Primary alkylsulphatase activities of the detergent-degrading bacterium *Pseudomonas* C12B

Purification and properties of the P1 enzyme

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The P1 primary alkylsulphatase of *Pseudomonas* C12B was purified 1500-fold to homogeneity by a combination of streptomycin sulphate precipitation of nucleic acids, $(\text{NH}_4)_2\text{SO}_4$ fractionation and chromatography on columns of DEAE-cellulose, Sephacryl S-300 and butyl-agarose. The protein was tetrameric with an M_r of 181 000–193 000, and exhibited maximum activity at pH 6.1. Primary alkyl sulphates of carbon-chain length C_1 – C_5 or above C_{14} were not substrates, but the intermediate homologues were shown to be substrates, either by direct assay (C_6 – C_9 and C_{12}) or by gel zymography (C_{10} , C_{11} , C_{13} and C_{14}). Increasing the chain length from C_6 to C_{12} led to diminishing K_m . Values of ΔG^0 for binding substrates to enzyme were dependent linearly on chain length, indicating high dependence on hydrophobic interactions. V_{max}/K_m values increased with increasing chain length. Inhibition by alk-2-yl sulphates and alkane-sulphonates was competitive and showed a similar dependence on hydrophobic binding. The P1 enzyme was active towards several aryl sulphates, including *o*-, *m*- and *p*-chlorophenyl sulphates, 2,4-dichlorophenyl sulphate, *o*-, *m*- and *p*-methoxyphenyl sulphates, *m*- and *p*-hydroxyphenyl sulphates and *p*-nitrophenyl sulphate, but excluding bis-(*p*-nitrophenyl) sulphate and the *O*-sulphate esters of tyrosine, nitrocatechol and phenol. The arylsulphatase activity was weak compared with alkylsulphatase activity, and it was distinguishable from the de-repressible arylsulphatase activity of *Pseudomonas* C12B reported previously. Comparison of the P1 enzyme with the inducible P2 alkylsulphatase of this organism, and with the Crag herbicide sulphatase of *Pseudomonas putida*, showed that, although there are certain similarities between any two of the three enzymes, very few properties are common to all three.

INTRODUCTION

The ability to biodegrade primary and secondary alkyl sulphate surfactants is a feature of a number of bacteria common to soils and waters (Dodgson *et al.*, 1982; Dodgson & White, 1983; White *et al.*, 1984). In the examples studied so far, biodegradation is initiated by primary or secondary alkylsulphatase enzymes, followed by the oxidation of the liberated primary or secondary alcohols by appropriate alcohol dehydrogenases (for a review see Dodgson & White, 1983). The bacteria involved are often capable of producing a multiplicity of alkylsulphatases. Thus *Pseudomonas* C12B can produce five such enzymes (Dodgson *et al.*, 1974), *Pseudomonas putida* FLA six (Lillis *et al.*, 1983) and *Pseudomonas* DES1 four (Hales, 1981).

Of the five alkylsulphatases produced by *Pseudomonas* C12B, two (designated P1 and P2) are active towards primary alkyl sulphates, whereas the other three (S1, S2 and S3) act on secondary alkyl sulphates (Matcham *et al.*, 1977a; Dodgson & White, 1983). The latter enzymes exhibit positional and stereo-specificity, S1 being active towards D-alk-2-yl sulphates, S2 towards L-alk-2-yl sulphates and S3 towards symmetrical and near-symmetrical secondary alkyl sulphates. Collectively, they

are able to initiate the degradation of commercial alkyl sulphate surfactant preparations, which generally contain a wide range of positional and stereoisomers and alkyl chain lengths. All three enzymes surprisingly operate by rupture of the C–O bond of the ester sulphate linkage, and this is accompanied by inversion of configuration (Bartholomew *et al.*, 1978; Shaw *et al.*, 1980).

It is less obvious why *Pseudomonas* C12B has the capacity to produce two primary alkylsulphatases because, at first sight, they appear to act on the same substrates. The P2 enzyme is inducible, appears in mid-exponential growth phase and has been purified to homogeneity (Cloves *et al.*, 1980a,b). In common with the secondary enzymes, it operates by fission of the C–O bond (Cloves *et al.*, 1977) rather than the O–S bond (Bartholomew *et al.*, 1977). The P1 enzyme has been considered to be constitutive and appears during the early stationary phase of growth.

The present paper describes the purification and properties of the P1 alkylsulphatase and relates its properties to those of the P2 enzyme and to the sulphatase of *Ps. putida* FLA, which, by its action, is able to initiate the activation of the pre-emergent herbicide sodium 2-(2,4-dichlorophenoxy)ethyl sulphate (Crag herbicide; see Lillis *et al.*, 1983).

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MATERIALS AND METHODS

Materials

Primary alcohols and racemic secondary alcohols were purchased from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. Sulphate esters of primary and secondary alcohols were prepared respectively by the methods of Lloyd *et al.* (1961) and Matcham & Dodgson (1977). 'Dec-5-yl sulphate' was prepared as described by Dodgson *et al.* (1974). Quotation marks are used to draw attention to the heterogeneity of the preparation. Substantial amounts of other positional isomers are present, and the preparation is used to detect secondary alkylsulphatase activities on polyacrylamide gels. Esters were recrystallized from methanol as potassium salts. Sodium salts of primary alkanesulphonates were purchased from Cambrian Chemicals, Croydon, Surrey, U.K., and sodium [1-¹⁴C]dodecyl sulphate (specific radioactivity 81 GBq/mmol) was a gift from Dr. A. H. Olavesen of this department. Aryl sulphate esters were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K., or were prepared by sulphonation of parent phenols by the method of Burkhardt & Lapworth (1926). Protein standards for M_r determinations were from the Boehringer Corporation (London), Lewes, East Sussex, U.K., Sigma (London) Chemical Co. and BDH Chemicals, Poole, Dorset, U.K. All other chemicals were the purest available from BDH Chemicals or Sigma (London) Chemical Co.

Growth of *Pseudomonas* C12B

Studies not reported in the present paper have shown that maximum yields of the P1 primary alkylsulphatase were obtained if the organism was grown on 0.3% nutrient broth (Difco Laboratories, Detroit, MI, U.S.A.). Growth was at 30 °C in 350 litres of broth containing 50 p.p.m. of Anti-Foam A Concentrate (Sigma). The fermentor (400 litres, New Brunswick Fermentor) contents were aerated at a rate of 1.8 m³ of air/h and agitated at 100 rev./min. Cell harvest was initiated at early stationary phase ($A_{650} = 0.4-0.45$) with a Sharples centrifuge operating at a flow rate of 90 litres/h. The wet cells (360 g) were resuspended in 400 ml of 10 mM-Tris/HCl buffer, pH 7.5, freeze dried and stored at -20 °C.

Assays

Alkylsulphatase. With alkyl sulphates other than sodium dodecyl sulphate (SDS), enzymically liberated SO_4^{2-} ions were measured by the BaCl_2 /gelatin method of Dodgson (1961) as modified by Thomas & Tudball (1967). Potassium octyl sulphate (12.5 mM final concentration) was employed as routine assay substrate and, for preliminary experiments and during enzyme purification, was incubated with enzyme preparations at 30 °C in the presence of 50 mM-Tris/HCl buffer, pH 7.0. Initial reaction rates were measured and appropriate control determinations were made. A unit of enzyme activity is defined as the amount of enzyme that liberates 1 μmol of SO_4^{2-} /min from octyl sulphate under these conditions. Subsequent work showed that the pH optimum for the P1 enzyme was 6.1. Consequently all kinetic studies with the purified enzyme were performed in 0.1 M-Tris/maleate buffer, pH 6.1, unless stated otherwise.

The BaCl_2 /gelatin method cannot be used in the presence of alkyl sulphates with chain lengths greater than C_8 . Enzyme activity towards SDS was therefore

determined by using the 1-¹⁴C-labelled ester. Equal volumes (100 μl) of suitable concentrations of enzyme and substrate solutions in 0.1 M-Tris/maleate buffer, pH 6.1, were mixed and incubated at 30 °C for various periods. The reaction was terminated by adding 50 μl of 15% (w/v) trichloroacetic acid, and the mixture was extracted with 3 $\times$ 1 ml portions of hexane. Pooled extracts were mixed with 18 ml of Optiphase X (LKB Scintillation Products, Loughborough, Leics., U.K.) and their radioactivities counted in an Inter technique scintillation counter. Appropriate control determinations were made, and further controls showed that SDS, dodecanol or hexane did not cause quenching of a [¹⁴C]hexadecanol standard over the range of substrate concentrations examined.

Arylsulphatase. Enzyme activity towards potassium *p*-nitrophenyl sulphate was assayed at 30 °C in the presence of 50 mM-Tris/HCl buffer, pH 7.2, by direct spectrophotometric measurement of liberated *p*-nitrophenol at 400 nm. Activity towards all other aryl sulphates was determined by measuring liberated SO_4^{2-} ions by the BaCl_2 /gelatin method.

Nucleic acid and protein. Nucleic acid was determined by the method of Layne (1957). The protein contents of extracts and column fractions were determined by the method of Bradford (1976), with bovine serum albumin (fraction V) as standard.

Polyacrylamide-gel electrophoresis

Alkylsulphatase activities on gels were separated and detected according to the directions of Payne *et al.* (1974), with the use of SDS to detect primary activity and 'dec-5-yl sulphate' for secondary activity. On some occasions octyl sulphate was employed as the primary enzyme substrate and detection of activity involved precipitation of liberated SO_4^{2-} ions as BaSO_4 (Cloves *et al.*, 1980b).

Arylsulphatase activities on gels were detected with 6-bromo-2-naphthyl sulphate and tetra-azotized di-*o*-anisidine (Fast Blue B dye) according to the method of van der Loos *et al.* (1981).

During enzyme purification, protein was detected with Coomassie Blue R-250 (Meyer & Lamberts, 1965) after non-denaturing anodic discontinuous gel electrophoresis according to the method of Davis (1964).

¹⁸O-incorporation experiments

The position of cleavage of the ester sulphate bond by the P1 alkylsulphatase was determined by examining the incorporation of ¹⁸O from H_2^{18}O into one or other of the products of the reaction. A 25 mM solution of potassium octyl sulphate in 250 μl of 0.4 M-Tris/maleate buffer, pH 6.1, was freeze-dried and then resuspended in the same volume of H_2^{18}O (70 atom% excess; Amersham International, Amersham, Bucks., U.K.). Pure P1 primary alkylsulphatase (250 μl containing 0.06 enzyme unit) was added and the whole was incubated at 30 °C overnight in a stoppered vessel. The reaction was terminated by the addition of 100 μl of 10 M-HCl, and 200 μl of a hot saturated solution of BaCl_2 was added to precipitate inorganic sulphate. This was pelleted by centrifuging and retained. The supernatant was shaken with diethyl ether (10 $\times$ 1 ml portions) to extract the enzymically liberated octanol. After removal of ether in

a stream of N_2 , the residue was dissolved in a minimal volume of diethyl ether and analysed in a Finnegan 4500 gas chromatograph-mass spectrophotometer (Finnegan Instruments, Hemel Hempstead, Herts., U.K.) to determine the extent of ^{18}O incorporation into octanol as described by Cloves *et al.* (1977).

The $BaSO_4$ precipitate that had been retained was washed with water (3×1 ml), methanol (3×1 ml) and diethyl ether (3×1 ml) and mixed with finely ground KBr before being dried at $100^\circ C$. The dried material was pressed into a disc and its i.r. spectrum determined (Perkin-Elmer 257 grating infra-red spectrophotometer) in order to assess ^{18}O incorporation from the relative absorptions at 970 and 990 cm^{-1} (see Spencer, 1959).

De-repressible arylsulphatase of *Pseudomonas* C12B

When grown in the absence of SO_4^{2-} or cysteine but in the presence of L-methionine or choline *O*-sulphate, *Pseudomonas* C12B produces an arylsulphatase enzyme (Fitzgerald & Payne, 1972) that has been purified and characterized (George & Fitzgerald, 1981). A preparation of the pure enzyme was kindly donated by Dr. J. W. Fitzgerald.

Crag herbicide sulphatase

Methods for the purification and assay of the Crag herbicide [2-(2,4-dichlorophenoxy)ethyl sulphate] sulphatase have been described elsewhere (Lillis *et al.*, 1983). The purified enzyme was used to determine K_m and V_{max} values for C_6 - C_9 primary alkyl sulphates at the optimum pH, 6.0.

Purification of the P1 alkylsulphatase

Stage 1. Freeze-dried cells (20 g) of the bacterium were resuspended in 250 ml of cold 10 mM-Tris/HCl buffer, pH 7.5 (suspension buffer), and passed three times through a chilled French press at a pressure of 126 MPa. Cell debris was removed by centrifuging ($31\,000\text{ g}_{av}$ at $4^\circ C$; Sorvall RC-5B centrifuge, SS34 rotor) for 1 h and the clear supernatant was dialysed against the suspension buffer (4×10 litres) for a total of 12 h at $4^\circ C$.

Stage 2. The cold cell-free extract (222 ml) was treated with a 50% (w/v) solution of streptomycin sulphate in the suspension buffer, to give a final concentration of the antibiotic of 2 mg/mg of protein. After stirring at $4^\circ C$ for 30 min precipitated nucleic acid was removed by centrifuging as before and the supernatant was dialysed at $4^\circ C$ against the suspension buffer (3×1 litre) for a total of 3 h, followed by 2×10 litres overnight. A further precipitate that sometimes appeared after dialysis was removed by centrifuging.

Stage 3. Finely ground $(NH_4)_2SO_4$ was added to 260 ml of the stage 2 preparation with gentle stirring over a period of 30 min, to give a final concentration equivalent to 60% saturation. After the slurry had been stirred for a further 1 h the precipitated protein was removed by centrifuging. The supernatant was dialysed against suspension buffer until all traces of inorganic sulphate had been removed.

Stage 4. The dialysed stage 3 material (378 ml) was applied to a DEAE-cellulose (DE52) ion-exchange column ($3\text{ cm} \times 12\text{ cm}$) that had previously been equilibrated with suspension buffer. After the column had been

washed with a further 200 ml of buffer the column was eluted with a linear salt gradient of 0 - 0.4 M -NaCl in 600 ml of the usual buffer. The flow rate of the column was maintained at 28.6 ml/h , and 5 ml fractions were collected and assayed for protein and P1 alkylsulphatase activity. The enzyme was eluted at a salt concentration of about 0.17 M (see Fig. 1a), and the most active fractions (55-63) were pooled.

Stage 5. The pooled sample (49 ml) was concentrated to 13.5 ml (Millipore CX-10 immersible membrane) and then applied to a Sephacryl S-300 column ($2\text{ cm} \times 73\text{ cm}$) that had been equilibrated with suspension buffer. The flow rate was adjusted to 18 ml/h , and 3.2 ml fractions were collected and assayed for protein and enzyme activity (Fig. 1b). Fractions 33-41 were pooled.

Stage 6. The pooled fractions were applied to a butyl-agarose column ($1.5\text{ cm} \times 40\text{ cm}$) that had been equilibrated with suspension buffer. The column was

Fig. 1. Elution profiles for P1 primary alkylsulphatase from DEAE-cellulose, Sephacryl S-300 and butyl-agarose

(a) Elution from DEAE-cellulose (DE52) at stage 4 of the purification; (b) elution from Sephacryl S-300 at stage 5; (c) elution from butyl-agarose at stage 6. —, Protein; ·····, P1 alkylsulphatase activity; ----, NaCl concentration gradient. Horizontal bars indicate pooled fractions.

washed with a further 100 ml of buffer, and the enzyme was eluted with a linear gradient of 0–0.1 M-NaCl in a total volume of 600 ml of buffer. Flow rate was maintained at 28.8 ml/h, and 5.1 ml fractions were collected. The enzyme eluted at a salt concentration of about 0.03 M. Active fractions showing one protein band only after SDS/polyacrylamide-gel electrophoresis (fractions 44–51, see Fig. 1c) were pooled and dialysed against suspension buffer before storage in 5 ml portions at 4 °C in sterile vials. The enzyme was not stable to freezing.

RESULTS

Purification of the P1 alkylsulphatase

The activities and recoveries of the enzyme during the course of a typical purification run are recorded in Table 1. The preparation was freed from S1 and S2 alkylsulphatases during ion-exchange chromatography, these secondary enzymes eluting at low ionic strength. Multiple preparations were consistently successful on 5 g, 10 g or 20 g scales, with final specific activities of the P1 enzyme varying by less than $\pm 3\%$. An overall purification of approx. 1500-fold from the crude cell extract to the pure enzyme is typical of other alkylsulphatases that appear to be constitutive (Matcham *et al.*, 1977b; Bartholomew *et al.*, 1978) and contrasts with the 250-fold overall purification of inducible alkylsulphatases (Cloves *et al.*, 1980b; Shaw *et al.*, 1980).

Determination of M_r

Polyacrylamide-gel electrophoresis of the purified enzyme under non-denaturing conditions (Bryan, 1977) indicated an M_r of 193 000. Standard proteins used were chick egg albumin, jack-bean urease (dimer and tetramer), bovine α -lactalbumin, erythrocyte carbonic anhydrase and serum albumin (monomer and dimer). Gel filtration on Sephacryl S-300 gave an M_r of 181 000 with as standards liver catalase, chymotrypsinogen A and serum albumin (all bovine), ferritin, rabbit muscle aldolase, hen's-egg albumin and horse heart cytochrome *c*.

SDS/polyacrylamide-gel electrophoresis under denaturing conditions gave a single band corresponding to an M_r of 53 700. Rabbit muscle phosphorylase *b*, hen's-egg ovotransferin and ovalbumin and bovine serum albumin and chymotrypsinogen A were the standards. The results suggest that the native enzyme is probably tetrameric.

Optimal assay conditions

Maximum enzyme activity was achieved at 30 °C with the standard substrate (potassium octyl sulphate) at

pH 6.1 in the presence of 0.1 M-Tris/maleate buffer and 12.5 mM substrate. The enzyme tended to be increasingly unstable at higher temperatures.

Position of bond cleavage

There was no significant incorporation of ^{18}O into octanol when octyl sulphate was used as substrate for the enzyme in the presence of H_2^{18}O . In contrast, the ^{18}O enrichment of the enzymically liberated SO_4^{2-} ions was 6.98 atom% excess, a value in reasonable agreement with the theoretical value 8.75 atom% excess for cleavage of the O–S bond of the C–O–S linkage. Previous work has shown that non-enzymic exchange from H_2^{18}O into intact alkyl sulphates or the alcohol or SO_4^{2-} hydrolysis products is negligible under comparable conditions (Bartholomew *et al.*, 1977; Cloves *et al.*, 1977; Shaw *et al.*, 1980). The finding that the P1 enzyme ruptured the O–S bond provided a notable contrast with the C–O bond rupture catalysed by the bacterium's P2 inducible enzyme, but not with the O–S bond rupture that characterizes the de-repressible arylsulphatase of the organism (Cloves *et al.*, 1977).

Effect of alkyl chain length on activity

A range of primary alkyl sulphates with chain lengths ranging from C_1 to C_{12} were tested as potential substrates for the P1 enzyme. Experimental conditions of pH and temperature were identical with those employed in the optimal assays procedure, and substrate concentrations were varied so that K_m and $V_{\max}$ values could be determined. Sulphate esters with chain lengths of C_5 and less did not serve as substrates under the conditions used (up to 30 mM substrate concentration), whereas esters with longer chain lengths all served as substrates. K_m values (determined by the method of Wilkinson, 1961) decreased with increasing chain length (Table 2) and a plot of ΔG° (apparent standard free energy change for the association between enzyme and substrate at pH 6.1) against alkyl chain length gave a linear relationship (Fig. 2a). Values of $V_{\max}$ increased with increasing chain length from C_6 to C_9 but declined sharply with the C_{12} ester. Pronounced inhibition by excess substrate occurred with the C_{12} compound, there being no detectable activity with 2 mM-SDS. Nevertheless of all substrates tested SDS was the 'optimum', judged from values of $V_{\max}/K_m$ (Fig. 2b).

Limitations of assay methods prevented determination of kinetic data for the C_{10} , C_{11} , C_{13} and C_{14} compounds, but it was possible to show by gel zymography that they could serve as substrates for the enzyme. Compounds

Table 1. Purification of the P1 alkylsulphatase from *Pseudomonas* C12B

See the text for experimental details and for definition of unit of enzyme activity.

Purification stage	Volume (ml)	Nucleic acid (%)	Protein (mg)	Enzyme activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
1. Cell extracts	222	> 20	3907	128.9	0.033	100	1.0
2. Streptomycin sulphate	261	0.75	2297	91.9	0.04	71	1.2
3. $(\text{NH}_4)_2\text{SO}_4$	378	—	605	145.1	0.24	112	7.3
4. DEAE-cellulose	48	—	50	74.9	1.50	58	45
5. Sephacryl S-300	26	—	3.8	41.5	10.9	32	331
6. Butyl-agarose	40	—	0.48	24.8	51.6	19	1562

Table 2. K_m and V_{max} values for the P1 primary alkylsulphatase of *Pseudomonas* C12B acting on a series of primary alkyl sulphate esters and on various aryl sulphate esters

Experimental details are given in the text.

Substrate	K_m (mM)	V_{max} . (μ mol of SO_4^{2-} liberated/min per mg of protein)
Alkyl sulphates* (potassium salts)		
Hexyl sulphate	4.70	43.9
Heptyl sulphate	2.22	110.5
Octyl sulphate	1.34	131.6
Nonyl sulphate	0.98	172.1
Dodecyl sulphate	0.068	27.4
Aryl sulphates† (potassium salts)		
<i>p</i> -Nitrophenyl sulphate	1.72	2.6
<i>o</i> -Chlorophenyl sulphate	14.80	7.0
<i>m</i> -Chlorophenyl sulphate	11.80	13.2
<i>p</i> -Chlorophenyl sulphate	6.05	29.5
<i>o</i> -Methoxyphenyl sulphate	8.30	3.5
<i>m</i> -Methoxyphenyl sulphate	7.00	3.4
<i>p</i> -Methoxyphenyl sulphate	6.20	20.7
<i>m</i> -Hydroxyphenyl sulphate	11.16	15.1
<i>p</i> -Hydroxyphenyl sulphate	8.00	26.1
2,4-Dichlorophenyl sulphate	1.00	17.1

* There was no activity towards C_1 – C_5 alkyl sulphates.

† There was no activity towards 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate), tyrosine *O*-sulphate, phenyl sulphate or bis-(*p*-nitrophenyl) sulphate.

with longer chain lengths were not substrates under similar circumstances.

Although, strictly speaking, butoxyethyl sulphate cannot be regarded as a true alkyl sulphate, it nevertheless served as a substrate for the P1 alkylsulphatase (K_m 21.3 mM; V_{max} 84.7 μ mol/min per mg of protein). In contrast, sodium 2-(2,4-dichlorophenoxy)-ethyl sulphate (Crag herbicide), 'sodium dodecyltriethoxy sulphate' ($C_{12}H_{25}[OCH_2CH_2]_3OSO_3^-Na^+$) and sodium diethylene glycol sulphate were not hydrolysed at concentrations up to 30 mM.

To allow comparison of the P1 and P2 enzymes with a primary alkylsulphatase from a different organism, analogous determinations were made of K_m and V_{max} for the Crag herbicide sulphatase acting on C_6 – C_9 primary alkyl sulphates. Values of $\Delta G^{0'}$ for enzyme–substrate association and V_{max}/K_m are included in Fig. 2.

Inhibition by alkyl sulphates and alkanesulphonates

Several secondary alkyl sulphates and alkanesulphonates were tested as potential inhibitors of the P1 enzyme. Secondary alkyl sulphates with alkyl chain lengths greater than C_5 were competitive inhibitors, K_i and $\Delta G^{0'}$ values decreasing with increasing chain length (Table 3). The D- and L-isomers of oct-2-yl sulphate gave K_i values that were close to that given by the racemic preparation.

Alkanesulphonates also served as competitive inhibitors provided the chain length was greater than C_5 . Values of K_i and $\Delta G^{0'}$ again decreased with increasing chain length (Table 3).

Short-chain primary alkyl sulphates ($\leq C_5$) were not inhibitors of P1 enzyme activity towards nonyl sulphate.

Activity towards arylsulphates

Surprisingly, the pure P1 alkylsulphatase exhibited activity towards a number of aryl sulphates under the

experimental conditions appropriate for alkyl sulphates. Table 2 shows the K_m and V_{max} values obtained for several such aryl sulphates. There was no correlation of kinetic constants with aromatic-substitution constants (σ) and values of V_{max}/K_m [all ≥ 5 (μ mol/min per mg) $\cdot$ mM $^{-1}$] showed that aryl sulphates are relatively inferior substrates of the P1 enzyme, compared with most alkyl sulphates (see Fig. 2b). No activity could be detected towards L-tyrosine *O*-sulphate (the substrate for the tyrosine *O*-sulphate sulphatase of *Comamonas terrigena*; Fitzgerald *et al.*, 1979), nitrocatechol sulphate (a substrate for the de-repressible arylsulphatase of *Pseudomonas* C12B; George & Fitzgerald, 1981), phenyl sulphate (a substrate for the *Alcaligenes metalcaligenes* arylsulphatase; Dodgson *et al.*, 1956) or bis-(*p*-nitrophenyl) sulphate.

After gel electrophoresis arylsulphatase activity towards 2-bromonaphthyl sulphate was detected as a single band that coincided with the band of P1 alkylsulphatase activity. Furthermore octyl sulphate served as a competitive inhibitor ($K_i = 0.12$ mM) of arylsulphatase activity towards *p*-nitrophenyl sulphate.

Relationship between arylsulphatase activities

Evidence already available pointed to the likelihood that the arylsulphatase activity exhibited by the P1 alkylsulphatase could not be related to the de-repressible arylsulphatase produced by the bacterium (Fitzgerald *et al.*, 1977). Thus the growth conditions required for the production of the enzymes are very different; only one of them (the de-repressible arylsulphatase) can hydrolyse nitrocatechol sulphate, and the M_r values are very different (Table 4). However, the M_r of the subunit of the P1 alkylsulphatase (53 700) is similar to that of the intact de-repressible arylsulphatase (52 000), and the two co-migrated as a single band on SDS/polyacrylamide-gel

Fig. 2. Effects of alkyl chain length on kinetic parameters for substrates of the P1, P2 and Crag herbicide primary alkylsulphatases

Effects of alkyl chain length on (a) apparent free energies (ΔG^0) for binding primary alkyl sulphates to primary alkylsulphatases and (b) values of $V_{\max}/K_m$ are shown. —, P1 primary alkylsulphatase of *Pseudomonas* C12B; ---, P2 primary alkylsulphatase of *Pseudomonas* C12B; ·····, Crag herbicide sulphatase of *Ps. putida*.

electrophoresis under denaturing conditions. Moreover, both enzymes catalyse the rupture of the O–S bond of their respective substrates. The possibility that the P1 enzyme subunit was identical with or related to the de-repressible arylsulphatase could not be dismissed. In order to throw further light on the matter, freeze-dried samples of the two native enzymes were resuspended in 8 M-urea containing 0.1% (w/v) 2-mercaptoethanol and subjected to electrophoresis in 7% polyacrylamide gels containing 2 M-urea. The two proteins did not co-migrate (mobilities relative to tracker dye 0.33 for P1 enzyme subunit and 0.46 for the de-repressible arylsulphatase).

In a further experiment, partial CNBr degradation of the P1 enzyme subunit and the de-repressible arylsulpha-

Table 3. Inhibition of the P1 primary alkylsulphatase of *Pseudomonas* C12B by secondary alkyl sulphates and alkanesulphonates

See the text for details.

Inhibitor	Average K_i (mM)
Secondary alkylsulphates (potassium salts)	
Hex-2-yl sulphate	11.15
Hept-2-yl sulphate	4.28
Oct-2-yl sulphate	1.92
Non-2-yl sulphate	1.30
Alkanesulphonates (sodium salts)	
Hexanesulphonate	6.30
Heptanesulphonate	5.60
Octanesulphonate	4.10
Nonanesulphonate	1.00

tase was carried out according to the procedure of Nicodem & Fresco (1979). Fragments were separated on 15% polyacrylamide gels containing SDS, and gels were stained for polypeptide material by the AgNO_3 method of Adams & Sammons (1982). The P1 enzyme yielded six fragments and the de-repressible arylsulphatase yielded three.

DISCUSSION

The present study was initiated in the hope of obtaining information that might help to explain why *Pseudomonas* C12B should have the capacity to produce two enzymes with the ability to catalyse the desulphation of primary alkyl sulphate esters. In the event, although most interesting differences between the P1 and P2 enzymes have emerged as a result of the study, little further light has been thrown on the need for two enzymes.

Table 4 reveals the differences and similarities among these two enzymes and the alkylsulphatase of *Ps. putida* FLA, an enzyme that also has the capacity to hydrolyse Crag herbicide. The most striking difference between the two *Pseudomonas* C12B enzymes is that relating to their modes of action. The P1 enzyme ruptures the O–S bond of the C–O–S linkage and hence would be regarded as exhibiting the normal behaviour for a hydrolytic enzyme active towards esters of the type alcohol–O–acid. Dodgson *et al.* (1982) have previously drawn attention to the fact that of six other known classes of hydrolytic enzymes active towards this type of ester, all operate by scission of the O–acid bond. In sharp contrast with the P1 enzyme, the P2 enzyme attacks the C(alcohol)–O bond, in common with the mechanisms of action of the secondary alkylsulphatases of *Pseudomonas* C12B that have so far been studied in detail (the S1 and S3 enzymes). These C–O–bond splitters are thus emerging as atypical of sulphatases in particular and hydrolytic enzymes in general.

Although the P1 and P2 alkylsulphatases exhibit entirely different mechanisms of action, they are both able to hydrolyse primary alkyl sulphate esters of chain lengths C_6 – C_{12} . Furthermore, both enzymes exhibit very similar behaviour in relation to increasing chain length. As shown in Fig. 2(a), plots of ΔG^0 values (relating to the association of enzyme and substrate) against carbon

Table 4. Comparison of the properties of the P1 and P2 alkylsulphatases of *Pseudomonas* C12B with its de-repressible arylsulphatase, and with the Crag herbicide sulphatase of *Ps. putida* FLA

Property	P1 alkyl sulphatase	P2 alkyl sulphatase*	Crag herbicide sulphatase†	De-repressible arylsulphatase‡
Appearance in batch culture	Stationary phase	Exponential phase (transient)	Stationary phase	
Regulation of synthesis	De-repressible§	Inducible	Constitutive	De-repressible
M_r	181 000–193 000	158 000–162 000	295 000	51 500
Tertiary structure	Tetramer	Dimer	Tetramer	Monomer
Cleavage site	O–S	C–O	O–S	O–S
K_m for octyl sulphate (mM)	1.34	1.50	2.1	Not active
Optimum pH	6.1 (octyl sulphate)	8.3 (hexyl sulphate)	6.0 (octyl sulphate)	8.8 (<i>p</i> -nitro-phenyl sulphate)
Minimum carbon-chain length for substrate	C ₆	C ₄	C ₆	Not active
Active towards:				
Aryl sulphates	Yes	No	No	Yes
Crag herbicide	No	No	Yes	—
Butoxyethyl sulphate	Yes	No	Yes	—
Inhibition by alk-2-yl sulphates	Competitive	Competitive	Not known	Not known
ΔG^0 (binding) versus chain length	Linear, -1.7 kJ/mol per CH ₂	Linear, -2.5 kJ/mol per CH ₂	Non-linear/independent	—

* From Cloves *et al.* (1980a,b).
† From Lillis *et al.* (1983) and present paper.
‡ From Fitzgerald & Payne (1972); Fitzgerald *et al.* (1977); Cloves *et al.* (1977); George & Fitzgerald (1981).
§ T. J. Bateman, K. S. Dodgson & G. F. White (unpublished work).

chain length are linear and very similar for both enzymes, although the standard free-energy change involved in transferring one methylene ($-\text{CH}_2-$) group of the substrate from the free solution situation to the enzyme-bound situation are somewhat different (-2.5 kJ/mol for the P2 enzyme and -1.7 kJ/mol for the P1 enzyme).

The Crag herbicide alkylsulphatase of *Ps. putida* FLA exhibits very different behaviour towards substrates of increasing chain length, and no differences in ΔG^0 or $V_{\text{max.}}/K_m$ values were observed between C₇, C₈ and C₉ primary alkyl sulphates (Fig. 2). This enzyme operates by O–S-bond fission, does not exhibit arylsulphatase activity but is able to hydrolyse 2-(2,4-dichlorophenoxy)ethyl sulphate (Crag herbicide). It seems likely that the ester sulphate group plays a major role in binding substrates to this particular enzyme.

Other interesting differences between the P1 and P2 enzymes should be noted. Thus the former appears to be tetrameric and the latter dimeric; the optimum pH of the former (pH 6.1) is on the acid side of neutrality whereas that of the latter (pH 8.3) is on the alkaline side. The alkylsulphatase of *Ps. putida* FLA is also a tetramer and, like the P1 enzyme, operates optimally at an acid pH (6.0). The P1 enzyme has always been regarded as constitutive; however, recent studies (T. J. Bateman, K. S. Dodgson & G. F. White, unpublished work) suggest that it appears (in the stationary phase of growth) in response to scarcity of carbon/energy sources and is repressed by a surfeit of such sources. In contrast, the P2 enzyme is an inducible enzyme (Cloves *et al.*, 1980a), appears in mid-exponential growth phase and has a relatively transient existence. Like the P1 enzyme the alkylsulphatase of *Ps. putida* FLA appears in the stationary phase of growth, but it appears

to be truly constitutive (Lillis *et al.*, 1983). One feature of the P2 enzyme, not shared by the others, is its ability to exhibit weak activity towards pentyl sulphate and (very weak) towards butyl sulphate.

The mystery of why *Pseudomonas* C12B should have the capacity to produce two enzymes that, with minor differences, will hydrolyse the same primary alkyl sulphates clearly remains unresolved. Certainly the P1 enzyme exhibits a wider specificity than the P2 in terms of other types of sulphate ester substrates, adding further to a growing store of evidence that some sulphatase enzymes exhibit surprising multiple specificities (Dodgson *et al.*, 1982).

The significance of the very different mechanisms of action exhibited by the P1 and P2 enzymes also remains obscure. The operation of an O–S-bond-splitting mechanism requires that the P1 enzyme functions by transferring the $-\text{SO}_3^-$ moiety (in the present instance, to water), whereas the C–O-bond-splitting P2 is concerned with the transfer of the alkyl chain to water. Alternative acceptors may exist *in vivo*, in which case the roles of the enzymes would need to be reassessed in terms of their sulphotransferase and alkyltransferase activities respectively.

It may well be that a rationale for the ability to produce two alkylsulphatases lies not in mechanistic complexity or specificity but in the difference in timing of their appearance during batch culture. In short, the P1 enzyme serves as a scavenging enzyme (using alkyl and aryl sulphate esters, both types of which are present in the environment) when carbon/energy supplies become depleted, whereas the inducible P2 enzyme is necessary to initiate and sustain growth when alkyl sulphates are the sole source of carbon/energy.

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