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Purification and properties of *S*-adenosylmethionine: aldoxime *O*-methyltransferase from *Pseudomonas* sp. N.C.I.B. 11652

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1. An enzyme catalysing the *O*-methylation of isobutyraldoxime by *S*-adenosyl-L-methionine was isolated from *Pseudomonas* sp. N.C.I.B. 11652. 2. The enzyme was purified 220-fold by DEAE-cellulose chromatography, $(NH_4)_2SO_4$ fractionation, gel filtration on Sephadex G-100 and chromatography on calcium phosphate gel. Homogeneity of the enzyme preparation was confirmed by isoelectric focusing on polyacrylamide gel and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 3. The enzyme showed a narrow pH optimum at 10.25, required thiol-protecting agents for activity and was rapidly denatured at temperatures above 35°C. 4. The K_m values for isobutyraldoxime and *S*-adenosyl-L-methionine were respectively 0.24mM and 0.15mM. 5. Studies on substrate specificity indicated that attack was mainly restricted to oximes of C_4–C_6 aldehydes, with preference being shown for those with branching in the 2- or 3-position. Ketoximes were not substrates for the enzyme. 6. Gel filtration on Sephadex G-100 gave an M_r of 84000 for the intact enzyme, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated an M_r of 37500, suggesting the presence of two subunits in the intact enzyme. 7. *S*-Adenosylhomocysteine was a powerful competitive inhibitor of *S*-adenosylmethionine, with a K_i of 0.027mM. The enzyme was also susceptible to inhibition by thiol-blocking reagents and heavy-metal ions. Mg^{2+} was not required for maximum activity.

A number of psychotrophic bacteria can produce the volatile secondary metabolite IBME when cultured under a wide range of environmental conditions (Harper & Gibbs, 1979; Harper & Nelson, 1982). Although oximes have been implicated in plants as intermediates in the biosynthesis of secondary metabolites such as cyanogenic glycosides (Conn, 1981) and glucosinolates (Underhill, 1980), their endogenous concentrations *in vivo* are so low that their formation from the corresponding ^{14}C-labelled amino acid has only been demonstrated by means of trapping experiments or the use of specific inhibitors. It has not been possible to characterize cell-free systems that are capable of forming or acting on oximes in such pathways, primarily because the entire enzyme complex responsible is located in the particulate fraction and the pathway appears to constitute an example of metabolic channelling in which intermediates remain associated with the enzyme complex throughout the biosynthetic sequence (Møller & Conn, 1980; Conn, 1983). Thus the bacterial formation of IBME is of particular significance, as it not only provides a microbial parallel to some of the reactions involved in the biosynthesis of cyanogenic glycosides, but is an alternative and more accessible system from which to attempt to isolate the individual enzymes responsible for the conversion of amino acids and oximes into this type of secondary metabolite.

The amino acid valine was established by Harper & Nelson (1982) as the precursor of IBME, and a possible biosynthetic route involving *N*-hydroxylation, decarboxylation and methylation was postulated, although the exact sequence in which these transformations occur was not clear. In the present paper the enzymology of the final stage in the biosynthesis of IBME, namely the methylation of isobutyraldoxime, is described, and the purification and properties of a new class of transmethylase, *S*-adenosylmethionine: aldoxime

Abbreviations used: IBME, isobutyraldoxime methyl ether; SDS, sodium dodecyl sulphate.

O-methyltransferase, are reported. Although the enzyme catalyses a reaction that does not have an exact parallel in the biosynthesis of cyanogenic glycosides, this report represents the first description in the literature of the purification of an enzyme capable of using oximes as substrates.

Materials and methods

Culture of micro-organism

Pseudomonas sp. N.C.I.B. 11652, which was employed in these studies, was initially isolated from spoiling meat and identified by Patterson & Gibbs (1977) as an *Alcaligenes* sp., although subsequently the organism was reclassified by the National Collection of Industrial Bacteria (N.C.I.B.), Torry Research Station, Aberdeen, U.K.

The organism was grown on a defined medium of the following composition (g/l): KH_2PO_4 (4.5); $K_2HPO_4,3H_2O$ (10.5); $MgSO_4,7H_2O$ (0.15); NH_4Cl (1.0); glucose (5.0); L-valine (2.0). The pH of the solution was adjusted to 7.0, and the medium was supplemented with a trace-element solution (10ml/l) containing (g/l): $ZnSO_4,7H_2O$ (0.04); $Na_2MoO_4,2H_2O$ (0.02); $FeCl_3$ (0.04); KI (0.01); $CuSO_4,5H_2O$ (0.004); H_3BO_3 (0.05); $MnSO_4,4H_2O$ (0.04). Conical flasks (2 litre) plugged with cotton-wool and containing 1 litre of culture medium were normally incubated at 15°C on an orbital shaker with an eccentricity of 2.5cm at 120rev./min. The growth of the organism was monitored by measuring the A_{690} of the culture medium, and assay of IBME in the medium was performed as described below.

Preparation of cell-free extracts

The organism was harvested by using a Sharples Super centrifuge (25000rev./min, flow rate 100ml/min) soon after the cultures had entered the stationary phase, when IBME concentration in the culture medium had attained 8μg/ml, which usually occurred after about 75h incubation (Harper & Nelson, 1982). A yield of 23g wet wt. of cell paste was obtained per litre of culture medium. Cells were washed with 75mM-potassium phosphate buffer, pH7.0, harvested by centrifugation (15000g, 30min, 3°C) and stored at −15°C until required for preparation of extracts.

To the paste of cells (50g wet wt.) was added 150mM-potassium phosphate buffer, pH7.5 (5ml), and 100mM-dithioerythritol (3ml), and the cells were disrupted by sonication for a total duration of 8min with an MSE 150W ultrasonic disintegrator at maximum amplitude. Cooling in an ice/NaCl bath ensured that the temperature did not rise above 14°C. The resulting homogenate was centrifuged (60000g, 30min, 2°C), and the clear orange–pink supernatant (54ml) was decanted and dialysed against 25mM-potassium phosphate buffer, pH7.5, containing 5mM-dithioerythritol.

Chemicals

Oximes were prepared by the standard method from the corresponding aldehyde or ketone by treatment with hydroxylamine hydrochloride and purified by distillation. The following were synthesized (b.p. in parentheses): n-propionaldoxime (94–96°C at 26.7kPa), n-butyraldoxime (73–74°C at 3.5kPa), isobutyraldoxime (118–120°C at 38.9kPa), methacrylaldoxime (77–79°C at 7.5kPa), 2-methylbutyraldoxime (82–83°C at 4.5kPa), 3-methylbutyraldoxime (168–170°C at 100.3kPa), n-valeraldoxime (84–85°C at 2.5kPa), n-hexanaldoxime (104–105°C at 3.9kPa), benzaldoxime (134–136°C at 3.3kPa) and 2-butoxime (147.5–148.5°C at 87.1kPa).

Methyl ethers of oximes were prepared as described by Harper & Nelson (1982) from the corresponding aldehyde or ketone by reaction with methoxyamine hydrochloride and purified by distillation. The following were synthesized (b.p. in parentheses): n-propionaldoxime methyl ether (74–76°C at 100.3kPa), n-butyraldoxime methyl ether (98–100°C at 99.2kPa), isobutyraldoxime methyl ether (91–93°C at 99.0kPa), methacrylaldoxime methyl ether (105–107°C at 98.7kPa), 2-methylbutyraldoxime methyl ether (114–116°C at 101.2kPa), 3-methylbutyraldoxime methyl ether (94°C at 51.5kPa), n-valeraldoxime methyl ether (94–96°C at 41.4kPa), n-hexanaldoxime methyl ether (56°C at 2.7kPa), benzaldoxime methyl ether (108–110°C at 7.5kPa) and 2-butoxime methyl ether (90–92°C at 90.0kPa).

S-Adenosyl-L-methionine was obtained in the form of the chloride salt from Sigma Chemical Co., Poole, Dorset, U.K., as was *S*-adenosyl-L-homocysteine. Calcium phosphate (type II, neutral; brushite), was also purchased from the above supplier.

Proteins employed for calibration of Sephadex G-100 columns and used as standards in SDS/polyacrylamide-gel electrophoresis were acquired from either Sigma Chemical Co. or Boehringer Corp., Lewes, East Sussex, U.K. Ampholines of various pH ranges used in isoelectric focusing were obtained from LKB Instruments, South Croydon, Surrey, U.K.

Protein and enzyme assays

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

O-Methyltransferase activity of the extracts of the microbial enzyme was assayed by measuring IBME production when the extract was incubated

with isobutyraldoxime in the presence of *S*-adenosylmethionine. The concentration of IBME was determined by gas chromatography, with a headspace technique. The standard assay was performed at 25°C in duplicate 25 ml screw-capped septum vials sealed with Teflon-lined silicone discs (Tuf-bond; Pierce and Warriner, Chester, U.K). Vials contained, in a total volume of 2 ml, 1 mmol of carbonate buffer, pH 10.25, 10 μmol of dithioerythritol, 4.8 μmol of isobutyraldoxime, 4.8 μmol of *S*-adenosylmethionine and 0.1 ml of sample extract to be assayed. After incubation at 25°C for 1 h the reaction was terminated by placing the vials in a boiling-water bath for 2 min. The assay mixture was allowed to cool and then equilibrated for 30 min at 25°C in a water bath with shaking, and the IBME concentration was measured as described by Harper & Nelson (1982). A 2 ml sample of headspace was extracted with a syringe and injected into a Pye–Unicam 104 (model 74) chromatograph equipped with a glass column (1.5 m × 2 mm internal diam.) packed with Tenax GC (60–80 mesh) and operated at an N_2 gas flow of 20 ml/min. The temperature of the chromatograph oven was programmed at a rate of 24°C/min from 100°C to 200°C. Compounds that were eluted were detected by using a flame ionization detector. Harper & Nelson (1982) have shown, using standard solutions of IBME (up to 1 mg/ml), that a linear relationship exists between the concentration of IBME in the solution in the vial and the peak height obtained on gas chromatography of the headspace in the vial, with a lower limit of detection of 0.2 μg/ml.

In experiments on substrate specificity where the products of reaction were oxime methyl ethers other than IBME, the technique could be readily adapted to quantify such compounds provided that standard solutions of the authentic compounds were available.

Isoelectric focusing on polyacrylamide gel

Analytical thin-layer electrofocusing in polyacrylamide gel was performed with an LKB Multiphor apparatus by the method described by Karlsson *et al.* (1973) for isoelectric focusing in the pH range 2.5–6.0, with riboflavin as the catalyst for polymerization of the acrylamide. Before application to the gel the protein fraction was dialysed against 10 mM-sodium phosphate buffer, pH 7.0. Volumes of sample solution containing 10–50 μg of protein were applied to the surface of gel absorbed on 5 mm × 10 mm pieces of Whatman 3MM chromatography paper. The pH gradient on the gel after electrofocusing at 2°C was determined by means of an Activion surface electrode. The staining technique of Vesterberg (1972) was used for localizing protein bands with Coomassie Brilliant Blue R-250.

SDS/polyacrylamide-gel electrophoresis

M_r determinations on the enzyme were performed by electrophoresis on polyacrylamide gel in the presence of SDS by the thin-layer technique described by Harper (1977), an LKB 2117 Multiphor apparatus being used. Thin-layer gels of 10% acrylamide containing 0.2% (w/v) SDS dissolved in 100 mM-sodium phosphate buffer, pH 7.2, were polymerized, with ammonium persulphate as catalyst and *NNN'N'*-tetramethylenediamine as accelerator. Samples were prepared for application to the gel as described by Weber *et al.* (1972) in 10 mM-sodium phosphate buffer, pH 7.0, containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol at 100°C. The gels were subjected to transverse electrophoresis, the reservoir buffer in the anode and cathode compartments being 50 mM-sodium phosphate buffer, pH 7.2, containing 0.1% SDS, and later stained with Coomassie Brilliant Blue as described by Vesterberg (1972).

The following standard proteins were used for calibration in SDS/polyacrylamide-gel electrophoresis (M_r of subunit in parentheses): bovine serum albumin (67500), catalase (58000), glutamate dehydrogenase (55400), ovalbumin (43000), fructose bisphosphate aldolase (41000), lactate dehydrogenase (36500) and carbonic anhydrase (29000).

Results and discussion

Purification of O-methyltransferase

All stages of enzyme purification were conducted at 0–3°C. Stages in purification of the dialysed cell-free extract (1) are described below and the results are summarized in Table 1.

(2) *DEAE-cellulose chromatography*. The cell-free extract was applied to a DEAE-cellulose column (2.5 cm × 50 cm) equilibrated with 25 mM-potassium phosphate buffer, pH 7.5, containing 5 mM-dithioerythritol. The column was eluted successively with 300 ml of 25 mM-, 50 mM- and 100 mM-potassium phosphate buffers, pH 7.5, containing 5 mM-dithioerythritol, and 10 ml fractions were collected. *O*-Methyltransferase activity was mainly confined to the protein fractions eluted with 100 mM buffer, and those fractions possessing high enzyme activity were pooled.

(3) *Precipitation with* $(NH_4)_2SO_4$. To the enzyme solution (108 ml) was added solid $(NH_4)_2SO_4$ (33.48 g) with stirring to give a 50%-saturated solution. After 30 min the solution was centrifuged (30000 *g*, 30 min) and the precipitate discarded. The supernatant was adjusted to 65% saturation by the addition of a further 12.96 g of $(NH_4)_2SO_4$,

Table 1. *Purification of aldoxime O-methyltransferase from Pseudomonas sp. N.C.I.B. 11652*
The results shown are of a typical enzyme purification as described in the text. One unit of enzyme activity is defined as the amount of enzyme required to catalyse the formation of 1 nmol of IBME/min at 25°C under the conditions of the standard assay in 0.5M-sodium carbonate buffer, pH 10.25, in the presence of 2.4mM-S-adenosylmethionine and 2.4mM-isobutyraldoxime.

Stage of purification	Volume (ml)	Total enzyme activity (units)	Yield (%)	Protein (mg/ml)	Specific activity (units/mg of protein)
1. Dialysed cell-free extract	56	728.0	100	11.64	1.12
2. DEAE-cellulose column chromatography	108	352.1	48.4	0.15	21.73
3. $(NH_4)_2SO_4$ fractionation	5.7	273.0	37.5	1.23	38.94
4. Gel filtration on Sephadex G-100	18.0	185.4	25.5	0.102	101.0
5. Chromatography on calcium phosphate	8.5	56.8	7.8	0.027	247.4

stirred for 30min and again centrifuged (50000g, 30min). The white precipitate was dissolved in 50mM-potassium phosphate buffer, pH 7.5, containing 10mM-dithioerythritol (5ml).

(4) *Gel filtration on Sephadex G-100.* The extract was applied to a Sephadex G-100 column (2.5cm × 60cm) equilibrated with 50mM-potassium phosphate buffer, pH 7.5, containing 10mM-dithioerythritol. On elution with this buffer, 4.8ml fractions were collected, and those fractions containing the highest enzyme activity at relative elution volumes between 1.47 and 1.60 were combined.

(5) *Chromatography on calcium phosphate.* The purified enzyme from the previous stage was dialysed against 10mM-potassium phosphate buffer, pH 6.8, containing 5mM-dithioerythritol, and the resulting solution was applied to a column of calcium phosphate gel (1cm × 6.5cm) equilibrated with the same buffer as used in dialysis. Brushite rather than hydroxyapatite was found to be a more suitable form of calcium phosphate gel for this stage of purification because of the substantially faster flow rate that could be achieved on column chromatography. The column was eluted successively with 30ml of 10mM-, 30ml of 25mM- and 20ml of 50mM-potassium phosphate buffer, pH 6.8, containing 5mM-dithioerythritol. Finally, the pure enzyme was eluted with 75mM-potassium phosphate buffer, pH 6.8, containing 5mM-dithioerythritol.

Samples from various stages of purification were examined by isoelectric focusing on polyacrylamide gel, which revealed the presence of only one protein band in the stage 5 fraction with pI 4.40. SDS/polyacrylamide-gel electrophoresis also confirmed the homogeneity of the preparation (see below under 'Determination of M_r'). Purification by the procedure described was 221-fold and the overall yield 7.8%.

Properties of O-methyltransferase

In the absence of thiol-protecting agents the enzyme was very labile, but in 100mM-potassium phosphate buffer, pH 7.5, containing 5mM-dithioerythritol the enzyme from stage 3 of the purification procedure exhibited a half-life of 10 weeks at 0°C and could be stored frozen at −15°C for several months without significant loss of activity. Preparations from stages 4 and 5 of the purification procedure, though possessing a similar half-life at 0°C, were totally inactivated by freezing. However, such preparations could be protected from denaturation by addition of 10% (v/v) glycerol before freezing and could then be stored indefinitely in this form at −15°C. In view of the low overall yield of the purification procedure, partially purified preparations from stage 3 were in general used for enzyme characterization. Pure enzyme was, however, employed for isoelectric focusing and the determination of the M_r of the enzyme.

Influence of enzyme concentration

The initial velocity of the enzyme reaction in the standard assay was directly proportional to enzyme concentration at final protein concentrations ranging from 20 to 450μg/ml. The velocity of the reaction was linear with respect to time over a period of at least 90min at 25°C provided that utilization of S-adenosylmethionine was not allowed to exceed 4%.

Influence of pH

The effect of pH on the activity of the O-methyltransferase was measured with the use of 0.5M-sodium carbonate, -Tris and -sodium phosphate buffers. Enzyme activity in Tris buffer at pH 9.0 was 132% of that in carbonate buffer at the same pH. In Fig. 1, which shows the effect of pH on O-methyltransferase activity, enzyme activities

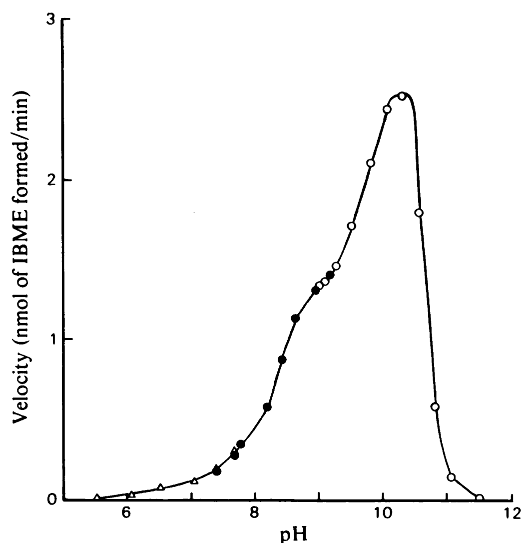

Fig. 1. *Effect of pH on aldoxime O-methyltransferase activity*

Enzyme was incubated at the appropriate pH under standard assay conditions at 25°C in 500mM-sodium phosphate buffer (△), 500mM-Tris buffer (●) or 500mM-sodium carbonate buffer (○). *S*-Adenosylmethionine and isobutyraldoxime concentrations in the assay mixture were both 2.4mM (K_m values at pH 10.25 for *S*-adenosylmethionine and isobutyraldoxime were respectively 0.15mM and 0.24mM). Enzyme activities in Tris buffer have been adjusted as described in the text.

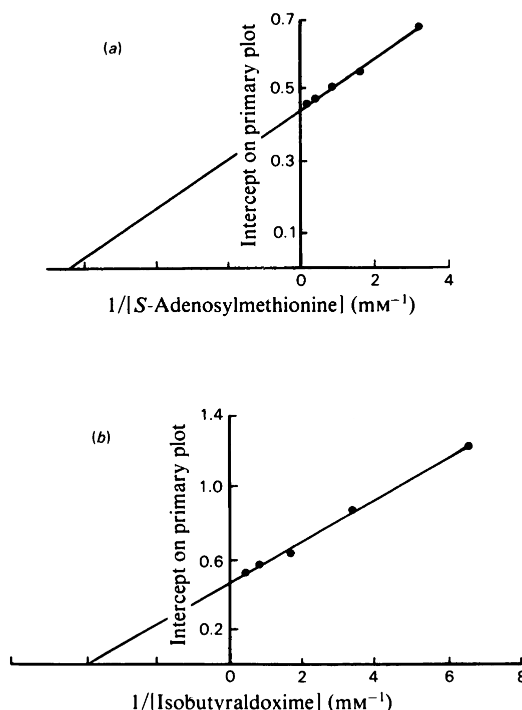

Fig. 2. *Secondary plots of primary-plot intercepts against the reciprocal of the concentration of (a) S-adenosylmethionine and (b) isobutyraldoxime*

The velocity of *O*-methylation was measured at concentrations of isobutyraldoxime between 0.15 and 2.4mM while the concentration of *S*-adenosylmethionine was held at a number of fixed values between 0.3 and 4.8mM. The usual double-reciprocal plots for two substrate reactions were obtained. The intercepts of such primary plots on the $1/v$ axis were then plotted against the reciprocal of substrate concentration for each substrate.

in Tris have therefore been adjusted to make them comparable with results obtained with carbonate and phosphate buffers. The enzyme exhibits a very narrow pH optimum between 10.15 and 10.35, with activity falling very sharply at higher pH. The optimum lies at a rather higher pH than that observed for other methyltransferases, though some phenolic *O*-methyltransferases have been reported to show optima above pH 9.0 (Poulton, 1981). Indeed, an enzyme purified from *Petroselinum hortense* cell cultures catalysing the methylation by *S*-adenosylmethionine of the *meta* position of dihydric phenols possessed an optimum around pH 9.7 (Ebel *et al.*, 1972).

Influence of temperature

The velocity of *O*-methylation was determined at temperatures between 5 and 45°C under standard assay conditions. The activation energy of the enzyme reaction between 5 and 30°C as determined by an Arrhenius plot was 54.7kJ/mol. Rapid denaturation of the enzyme occurred at temperatures above 35°C, inactivation being almost instantaneous at 45°C.

Effect of substrate concentration

The velocity of *O*-methylation was measured at concentrations of isobutyraldoxime between 0.15mM and 2.4mM while the concentration of *S*-adenosylmethionine was held at a number of fixed values between 0.3 and 4.8mM, and the usual double-reciprocal plots for two-substrate reactions were obtained (Dixon & Webb, 1979). When the intercepts of the primary plots (representing $1/V_{max.}$ for a series of concentrations of each substrate) were plotted against the reciprocal of substrate concentration for each substrate, the straight lines in Figs. 2(*a*) and 2(*b*) were obtained, indicating K_m values of 0.15mM for *S*-adenosylmethionine and 0.24mM for isobutyraldoxime.

Substrate specificity

The relative rates of methylation and apparent K_m values of a number of aldoxime substrates are

shown in Table 2. It is clear that attack is mainly restricted to oximes of C_4–C_6 aldehydes, with preference being shown for oximes with branching in the 2- or 3-position. The enzyme appears to have the highest affinity for 2-methylbutyraldoxime, which exhibits an apparent K_m of approx. 50% of that of isobutyraldoxime and has $V_{max.}$ 60% greater than that of the latter compound, which is presumably the natural substrate of the enzyme. The comparatively small decrease in $V_{max.}$ despite the large increase in apparent K_m on introduction of a double bond between C-2 and C-3 in isobutyraldoxime can probably be attributed to the promotion of ionization of the hydroxy-group hydrogen by conjugation.

Ketoximes such as 2-butoxime did not act as substrates for the enzyme, nor did oximes of aromatic aldehydes such as benzaldoxime.

Determination of M_r

The M_r of the enzyme was determined by gel filtration of the pure *O*-methyltransferase on a Sephadex G-100 column previously calibrated with the following reference proteins (M_r in parentheses): fructose bisphosphate aldolase (145000), bovine serum albumin (67500), carbonic anhydrase (29000) and myoglobin (17500). Purified enzyme (100 μg) was applied to the top of a column (2.5 cm × 70 cm) of Sephadex G-100 equilibrated with 50 mM-potassium phosphate buffer containing 10 mM-dithioerythritol and eluted with the same buffer. Fractions (4.5 ml) were collected and assayed for *O*-methyltransferase activity. The eluted enzyme protein emerged as a single discrete peak of activity at a relative elution volume, V_e/V_0, of 1.52, corresponding to an M_r value of 84000.

The M_r of the constituent subunits of the enzyme was measured by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. This technique indicated a subunit M_r of 37500. Thus it appears that the active enzyme is composed of two subunits with a combined M_r of approx. 75000. This value can be compared with M_r values of 48000 and 110000 found by gel filtration for the *o*-dihydricphenol *meta-O*-methyltransferase from *Petroselinum hortense* (Ebel *et al.*, 1972) and the isoflavone 4′-*O*-methyltransferase from *Cicer arietinum* (Wengenmayer *et al.*, 1974) respectively.

Table 2. *Substrate specificity of aldoxime O-methyltransferase*

Rates of methylation of different oxime substrates were measured at six concentrations under the conditions of the standard assay with *S*-adenosylmethionine at a concentration of 2.4 mM. The enzyme preparation employed was that from stage 3 of the purification procedure at a final concentration in the assay mixture of 60 μg/ml. $V_{max.}$ and apparent K_m values for the oxime substrates were determined by the Lineweaver–Burk method. K_m values shown represent apparent K_m values for the ssubstrates under standard assay conditions with *S*-adenosylmethionine at a concentration of 2.4 mM.

Substituent group R– in substrate R–CH=NOH	$V_{max.}$ (% of $V_{max.}$ with isobutyraldoxime)	K_m (mM)
CH_3–CH_2–	5.3	1.54
CH_3–$[CH_2]_2$–	33.9	0.98
CH_3–$[CH_2]_3$–	25.8	0.67
CH_3–$[CH_2]_4$–	0	—
CH_3–CH(CH_3)–	100.0	0.27
CH_3–CH_2–CH(CH_3)–	161.2	0.14
CH_3–CH(CH_3)–CH_2–	76.2	0.16
CH_2=C(CH_3)–	66.9	1.11

Effects of inhibitors and metal ions

A typical feature of transmethylation reactions involving *S*-adenosylmethionine is that the reaction is strongly inhibited by low concentrations of the product, *S*-adenosylhomocysteine (Poulton, 1981). The effect of *S*-adenosylhomocysteine on the rate of oxime methylation was determined at a number of different concentrations of *S*-adenosylmethionine, and the results are presented in Fig. 3 in the form of a Dixon (1953) plot.

S-Adenosylhomocysteine acted as a powerful competitive inhibitor of the reaction, K_i for the compound of 0.027 mM being considerably less than the K_m of 0.15 mM for *S*-adenosylmethionine. The ratio between these parameters is very similar to that noted for isoflavone 4′-*O*-methyltransferase by Wengenmayer *et al.* (1974), who observed 0.030 mM as K_i for *S*-adenosylhomocysteine and 0.16 mM as K_m for *S*-adenosylmethionine.

The sensitivity of the enzyme to thiol-blocking reagents, metal ions and chelating agents was also investigated after dialysis against 50 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-dithioerythritol. The influence of the various compounds and ions on activity is illustrated in Table 3. As might be expected from the lability of the enzyme in the absence of thiol-protecting agents, the enzyme was strongly inhibited by thiol-blocking reagents such as phenylmercuriacetate and low concentrations of heavy-metal ions, suggesting the presence of thiol groups at the active

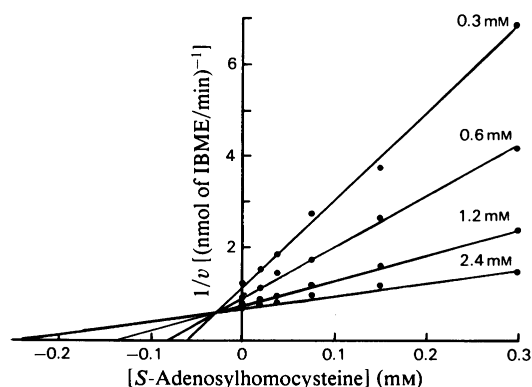

Fig. 3. *Dixon plot of 1/v against S-adenosylhomocysteine concentration at different concentrations of S-adenosylmethionine*

Enzyme was incubated with various concentrations of *S*-adenosylhomocysteine at the four different concentrations of *S*-adenosylmethionine indicated on the Figure. Other parameters were as described in the standard assay procedure.

Table 3. *Effect of possible inhibitors and metal ions on aldoxime O-methyltransferase activity*

The dialysed enzyme was preincubated in the presence of possible inhibitor for 10min before addition of substrate. Assay was performed by the standard procedure except that incubation was for 30 min only and was performed in the absence of added dithioerythritol.

Inhibitor or metal ion added	Final concn. (mM)	Relative activity (enzyme without addition = 100%)
N-Ethylmaleimide	0.05	44
Iodoacetamide	0.5	45
Phenylmercuriacetate	0.025	5
Hg^{2+}	0.05	0
Ag^{+}	1.0	0
EDTA	1.0	107
Mg^{2+}	1.0	102

site. Animal catechol *O*-methyltransferase often requires bivalent ions such as Mg^{2+} for activity (Axelrod & Tomchick, 1958), and Mg^{2+} ion has been shown to be necessary for maximum activity of some *o*-dihydricphenol *meta*-*O*-methyltransferases involved in flavonoid biosynthesis in plants (Ebel *et al.*, 1972; Poulton *et al.*, 1977; Sutfield & Wiermann, 1978). However, it is clear from Table 3 that Mg^{2+} has no significant effect on aldoxime *O*-methylation, and in this respect the enzyme appears to be similar to the *O*-methyltransferase involved in lignin biosynthesis (Poulton, 1981). The lack of a metal-ion requirement was confirmed by the failure of chelating agents such as EDTA to affect activity to any appreciable extent.

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