THE *d*-AMINO ACID OXIDASE OF NEUROSPORA

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Among artificially produced mutants of the mold *Neurospora* have been found strains lacking the ability to synthesize specific amino acids (1, 2). In the course of biochemical and genetic studies of this group of mutants it was observed that some of the mutants, *e.g.* those deficient in methionine, leucine, and arginine,[1] are able to utilize racemic mixtures of the amino acids with the same efficiency as the *l*, or physiologically occurring, forms. In the cases of the leucine- and the methionine-requiring mutants it was also possible to show utilization of the α-keto analogues. It thus appeared possible that the mode of conversion of the *d* to the *l* isomers consists in oxidative deamination, followed by resynthesis. A study was therefore undertaken to test the ability of *Neurospora* to oxidize the "unnatural" optical isomers of the amino acids. It was found that extracts of the mold contain a *d*-amino acid oxidase similar in its action to the *d*-amino acid oxidase of mammalian kidney and liver (3). This finding supports the above hypothesis for the conversion of the *d*- to the *l*-amino acids.

Since it appears that the *d*-amino acid oxidase has not been previously described in fungi, a number of experiments were performed on the *Neurospora* enzyme, the results of which are reported here.

Methods

Wild type *Neurospora crassa* was grown in Fernbach flasks containing 500 ml. of the salt-sucrose-biotin medium previously described (4). After 7 to 14 days at 25° the pads were harvested and washed in several changes of the basal salt medium. They were then pressed out through a cloth to remove excess water and weighed. At this stage the pads weighed 4 to 6 gm. each and contained 70 to 75 per cent of water. They were next ground in a mortar, with sand and 2 ml. of M/60 pyrophosphate buffer, pH 8.5, per gm. of wet tissue. The resulting paste was centrifuged at high speed for several minutes, and the supernatant, containing the enzyme, was poured off and diluted with 0.25 volume of 0.25 M pyrophosphate, pH 8.5. The final pH, determined with the glass electrode, was 8.0 to 8.2.

Oxygen consumption was measured in the Warburg apparatus at 28.6°.

[1] See the papers on the *leucineless* mutant (Regnery, D. C., *J. Biol. Chem.*, **154,** 151 (1944)) and on the *argininеless* mutants (Srb, A. M., and Horowitz, N. H., *J. Biol. Chem.*, **154,** 129 (1944)).

2 ml. of the enzyme solution were placed in the main compartment and 0.2 ml. of a M/15 solution of the racemic amino acid in the side arm. In the case of insoluble amino acids, a solution of the sodium salt was used. KOH was placed in the well; the atmosphere was air.

In all experiments the small autorespiration was automatically corrected for by placing enzyme solution in the thermobarometer vessel.

Results

Stoichiometric Relations—In the absence of added substrates the oxygen consumption of the preparation is slight but measurable. On the addition of *dl*-methionine a rapid oxidation was observed. The rate of oxygen con-

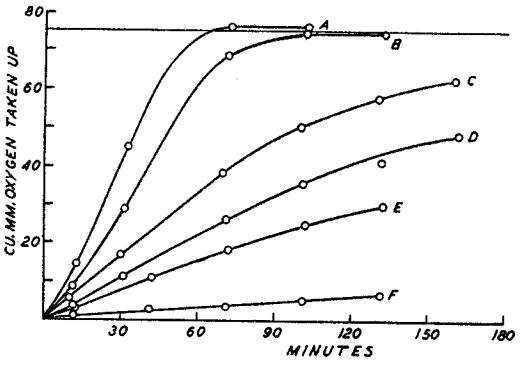

FIG. 1

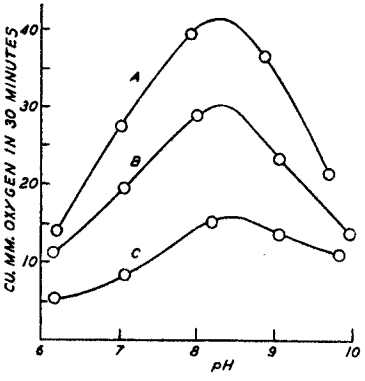

FIG. 2

FIG. 1. Oxidation of some amino acids by *Neurospora* *d*-amino acid oxidase. Curve A, *dl*-methionine; Curve B, *dl*-leucine; Curve C, *dl*-isoleucine; Curve D, *dl*-valine; Curve E, *dl*-lysine; Curve F, *dl*-ornithine. The horizontal line is the theoretical for the uptake of 1 atom of oxygen per molecule of one optical isomer.

FIG. 2. pH curves of *Neurospora* enzyme on *dl*-methionine (Curve A), *dl*-alanine (Curve B), and *dl*-α-amino-*n*-caprylic acid (Curve C).

sumption remained almost constant until 0.25 mole of oxygen per mole of *dl*-methionine was taken up, and then it rapidly dropped to zero. When *l*-methionine was substituted for the racemic mixture, no oxidation occurred. It is thus evident that the reaction involves the oxidation of *d*-methionine only, with the uptake of 1 atom of oxygen per molecule (Fig. 1). The same relation was found to hold for all other *dl*-amino acids whose oxidation rate was high enough to make an accurate determination of the end-point readily possible; namely, *dl*-phenylalanine, *dl*-norvaline, *dl*-citrulline, *dl*-arginine, *dl*-α-amino-*n*-butyric acid, *dl*-leucine, *dl*-norleucine, *dl*-isoleucine, and *dl*-glutamic acid.

The keto acid analogue of methionine, α-keto-γ-methiolbutyric acid, was found to be a product of the oxidation of *d*-methionine by the enzyme. It

was isolated from the reaction mixture in the form of its 2,4-dinitrophenylhydrazone, melting at 149°, in agreement with the melting point published by Waelsch and Borek (5) and by Cahill and Rudolph (6). When dissolved in alkali the compound gave the red color characteristic of the 2,4-dinitrophenylhydrazones of α-keto acids. Sulfur (by sodium fusion) was present, and sulfhydryl (by the nitroprusside test) was absent.

pH Optimum—The effect of pH changes in the range pH 6 to 10 on the activity of the enzyme was determined. Phosphate buffer was used at pH 6 to 8, pyrophosphate at pH 9 to 10. Determinations were made on three different substrates, *dl*-methionine, *dl*-alanine, and *dl*-α-amino-*n*-caprylic acid, respectively. In all cases a marked optimum at pH 8.0 to 8.5 was observed (Fig. 2).

Effect of Substrate Concentration—The relation between substrate concentration and reaction rate, with *dl*-methionine as substrate, was found to follow the usual hyperbolic law, within experimental limits. The Michaelis constant was approximately 2.5×10^{-4}. This value represents the concentration of *d*-methionine which produces the half maximum velocity, and is equal to the dissociation constant of the enzyme-substrate complex.

Inhibitors—The system is not significantly inhibited by cyanide (0.001 M), iodoacetate (0.001 M), or benzoate (0.01 M). Benzoate has been reported to produce complete inhibition of the kidney *d*-amino acid oxidase at a concentration of 0.01 M (7). On the other hand, drying the tissue with acetone and ether before extracting does not affect the activity of the mammalian enzyme, but in the case of *Neurospora* this treatment results in inactive preparations. The *Neurospora* enzyme is competitively inhibited by isovaline (see below).

Specificity—The enzyme was found to oxidize the *d* forms of most of the amino acids tested. Glycine and *l*-amino acids, with the exception of *l*-glutamate, are not oxidized. *l*-Glutamate is oxidized at less than one-fifth the rate of *d*-glutamate under the conditions of these experiments and presumably by a different enzyme system.

As is the case with the *d*-amino acid oxidase of kidney, *d*-methionine is the substrate most readily attacked by the *Neurospora* enzyme. The oxygen uptake on *dl*-methionine (6.06×10^{-3} M) of sixteen different preparations varied from 64.2 to 148 c.mm. of oxygen per hour per gm. of wet weight of mold, with a mean value of 107 c.mm. The cause of the variability is not definitely known. The experiments have indicated, however, that the variation in activity does not affect the relative rates of oxidation of the amino acids. In the determination of the oxidation rates presented in Table I the activity of each new enzyme preparation was standardized on *dl*-methionine as substrate, to which all other substrates were then referred.

As can be seen from Table I, the following changes in the structure of the substrates destroy their reactivity: shift of the amino group from the α to the β position; replacement of the hydrogen attached to the α-carbon atom by an alkyl group; replacement by methyl groups of both hydrogens attached to the amino nitrogen atom; replacement by methyl groups of the hydrogens attached to the β-carbon atom; substitution of a hydroxyl group on the β-carbon atom; and peptide bond formation through the carboxyl group. The effect of substitutions on the β-carbon atom in

TABLE I

Relative Rates of Oxidation of Amino Acids by d-Amino Acid Oxidase of Neurospora

The mean rate of oxidation of *dl*-methionine = 107 c.mm. of O_2 per hour per gm. of wet mold. All amino acids were tested in a final concentration of 3.03×10^{-3} M in terms of one optical isomer.

Substrate	Relative rate	Substrate	Relative rate
dl-Methionine	100	*dl*-N-Methylleucine	13
dl-Phenylalanine	85	*dl*-α-Aminophenylacetic acid	About 9
dl-Norvaline	85	*dl*-Tryptophane	" 5
dl-Citrulline	81	*dl*-Ornithine	" 4
dl-Arginine	80	*dl*-Serine	0
dl-α-Amino-*n*-butyric acid	74	*dl*-Threonine	0
dl-Leucine	66	*dl*-Proline	0
dl-Norleucine	52	β-Alanine	0
dl-Glutamic acid	41	*dl*-β-Amino-*n*-butyric acid	0
dl-Isoleucine	38	*dl*-α-Amino-α-methylbutyric acid	0
d(−)-Alanine	33	*dl*-α-Amino-α-ethylbutyric acid	0
dl-Aspartic acid	29	*dl*-β,β-Dimethyl-α-amino-*n*-butyric acid	0
dl-Alanine	26	*dl*-N,N-Dimethylleucine	0
dl-Valine	26	*dl*-Leucylglycine	0
dl-α-Amino-*n*-caprylic acid	22	Glycine	0
dl-Lysine	14		

lowering the reactivity of the substrate has also been noted in studies of the mammalian *d*-amino acid oxidase (8–10).

Inhibition by Isovaline—A number of the non-reactive amino acids were tested for their effect on the oxidation of methionine. If these substances attach to the enzyme to form an inactive complex, they should competitively inhibit the oxidation of other amino acids. If, on the other hand, no or only slight complex formation occurs, no inhibition is expected. The following compounds were tested: *dl*-serine, *dl*-N,N-dimethylleucine, *dl*-β-amino-*n*-butyric acid, and *dl*-isovaline (α-amino-α-methylbutyric acid). No inhibition of methionine oxidation was found with the first three, even at concentrations which were 10 times higher than the concentration of

methionine. It is concluded that in these cases complex formation with the enzyme does not occur.

In the case of the fourth substance tested, isovaline, an inhibition of methionine oxidation was observed. The competitive nature of the inhibition is indicated by its dependence on the concentration of methionine (Table II). The dissociation constant of the enzyme-isovaline complex was calculated by a modification of the equation of Lineweaver and Burk (11),

$$v' = \frac{V(S)K_i}{K_s K_i + K_s(I) + K_i(S)} \quad (1)$$

TABLE II

Inhibition of Neurospora Enzyme by Isovaline

The concentrations of amino acids are given in terms of one optical isomer. The isovaline concentration was 3.0×10^{-2} M in all experiments. A fresh preparation of enzyme was used for each experiment.

Experiment No.	Methionine concentration	p	K_i
	$M \times 10^3$		$\times 10^3$
1	3.0	0.36	4.1
2	3.0	0.36	4.1
	1.5	0.52	4.0
3	1.5	0.44	5.4
	0.75	0.46	8.8
4	3.0	0.36	4.1
	1.5	0.53	3.8
	0.75	0.69	3.4
5	0.75	0.69	3.4
Mean			4.6

where v' = the rate of inhibited reaction, V = the maximum rate (proportional to the enzyme concentration), K_i = the dissociation constant of the enzyme-inhibitor complex, K_s = the dissociation constant of the enzyme-substrate complex, (S) = the substrate concentration, and (I) = the inhibitor concentration. In the absence of inhibitor the rate is given by the Michaelis-Menten equation,

$$v = \frac{V(S)}{K_s + (S)} \quad (2)$$

Combining the above equations, one obtains for the inhibited fraction of the rate, p,

$$p = \frac{v - v'}{v} = \frac{K_s(I)}{K_s K_i + K_s(I) + K_i(S)}$$

from which

$$K_i = \frac{K_s(I)(1 - p)}{p(K_s + (S))} \quad (3)$$

Table II shows values of K_i calculated by means of Equation 3, with $K_s = 2.5 \times 10^{-4}$ (see above). The constancy of K_i may be considered good in view of the errors involved in the determination of K_s and of p at low concentrations of substrate.

The failure of isovaline to be oxidized by the enzyme is ascribable to the impossibility of forming the imino structure,

$$R{-}CHNH_2{-}COOH + \tfrac{1}{2}O_2 \rightarrow R{-}\underset{\underset{NH}{\|}}{C}{-}COOH + H_2O \rightarrow R{-}CO{-}COOH + NH_3$$

TABLE III

Effect of Chain Length on Reactivity of Straight Chain Amino Acids toward Neurospora Enzyme

Final concentration of amino acids, 3.03×10^{-3} M in terms of one optical isomer. Atmosphere, air; temperature, 28.6°.

Substrate	Oxygen consumed in 15 min.
	c. mm.
dl-Alanine	9.4
dl-α-Amino-*n*-butyric acid	17.2
dl-Norvaline	21.4
dl-Norleucine	16.9
dl-α-Amino-*n*-caprylic acid	6.2

In the cases of serine, N,N-dimethylleucine, and β-amino-*n*-butyric acid, imino formation, or its equivalent,[2] is theoretically possible, but the reaction is blocked by factors which prevent attachment of the molecule to the enzyme. In the mammalian *d*-amino acid oxidase, Keilin and Hartree (12) have shown that neither α-methylalanine nor N,N-dimethylalanine is able to form a complex with the enzyme.

Effect of Chain Length—An important relation shown in Table I concerns the effect of chain length on the reactivity of substrates toward *Neurospora* enzyme. With increasing length of the carbon chain in the

[2] The corresponding oxidation product of N,N-dimethylleucine would be the quaternary ammonium salt, $R{-}\underset{\underset{CH_3{-}\overset{+}{N}{-}CH_3}{\|}}{C}{-}COOH$.

homologous series of straight chain, monoaminomonocarboxylic acids, the oxidation rate first rises to a maximum at a length of 5 carbon atoms (norvaline) and then drops off. Since the data in Table I were obtained at different times, with a fresh enzyme preparation each time, it appeared desirable to check this relation on a single preparation. This was done with the results shown in Table III. These data corroborate the previous result.

It seems clear from these findings that an optimum chain length exists among the substrates of the *Neurospora* enzyme. The effect of various substitutions and internal rearrangements on the reactivity of the substrate may thus in part be ascribed to the changes they produce in the length of the molecule. Published reports do not indicate a similar dependence in the case of crude mammalian *d*-amino acid oxidase. In the case of the purified mammalian enzyme, it appears that rate data are not available for a sufficient number of substrates to decide the point.

DISCUSSION

The function of *d*-amino acid oxidase in the metabolism of *Neurospora* is unknown. Any explanation which is based on the hypothesis that the organism may encounter racemic amino acids in nature, or that it may produce them in the course of the digestion and assimilation of proteins, appears unacceptable, since the wild type of *Neurospora* is able to synthesize all of its amino acids from carbohydrates and inorganic nitrogen; it is consequently independent of external supplies of amino acids. If the enzyme serves a useful purpose, it would therefore seem to be concerned with products of the organism's own metabolism. This suggests the possibility of symmetric synthesis of amino acids by the mold. The applicability to *Neurospora* of the recent finding by Shemin and Rittenberg (13) that *d*-glutamic acid and *d*-tyrosine are not synthesized by the riboflavin-deficient rat is an open question.

In amino acid-deficient mutants of *Neurospora*, present evidence suggests that the *d*-amino acid oxidase plays an essential part in the transformation of *d*-amino acids (supplied from the outside in racemic mixtures) to *l*-amino acids. Thus, *d*-methionine, *d*-leucine, and *d*-arginine are all rapidly oxidized by the enzyme and are efficiently utilized by the corresponding mutant strains. In the cases of methionine and leucine the evidence is more complete, in that utilization of the α-keto analogues has also been found. The α-keto analogue of arginine has not been tested. Further evidence, of an indirect kind, comes from the tryptophane-requiring mutants. Tatum and Bonner (14) have shown that tryptophane synthesis in *Neurospora* occurs by a condensation of indole with *l*-serine. *dl*-

Serine is only one-half as effective as *l*-serine in promoting this reaction in experiments *in vivo* (15), indicating that *Neurospora* is unable to convert *d*- to *l*-serine. This finding is in harmony with the observation that *d*-serine is not attacked by the *Neurospora* enzyme. Similar evidence for other amino acids has been obtained with mutants currently under investigation and will be published at a later date.

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SUMMARY

1. Extracts of *Neurospora* contain a *d*-amino acid oxidase similar in its action to the *d*-amino acid oxidase of mammalian tissues.

2. The pH optimum of the system lies at pH 8.0 to 8.5.

3. The enzyme is destroyed by drying, but is not inhibited by cyanide, iodoacetate, or benzoate. It is competitively inhibited by isovaline.

4. The *d* forms of the following amino acids are rapidly oxidized: methionine, phenylalanine, norvaline, citrulline, arginine, α-amino-*n*-butyric acid, leucine, norleucine, isoleucine, and glutamic acid. The following are slowly oxidized: aspartic acid, valine, alanine, α-amino-*n*-caprylic acid, lysine, α-aminophenylacetic acid, tryptophane, ornithine, N-methylleucine. The following are not oxidized: glycine, serine, threonine, proline, β-alanine, β-amino-*n*-butyric acid, α-amino-α-ethylbutyric acid, β,β-dimethyl-α-amino-*n*-butyric acid, N,N-dimethylleucine, leucylglycine, and isovaline.

5. The activity of the enzyme shows a marked dependence on the chain length of the substrate. It was found that an optimum chain length exists.

6. The rôle of *d*-amino acid oxidase in the wild type and in mutants of *Neurospora* is discussed.

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