Purification and Properties of the P2 Primary Alkylsulphohydrolase of the Detergent-Degrading Bacterium *Pseudomonas* C12B

Jillian M. CLOVES,* Kenneth S. DODGSON,*† Graham F. WHITE* and John W. FITZGERALD†

*Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, Wales, U.K.,
and †Department of Microbiology, University of Georgia, Athens, GA 30602, U.S.A.

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The P2 primary alkylsulphohydrolase of the soil bacterium *Pseudomonas* C12B was purified to homogeneity (200–250-fold) by column chromatography on DEAE-cellulose, Sephadex G-100 and butyl-agarose. The intact protein is a dimer with a mol. wt. of 160 000. Activity towards primary alkyl sulphate esters was maximal at pH 8.3, varied little in the range pH 7.8–8.7, but decreased sharply at higher pH. For a homologous series of primary alkyl sulphate substrates (C₆–C₁₂), log *K_m* decreased linearly with increasing chain length, corresponding to a contribution to the free energy of association between enzyme and substrate of –2.5 kJ/mol for each additional CH₂ group in the alkyl chain. log *K_i* for the competitive inhibition by secondary alkyl 2-sulphate esters followed a similar pattern (–2.4 kJ/mol for each additional CH₂ group) except that only *n* – 1 carbon atoms effectively participate in hydrophobic bonding, implying that the C-1 methyl group is not involved. log *K_i* values for inhibition by primary alkanesulphonates also depended linearly on chain length but with a diminished gradient, indicating a free-energy increment of –1.2 kJ/mol per additional CH₂ group. The collective results showed the presence of a hydrophobic site on the enzyme capable of accommodating an alkyl chain of considerable length. Cationic structures (in the form of arginine, lysine or histidine), whose presence might be expected for binding the anionic sulphate group, were not detectable at the active site.

Primary and secondary long-chain alkyl sulphate esters form two commercially important groups of readily biodegradable detergents. However, in spite of their widespread use, little information has been available at the molecular level concerning the mechanisms by which these detergents are degraded by micro-organisms. The collective results suggest that both types of ester are first hydrolysed by alkylsulphohydrolases of appropriate specificity (Payne *et al.*, 1965; Williams *et al.*, 1966; Dodgson *et al.*, 1974; Matcham *et al.*, 1977a), and the oxidation of the liberated primary or secondary alcohol is then initiated by alcohol dehydrogenase-type enzymes (Williams *et al.*, 1966; Lijmbach & Brinkhuis, 1973).

Pseudomonas C12B is a micro-organism that, under certain circumstances, can produce two distinct primary alkylsulphohydrolases and three distinct secondary alkylsulphohydrolases (Dodgson *et al.*, 1974), all of which appear to have a periplasmic localization in the cell (Fitzgerald & Laslie, 1974, 1975; Dodgson *et al.*, 1978). The explanation for the curious multiplicity of secondary alkylsulphohydrolases in this organism has been provided by Matcham *et al.* (1977a) and Bartholomew *et al.* (1978), and

stemmed from the recognition that enzyme stereospecificity is involved (see Matcham *et al.*, 1977b; Matcham & Dodgson, 1977). However, there is as yet no explanation why the micro-organism should produce two primary alkylsulphohydrolases. One of these two enzymes (designated P1) appears to be constitutive and appears in maximum quantities during the stationary phase of growth [nutrient broth, batch culture (Dodgson *et al.*, 1974)]. The other (designated P2) is an inducible enzyme that is synthesized during the mid-exponential growth phase and has a somewhat transient existence (Fitzgerald, 1974). As part of the effort to provide an explanation for the existence of the P1 and P2 primary alkylsulphohydrolases, the latter enzyme has now been purified to homogeneity and some observations on its specificity and general properties have been made.

Experimental

Materials

Primary alcohols and racemic secondary alcohols were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. D- and L-Octan-2-ols were prepared from racemic octan-2-ol as described by Matcham & Dodgson (1977). The sulphate esters of

† To whom reprint requests should be addressed.

primary alcohols were prepared as described by Dodgson *et al.* (1974) and sulphate esters of secondary alcohols by the pyridine/SO₃ procedure of Matcham & Dodgson (1977). All esters were recrystallized as potassium salts. Sodium salts of alkanesulphonates were purchased from Cambrian Chemicals, Croydon, Surrey, U.K. Sodium dodecyl [³⁵S]sulphate (specific radioactivity 25.2 μ Ci/ μ mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Protein standards for molecular-weight determinations came from Sigma (London) Chemical Co., Poole, Dorset, U.K., BDH Chemicals, Poole, Dorset, U.K., and Boehringer Corp. (London), Lewes, Sussex, U.K. All other chemicals used were the purest available from BDH Chemicals or Sigma (London) Chemical Co.

Growth of Pseudomonas C12B

Although maximum yields of the P2 alkylsulphohydrolase were synthesized when resting cells of this bacterium were exposed to sodium dodecyl sulphate (Cloves *et al.*, 1980), preliminary studies established that the micro-organism grew poorly on this ester and synthesized smaller amounts of this enzyme compared with those obtained during growth on potassium hexyl sulphate. Moreover previous work (Fitzgerald, 1974) had shown that batch-cultures of *Pseudomonas* C12B did not synthesize the P1 primary alkylsulphohydrolase when growth occurred on sodium citrate as the carbon source and hexyl sulphate as sulphur source. This latter finding was confirmed in the present instance for cells grown in fermenter conditions. For enzyme purification purposes therefore the organism was grown at 30°C (400-litre-capacity New Brunswick Fermentor) in 350 litres of basal salts medium (Fitzgerald & Payne, 1972), supplemented with 1% (w/v) sodium citrate and 4 mM-potassium hexyl sulphate. Aeration was at a rate of 1.8 m³ of air/h and cultures were agitated at 100 rev./min. Cells were harvested at mid-exponential growth phase ($A_{420} = 0.6-0.7$; see Fitzgerald, 1974) by means of a Sharples centrifuge (flow rate 90 litres/h), and the wet cells were resuspended in 5 mM-Tris/HCl buffer, pH 7.5 (lg of cells/ml), and ruptured by continuous passage through a Manton-Gaulin laboratory homogenizer operating at 60 MPa (type 15M8; Manton-Gaulin Manufacturing Co., Everett, MA, U.S.A.). The broken-cell suspension was freeze-dried and stored at -20°C until required.

Assay of sulphohydrolase activity

Standard assay procedures. With all substrates other than sodium dodecyl sulphate, enzymically liberated SO₄²⁻ was determined by the BaCl₂/gelatin method of Dodgson (1961), as modified by Thomas & Tudball (1967). Suitable control determinations were made. In kinetic experiments, care was taken to

determine initial rates by assaying enzyme activity for 5 min, 10 min and 15 min periods (cf. Matcham *et al.*, 1977b). Routinely, the P2 primary alkylsulphohydrolase activity was assayed with potassium hexyl sulphate at a final concentration of 18 mM and at pH 8.2. It is shown below that this ester is not the best substrate for the enzyme. The S1 and S2 secondary alkylsulphohydrolases were assayed with potassium D-octan-2-yl (D-1-methylheptyl) sulphate and potassium L-octan-2-yl sulphate as respective substrates (Bartholomew *et al.*, 1978). For all the enzymes, a unit of activity is defined as 1 μ mol of SO₄²⁻ liberated/min at 30°C.

Assay procedure for use with sodium dodecyl sulphate. The BaCl₂/gelatin method cannot be used with alkyl sulphates of chain length greater than C₁₀ because of the relative insolubility of the barium salts of those esters. It has always been clear that the P2 alkylsulphohydrolase can use sodium dodecyl sulphate as a substrate (e.g. Dodgson *et al.*, 1974), but a number of problems have been associated with attempts to make a precise quantitative assessment of its suitability as a substrate for primary alkylsulphohydrolase. Williams & Payne (1964) approached the problem by using sodium dodecyl [³⁵S]sulphate, precipitating unreacted substrate with 15% (w/v) KCl in 4% (w/v) trichloroacetic acid and counting the radioactivity remaining in solution. However, this method proved to be unsatisfactory in our hands because of the failure to precipitate the unreacted substrate completely.

A number of different approaches to the problem were tried during the present study, and a satisfactory method, based on ³⁵S-labelled substrate, was finally developed by taking advantage of the ability of sodium dodecyl sulphate to form a chloroform-soluble complex with Methylene Blue (see Hsu, 1965). Unreacted substrate was complexed with Methylene Blue and the complex was extracted into chloroform. The radioactivity of the enzymically liberated ³⁵SO₄²⁻ remaining in the aqueous phase was then counted. The following procedure was used. Equal volumes (500 μ l) of enzyme and substrate solutions in 0.1 M-Tris/HCl buffer pH 8.3, were mixed and incubated at 30°C. The substrate solution contained unlabelled sodium dodecyl sulphate and 0.347 μ Ci of sodium dodecyl [³⁵S]sulphate. Samples (200 μ l) were transferred at 3 min, 5 min and 8 min intervals into test tubes containing 50 μ l of 15% (w/v) trichloroacetic acid. After the solutions had been mixed, 250 μ l of a 2 mM-Methylene Blue solution (in water) was added to each and, after further mixing, tubes were left for 5 min in order to ensure that formation of the complex between dye and unreacted substrate was complete. Chloroform (500 μ l) was added and the whole thoroughly mixed in order to extract the complex into the chloroform phase. After centrifuging briefly in order to facilitate separation of the

two liquid phases, 250 μ l portions were taken from the aqueous phase and added to 4 ml of PCS (phase-combining system; Amersham Corp., Arlington Heights, IL, U.S.A.) before the radioactivity was counted in an Intertechnique scintillation counter. Appropriate control determinations were treated in the same way. The effect of the presence of the excess Methylene Blue in the aqueous phase was taken into account by means of a quench correction curve. By employing this method good recoveries of added $^{35}\text{SO}_4^{2-}$ ions could be made from mixtures of enzyme, substrate and trichloroacetic acid at substrate concentrations that ranged from 0.005 to 1.0 mM.

Determination of nucleic acid and protein

Nucleic acid was determined as a percentage of total u.v.-absorbing material (A_{280}/A_{260} ratio) by the method of Layne (1957). Protein was generally determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard. The protein contents of the final purified enzyme preparations were assayed by the Coomassie Brilliant Blue G-250 technique of Sedmark & Grossberg (1977), with bovine serum albumin again being used as standard.

Polyacrylamide-gel electrophoresis

Detection of primary and secondary alkylsulphohydrolases and protein during enzyme purification was achieved by the modified polyacrylamide-gel electrophoresis technique described by Payne *et al.* (1974). However, during the later stages of the purification procedure the secondary alkylsulphohydrolase activity became so low that it could no longer be detected by this technique. Greater sensitivity was obtained by altering the procedure as follows. The running buffer was modified by including 10 mM-barium acetate and, after electrophoresis, the extruded gels were incubated for several hours at 30°C with an aqueous solution of 5 mM-barium acetate containing either 10 mM-potassium D-octan-2-yl sulphate (substrate for the S1 secondary alkylsulphohydrolase) or potassium L-octan-2-yl sulphate (substrate for the S2 secondary alkylsulphohydrolase). Under these circumstances liberated SO_4^{2-} ions were precipitated as BaSO_4 , giving a band of very small white spots.

Purification of the P2 primary alkylsulphohydrolase

Stage 1. Freeze-dried broken cells (10 g) were resuspended in 70 ml of 0.01 M-Tris/HCl buffer, pH 8.2, and stirred for 30 min at 4°C. The whole was then passed three times through a chilled French press (continuous-flow attachment) at a pressure of 126 MPa. The suspension was then centrifuged for 1 h at 4°C and 23 000 $g_{av.}$ in the no. 69180 angle head on an MSE High Speed 18 centrifuge, and the super-

natant was dialysed for 18 h at 4°C against several changes of 0.01 M-Tris/HCl buffer, pH 8.2. At this and later stages, samples were withdrawn for protein and nucleic acid determination, enzyme assays and gel electrophoresis. All subsequent operations were performed at 4°C.

Stage 2. The dialysed solution (95 ml) was treated with 9.5 ml of a freshly prepared aqueous solution of 5% (w/v) streptomycin sulphate, which was added slowly with gentle stirring. After a further 30 min stirring, the precipitated nucleic acid was removed by centrifuging as before and the clear supernatant was dialysed for 24 h against several changes of 0.05 M-Tris/HCl buffer, pH 7.0.

Stage 3. The supernatant was applied to a DEAE-cellulose (DE-52) column (30 cm $\times$ 4.25 cm) that had been equilibrated with 0.05 M-Tris/HCl buffer, pH 7.0. The column was washed with 750 ml of the same buffer, followed by a linear Tris/HCl (pH 7.0) gradient (from 0.05 M to 0.2 M in a total volume of 1.5 litres). The flow rate was adjusted to 12 ml/h and 13 ml fractions were collected. Protein was monitored at 280 nm and the elution pattern is shown in Fig. 1(a).

Stage 4. The combined fractions containing the P2 enzyme were concentrated to about 13 ml in dialysis sacs immersed in a solution of 50% (w/v) poly(ethylene glycol) 6000 in 0.01 M-Tris/HCl buffer, pH 8.2, and 11.5 ml of the concentrate was applied to a Sephadex G-100 (superfine grade) column (85 cm $\times$ 2.5 cm) that had been equilibrated with 0.01 M-Tris/HCl buffer, pH 8.2. The column was eluted with the same buffer at a flow rate of 5 ml/h and 5 ml fractions were collected. The elution profile is shown in Fig. 1(b).

Stage 5. Preliminary studies with exploratory hydrophobic chromatography columns of alkyl agarose [Shaltiel hydrophobic chromatography kit, agarose- C_n series; Miles Yeda, Miles Laboratories (U.K.), Stoke Poges, Bucks., U.K.] established that almost the whole of the enzyme activity became strongly bound to columns (equilibrated with 0.01 M-Tris/HCl buffer, pH 8.2) where n was 6, 8 or 10, whereas more than 70% was bound when n was 4. Accordingly the C_4 homologue (butyl-agarose) was selected for this purification step. The remainder of the enzyme eluate from stage 4 (30 ml) was applied to a butyl-agarose column (10 cm $\times$ 1 cm) that had been equilibrated with 0.01 M-Tris/HCl buffer, pH 8.2, and the column was washed with 30 ml of the same buffer. All the P2 alkylsulphohydrolase activity was retained on this larger column and was subsequently eluted with a linear gradient (0–0.16 M in a total volume of 100 ml) of NaCl in 0.01 M-Tris/HCl buffer, pH 8.2 (Fig. 1c). Column fractions (3 ml) that contained the P2 enzyme as the only protein (single band on gel electrophoresis) were combined and used for molecular-weight determinations and

Fig. 1. Elution profiles for P2 alkylsulphohydrolase from DEAE-cellulose, Sephadex G-100 and butyl-agarose (a) Elution from DEAE-cellulose (DE-52) at stage 3 of the purification procedure; (b) elution from Sephadex G-100 at stage 4; (c) elution from butyl-agarose at stage 5. See the text for details. $\cdots$, P2 enzyme activity; —, protein elution; — —, eluent concentration gradient.

enzyme specificity studies. Other fractions contained traces of a second protein, and these were used in other experiments in which absolute purity was not essential. All enzyme preparations were stored in small quantities in glass tubes at -20°C .

Results

Table 1 provides details of the activities and recoveries of the P2 enzyme during a typical purification procedure. Because of the relatively low

yields of cells obtained by harvesting at mid-point of the exponential growth phase, it was necessary to grow the organism on a large scale on several occasions during the development of the final purification procedure. It became apparent that some variation existed between the behaviour of different batches of cells during enzyme purification. The appearance and disappearance of the P2 enzyme during batch culture occurs over a comparatively short period of about 6 h, and peak activity is maintained for a matter of 1–2 h only (Fitzgerald, 1974) at

Table 1. Purification of the P2 primary alkylsulphohydrolase of *Pseudomonas* C12B

The starting point for this particular preparation was 10g of freeze-dried cells. The S1 and S2 enzyme activities were assayed with D- or L-octan-2-yl sulphate, and the P2 enzyme activity was assayed with hexyl sulphate. See the text for definition of enzyme units and for further details.

Purification stage	Protein (mg)	P2 enzyme activity		S1 enzyme activity (units)	S2 enzyme activity (units)	P2 enzyme specific activity (units/mg of protein)	P2 enzyme purification (fold)
		(units)	(% yield)				
1	2043	65.4	100	0.93	2.8	0.032	1
2	1715	63.4	97	6.40	3.3	0.037	1.15
3	29.2	56.1	85	0	0.54	1.92	52
4	7.3	50.4	77	0	0.26	6.9	215
5	5.6	42.6	65	0	0	7.6	237

a time when rapid growth (rapid alterations in protein and nucleic acid contents) is taking place. Problems therefore arise with the large-scale fermentation of the organism where, for example, the harvesting of cells takes about 4h. In consequence, the batches of cells from each of the large-scale fermentations might be expected to behave differently during purification. This proved to be the case and it became necessary to make minor modifications in the experimental procedure (in particular, the fractions pooled from column separations) as the purification progressed. The results presented in Table 1 are those from one purification run only, and some variations in enzyme activities at different purification stages were observed in other runs. Overall purification achieved varied between 200- and 250-fold. This degree of purification should be considered in the light of that obtained (> 5000-fold) for the S1 secondary alkylsulphohydrolase (Bartholomew *et al.*, 1978) and presumably reflects the difference between the cellular concentrations of a constitutive (S1) and an inducible (P2) alkylsulphohydrolase.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by two different methods. Gel filtration on Sephadex G-200, with as standards bovine cytochrome *c*, catalase and serum albumin, yeast alcohol dehydrogenase, pig heart aspartate amino-transferase (gift from Dr. R. A. John) and *Saccharomyces fragaellis* β -galactosidase, gave a mol.wt. of 162000. Polyacrylamide-gel electrophoresis with two different concentrations of Cyanogum 41, by the method of Zwann (1967), gave a mol.wt. of 158000. Soya-bean trypsin inhibitor, pig pepsinogen, bovine serum albumin and catalase were used as standards.

Difficulty was experienced in attempting to subject the enzyme to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis because the enzyme was

still able to use the sodium dodecyl sulphate as a substrate during and after the usual initial mercapto-ethanol treatment. Interference with the electrophoretic movement of the treated enzyme resulted, this probably being due to the insoluble dodecanol liberated during the initial treatment (cf. Takagi *et al.*, 1975). However, satisfactory runs were obtained if the enzyme solution was heated at 100°C for a few seconds before the initial treatment. The standards used (papain, yeast alcohol dehydrogenase, bovine serum albumin, catalase and *S. fragaellis* β -galactosidase) were not subjected to the heat treatment before exposure to sodium dodecyl sulphate and mercaptoethanol. A molecular weight of 79400 was obtained by this method, indicating that the native enzyme is a dimer.

Optimum pH for enzyme activity

Potassium hexyl sulphate (18mm) was employed as assay substrate in the presence of 0.1M-Tris/HCl buffers (pH 6.8–8.3 inclusive) and 0.1M-glycine/NaCl/NaOH buffer (pH 8.3–9.8). Maximum activity was obtained in the region of pH 8.3 with relatively little change between pH 7.8 and 8.7. Activity declined sharply at pH values above 8.7. The same pH optimum was obtained for all other primary alkyl sulphates used as substrates for specificity studies.

Relative substrate specificity

A number of primary alkyl sulphates (potassium salts) were tested for their ability to serve as substrates for the P2 enzyme. The following concentration ranges were used: pentyl sulphate (0–200mm), hexyl sulphate (0–25mm), heptyl sulphate (0–20mm), octyl sulphate (0–3.0mm), nonyl sulphate (0–1.5mm) and dodecyl sulphate (sodium salt, 0–1.0mm). All the esters could serve as substrates and gave normal Michaelis–Menten curves (see Figs. 2a and 2b for examples), with the exception of dodecyl sulphate, where inhibition occurred as the concentration of substrate was increased beyond about 0.21mm

(Fig. 2c). Determination of the critical micelle concentration (see Cloves *et al.*, 1980) of sodium dodecyl sulphate under the specific incubation conditions employed for the enzyme experiments (0.1M-Tris/HCl buffer, pH8.3, and at 30°C) gave a value of 0.25mM. Enzyme inhibition by excess substrate may therefore reflect detergent micelle formation to some extent, but other factors are probably also involved. None of the other substrates examined formed micelles under the experimental conditions in which they were used.

Table 2 shows that the K_m values (double-reciprocal plots) for the different substrates decreased as the chain length of the substrate increased. When $\log K_m$ was plotted against the number of carbon atoms in the alkyl chains, a straight-line relationship was obtained (not shown), in accord with the characteristic behaviour of an homologous series. V_{max} values increased as chain length increased from C_5

Table 2. K_m and V_{max} values for the P2 primary alkylsulphohydrolase of *Pseudomonas* C12B acting on a series of primary alkyl sulphate esters

Experimental details are given in the text.

Substrate (potassium salt)	K_m (mM)	V_{max} (μ mol of SO_4^{2-} liberated/min per mg of protein)
Pentyl sulphate	30.3	3.6
Hexyl sulphate	12.5	9.1
Heptyl sulphate	3.77	16.3
Octyl sulphate	1.50	20.8
Nonyl sulphate	0.49	40.8
Dodecyl sulphate	0.03	7.2

Fig. 2. Typical enzyme activity—substrate concentration curves for the action of P2 alkylsulphohydrolase on primary alkyl sulphate esters

(a) Hexyl sulphate; (b) heptyl sulphate; (c) dodecyl sulphate. See the text for details.

to C_9 , but there was a sharp decline in V_{max} with sodium dodecyl sulphate (Table 2).

Although no precise kinetic data were obtained, it was also possible to establish that the enzyme was active towards the C_4 and C_{14} alkyl sulphates, butyl sulphate and tetradecyl sulphate. The former compound was hydrolysed at a rate equivalent to 1.23μ mol of SO_4^{2-} /min per mg of protein when present in incubation mixtures at a concentration of 200mM. It was not possible to assay enzyme activity towards tetradecyl sulphate because of the limitations of the assay method. However, substitution of this ester for sodium dodecyl sulphate in the detection of the P2 enzyme on polyacrylamide-gel zymograms gave the white band characteristic of the liberated insoluble alcohol.

Inhibition by secondary alkyl sulphates and alkane-sulphonates

Potassium octyl sulphate (at concentrations up to 3mM) was employed as assay substrate in these experiments and a range of racemic secondary alk-2-yl sulphate esters, and in the case of octan-2-yl sulphate the separate enantiomers also were tested as potential inhibitors. Double-reciprocal plots were used to calculate K_i values, which are recorded in Table 3. All the secondary alkyl sulphates with the sulphate group on C-2 were competitive inhibitors of the enzyme and, because both optical isomers of octan-2-yl sulphate inhibited to the same extent, it was assumed that this was also true of other optically active esters. Increase in carbon chain length was accompanied by a decrease in K_i values, and the plot (not shown) of $\log K_i$ against the number of carbon atoms in the alkyl chain gave the straight-line relationship characteristic of an homologous series (cf. Bartholomew *et al.*, 1978).

Alkanesulphonates (sodium salts were used) also served as competitive inhibitors of P2 alkylsulphohydrolase activity towards potassium octyl sulphate. Again the K_i values decreased with increasing

Table 3. Inhibition of the P2 primary alkylsulphohydrolase by secondary alkyl sulphates and alkanesulphonates. See the text for details. Inhibitor constants were calculated from the double-reciprocal plot of Lineweaver & Burk (1934).

Inhibitor	Concn. of inhibitor (mM)	Average K_i (mM)
Secondary alkyl sulphates (potassium salts)		
DL-Hexan-2-yl sulphate	100	46.7
DL-Heptan-2-yl sulphate	50	13.08
DL-Octan-2-yl sulphate	20	2.52
D-Octan-2-yl sulphate	10	2.88
L-Octan-2-yl sulphate	10	3.68
DL-Decan-2-yl sulphate	5	0.70
Alkanesulphonates		
Butanesulphonate	10	22.3
Pentanesulphonate	10	10.0
Hexanesulphonate	5, 10	6.7*
Heptanesulphonate	2.5, 5	5.2*
Octanesulphonate	1.0, 2.0	2.5*
Nonanesulphonate	0.5, 1.0	0.8*
Decanesulphonate	0.5, 1.0	1.0*

* Average values.

inhibitor chain length (Table 3), and a linear relationship (not shown) was obtained between $\log K_i$ and the number of carbon atoms in the alkyl chain.

Collectively these results, together with those obtained for the primary alkyl sulphate substrates, emphasize the importance of the carbon chain in enzyme-substrate or enzyme-inhibitor interactions. For each of the three series of compounds (primary alkyl sulphate substrates, secondary alkyl sulphate inhibitors with the sulphate at C-2 and alkanesulphonate inhibitors), linear relationships were obtained when ΔG^0 (the standard free-energy change for the association between enzyme and substrate or competitive inhibitor) was plotted against the number of carbon atoms in the alkyl chains (Fig. 3). The slopes of the curves give the ΔG^0 values corresponding to the free-energy change involved in transferring one $-\text{CH}_2-$ group from its free state in solution to the state of adsorption on to the enzyme, a relationship first described by Bergmann & Segal (1954) during studies on 'true' and 'pseudo' cholinesterase. The values obtained for the primary alkyl sulphate substrates and the secondary alkyl sulphate inhibitors were in close agreement (-2.50 kJ/mol and -2.40 kJ/mol respectively), and this finding was not unexpected in view of the close similarity of the two types of compound. However, the respective plots of ΔG^0 against chain length were parallel rather than co-incident, with an energy difference between the two corresponding to 3 kJ/mol . This value is similar to the energy involved in adding one $-\text{CH}_2-$ group to the alkyl chain.

Fig. 3. Linear free-energy relationships for the binding of substrate and inhibitor homologous series to the P2 alkylsulphohydrolase

●, Primary alkyl sulphate substrates; ○, secondary alk-2-yl sulphate inhibitors; □, primary alkanesulphonate inhibitors. See the text for details.

Secondary alkyl sulphates (with sulphate on C-2) therefore appear to bind to the P2 enzyme in the same way as the primary alkyl sulphates so far as the alkyl chain is concerned, but only $n-1$ carbon atoms can bind. That is to say that the C-1 methyl group almost certainly is not involved in the binding. Hansch & Dunn (1972) have defined three classes of biochemical response according to whether there is high, intermediate or low sensitivity to hydrophobic interactions. It can be shown that the free energies of association of the primary and secondary alkyl sulphate esters with the P2 enzyme place these interactions at the lower limit of the highly sensitive group.

In the cases of the alkanesulphonate inhibitors the ΔG^0 value (-1.2 kJ/mol) resulting from the introduction of an additional $-\text{CH}_2-$ group into the alkyl chain was approximately one-half that calculated for the alkyl sulphates, placing the enzyme-alkanesulphonate interaction in the class showing intermediate sensitivity to hydrophobic effects. This indicates that the alkyl chains of the alkanesulphonates do not bind to the enzyme in precisely the same way as that of an alkyl sulphate. Presumably this reflects the effect of replacing the C-O-S structure of the ester sulphate group with the C-S structure of the sulphonate group. Interestingly, the intercepts obtained by extrapolating the plots in Fig. 3 to zero carbon-chain length imply that the sulphonate $-\text{SO}_3^-$ group contributes relatively much more to the

binding of alkanesulphonates than does the $-O-SO_3^-$ group for the alkyl sulphates. Whether or not tighter binding of the $-SO_3^-$ in the alkanesulphonates is responsible for displacing the alkyl chain from the position required for ideal binding, and so decreasing the sensitivity to hydrophobic interactions, is not yet clear.

Preliminary active-site studies

In view of the apparent differences in binding between the primary and secondary alkyl sulphates on the one hand, and the alkanesulphonates on the other, a preliminary examination of the active site was made for groups that may be responsible for binding these compounds. At all pH values where the P2 alkylsulphohydrolase remains active the alkyl sulphate substrate is always present in a fully ionized anionic form. The possibility therefore existed that a cationic group may be necessary in the 'active site' for enzyme-substrate association to occur. Thus a number of enzymes acting on anionic substrates have been shown to possess an arginine residue that is involved in substrate binding (see Riordan *et al.*, 1978). Other similar possibilities could involve the ϵ -amino group of lysine or the imidazole nitrogen of histidine. The P2 enzyme was therefore treated for various times with 'group-specific' reagents for these amino acids as follows: (a) with 0.01 M-butanedione in 0.1 M-Tris/HCl buffer, pH 8.3, and 0.01 M-butanedione in 0.05 M-sodium borate buffer, pH 8.3 [group-specific for arginine (Riordan, 1973; Riordan *et al.*, 1978)]; (b) with 6 mM-diethyl pyrocarbonate in 0.1 M-sodium acetate buffer, pH 6.0 [group-specific for histidine (Melchior & Fahrey, 1970)]; (c) 2.0 mM-pyridoxal 5'-phosphate in 0.1 M-Tris/HCl buffer, pH 8.3 [group-specific for lysine residues (e.g. Ramponi *et al.*, 1975)]. After treatment, enzyme activity was assayed as usual with potassium octyl sulphate except in the case of (c), where 2.0 mM-pyridoxal 5'-phosphate was also included in the substrate solution. No significant effects were observed with any of the reagents, indicating that no guanidinium, imidazole or lysine ϵ -amino moieties were accessible for modification as essential components of the active site.

Discussion

The P2 primary alkylsulphohydrolase of *Pseudomonas* C12B exhibits activity towards a wide range of primary alkyl sulphates. The enzyme will not hydrolyse secondary alkyl sulphate esters; rather do such esters serve as competitive inhibitors. In cells grown in batch culture in the presence of an appropriate inducer the enzyme has a relatively transient existence, with maximum activity being attained during the mid-exponential growth phase (Fitzgerald, 1974). Nevertheless, its appearance presumably

enables the micro-organism to hydrolyse any alkyl sulphates present in the environment, thereby eliminating the potential toxicity of such compounds.

A number of microbial sulphatases (e.g. choline sulphohydrolase, arylsulphohydrolase) are produced as a result of the need to acquire sulphate for growth purposes, and the presence of sulphate or sulphur-containing intermediates on the pathway to cysteine is sufficient to repress the production of the enzymes (see Dodgson & Rose, 1975). However, this is not the case with the P2 alkylsulphohydrolase, the appearance of which is not affected by the presence of either sulphate or cysteine in the growth medium (Fitzgerald & Payne, 1972). The role of the enzyme is therefore not concerned with the need to scavenge sulphur. In support of the possibility that the enzyme can serve in a protective role is the fact that the enzyme is localized in the periplasmic region of the bacterial cell (Fitzgerald & Laslie, 1975). Contact with a detergent such as sodium dodecyl sulphate in reasonably small concentrations does not present any undue hazard for the organism, which grows well, for example, on nutrient broth containing that compound at a concentration of 0.1% (Williams & Payne, 1964).

The question now arises as to the relationship between this inducible enzyme and the P1 primary alkylsulphohydrolase that appears in nutrient-broth-grown cells during the stationary phase of growth. Both enzymes are clearly distinguishable from each other, not only because of the timing of their appearance in batch culture but also because they can be readily separated by polyacrylamide-gel electrophoresis (Dodgson *et al.*, 1974). No attempt has yet been made to purify the P1 enzyme, but it is known that it is capable of hydrolysing a number of primary alkyl sulphate esters, including pentyl, hexyl, octyl and dodecyl sulphates. The relative specificity of the enzyme may not therefore be very different from that of the P2 enzyme. In the cases of the three secondary alkylsulphohydrolases produced by *Pseudomonas* C12B, the reason for the multiplicity of enzymes is now clear. Thus the S1 secondary alkylsulphohydrolase is specific for D-alk-2-yl sulphate esters, the S2 enzyme is specific for L-alk-2-yl sulphate esters and the S3 enzyme for symmetrical secondary sulphate esters (e.g. heptan-4-yl sulphate) and certain other esters where the sulphate group is present at a position in the chain other than C-2 (Matcham *et al.*, 1977a). Collectively, therefore, the three secondary enzymes should enable the metabolism of a complex secondary alkyl sulphate detergent such as Oronite (a commercial detergent consisting of a mixture of C_{10} - C_{20} secondary alkyl sulphate esters) to be initiated. No analogous reason for the duplication of primary alkylsulphohydrolase enzymes in *Pseudomonas* C12B has yet emerged. Clearly, it will now be necessary to purify the P1 enzyme and to examine in

detail its general properties and specificities before further progress can be made.

Meanwhile, the P2 enzyme possesses a number of interesting features that provide it with a degree of uniqueness. Its ability to hydrolyse a relatively toxic anionic detergent such as sodium dodecyl sulphate without being inactivated is particularly noteworthy. No attempt has yet been made to establish whether the binding of dodecyl sulphate is confined to the active site or whether other sites can also bind the ester, especially at higher concentrations. That the latter situation may be the case is perhaps indicated by the inhibition of enzyme activity that occurs with excess substrate, and the fact that the $V_{\max}$ for the substrate is no longer in accord with that expected from a consideration of the values obtained for the lower members of the homologous substrate series (see Table 2). In any event it is clear that the enzyme possesses a region of hydrophobicity at the active site that is sufficiently large to accommodate an alkyl chain of substantial length. Furthermore this hydrophobic pocket plays a major role in the binding of substrate. The strongly anionic and fully dissociated ester sulphate group appears at first sight to play a less important role in the substrate-binding phenomenon, since secondary alkyl sulphate esters appear to bind equally well to the enzyme when one discounts the terminal methyl group as part of the alkyl chain (Tables 2 and 3 and Fig. 3). Moreover there are only minor differences between the binding affinities (K_i) of the D- and L-isomers of octan-2-yl sulphate. In the light of the fully dissociated sulphate ester group of the substrate it is noteworthy also that there is no apparent involvement of the guanido group of arginine in substrate binding, although such involvement has now been established for many other enzymes that act on anionic substrates (see Riordan *et al.*, 1978). Other possible cationic structures, lysine ϵ -amino and imidazole nitrogen, also appear not to be involved in the binding process.

The absence of a cationic structure was somewhat unexpected, and clearly further work is necessary before the details of the mechanisms of substrate binding, including the contribution made by the sulphate group, become apparent. Another unusual, but central, feature of the catalytic action of the P2 enzyme is that, in common with the S1 and CS2 secondary alkylsulphohydrolases of *Pseudomonas* C12B and *Comamonas terrigena* respectively, it operates by fission of the alcohol-O, as opposed to acid-O, bond of the ester linkage (Bartholomew *et al.*, 1977; Cloves *et al.*, 1977). Whatever mechanism eventually emerges for the P2 enzyme, it must clearly be consistent with both of these unusual features.

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