Further studies on the substrate specificity and inhibition of the stereospecific CS2 secondary alkylsulphohydrolase of *Comamonas terrigena*

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A series of D-alkan-2-yl sulphate esters (C_7 – C_{14}) were prepared by sulphation of the resolved parent alcohols by a method that entails complete retention of configuration. These sulphate esters were tested as substrates for the stereospecific CS2 secondary alkylsulphohydrolase of *Comamonas terrigena*. $V_{\max}$ reached a maximum with the C_9 compound, whereas $\log K_m$ decreased linearly as the alkyl-chain length was increased from C_7 to C_{14} . A parallel series of L-alkan-2-yl sulphates was also prepared, and these esters, together with homologous series of primary alkyl sulphates and primary alkanesulphonates, were shown to be competitive inhibitors of the CS2 enzyme. For each series of compounds, $\log K_i$ values decreased linearly with increasing alkyl-chain length. Plots of chain length against the standard free energy of binding (ΔG^0) of substrate and inhibitors to the CS2 enzyme showed that the standard free energy of association of a $-\text{CH}_2-$ group with the enzyme was 2.0–2.4 kJ/mol for all classes of compound studied, indicating an important contribution from hydrophobic interactions to the overall binding. Plots for D-alkan-2-yl sulphate substrates and primary alkyl sulphate inhibitors were nearly coincident, suggesting that the overall interaction between a primary ester and the enzyme is the same as that between the isomeric secondary substrate and the enzyme. Plots for L-alkan-2-yl sulphate and alkane-sulphonate inhibitors were very similar to each other, but were displaced by 1.5–3.0 kJ/mol from that for substrate binding. This indicates that the binding of any one of these particular inhibitors involves one carbon atom fewer than the number involved in binding a substrate of the same chain length. These observations are discussed in terms of a three-point attachment of substrate to the enzyme involving the alkyl chain, sulphate group and the C-1 methyl group.

Primary and secondary alkyl sulphate esters are surfactant components of commercially important detergents that are readily biodegradable. However, despite their widespread usage, there is a scarcity of information on the molecular basis for the degradation of these surfactants by micro-organisms. The existing evidence suggests that both types of alkyl sulphate initially undergo ester-bond cleavage via the agency of an alkylsulphohydrolase of appropriate specificity (Payne *et al.*, 1965, 1967; Dodgson *et al.*, 1974; Matcham *et al.*, 1977a), and this is followed by oxidation of the liberated alcohol, initiated by appropriate alcohol dehydrogenases (Williams *et al.*, 1966; Lijmbach & Brinkhuis, 1973).

In the Cardiff laboratories attention has focused on the ability of two soil bacteria to degrade alkyl

sulphate surfactants. One of the organisms, *Pseudomonas* C12B, under certain conditions, produces up to five distinct alkylsulphohydrolase enzymes (Dodgson *et al.*, 1974). Two of these (designated P1 and P2) are specific for primary alkyl sulphates, and the other three (designated S1, S2 and S3) are specific for secondary alkyl sulphates. The other bacterium, *Comamonas terrigena*, produces only two alkylsulphohydrolases (designated CS1 and CS2), and these are specific for secondary alkyl sulphate esters (Fitzgerald *et al.*, 1975). The S1 and S2 enzymes of *Pseudomonas* C12B and the CS1 and CS2 enzymes of *C. terrigena* appear to be specific for secondary alkyl sulphates in which the sulphate group is attached at C-2 of the alkyl chain. Furthermore, the enzymes are stereospecific, with S1 and CS2 hydrolysing D-alkan-2-yl sulphates, and S2 and CS1 hydrolysing the corresponding L-isomers

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(Matcham *et al.*, 1977b; Bartholomew *et al.*, 1978). The S3 alkylsulphohydrolase is rather different in that it hydrolyses secondary alkyl sulphates in which substitution occurs at carbon atoms more remote from the ends of the alkyl chain and, although there is a preference for L-isomers, it will also hydrolyse certain D-isomers, albeit relatively slowly (Shaw *et al.*, 1980).

Although the positional and optical stereospecificities of the secondary alkyl sulphohydrolases are now well established, the relationship between substrate specificity and the length of the alkyl chain is less clear. Such a relationship has been studied in the case of the P2 primary alkylsulphohydrolase of *Pseudomonas* C12B, where substrate specificity in terms of $V_{\max}$ and K_m for a homologous series of primary alkyl sulphates has been reported (Cloves *et al.*, 1980). Parallel studies for the secondary enzymes have hitherto been hindered by the unavailability of homologous series of resolved secondary alkyl sulphate esters. Racemic esters are not suitable, because the kinetics of hydrolysis of each isomer are complicated by inhibition by the other enantiomer (Matcham *et al.*, 1977b). A study of the competitive inhibition of the S1 enzyme by homologous series of primary alkyl sulphates and alkane-sulphonates (Bartholomew *et al.*, 1978) has established the close relationship between the degree of inhibition and alkyl-chain length. However, there is no guarantee that the members of either class of inhibitor bind in precisely the same way as the substrates.

The present paper describes the preparation of a series of resolved D- and L-alkan-2-yl sulphate esters that has allowed, for the first time, a detailed examination of the specificity of a secondary alkylsulphohydrolase (the CS2 enzyme of *C. terrigena*) for defined substrates of various alkyl-chain lengths, and a comparison of this specificity with that for the inhibitory L-alkan-2-yl sulphates, primary alkyl sulphates and alkanesulphonates.

Materials and methods

Chemicals

Alkan-2-ols, primary alcohols and (+)- α -methylbenzylamine were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Sodium salts of primary alkanesulphonates were supplied by Cambrian Chemicals, Croydon, Surrey, U.K. ^{35}S -labelled chlorosulphonic acid (sp. radioactivity 48.6 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and diluted with unlabelled material to 2 Ci/mol before use. D- and L-Isomers of tetradecan-2-ol ($\alpha_D^{27} + 5.33^\circ$ and -4.76° respectively, in ethanol) were kindly resolved from racemic alcohol by Professor A. R. Pinder, Clemson University, Clemson, SC, U.S.A.

Unlabelled and ^{35}S -labelled triethylamine- SO_3 complexes were prepared by the method of Cherniak & Davidson (1964), except that for labelled complexes the scale was decreased 20-fold. In order to maximize the yield in the smaller-scale preparation, the chloroform was pre-treated with activated alumina in order to remove ethanol, and with Linde molecular sieve [0.4 nm (4Å); BDH Chemicals, Poole, Dorset, U.K.] to remove water. Triethylamine was also dried in the same way before use. The radiolabelled product was obtained in 38–47% yield, with a melting point of 88–91°C (uncorrected; literature cites 90–91°C).

All other chemicals were the purest available from either BDH Chemicals or Sigma (London) Chemical Co., Poole, Dorset, U.K.

Analytical methods

Optical rotations were measured with an NPL type 243 automatic polarimeter (Thorn Automation, Nottingham, U.K.), with ethanol as solvent for both resolved alkyl hydrogen phthalate esters and alcohols.

G.l.c. was performed with a Perkin-Elmer F11 gas chromatograph fitted with a glass column (180 cm $\times$ 0.65 cm) containing 15% (w/w) EGSS on Gas-Chrom Q, 80–100 mesh (Chromatography Services, Merseyside, U.K.).

I.r. spectra of alcohols were obtained with a Perkin-Elmer Infracord spectrophotometer, and potassium contents of alkyl sulphate esters were determined by flame photometry (EEL flame photometer; Evans Electroselenium, Halstead, Essex, U.K.), with K_2SO_4 as standard.

Preparation of optically active alkan-2-ols

Racemic alkan-2-ols were resolved by the method of Pickard & Kenyon (1911) as described by Vogel (1956), and which involves the formation of a DL-alkan-2-yl hydrogen phthalate and its resolution by fractional crystallization from acetone as the brucine salt. This procedure yields optically pure D-alkan-2-ols, but the L-alkan-2-ols are invariably contaminated with the D-isomer. Optically pure L-alkan-2-ols were therefore prepared by fractional crystallization from acetone of the (+)- α -methylbenzylamine salt of residual alkan-2-yl hydrogen phthalate recovered during the brucine resolution of the D-isomer. This method has previously been employed for preparation of L-decan-2-ol (Prout *et al.*, 1948). As several modifications were made to these methods in the light of experience, the procedure for a 0.2 mol-scale preparation is described in detail.

Synthesis of racemic alkan-2-yl hydrogen phthalate esters

Equimolar quantities of the racemic alkan-2-ol

and phthalic anhydride were refluxed in 25 ml of pyridine (distilled from KOH and dried over CaH_2) for 1–2 h at 100°C , until all solids had disappeared. The resulting oil was cooled and dissolved in acetone. A mixture of 50 ml of conc. HCl and 50 ml of ice was gradually added to the stirred acetone solution. A homogeneous solution was maintained by addition of extra acetone if an oil separated out, or extra water if an acetone layer appeared. Then 1 litre of ice/water was added and the solution kept at 4°C overnight in order to allow the alkan-2-yl hydrogen phthalate ester to form a precipitate. If the ester was a waxy solid, it was filtered off, crushed and air-dried. However, on several occasions it appeared as an oil, in which case it was extracted into chloroform or diethyl ether and dried over anhydrous CaSO_4 . Solvent was removed by rotary evaporation to yield the oily ester.

Resolution of D-alkan-2-yl hydrogen phthalate esters

Each racemic alkan-2-yl hydrogen phthalate was dissolved in cold acetone, and an equimolar amount of anhydrous brucine was added. The mixture was refluxed at 70°C until all the brucine had dissolved, with addition of extra acetone if necessary. Any insoluble residue was removed by filtration and the solution was left to cool to room temperature in order to allow crystallization of brucine D-alkan-2-yl phthalate. Crystals were harvested and washed in cold acetone. The mother liquor was concentrated by rotary evaporation and left to produce a second crop of crystals, which was collected and washed as before. The mother liquor was retained for subsequent recovery of L-alkan-2-yl hydrogen phthalate. The brucine D-alkan-2-yl phthalate crystals were finely crushed, covered with acetone and acidified with a mixture of conc. HCl and water (1 : 1, v/v). A homogeneous solution was maintained as described above by appropriate additions of acetone or water. The D-alkan-2-yl hydrogen phthalate was subsequently precipitated with ice/water.

Resolution of L-alkan-2-yl hydrogen phthalate esters

The residual alkan-2-yl hydrogen phthalate in the mother liquor (mainly L-isomer) was recovered from its brucine salt as described for the D-isomers. Fractional crystallization of the (+)- α -methylbenzylamine salt of L-alkan-2-yl hydrogen phthalate was then performed as described by Prout *et al.* (1948), and the acid monoester recovered as described above.

Recrystallization and characterization of D- and L-alkan-2-yl hydrogen phthalate esters

The D- and L-alkan-2-yl hydrogen phthalates were recrystallized by dissolving each in a minimum volume of warm acetic acid, and adding water, with thorough mixing until the solution just became

translucent. If no crystals appeared when the solution was kept at room temperature overnight, the temperature was lowered to 4°C and, if necessary, a little more water was added to induce crystallization. The purity of the resolved alkan-2-yl hydrogen phthalates was assessed by comparing their melting points and optical rotations with literature values (Pickard & Kenyon, 1911). Alcohols were recovered only from those alkan-2-yl hydrogen phthalates with optical purities of at least 96%.

Recovery of D- and L-alkan-2-ols from their respective phthalate esters

The D- and L-alkan-2-ols were recovered by steam-distillation (Vogel, 1956). The alcohols were extracted into diethyl ether from the steam-distillate, dried over K_2CO_3 and the solvent was removed by rotary evaporation. The optical and chemical purities of the resolved alcohols were assessed as described by Matcham & Dodgson (1977). The i.r. spectra of the resolved alcohols and the racemic mixture were indistinguishable, and the g.l.c. retention times of the resolved and unresolved alcohols were identical. Comparison of the optical rotations with those reported by Pickard & Kenyon (1911) indicated that the resolved alcohols were at least 95% optically pure.

Preparation of alkyl sulphate esters

Alkan-2-yl sulphate esters were prepared by sulphation of the sodium alkoxide of the parent alcohol by using triethylamine- SO_3 complex (White *et al.*, 1980), a method that ensures retention of configuration. D-Decan-2-yl sulphate, D-dodecan-2-yl sulphate and D-tetradecan-2-yl sulphate were synthesized by using ^{35}S -labelled sulphating agent. Primary alkyl sulphates were prepared by the method of Dodgson *et al.* (1974).

Preparation of potassium alkanesulphonates

Sodium salts of alkanesulphonates were passed through a Dowex 50W ion-exchange column (H^+ form) and converted into potassium salts by neutralizing the acid eluate with KOH. After freeze-drying, the potassium alkanesulphonates were recrystallized from methanol. Potassium contents of the alkanesulphonates, determined by flame photometry, were close to theoretical values, and i.r. spectra were satisfactory.

Purification and assay of the CS2 alkylsulphohydrolase

The purification and assay of the CS2 secondary alkylsulphohydrolase enzyme of *C. terrigena* have been described previously (Matcham *et al.*, 1977b). One unit of enzyme activity is defined as the amount of enzyme that liberates $1\text{ }\mu\text{mol}$ of SO_4^{2-} /min with

D-octan-2-yl sulphate as substrate. For all kinetic experiments, initial rates of reaction in the presence of 0.02 enzyme unit were determined by performing incubations as described by Matcham *et al.* (1977b), except that the nature and concentrations of the substrates and inhibitors, were varied as necessary. Appropriate controls were included in all cases. When D-alkan-2-yl sulphates containing less than ten carbon atoms per molecule were used as substrates, the SO_4^{2-} ions liberated were assayed by the BaCl_2 /gelatin method B of Dodgson (1961) as modified by Thomas & Tudball (1967). However, this method is unsuitable for use with alkyl sulphates containing longer alkyl chains, because they tend to form insoluble barium salts under the conditions of the assay. In these cases the rate of hydrolysis was measured by using ^{35}S -labelled long-chain alkan-2-yl sulphate esters. Liberated $^{35}\text{SO}_4^{2-}$ was determined by liquid-scintillation counting after the removal of residual labelled alkyl sulphate ester as a chloroform-soluble Methylene Blue complex. This method, which was originally devised for use with sodium dodecyl sulphate, has been described fully elsewhere (Cloves *et al.*, 1980). For the present purposes, appropriate control experiments showed that C_{10} – C_{14} alkan-2-yl sulphates were completely extractable as Methylene Blue complexes, and that the recovery of $^{35}\text{SO}_4^{2-}$ ions from solutions containing these esters was also complete.

Kinetic constants were determined from double-reciprocal plots (Lineweaver & Burk, 1934).

Results

Substrate specificity of the CS2 secondary alkylsulphohydrolase

The initial rates of enzymic hydrolysis of several D-alkan-2-yl sulphates were measured over the following concentration ranges: D-heptan-2-yl sulphate (0–9.0 mM), D-octan-2-yl sulphate (0–4.0 mM), D-nonan-2-yl sulphate (0–3.0 mM), D-decan-2-yl sulphate (0–0.85 mM), D-dodecan-2-yl sulphate (0–0.086 mM), D-tetradecan-2-yl sulphate (0–0.064 mM).

Because of limitations inherent in the BaCl_2 /gelatin method for SO_4^{2-} determination, it was necessary to use the radioisotopic assay method referred to in the Materials and methods section for measuring $^{35}\text{SO}_4^{2-}$ liberated from the C_{10} – C_{14} ^{35}S -labelled substrates.

At the concentrations used, all the esters showed normal Michaelis–Menten kinetics and K_m values were obtained from double-reciprocal plots (Table 1). The K_m value for different substrates decreased as the chain length of the substrate increased, and the plot of $\log K_m$ against the number of carbon atoms in the alkyl chain (not shown) gave the straight-line relationship characteristic of a homologous series. The K_m value for D-octan-2-yl sulphate of 1.02 mM was in reasonable agreement with the value of 1.38 mM obtained by Matcham *et al.* (1977b). Values of $V_{\max}$ increased as chain length increased from C_7 to C_9 , followed by a rapid decline with further increase in chain length (Table 1).

Inhibition of the CS2 alkylsulphohydrolase by L-alkan-2-yl sulphates, primary alkyl sulphates and alkanesulphonates

Previous work has shown that L-octan-2-yl sulphate was a competitive inhibitor of CS2 (Matcham *et al.*, 1977b) and S1 alkylsulphohydrolases, the latter enzyme also being subject to competitive inhibition by primary alkyl sulphates and alkanesulphonates (Bartholomew *et al.*, 1978). In order to obtain a direct comparison between the substrate specificity of a secondary alkylsulphohydrolase and its susceptibility to inhibition by related compounds, a range of L-alkan-2-yl sulphates, primary alkyl sulphates and alkanesulphonates were tested as inhibitors of the CS2 enzyme at the concentrations shown in Table 2. It has previously been shown that at these concentrations primary alkyl sulphates of chain lengths not exceeding C_{10} and sulphonates of chain lengths not exceeding C_9 do not interfere with the assay of liberated SO_4^{2-} ions by the BaCl_2 /gelatin assay method (Bartholomew *et al.*, 1978; K. S. Dodgson, unpublished work). For the present work it was

Table 1. K_m and $V_{\max}$ values for a series of D-alkan-2-yl sulphate substrates of the CS2 alkylsulphohydrolase of *C. terrigena*

See the text for details. $V_{\max}$ is expressed relative to that for D-nonan-2-yl sulphate, which is taken as 100.

Substrate (potassium salt)	K_m (mM)	Relative $V_{\max}$
D-Heptan-2-yl sulphate	6.80	18
D-Octan-2-yl sulphate	1.02	80
D-Nonan-2-yl sulphate	0.73	100
D-Decan-2-yl sulphate	0.25	22
D-Dodecan-2-yl sulphate	0.055	1.2
D-Tetradecan-2-yl sulphate	0.006	0.02

Table 2. *Inhibition of the CS2 alkylsulphohydrolase by various alkyl sulphate esters and alkanesulphonates*
D-Octan-2-yl sulphate was employed as substrate and all inhibitors were of the competitive type. For further details see the text.

Inhibitor (potassium salt)	Concn. of inhibitor (mM)	Average K_i (mM)
L-Alkan-2-yl sulphates		
L-Octan-2-yl sulphate	2.0, 4.0	2.29
L-Nonan-2-yl sulphate	1.5, 3.0	1.36
L-Decan-2-yl sulphate	1.0, 2.0	0.30
L-Tetradecan-2-yl sulphate	0.05, 0.10	0.02
Alkan-1-yl sulphates		
Propan-1-yl sulphate	15.0, 30.0	59.0
Butan-1-yl sulphate	10.0, 20.0	46.8
Pentan-1-yl sulphate	10.0, 20.0	14.3
Hexan-1-yl sulphate	4.5, 9.0	6.4
Heptan-1-yl sulphate	3.0, 6.0	3.1
Octan-1-yl sulphate	2.0, 4.0	1.3
Nonan-1-yl sulphate	1.0, 2.0	0.73
Decan-1-yl sulphate	1.0, 2.0	0.30
Alkanesulphonates		
Butanesulphonate	15.0, 30.0	161.5
Pentanesulphonate	8.0, 16.0	66.0
Hexanesulphonate	5.0, 10.0	19.2
Heptanesulphonate	4.0, 8.0	8.9
Octanesulphonate	4.0, 8.0	4.5
Nonanesulphonate	2.0, 4.0	1.6

further demonstrated that L-tetradecan-2-yl sulphate did not interfere at the concentrations used. All compounds tested inhibited the enzyme's activity towards D-octan-2-yl sulphate, and double-reciprocal plots showed this inhibition to be competitive in each case.

For each series of compounds values of K_i (Table 2) decreased as the carbon-chain length increased, and, when $\log K_i$ was plotted against the number of carbon atoms in the alkyl chain, a linear relationship was obtained, characteristic of a homologous series (cf. Bartholomew *et al.*, 1978; Cloves *et al.*, 1980). The value of K_i (2.29 mM) for L-octan-2-yl sulphate agreed reasonably with the value of 3.6 mM reported previously (Matcham *et al.*, 1977b).

Discussion

The results obtained for D-alkan-2-yl sulphate substrates and the competitive inhibitors of the CS2 enzyme (Tables 1 and 2) respectively reveal the importance of the alkyl chain in enzyme-substrate and enzyme-inhibitor interactions. It is noteworthy that, even with the longer chain lengths used, the K_m and K_i values are still decreasing, indicating that the hydrophobic alkyl-chain-binding site is very extensive. This situation is similar to that previously reported for the P2 primary alkylsulphohydrolase of

Fig. 1. *Influence of carbon-chain length on V_{max} for the CS2 secondary alkylsulphohydrolase of C. terrigena and P2 primary alkylsulphohydrolase of Pseudomonas C12B*
Values of V_{max} are relative to those of D-nonan-2-yl sulphate for the CS2 enzyme (●), and nonan-1-yl sulphate for the P2 enzyme (○). Data for the latter are taken from Cloves *et al.* (1980).

Pseudomonas C12B (Cloves *et al.*, 1980). However, D-alkan-2-yl sulphates with more than nine carbon atoms show a sharp decrease in V_{max} with increasing alkyl-chain length (Fig. 1). Values of V_{max}

for the P2 enzyme have also been included, and the striking similarity of the two curves adds further weight to the idea that their mechanisms of action probably incorporate common features, despite their different stereospecificities and origins.

For each of the four homologous series of compounds investigated in the present study, a linear relationship was observed 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 chain (Fig. 2). The gradients of the plots give the ΔG^0 value corresponding to the change in free energy that occurs when one $-\text{CH}_2-$ group is transferred from a free state in solution to its state in the active-site environment of the enzyme. Such a relationship was first described by Bergmann & Segal (1954) during studies on 'true' and 'pseudo' cholinesterase. It has also been reported for substrates and competitive inhibitors of the P2 primary alkylsulphohydrolase of *Pseudomonas* C12B (Cloves *et al.*, 1980) and can be computed from published data (Bartholomew *et al.*, 1978) for the competitive inhibitors of the secondary alkylsulphohydrolase, S1. However, this is the first time that the relationship for substrates, in addition to com-

petitive inhibitors, has been described for a secondary alkylsulphohydrolase. For the CS2 enzyme, the ΔG^0 values for binding $-\text{CH}_2-$ units are similar for substrates and all classes of competitive inhibitors tested (-2.4 kJ/mol , -2.0 kJ/mol , -2.0 kJ/mol and -2.3 kJ/mol for D-alkan-2-yl sulphates, L-alkan-2-yl sulphates, primary alkyl sulphates and alkanesulphonates respectively). According to the classification of sensitivity of biochemical response to hydrophobic interactions (Hansch & Dunn, 1972), these values lie at the lower range of the high-sensitivity group. The similarity of the ΔG^0 values for $-\text{CH}_2-$ group binding indicates that, for all these types of compound, the alkyl chain binds to the CS2 alkylsulphohydrolase in a similar manner.

The plot of ΔG^0 for binding against chain length for L-alkan-2-yl sulphate inhibitors is parallel to the corresponding plot for D-alkan-2-yl sulphate substrates, but displaced from it in the direction of weaker binding by an extent equivalent to 1.5–2.5 kJ/mol (Fig. 2). This value corresponds to the contribution to binding of a single methylene unit. It is therefore possible that each L-alkan-2-yl sulphate is attached to the CS2 enzyme with one less $-\text{CH}_2-$ group contributing to binding than is the case for the corresponding D-isomer. It has previously been postulated (Matcham *et al.*, 1977b) that the substrate binds to the active site of the enzyme by a three-point attachment involving the alkyl chain, the sulphate moiety and the C-1 methyl group. It has been established above that the alkyl chains of D- and L-isomers bind in similar ways. If the sulphate group of an inhibitory L-alkan-2-yl sulphate also occupies its normal site, the methyl moiety would be orientated away from its normal binding site. This would explain the displacement of the L-alkan-2-yl sulphate binding curve from the D-isomer substrate curve by a ΔG^0 value representing one $-\text{CH}_2-$ group.

The plot of ΔG^0 for binding against alkyl-chain length for the primary alkyl sulphates is almost coincident with that for the substrates. This implies that, for isomeric primary and D-secondary alkyl sulphates, the same number of carbon atoms interact with the CS2 enzyme, presumably with an extra carbon atom in the alkyl chain of the primary esters compensating for the vacant methyl-binding site. On the other hand, the primary alkanesulphonates behave in a manner similar to that of the L-alkan-2-yl sulphate inhibitors with values displaced by 1.5–3.0 kJ/mol from the corresponding substrate values. If alkanesulphonates are considered as analogues of primary alkyl sulphates with the C–O–S ester link replaced by C– CH_2 –S, then it is reasonable to suppose that the C-1 atom in the sulphonates is unable to bind at the alkyl-binding site. This would produce the observed displacement of the linear free-energy plots.

Fig. 2. Linear free-energy relationships for the binding of substrate and inhibitor homologous series to the CS2 secondary alkylsulphohydrolase

▲, D-Alkan-2-yl sulphate substrates; △, L-alkan-2-yl sulphate inhibitors; □, primary alkyl sulphate inhibitors; ○, primary alkanesulphonate inhibitors.

It has been reported for the P2 enzyme of *Pseudomonas* C12B that the free-energy increment for binding a $-\text{CH}_2-$ group is -2.5 kJ/mol for primary alkyl sulphate substrates and -2.4 kJ/mol for the competitive alkan-2-yl sulphate inhibitors (Cloves *et al.*, 1980). The ΔG° curves for the two types of compound are displaced from each other by 3 kJ/mol , and it was suggested that one less $-\text{CH}_2-$ group (probably the C-1 methyl group) was binding to the enzyme in the case of the secondary ester compared with its primary isomer. Competitive inhibition by alkanesulphonates showed a very different behaviour, with a ΔG° increment per $-\text{CH}_2-$ group of -1.2 kJ/mol . This was interpreted as indicating that alkanesulphonates bind to the P2 enzyme with a lower contribution from hydrophobic interactions and that this reflects the displacement of the alkyl chain from an ideal position when the sulphate group is replaced by the sulphonate group. This is in marked contrast with the situation suggested here for the CS2 enzyme, which binds alkyl chains of all sulphate esters and sulphonates in the same way.

Similar data have not been published for the S1 alkylsulphohydrolase, but computation of the results obtained by Bartholomew *et al.* (1978) reveals that, for the competitive inhibitors, primary alkyl sulphates and alkanesulphonates, the free-energy increments for $-\text{CH}_2-$ -group-binding are -2.2 and -3.3 kJ/mol respectively. Thus primary alkyl sulphates appear to bind to alkylsulphohydrolases in a consistent way, whether as substrates of the P2 enzyme from *Pseudomonas* C12B or as competitive inhibitors of the S1 secondary alkylsulphohydrolase from the same organism or the analogous CS2 enzyme from *C. terrigena*. However, the binding of alkanesulphonates to the S1 enzyme shows a very high sensitivity to hydrophobic interaction, and consequently this competitive inhibitor exhibits different hydrophobic interactions with each of the three alkylsulphohydrolases so far studied.

As a final comment, it is intriguing that, although both primary alkyl sulphates and L-alkan-2-yl sulphates apparently interact with the CS2 enzyme with their sulphate moieties in the correct position at the active site, they do not serve as substrates for the enzyme. This indicates that a methyl group is required adjacent to the ester bond, in a specific conformation to allow the enzyme-catalysed hydro-

lysis to proceed, and may represent a novel mechanism of enzymic hydrolysis.

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