Stereospecificity of Pig Kidney and Pea Seedling Diamino Oxidases on 2-Methyl-1,4-diaminobutane

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Diamine oxidase from pea seedlings (PDAO) oxidizes both (*R*)- and (*S*)-2-methylputrescine at the less hindered C-4, whereas pig-kidney diamine oxidase (KDAO) shows a dependence on the stereochemistry of the substrate, since the (*R*)-isomer is oxidized at C-1 and the (*S*)-isomer at the less hindered amino group at C-4.

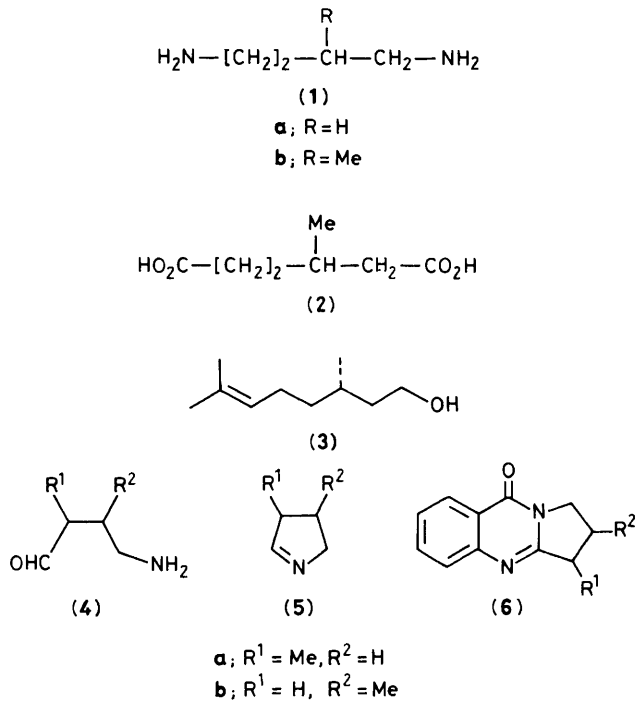
Diamine oxidases (E.C.1.4.3.6, diamine:oxygen oxidoreductase, deaminating) catalyse the oxidative deamination of various substrates[1] including diamines, *e.g.* 1,4-diaminobutane (putrescine) (**1a**). Using racemic 2-methyl-1,4-diaminobutane (**1b**) and two diamine oxidases from different sources, we have been able to show that the oxidation of methylputrescine occurred with different regioselectivity depending on the enzyme used.[2] This approach suggested that by replacement of hydrogen with a methyl group in the physiological substrate of an enzymic reaction, useful information on the active site of the enzymes examined can be obtained. We have now studied the stereospecificity of the reaction, using (*R*)- and (*S*)-(**1b**) as substrates and diamine oxidases from animal (pig kidney diamino oxidase, KDAO) and plant (pea seedling diamino oxidase, PDAO) sources.†

(*R,S*)- and (*R*)-(**1b**) were prepared from commercially available (*R,S*)- and (*R*)-methyladipic acid (**2**) according to a published procedure.[4] The (*R*)-isomer was 90% optically pure, as determined by the optical rotation of its dihydrochloride {$[\alpha]_D$ +5.1° (*c* 2.7, H_2O), lit.[4] $[\alpha]_D$ +5.6°}. The (*S*)-diamine (**1b**) was synthesized from (*S*)-methyladipic acid, in turn prepared from commercially available (*S*)-(−)-citronellol (**3**) (Fluka). Thus, (−)-citronellol (**3**) was converted into (*S*)-(−)-citronellic acid most satisfactorily with a two-step oxidation. The alcohol (**3**) was oxidized to the corresponding aldehyde with pyridinium chlorochromate in the presence of sodium acetate[5] and then the aldehyde was transformed into the acid with Ag_2O.[6] Ozonolysis at −78 °C of (−)-citronellic acid and oxidation of the ozonide with performic acid afforded (*S*)-(**2**) in 23% yield from (**3**). (*S*)-Diamine (**1b**) obtained from (*S*)-(−)-(**3**) was 95% optically pure ($[\alpha]_D$ −5.2° for its dichloride).‡

In preliminary experiments, the optimal pH for the oxidation of (*R,S*)-(**1b**) was determined as 9 and 8.5 for PDAO and KDAO, respectively. The relative activities of the two enzymes on (*R,S*)-(**1b**), each at their own optimal pH, were compared with those on (**1a**)[7] [pH 6.5 and 6.0 for the two enzymes when substrate was (**1a**)] and found to be 12.7 and 18.4% for PDAO and KDAO respectively.

Compounds (*S*)- and (*R*)-(**1b**) were incubated at their optimal pH with the two different enzymic preparations and quantitative analyses of the products of oxidative deaminations were performed by h.p.l.c. after conversion of the aminoaldehydes (**4a**) or (**4b**) [in equilibrium with the corresponding Δ^1-pyrrolines (**5a**) or (**5b**)] into quinazolones (**6a**) or (**6b**).§ The relative amounts of compounds (**6a**) and (**6b**) measured at high substrate concentration furnished the information on the site of oxidation of the substrate, since (**6a**) and (**6b**) correspond to the direct products of deamination, (**4a**) and (**4b**), at C-1 and C-4 respectively.

The results so far obtained show that PDAO catalyses the deamination of 2-methyl-1,4-diaminobutane at the less hindered amino group, independently of the configuration of the substrate used, since (**6b**) is preferentially formed (85%) by oxidation of (*R*)-(**1b**) and is practically the only product (95%) when (*S*)-(**1b**) is used as a substrate. On the other hand, when



† PDAO was extracted according to Hill's method (ref. 3), up to step IV, and stored at −20 °C in 10 mM phosphate buffer (pH 7.0). KDAO (Sigma) was purified by chromatography on Sephacryl S-200 using a 50 mM phosphate buffer (pH 7.5). The specific activities of the two enzyme preparations were 780 (PDAO) and 23 mU/mg (KDAO).

‡ All compounds have ^{1}H n.m.r., i.r., and mass spectra in complete accordance with assigned structures. All new compounds gave correct microanalyses.

§ After 10 minutes incubation at 37 °C in 1 ml total volume containing 5 mmol of substrate and 4 mU of enzyme, the reaction was stopped by addition of 0.15 ml of 10% trichloroacetic acid. A known amount of Δ^1-pyrroline (prepared by hydrolysis of γ-aminobutanal diethylacetal) was added as an internal standard and 0.05 ml of a saturated aqueous solution of *ortho*-aminobenzaldehyde added. After 2 h at room temperature the solutions were treated with 0.1 ml of 0.64 M CrO_3 solution in 0.8 M H_2SO_4. After 4 h at room temperature, the samples were made alkaline with 5 M NaOH and extracted twice with 3 ml of diethyl ether. The final organic phases, evaporated to dryness, were redissolved in 50 μl of ethyl acetate and 5 μl used for the h.p.l.c. The separation was performed on a 25 cm × 4.6 mm LiChrosorb Si-100 column (Merck) using ethyl acetate as eluent at a flow rate of 1 ml/min and spectrophotometric detection at 268 nm.

Quinazolones (**6a**) and (**6b**) were separated by preparative h.p.l.c. and identified by their 200 MHz ^{1}H n.m.r. spectra.

KDAO is the enzyme under study, the oxidation follows two different patterns, depending upon the configuration of the chiral substrate. With (*R*)-(**1b**) the C-1 primary amine is converted into the corresponding aldehyde [95% of (**6a**) formed], whereas with (*S*)-(**1b**), the less hindered position C-4 is the site of the oxidation [90% of (**6b**) formed].

These findings furnish some indications on the steric prerequisites of the active sites of two enzymes which belong to the same class but come from different sources. For both enzymes, substitution of hydrogen at C-2 in (**1a**) with a methyl group reduces the activity to a great extent, indicating that the region near the reacting amino group is rather sensitive to the steric hindrance. However, PDAO regioselectively oxidizes (**1b**) only at C-4 and shows no stereospecific distinction between (*R*)- and (*S*)-(**1b**). On the contrary, KDAO catalyses the oxidation of both (*R*)- and (*S*)-(**1b**) with opposite regioselectivity, thus suggesting that for this enzyme the deaminating site is more sensitive to the configuration of the substrate. When the hydrogen at C-2 in (**1a**) is substituted with a methyl group, only one enantiomer of (**1b**) is oxidized at C-1, showing that KDAO, unlike PDAO, is stereoselective.

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