THE ACTION OF L-AMINO ACID OXIDASES ON THE OPTICAL ISOMERS OF α,ε-DIAMINOPIMELIC ACID

by

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The bacterial amino acid, α,ε-diaminopimelic acid (WORK[1]) has recently been resolved into its three isomers, L-L, D-D and *meso* (internally compensated) (WORK, BIRNBAUM, WINITZ AND GREENSTEIN[2]). This paper describes the effect of L-amino acid oxidases from three different sources on the isomers and also on L-diaminopimelic acid-D-monoamide which was a product of the resolution.

Neurospora L-amino acid oxidase was shown by BURTON[3] (see also[1]) to attack the naturally occurring form of diaminopimelic acid isolated from *Corynebacterium diphtheriae*. Only one atom of oxygen was consumed and one mole of ammonia was produced per mole of amino acid and no unchanged amino acid was left in the reaction mixture. Synthetic unresolved diaminopimelic acid gave only about 1/2 this oxygen uptake (WORK[4]). Since the bacterial form of diaminopimelic acid showed no optical rotation, the suggestion was made, partly on the basis of BURTON's findings, that it was the *meso* form (WORK[1,4]). The comparison, reported in this paper, of the action of *Neurospora* L-amino acid oxidase on the bacterial amino acid and on the resolved isomers, supports this suggestion.

EXPERIMENTAL

Materials

Natural (bacterial) diaminopimelic acid was prepared from hydrolysed *Corynebacterium diphtheriae* (WORK[1]). Diaminopimelic acid isomers and derivatives were prepared as described (WORK, BIRNBAUM, WINITZ AND GREENSTEIN[2]).

Enzymes

Neurospora L-amino acid oxidase was prepared from the culture fluid of *Neurospora* strain 4A by the method of THAYER AND HOROWITZ[5]. It was dialyzed and lyophilized and contained 6.1% N. I am indebted to Dr. EINOSUKE OHMURA for this preparation. It was dissolved in 0.1 *M* phosphate buffer pH 6.8 to give concentrations of 5–15 mg of enzyme/ml. The following dried preparations of venom oxidases were used: Rattlesnake (*Crotalus adamanteus*) from Ross Allen's Reptile Institute, Silver Springs, Fla. containing 12.6% N; and Viper (*Bothrops jararaca*) from Istituto Butantan, São Paulo, containing 12.8% N. The venoms were dissolved in 0.2 *M* tris (hydroxymethyl) aminomethane buffer pH 7.2 (10–50 mg of venom/ml) and dialyzed overnight against the same buffer at 2°. Catalase was a dialyzed preparation from Worthington Chemical Corporation.

Method

The experiments were carried out in Warburg manometers at 37° in an atmosphere of O_2. 2 ml of buffer containing an appropriate amount of enzyme was placed in the main compartment with

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0.1 ml of 3 *N* NaOH and filter paper in the centre well. Catalase (0.2 ml) was added to each flask. Amino acid (0.25 *M* aqueous solution) was tipped from the side arm: 0.4 ml (10 μM) was generally used unless otherwise stated. An enzyme blank was included in every experiment. The results are expressed as Q_{O_2} = μl O_2 consumed/h/mg enzyme preparation.

RESULTS

Neurospora L-*amino acid oxidase.* The results are summarized in Table I and Fig. 1 and include L-lysine and L-methionine as controls. With *Neurospora* amino acid oxidase, theoretical oxygen uptake (10 μM or 1 atom/mole of amino acid) was obtained with *meso* and bacterial diaminopimelic acid and the oxidation rates (Q_{O_2} = 16.8) were identical. The rates were of about the same order of magnitude, but slightly higher, than those for L-lysine and L-methionine. DD-diaminopimelic acid was not oxidised, even when as much as 60 mg of enzyme was used.

TABLE I

EFFECT OF L-AMINO ACID OXIDASES ON ISOMERS AND AMIDES OF DIAMINOPIMELIC ACID (DAP)

Expressed as Q_{O_2} = μl O_2/h/mg enzyme preparation. Figures in brackets indicate number of experiments.

Compound	*Neurospora oxidase*	*Viper oxidase*	*Rattlesnake oxidase*
Bacterial DAP	16.7 (6)	0.98 (3)	0.18 (1)
Meso-DAP	16.8 (8)	0.96 (3)	0.18 (1)
L-L-DAP	15.7 (8)	0.97 (2)	0.18 (1)
D-D-DAP	0 (3)	0 (2)	—
L-DAP-D-monoamide	13.1 (4)	12.2 (2)	6.6 (3)
DAP-diamide (unresolved)	0 (2)	—	—
L-lysine	8.8 (4)	1.5 (3)	0.72 (3)
L-methionine	11.3 (3)	> 114.0 (2)	> 70.0 (1)

With the L-L isomer, oxidation occurred in two stages (see Fig. 1): the first stage showed a steady oxygen uptake at a rate only slightly lower than that of the *meso* form. When approximately 10 μM of oxygen had been consumed, the rate decreased suddenly, as shown by an abrupt change in slope of the time curve of oxygen uptake; this second stage continued at a fairly steady rate for some time. The final oxygen consumption reached 1.9–2.0 atoms per mole of diaminopimelic acid. The same result was obtained in the absence of added catalase, showing that the effect cannot have been due to the lack of catalase during the latter half of the experiment. The change in rate also occurred when the amount of L-L-diaminopimelic acid was halved; the initial rate of oxygen uptake was not affected by this change, but the reaction appeared to halt temporarily after consumption of 5 μM oxygen and resumed at a different speed (see Fig. 1).

Change in enzyme concentration, although affecting the initial rate of oxygen uptake in the expected way, did not eliminate the two-step oxidation of the L-L isomer. The change in slope occurred after approximately the same oxygen consumption, irrespective of enzyme concentration. The mean oxygen consumption at which the reaction rate changed for different enzyme and substrate concentrations are as follows: 10 μM of L-L isomer, 7 experiments using 10, 15 or 20 mg of enzyme, change occurred between 91 and 114 (mean 103) μl oxygen: 5 μM of L-L isomer, 3 experiments with 10 or 20 mg of enzyme, change occurred between 49 and 53 (mean 51) μl oxygen.

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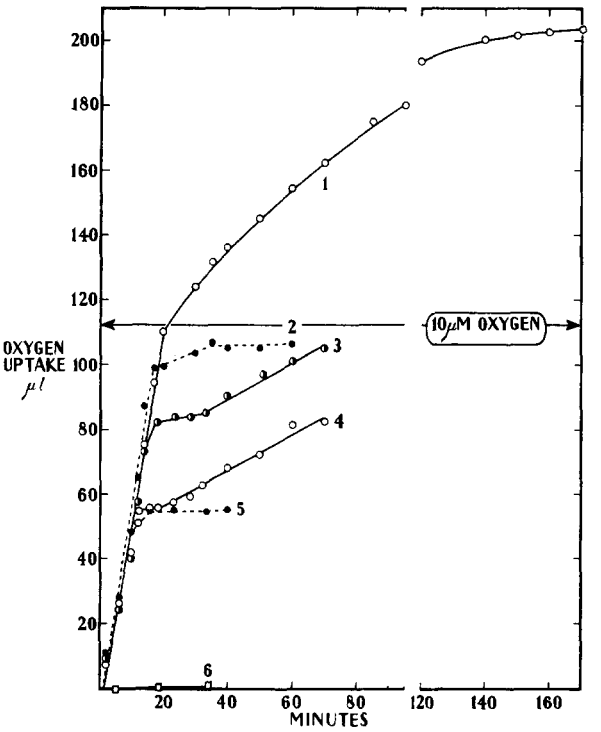

Fig. 1. Oxidation of diaminopimelic acid (DAP) isomers by *Neurospora* L-amino acid oxidase (20 mg) at pH 6.8; atmos. O_2. ○——○ L-L-DAP, 10 μM Curve 1, 5 μM Curve 4. ●----● *Meso*-DAP, 10 μM Curve 2, 5 μM Curve 5. ◑——◑ Mixture, *Meso*-DAP, 5 μM + LL-DAP, 2.5 μM Curve 3. □——□ D-D-DAP, 10 μM Curve 6.

With 10–20 mg of enzyme per flask, the L-L isomer was oxidised at a mean rate 7% lower than that of the *meso* form. This difference is hardly outside the experimental error, but as it was apparent in every experiment it is probably significant. With 30 mg of enzyme no difference between the isomers was observed, but the rates (80 μl/10 min) were too high for accurate measurement and are not included in any of the averages shown in the table.

D-D-Diaminopimelic acid had no effect on the course of oxidation of either of the other isomers. The L-L isomer had no effect on the initial rate of oxidation of the *meso* form, the total oxygen uptake was additive and the change in slope due to L-L isomer occurred at the usual 1 equivalent of oxygen. This can be seen in Fig. 1 (curve 3) where 5 μM of *meso* + 2.5 μM of L-L isomer showed change in slope at 81 μl O_2 (theoretical 84 μl). A mixture of all three isomers (5 μM meso, + 2.5 μM of each of the other isomers) was oxidised at the same initial speed as the *meso* isomer alone, and the secondary reaction was identical with that obtained without the D-D form.

L-diaminopimelic acid-D-monoamide was attacked at 77% of the speed of *meso* diaminopimelic acid. The oxygen uptake was constant until the maximum had been reached and stopped abruptly. The figures for oxygen consumption were variable and always well below theoretical (92, 80, 81 and 90 μl O_2 per 10 μM of amide being the actual figures obtained). An unresolved synthetic mixture containing all three isomers of diaminopimelic acid diamide was not attacked.

Venom L-amino acid oxidases. Meso, bacterial and L-L diaminopimelic acid were all attacked very slowly by both rattlesnake and viper venom oxidases (see Table I). 100 mg of rattlesnake venom had to be used in each flask to get a measurable oxygen uptake, but with the more active viper venom only 60 mg was used and reasonable oxygen uptakes were obtained. No differences between *meso* and L-L isomers and the bacterial material were found. The D-D isomer was not attacked by the viper oxidase; rattlesnake venom was not tried on this isomer because of its low activity towards the other isomers. Both venoms oxidised L-lysine slightly faster than diaminopimelic acid (Fig. 2); L-methionine was attacked at a rate more than 100 times that of diaminopimelic acid. L-diaminopimelic acid-D-monoamide proved to be much more susceptible to attack than

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the free amino acid, particularly in the case of rattlesnake venom where 20 mg of venom produced an oxygen uptake of 21 μl/10 min with the monoamide and no measurable uptake with the free amino acid. The oxygen uptake with the amide was approximately 1 atom when large amounts of venom were used. Total oxygen uptake with the free amino acid could not be measured as the reaction was so slow that catalase became inactivated before the end of the experiment. Even with as much as 100 mg of viper venom the oxygen uptake was more than theoretical.

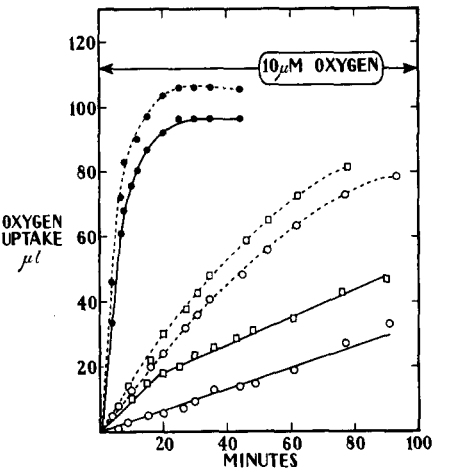

Fig. 2. Action of snake venom L-amino acid oxidases on 10 μM of substrates; pH 7.2, atmosphere O_2. ○ *meso*-diaminopimelic acid. ● L-diaminopimelic acid-D-monoamide. □ L-lysine. ------ Viper venom (75 mg). —— Rattlesnake venom (100 mg).

The effect of dialyzing the venoms was to accelerate slightly the rate of oxidation of lysine in comparison with diaminopimelic acid. This was particularly noticeable with viper venom where the Q_{O_2} values before dialysis were, for *meso*-diaminopimelic 0.80, and for L-lysine 0.82 (see Table I for corresponding values after dialysis). Dialysis reduced substantially the residual oxygen consumption in the absence of substrate.

DISCUSSION

Diaminopimelic acid differs structurally from lysine only by having one more carboxyl group. Its similarity is also apparent in its susceptibility to the action of the L-amino acid oxidases. Both these amino acids are attacked by *Neurospora* oxidase at rates comparable with the other amino acids (BENDER AND KREBS[6], BURTON[3]). The snake venoms oxidise both lysine and diaminopimelic acid much more slowly than the neutral amino acids; in fact, BENDER AND KREBS[6] and ZELLER[7] reported that lysine was not attacked by venom oxidases, but GREENSTEIN, BIRNBAUM AND OTEY[8] showed that large amounts of enzymes would cause slow oxidation. In the case of diaminopimelic acid, up to 100 mg of venom had to be used to obtain measurable oxidation. Since kidney L and D-amino acid oxidases are known to have no action on lysine[6,8] it was not thought profitable to test diaminopimelic acid with these enzymes.

As the initial rates of oxidation of L-L and *meso*-diaminopimelic acid were always essentially similar, it appears that the optical configuration at the ω-carbon atom has no effect on the action of any of the oxidases studied. It is known that the charge on the ω-carbon atom is of importance in determining susceptibility to venom oxidases, since any amino acid with an ω-polar group, such as ornithine, lysine, aspartic acid or glutamic acid, is oxidised slowly if at all (ZELLER[7]). Blocking of the polar group, as in citrulline, ε-L-benzoyl lysine, asparagine and glutamine, renders the derivatives very susceptible to oxidation. *Meso*-diaminopimelic acid, with two ω-polar groups, appears to belong to this class of amino acids; it is only slowly oxidised but is more susceptible following amidation of the D-ω-carboxyl. In the case of *Neurospora* amino acid oxidase susceptibility does not appear to be influenced by ω-polarity, since lysine, diaminopime-

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lic acid and L-diaminopimelic acid-D-monoamide are all oxidised at approximately the same rate as the neutral amino acid methionine.

The results described here on *Neurospora* oxidase, although in general agreement with those of BENDER AND KREBS, and BURTON, differ slightly in the relative rates of oxidation of diaminopimelic acid, lysine and methionine. As far as lysine and methionine go, they agree with the results of THAYER AND HOROWITZ; evidently the strain of *Neurospora* used by BENDER AND KREBS and BURTON produces an enzyme with a slightly different specificity from that of HOROWITZ. BURTON found bacterial diaminopimelic acid was oxidised at 3.6 times the rate of lysine; the enzyme used for this work, which was from HOROWITZ's strain, oxidised *meso*- or bacterial diaminopimelic acid only 1.9 times as fast as lysine. Amidation of diaminopimelic acid produced a slight decrease in susceptibility to attack by *Neurospora* enzyme. Evidently, polar groups in the ω-position are not responsible for any adverse effect on susceptibility to attack by this enzyme and change in polarity has little effect.

The oxygen uptake obtained by oxidation of L-L-diaminopimelic acid by *Neurospora* oxidase indicates that both amino groups are ultimately oxidised, but the abrupt change in slope half way through the reaction suggests that the amino acid must have a much greater affinity for the enzyme than the product produced by oxidation of only one amino group. The mono-α-keto acid formed as the primary oxidation product may cyclise immediately to form a 6-membered ring, as in the case of lysine where the α-keto acid cyclises spontaneously to Δ^1-dehydropipecolic acid (MEISTER[9], SCHWEET, HOLDEN AND LOWY[10]). Whatever its nature, the primary oxidation product from diaminopimelic acid evidently is not further oxidised until all the diaminopimelic acid originally present has been attacked by the enzyme.

The question of the identity of bacterial and *meso* (resolved) diaminopimelic acid has been fully dealt with in another communication (HOARE AND WORK[11]).

ACKNOWLEDGEMENTS

I am very grateful to the Anna Fuller Fund for a travelling grant, to Dr. J. P. GREENSTEIN for hospitality and advice, and to the National Institutes of Health for providing facilities for this work.

SUMMARY

The oxidation of the optical isomers and some amides of α,ε-diaminopimelic acid by L-amino acid oxidases from *Neurospora* and snake venoms has been investigated. *Neurospora* oxidase attacked *meso* and L-L-diaminopimelic acid, L-diaminopimelic acid-D-monoamide, L-lysine and L-methionine at rates which were of the same order of magnitude. The total oxygen uptake/mole of amino acid was 1 atom for *meso*-diaminopimelic acid and 2 atoms for L-L-diaminopimelic acid, but after 1 atom had been consumed by the L-L-isomer, a sudden reduction in speed occurred. *Meso* and L-L-diaminopimelic acid were oxidised very slowly by venom oxidases; L-diaminopimelic acid-D-monoamide was oxidised at about 12 times the rate of free *meso*-diaminopimelic acid. D-D-diaminopimelic acid was not attacked by any of the oxidases, and did not inhibit oxidation of the other isomers. The optically inactive diaminopimelic acid isolated from bacterial hydrolysates behaved identically with *meso*-diaminopimelic acid.

RÉSUMÉ

L'oxydation des isomères optiques et de quelques amides de l'acide α,ε-diaminopimélique par les L-aminoacide oxydases de *Neurospora* et des venins de serpent a été étudiée. L'oxydase de *Neuro-*

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spora attaque l'acide *méso* et l'acide L-L-diaminopimélique l'acide, L-diaminopimélique-D-monoamide, la L-lysine et la L-méthionine avec des vitesses qui sont du même ordre de grandeur. La consommation d'oxygène totale par mole d'amino-acide est de un atome pour l'acide *méso*-diaminopimélique et de deux atomes pour l'acide L-L-diaminopimélique, mais, après consommation d'un atome par l'isomère L-L, une diminution subite de vitesse a lieu. Les acides *méso* et L-L-diaminopiméliques sont oxydés très lentement par les oxydases du venin; l'acide L-diaminopimélique-D-monoamide est oxydé environ 12 fois plus vite que l'acide *méso*-diaminopimélique libre. L'acide D-D-diaminopimélique n'est attaqué par aucune des oxydases et n'inhibe pas l'oxydation des autres isomères. L'acide diaminopimélique optiquement inactif isolé des hydrolysats de bactéries se comporte de la même façon que l'acide *méso*-diaminopimélique.

ZUSAMMENFASSUNG

Es wurden Untersuchungen über die Oxydation der optischen Isomeren, sowie einiger Amide von α,ε-Diamino-Pimelinsäure durch L-Aminosäurenoxydase aus *Neurospora* und Schlangengiften durchgeführt. *Neurospora*-Oxydase oxydiert mit ähnlicher Geschwindigkeit *Meso*- und L-L-Diaminopimelinsäure, L-Diaminopimelinsäure-D-Monoamid, L-Lysin und L-Methionin. Die gesamte Sauerstoffaufnahme per Mol Aminosäure war 1 Atom im Falle von Meso-Diaminopimelinsäure und 2 Atome im Falle von L-L-Diaminopimelinsäure; nachdem jedoch ein Atom durch das L-L-Isomer verbraucht worden war, fand ein plötzliches Herabsinken der Geschwindigkeit statt. Meso- und L-L-Diaminopimelinsäure wurden sehr langsam durch Schlangengift oxydiert. L-Diaminopimelinsäure-D-Monoamid wurde ungefähr zwölfmal so schnell oxydiert, wie freie *Meso*-Diaminopimelinsäure. D-D-Diaminopimelinsäure wurde von keiner der Oxydasen angegriffen und hemmte die Oxydation der anderen Isomeren nicht. Optisch inaktive, aus Bakterienhydrolysaten isolierte Diaminopimelinsäure betrug sich genau so wie *Meso*-Diaminopimelinsäure.

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Received January 28th, 1955