Tetrahedron Letters,Vol.30,No.38,pp 5167-5170,1989 0040-4039/89 $3.00 + .00
Printed in Great Britain Pergamon Press plc

OXIDATION OF N-ALKYLPUTRESCINES BY DIAMINE OXIDASES

Alan Cooper, Angela M. Equi, Surjit K. Ner, Allan B. Watson and David J. Robins*

Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, Scotland.

Summary: N-alkylputrescines have been assayed as substrates for the diamine oxidases from pea seedlings and pig kidney using two independent techniques. K_M and V_{max} data are reported. In contrast with published observations, N-methylputrescine was oxidised by both enzymes with an efficiency intermediate between that of putrescine and N-ethylputrescine.

Diamine oxidase (DAO, EC 1.4.3.6) catalyses the oxidation of a range of primary diamines to yield the corresponding aminoaldehydes (Scheme 1).[1] This oxidation is a key step in the metabolism of biologically important polyamines [such as putrescine (1)] which are essential for growth and replication of all living cells.[2] Two forms of DAO are readily available - pig kidney (Sigma) and pea seedling (by extraction[1]). Frydman et al.[3] recently reported studies on the oxidation of putrescine and the N-alkylputrescines (2)-(4) catalysed by both forms of diamine oxidase. One puzzling feature of these results, for which no explanation was offered, was that although N-ethylputrescine and higher homologues were efficiently oxidised to the corresponding aminoaldehydes, N-methylputrescine showed little or no apparent substrate activity with either enzyme. This is a surprising result in view of the known susceptibility to oxidation of N-methylputrescine by oxidase enzymes present in a number of plants including Nicotiana tabacum, Datura stramonium, and Atropa belladonna.[4] As part of a systematic study of the activity and specificity of diamine oxidases we have repeated the experiments of Frydman et al. under their conditions and have found, in contrast, that N-methylputrescine is indeed a reasonable substrate for both forms of diamine oxidase. This has been confirmed using an alternative assay system which has an additional advantage of allowing more complete kinetic characterisation of the enzymic reactions.

$$RNH(CH_2)_4NH_2 + H_2O + O_2 \xrightarrow{DAO} RNH(CH_2)_3CHO + H_2O_2 + NH_3$$

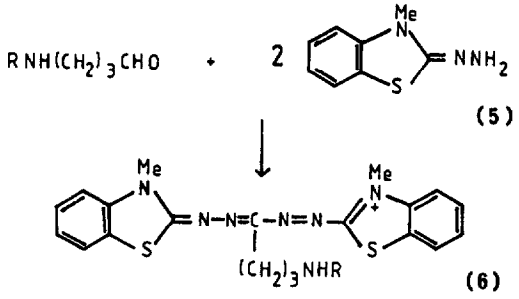
(1): R= H (3): R= C_2H_5

(2): R= CH_3 (4): R= n-C_3H_7

Scheme 1

The N-alkylputrescines (2)-(4) were prepared as described,[5] and purified by recrystallisation of the dihydrochlorides to give literature m.p.s and correct analytical data. Pig kidney diamine oxidase was purchased from Sigma with a nominal activity of 0.06

units per mg solid.[6] Pea seedling diamine oxidase was extracted from 10 day old pea seedlings as described by Hill and purified up to Step 4.[1] Enzyme activity was 1170 units per mg solid (at 25°C). SDS gel electrophoresis showed that the commercial pig enzyme is a heterogeneous mixture of many polypeptide species whereas the pea preparation, while not homogeneous, comprises just two major protein components. Enzyme activity for all substrates was assayed by two independent methods. The first, following Frydman et al.,[3] measures the extent of oxidation of putrescine and the N-alkylputrescines with diamine oxidase during a 30 minute incubation by quenching and trapping the aminoaldehydes with 3-methyl-2-benzothiazolinone hydrazone (5) and measuring the absorbance of the bis-hydrazone cation (6) at 660nm (Scheme 2). The values shown in Table 1 are an average of two determinations and are compared with putrescine (1) as standard, together with the results of Frydman et al. for comparison.[3]



Scheme 2

The second procedure involves a peroxidase-coupled assay (horseradish peroxidase, EC 1.4.3.6, from Sigma) to monitor continuously the hydrogen peroxide released during diamine oxidation (Scheme 1) at 25°C, pH 6.3, in the presence of 3-methyl-2-benzothiazolinone hydrazone (5) and 3-(dimethylamino)benzoic acid.[7] Oxidative coupling generates stoichiometric quantities of an indamine dye with a characteristic absorbance maximum at 595nm, and rates of reaction can be determined directly in the spectrophotometer. The reaction was calibrated using standard hydrogen peroxide solutions and control experiments showed that, at the considerably lower concentrations used in this assay, bis-hydrazone(6) formation by reaction with (5) was negligible. Initial rates were determined over a range of substrate concentrations and Michaelis-Menten kinetic behaviour was observed in all cases. Rate data were analysed for K_M and V_{max} (Table 2) by least-squares fitting of Eadie-Hofstee or Lineweaver-Burk plots. Data quoted are means of at least three determinations. (Complete kinetic data for pig kidney diamine oxidase were unobtainable using N-ethyl- and N-propylputrescine because of the very low activity of this enzyme preparation with these substrates.) Protein concentrations were determined by the Coomassie Blue method of Sedmak and Grossberg.[8]

It is apparent from our results in Table 1, confirmed by the independent peroxidase-coupled assay (Table 2), that oxidation of N-methylputrescine is catalysed by both enzymes. This in direct contrast to the reported results.[3] The reason for this discrepancy remains

Table 1. Oxidation rates of N-alkylputrescines relative to putrescine, catalysed by pea seedling and pig kidney diamine oxidase (DAO) at 37°C and pH 7.4[a]

Substrate	Pea seedling DAO		Pig kidney DAO	
	Activity[b] (nmol/30min)	Relative rate[b] (%)	Activity[b] (nmol/30min)	Relative rate[b] (%)
(1) Putrescine	122 (180)	100 (100)	106 (120)	100 (100)
(2) N-methylputrescine	119 (2)	98 (1)	90 (2)	85 (2)
(3) N-ethylputrescine	83 (125)	68 (69)	79 (58)	74 (48)
(4) N-propylputrescine	70 (105)	57 (59)	63 (30)	53 (25)

[a]Conditions of incubation as in ref.3 ; [b]Data from ref.3 in parentheses.

Table 2. K_M and V_{max} values for oxidation of putrescine and N-alkylputrescines catalysed by diamine oxidase from pea seedlings and pig kidney at 25°C and pH 6.3[a]

Substrate	Pea seedling DAO		Pig kidney DAO	
	K_M (mM)	V_{max} (μmol $mg^{-1}h^{-1}$)	K_M (mM)	V_{max} (μmol $mg^{-1}h^{-1}$)
(1) Putrescine	1.18(±.37)	1170(±220)	0.43(±.17)	0.030(±.005)
(2) N-methylputrescine	1.12(±.14)	211(±24)	1.34(±.28)	0.011(±.002)
(3) N-ethylputrescine	1.17(±.40)	3.1(±.3)	-	<0.002
(4) N-propylputrescine	0.68(±.22)	0.26(±.06)	-	<0.002

[a]Peroxidase-coupled assay (ref.7), standard deviations in parentheses.

unclear, but one possibility might be the presence of an inhibitory impurity in the original N-methylputrescine preparation. The kinetic curves of ref.3 show apparent inhibition phenomena after about 10 minutes of incubation under these conditions, with plateau levels different for different subtrates. It is unlikely that this is due to product inhibition or to depletion of substrates since, under apparently identical conditions, our data (Table 1) show essentially identical amounts of aminoaldehyde formation after 30 minutes for both putrescine and N-methylputrescine. This illustrates the unsatisfactory nature of the bis-hydrazone assay procedure for comparing substrate kinetics since for good substrates the reaction is essentially complete within the standard 30 minute incubation period, and even the poorer substrates react substantially within this time, so the data bear no relation to initial reaction rates in most cases.

Analysis of the kinetic parameters (K_M and V_{max}) for the pea-seedling DAO reaction using the more satisfactory peroxidase-coupled assay[7] (Table 2) reveals that the progressive decrease in catalytic efficiency with increasingly bulky substituents arises from changes in catalytic rate constant (V_{max}) rather than substrate binding affinity (K_M). K_M values are remarkably uniform at about 1mM over the range of substrates, but there is a sharp fall in V_{max} as the size of the N-alkyl group is increased. This seems to indicate that the active site of diamine oxidase is relatively uncrowded, at least at the distal end of the substrate. Substrate selectivity in this enzyme thus resides in the catalytic mechanism rather than the binding affinity. The progressive reduction in V_{max} might arise because the bulky substituents inhibit important conformational changes in the catalytic step of the enzyme, or because they restrict access of other reactants (oxygen and water) to the reactive site.

The situation with pig kidney DAO is less clear, and interpretation is limited because the poor specific activity of this enzyme has prevented study of an extensive range of substrates so far. It is not clear at this stage whether the greatly reduced specific activity of pig kidney diamine oxidase, compared to the pea seedling enzyme, is due to the impure state of this enzyme preparation or an intrinsic property of the enzyme itself. The substrate affinities (K_M) of the two enzymes, which are unaffected by protein impurities, are however very similar. But the change in reactivity of this enzyme for N-methyl-putrescine, as compared to putrescine, is much less marked than for the pea enzyme and arises from a combination of changes in both binding affinity and catalytic efficiency. It would seem possible that these two enzymes from widely divergent sources might employ somewhat different kinetic strategies for molecular recognition of diamine substrates.

Acknowledgements. We thank Miss Isabel Freer for assistance in the isolation of pea seedling diamine oxidase and the SERC for financial support.

References and Notes

1. J.M.Hill, Methods in Enzymology, 1971, 17B, 730.
2. D.Walters, Biologist, 1987, 34, 73.
3. R.B.Frydman, O.Ruiz, M.Kreisel and U.Bachrach, FEBS Lett., 1987, 219, 380.
4. F.Feth, V.Wray and K.G.Wagner, Phytochemistry, 1985, 24, 1653.
5. D.O.Alonso Garrido, G.Buldain and B.Frydman, J.Org.Chem., 1984, 49, 2021.
6. One standard unit will oxidise 1.0 μmol of putrescine (1) per hour at 37°C and pH 7.2
7. P.Stoner, Agents and Actions, 1985, 17, 5.
8. J.J.Sedmak and S.E.Grossberg, Anal.Biochem., 1977, 79, 544.

(Received in UK 27 July 1989)