The Mechanism of Action of Primary Alkylsulphohydrolase and Arylsulphohydrolase from a Detergent-Degrading Micro-organism

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Previous studies have shown that secondary alkylsulphohydrolases from certain detergent-degrading micro-organisms are unusual esterases in that they catalyse fission of the C–O bond of the alkyl sulphate ester linkage. The position of bond fission catalysed by a primary alkylsulphatase and an arylsulphohydrolase present in *Pseudomonas* C12B has now been investigated. The primary alkylsulphatase behaved like the secondary alkylsulphohydrolases in cleaving the C–O bond of potassium heptan-1-yl sulphate. In contrast, the arylsulphohydrolase, in common with other similar enzymes previously studied, catalysed the fission of the O–S bond of potassium *p*-nitrophenyl sulphate.

Microbial alkylsulphohydrolases appear to initiate the biodegradation of secondary and primary alkyl sulphate detergents. *Pseudomonas* C12B and *Comamonas terrigena* are micro-organisms that possess such enzymes. The former produces two primary (designated P1 and P2) and three secondary (S1, S2 and S3) enzymes under appropriate circumstances (Dodgson *et al.*, 1974), whereas the latter produces two secondary enzymes (designated CS1 and CS2; Fitzgerald *et al.*, 1975).

The work of Bartholomew *et al.* (1977) on the purified optically stereospecific (for *D*-isomers) S1 and CS2 secondary alkylsulphohydrolases has shown that they catalyse the scission of the C–O bond of the C–O–S ester linkage, with a simultaneous inversion of configuration about the asymmetric carbon atom. This mechanism appears to be novel in that other esterases so far studied catalyse scission of acid–oxygen, rather than alcohol–oxygen, bonds.

Previous studies on the position of bond scission by sulphohydrolases have been restricted to arylsulphohydrolases. Several different examples of such enzymes were studied by Spencer (1958, 1959), who found that the O–S bond was ruptured in each case. This perhaps points to the possibility that scission of the C–O bond by the S1 and CS2 secondary alkylsulphohydrolases might be an exceptional mechanism related to the optical stereospecificity of the enzymes. We decided to find out whether a primary alkylsulphohydrolase, where no question of optical stereospecificity arises, would operate in the same manner. This possibility was tested by using a highly purified preparation of the inducible P2 primary enzyme of *Pseudomonas* C12B. The organism can also produce an arylsulphohydrolase enzyme under certain growth

conditions (Fitzgerald & Payne, 1972a). An unusual opportunity was thus afforded to compare the position of bond scission by three different types of sulphohydrolase produced by a single micro-organism.

Materials and Methods

¹⁸O-enriched water (61.8 and 62.5 atom % excess of ¹⁸O) was purchased from the British Oxygen Co. (Prochem), London SW19 3UF, U.K. Heptan-1-ol and hexan-1-ol were purchased from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. Other chemicals used were the purest available from BDH Chemicals Ltd., Poole, Dorset, U.K., or Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. The potassium salts of heptan-1-yl sulphate and hexan-1-yl sulphate were prepared from the parent alcohols by the method of Dodgson *et al.* (1974). Potassium *p*-nitrophenyl sulphate was prepared as described by Burkhardt & Lapworth (1926).

Enzyme preparations

Arylsulphohydrolase. *Pseudomonas* C12B was grown to the stationary phase in a basal-salts medium containing citrate and methionine as described by Fitzgerald & Payne (1972a). The cells from a 3.5-litre culture were harvested and resuspended in a washing buffer consisting of 10 mM-Tris/HCl buffer, pH 8.0, 85 mM-sodium citrate and 20% (w/v) sucrose. The suspension was shaken at 30°C for 2 h to extract the arylsulphohydrolase. The cells were then separated by centrifuging (2200 *g*, 30 min), and the clear supernatant was dialysed at 2°C against 3 × 2 litres of 10 mM-Tris/HCl, pH 7.5, before concentration by

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Diaflow ultrafiltration (UM-10 membrane; Amicon Corp., MA, U.S.A.) to yield 56 ml (119 mg of protein) of enzyme solution, designated as Fraction 1. The arylsulphohydrolase activity of this fraction was 0.029 unit/mg (for definition of unit, see under 'Assay of sulphohydrolase activity' below). A portion (30 ml) of the fraction was treated with solid $(\text{NH}_4)_2\text{SO}_4$, and material that was precipitated between the limits 55 and 80% saturation was separated by centrifuging, re-dissolved in 1.0 ml of 10 mM-Tris/HCl buffer, pH 7.5, and exhaustively dialysed against the same buffer to give 1.4 ml (4.38 mg of protein) of Fraction 2. The activity of this fraction was 0.092 unit/mg. A further purified preparation of the enzyme (giving one major and two minor protein bands on polyacrylamide-gel electrophoresis; activity 1.27 units/mg; designated Fraction 3) was kindly supplied by Dr. J. W. Fitzgerald and Dr. J. R. George, Department of Microbiology, University of Georgia, Athens, GA, U.S.A. All three preparations gave one arylsulphohydrolase band only on gel electrophoretograms.

P2 primary alkylsulphohydrolase. *Pseudomonas* C12B was grown on basal-salts medium (Fitzgerald & Payne, 1972b) supplemented with 1% (w/v) sodium citrate and 2 mM-potassium hexan-1-yl sulphate. Cells were harvested at mid-exponential growth phase ($A_{420} = 0.7$; see Fitzgerald, 1974). The P2 enzyme was purified about 100-fold from cell extracts by a procedure involving: (a) removal of nucleic acid with streptomycin sulphate; (b) fractionation with $(\text{NH}_4)_2\text{SO}_4$ between the limits 25 and 52% saturation; (c) dialysis of the re-dissolved precipitate against 20 mM-Tris/HCl, pH 7.25, followed by chromatography on DEAE-cellulose (DE-52) with a linear gradient of 0–0.5 M-NaCl; (d) fractionation on Sephadex G-100 in 2.5 mM-Tris/HCl, pH 7.8; (e) re-chromatography on DEAE-cellulose with a linear gradient of 0–0.36 M-NaCl. This preparation had an activity of 5 units/mg of protein and gave one major and one minor band on polyacrylamide-gel electrophoresis (J. M. Cloves & K. S. Dodgson, unpublished work). Enzyme activity coincided with the major band.

Assay of sulphohydrolase activity

Arylsulphohydrolase activity towards potassium *p*-nitrophenyl sulphate was routinely measured essentially as described by Dodgson & Spencer (1957), except that 0.6 ml of a mixture containing substrate (25 mM), 10 mM-Tris/HCl, pH 7.5, and suitably diluted enzyme was incubated at 30°C for 15 min before adding 1.5 ml of 1 M-NaOH and measuring the absorbance of the liberated *p*-nitrophenol at 400 nm ($\epsilon = 18000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). One unit of activity is defined as 1 μmol of *p*-nitrophenol liberated/min.

P2 primary alkylsulphohydrolase activity was

routinely measured by the method of Dodgson *et al.* (1974). One unit of activity is defined as 1 μmol of SO_4^{2-} ions liberated/min at 30°C from 18 mM-potassium hexan-1-yl sulphate in 0.1 M-Tris/HCl, pH 7.8.

Detection of sulphohydrolases in polyacrylamide gels

The conditions used for polyacrylamide-gel electrophoresis and detection of primary alkylsulphohydrolase activity were as described by Payne *et al.* (1974). Arylsulphohydrolase activity was detected as a yellow band on similar gels by incubating the extruded gel for 5 min with 25 mM-potassium *p*-nitrophenyl sulphate in 10 mM-Tris/HCl, pH 7.5.

Isotope-incorporation experiments

Arylsulphohydrolase. The incubation mixture (total volume 0.7 ml) contained enzyme (0.0307, 0.142 or 0.425 unit of Fractions 1, 2 or 3 respectively), 0.2 ml of H_2^{18}O (61.8 or 62.5 atom% excess of ^{18}O), Tris/HCl buffer (0.5 M, Fractions 1 and 2; 0.1 M, Fraction 3), pH 7.5, and 50 μmol of potassium *p*-nitrophenyl sulphate. Incubations at 30°C were for 24, 2 and 1 h for Fractions 1, 2 and 3 respectively, and the corresponding extents of hydrolysis for these times were 21.4, 27.0 and 22.2% as determined on 5 μl samples. Reactions were terminated by adding 0.2 ml of 15% (w/v) trichloroacetic acid, and precipitated protein was removed by centrifuging. Liberated *p*-nitrophenol was extracted with diethyl ether (2 $\times$ 0.4 ml), and the ether extracts were re-extracted with 2 $\times$ 0.8 ml portions of 0.5 M-glycine buffer (0.5 M-glycine/0.5 M-NaCl, adjusted to pH 3.3 with HCl) to remove trichloroacetic acid. The ether extracts were then analysed for ^{18}O by mass spectrometry in a Finnegan 4000 GCMS instrument (Finnegan Instruments, Hemel Hempstead, Herts., U.K.), by using the selected ion-monitoring technique to measure ions with m/e 139 and 141 (molecular ions of $[\text{C}^{16}\text{O}]p\text{-nitrophenol}$ and $[\text{C}^{18}\text{O}]p\text{-nitrophenol}$ respectively). The SO_4^{2-} ions that remained in the aqueous layer after the initial ether extraction were then precipitated as BaSO_4 and analysed for ^{18}O -content by the i.r. method of Spencer (1959) as modified by Tudball & Thomas (1972).

Primary alkylsulphohydrolase. Potassium heptan-1-yl sulphate was used as substrate in these experiments because we found preliminary indications (J. M. Cloves & K. S. Dodgson, unpublished results) that it is a better substrate than hexan-1-yl sulphate. The incubation mixture (total volume 0.4 ml), containing 0.65 unit of enzyme, 25 mM-Tris/HCl, pH 7.5, 15 μmol of substrate and 0.2 ml of H_2^{18}O (61.8 atom% excess of ^{18}O), was kept at 30°C for 1 h. Preliminary experiments (with unlabelled water) showed that 94% of the substrate was hydrolysed under these condi-

tions. The reaction was terminated by adding 0.1 ml of 15% (w/v) trichloroacetic acid, and precipitated protein was removed by centrifugation. Liberated heptan-1-ol was extracted with diethyl ether (2×0.4 ml), and the pooled extract was analysed by mass spectroscopy as before, measuring the intensities of ions of m/e 31 and 33 ($\text{CH}_2^{16}\text{OH}$ and $\text{CH}_2^{18}\text{OH}$ respectively). The SO_4^{2-} ions remaining in the aqueous layer after ether extraction were precipitated as BaSO_4 and analysed for ^{18}O -content as described for arylsulphohydrolase.

Control experiments

For both enzymes, control incubations were carried out, substituting unlabelled water for H_2^{18}O . For the arylsulphohydrolase an exchange control was carried out in which Na_2SO_4 was substituted for substrate and incubated in the presence of Fraction-1 enzyme for 24 h to check the extent of any ^{18}O exchange between H_2^{18}O and SO_4^{2-} ions. No exchange was noted.

Calibration curve for ^{18}O incorporation into SO_4^{2-}

Samples of $\text{BaS}^{16}\text{O}_3^{18}\text{O}$ containing various atom% excesses of ^{18}O were prepared by mixing various amounts of two samples of BaSO_4 prepared after acid hydrolysis of potassium *p*-nitrophenyl sulphate in unlabelled and ^{18}O -enriched water. These samples were subjected to i.r. analysis as before, and a calibration curve was obtained. A control experiment in which Na_2SO_4 was substituted for potassium *p*-nitrophenyl sulphate showed that no ^{18}O exchange into SO_4^{2-} ions occurred under the hydrolysis conditions used.

Results and Discussion

Table 1 records the ^{18}O contents of the products of the reactions catalysed by the P2 and arylsulphohydrolase enzymes. Clearly the P2-enzyme reaction proceeds by C–O fission and the arylsulphohydrolase by O–S fission. ^{18}O -incorporation values for the latter enzyme were closer to theoretical the more pure the enzyme preparation used. In the Fraction-3-enzyme experiment, the i.r. spectrum of the $\text{BaS}^{16}\text{O}_3^{18}\text{O}$ was repeated ten times to assess the repeatability of what is really a relatively insensitive method (see Spencer, 1959). When the variability is taken into account the value obtained (Table 1) is very close to theoretical. Deviation from theory of the Fractions 1 and 2 results may be due to some slight ^{18}O exchange between substrate and water. Exchange between water and SO_4^{2-} ions was eliminated by the control experiment.

In the P2-alkylsulphohydrolase experiment, the atom% excess of ^{18}O in the liberated heptan-1-ol is slightly lower than theoretical. This may well reflect the low intensity of the peak due to the CH_2OH ion used in the gas chromatography–mass spectroscopy analysis and, in consequence, the relatively poor sensitivity of the method when applied to primary alcohols (cf. Bartholomew *et al.*, 1977).

These results, together with those of Bartholomew *et al.* (1977) for the S1 secondary alkylsulphohydrolase of *Pseudomonas* C12B, show that, within the same micro-organism, sulphohydrolases are operating through different types of mechanism. The attack on the O–S bond by the arylsulphohydrolase conforms with the results obtained on a number of other such enzymes reported by Spencer (1958, 1959). However, it is somewhat surprising to find that, in common with secondary alkylsulphohydrolases, the

Table 1. Incorporation of ^{18}O into the reaction products of the P2 alkylsulphohydrolase and the arylsulphohydrolase of *Pseudomonas* C12B

The H_2^{18}O used in the experiments contained 61.8 atom% excess of ^{18}O , except for the Fraction-3 arylsulphohydrolase, when the value was 62.5.

Enzyme preparation	H ₂ O in incubation mixture	Atom% excess of ¹⁸ O					
		Calculated for C–O split		Calculated for O–S split		Found	
		Phenol or alcohol	SO ₄ ^{2–}	Phenol or alcohol	SO ₄ ^{2–}	Phenol or alcohol	SO ₄ ^{2–}
Arylsulphohydrolase							
Fraction 1	17.7	17.7	0	0	4.4	0	5.4
Fraction 2	17.7	17.7	0	0	4.4	0	5.2
Fraction 3	17.8	17.8	0	0	4.5	0	4.9 ± 0.3*
Alkylsulphohydrolase							
P2 enzyme	30.9	30.9	0	0	7.7	26.3	0

* s.d. calculated after repeating the i.r. scan ten times (see the text).

P2 primary enzyme attacks the C–O bond, even though optical stereospecificity or inversion of configuration is not a feature of the enzyme's action. This finding presumably reflects a similar evolutionary history for primary and secondary alkylsulphohydrolases which, in turn, is different from that for arylsulphohydrolases. Similar considerations may apply to the metabolic roles of the enzymes. The interesting possibility is also raised that other types of sulphohydrolase may operate by C–O-bond splitting, a mechanism not generally associated with hydrolytic enzymes acting on ester bonds (cf. Bartholomew *et al.*, 1977).

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