in genetic experiments.

Aromatic D-amino acids are toxic for Neurospora at relatively low concentrations, and it was therefore interesting to compare their effect on wild type and the mutants. It was found that D-phenylalanine and D-tyrosine are significantly more inhibitory to the growth of *oxD¹* than to wild type (Table IV). The difference indicates a protective action of the D-amino acid oxidase.

Genetics—oxD¹ behaves as a single gene in inheritance. It is located in the fourth linkage group, very close to pyridoxineless-1 (no recombinants in 55 tetrads). Its linkage relations, based on an analysis of 226 complete tetrads, are mapped in Fig. 2. Pyridoxineless-1 is tentatively placed proximal to *oxD¹*, pending further studies.

In attempting to separate the two biochemical defects of *oxD¹* (see above), 24 phenotypically wild type progeny were tested from the cross *oxD¹* × *arg-2*, 13 from *oxD¹* × *pyr-1*, and 4 from

TABLE IV

Effect of aromatic D-amino acids on growth of wild type and oxD¹

The medium contained 0.5 mM thiosulfate as a source of sulfur.

Supplement	Concentration	Per cent of maximal growth	
		Wild type	*oxD¹*
	mM		
None		100	100
D-Phenylalanine	0.15	89.0	16.5
	0.30	53.5	13.0
	1.20	17.5	9.9
	1.80	14.3	9.4
D-Tyrosine	0.138	86.0	29.8
	0.276	86.6	15.5
	1.10	18.3	2.0
	1.65	8.0	1.0

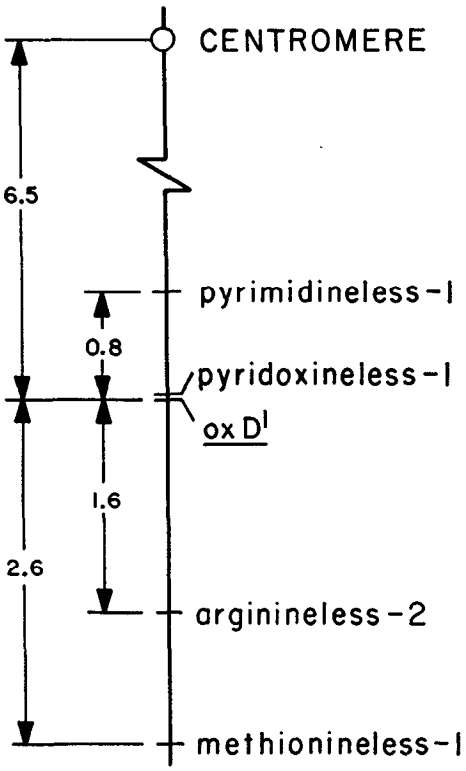

FIG. 2. Map of the oxD^1 region, linkage Group IV, of *Neurospora crassa*. Distances in standard map units.

oxD^1 *arg-2* × *pdx-1*. All of these must have undergone recombination close to the oxD^1 locus, yet all showed normal D-amino acid oxidase activity, indicating the close association between oxD^1 and the "sulfiteless" character.

Preliminary evidence indicates that all of the *oxD* mutants obtained in this investigation are allelic or closely linked. Twelve of the 13 mutants (all except oxD^1) were obtained in the same *me-1 inos* parental strain and are presumably homogeneous with respect to incompatibility alleles. Nevertheless, we were unable to detect any heterocaryotic complementation among them, a result suggesting allelism of the mutants. Likewise, no complementation occurred between oxD^1 and any of the other 12 mutants, but since the genetic backgrounds differ in this case, this is not evidence for allelism. The cross $oxD^1 \times oxD^8$ *me-1 inos* was therefore carried out, and 631 random spores were tested for their ability to grow on D-methionine as the sole sulfur source. Two such spores were found; these were also wild type with respect to methionineless and inositolless. It is evident from this result that oxD^1 and oxD^8 are closely linked, but further work is needed before it can be decided whether or not they are functionally allelic.

DISCUSSION

The experiments reported here confirm the role of the D-amino acid oxidase in the inversion of exogenous D-amino acids. They also show that the enzyme protects Neurospora against the toxic effects of D-phenylalanine and D-tyrosine and that it has sufficient capacity *in vivo* to provide the sulfur (but not the nitrogen) requirements of the organism from exogenous D-methionine. How important these functions are in nature is, however, unknown.

The oxidase-deficient mutants show normal growth and fertility, except when stressed with exogenous D-aromatic amino acids. The block to sulfate utilization characteristic of oxD^1, the first mutant of this series to be studied (1), is not found in other mutants and is presumed to be due to the coincident mutation (or overlapping deletion) of a closely linked gene concerned with sulfate metabolism. The failure to detect any accumulation of D-amino acids in oxidase-deficient cultures is further evidence of the essential normality of the mutants. It thus appears that the enzyme has no essential function under laboratory conditions. It can hardly be doubted, however, that it is in some way useful to the organism in nature. We have tested twenty wild strains of Neurospora from various regions of the world and have found D-amino acid oxidase activity in all of them. If the enzyme conferred no selective advantage on its possessors, one would expect it to have been lost by mutation in at least some natural strains.

It is difficult to evaluate the significance of the residual oxidase activity shown by some mutant extracts without further purification of the enzyme. This residual activity, which is always very small, may be due to other enzymes. Preliminary attempts to fractionate extracts have indicated that the oxidase is unusually labile.

SUMMARY

The role of the D-amino acid oxidase in metabolism was investigated in five mutants of Neurospora in which the D-amino acid oxidase activity is less than 5% of normal. With one exception, the mutants grow at the normal rate on minimal medium and are sexually fertile. The exceptional mutant is unable to utilize sulfate for growth; this effect is probably due to the coincident mutation of another gene closely linked to the gene governing D-amino acid oxidase synthesis. It is shown that the D-amino acid oxidase is essential for the inversion of exogenous D-amino acids, that it protects the organism against the toxic effects of D-aromatic amino acids, and that it attacks exogenous D-methionine at a rate sufficient to provide the sulfur, but not the nitrogen, needed for growth. Deficiency of the D-amino acid oxidase did not result in a detectable accumulation of D-amino acids in the cultures. It is concluded that the enzyme performs no essential function under laboratory conditions, although it appears to be useful to Neurospora in nature. A total of 13 D-amino acid oxidase-deficient mutants of independent origin were obtained in this investigation. All of them appear to be alleles or closely linked in linkage Group IV.

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