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Initiation of Activation of a Preemergent Herbicide by a Novel Alkylsulfatase of *Pseudomonas putida* FLA

VERONICA LILLIS,[1] KENNETH S. DODGSON,[1]* GRAHAM F. WHITE,[1] AND WILLIAM J. PAYNE[2]

Department of Biochemistry, University College, Cardiff CF1 1XL, United Kingdom,[1] and Department of Microbiology, University of Georgia, Athens, Georgia 30602[2]

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The activation of the preemergent herbicide 2-(2,4-dichlorophenoxy)ethyl sulfate (Crag herbicide) is initiated by soil microorganisms that are presumed to act by removing the ester sulfate group via some type of sulfatase enzyme. An enrichment technique with the herbicide as the sole source of sulfur led to the isolation of several pure cultures that could produce 2-(2,4-dichlorophenoxy)ethanol from the herbicide. One of these, a strain of *Pseudomonas putida*, was particularly active. Polyacrylamide gel zymograms of extracts of cells grown on nutrient broth showed the presence of three secondary and three primary alkylsulfatases. One of the latter enzymes was active toward Crag herbicide as well as sodium dodecyl sulfate. Maximum activity was obtained in the late-stationary phase of growth, and enzyme yields were not affected by either the presence or the absence of the herbicide in the growth medium. The enzyme was purified 2,670-fold to homogeneity by a combination of streptomycin sulfate treatment, heat treatment, and column chromatography on DEAE-cellulose, Sephacryl 200-S, and butyl agarose. The pure enzyme was tetrameric (molecular weight, 295,000) and most active at pH 6.0. Saturation kinetics with inhibition by excess substrate were observed for Crag herbicide and octyl sulfate. 2-Butoxyethyl sulfate was a relatively poor substrate, and dodecyltriethoxy sulfate was not hydrolyzed at all. Enzymatic hydrolysis of each substrate in the presence of $H_2{}^{18}O$ led to incorporation of ^{18}O exclusively into SO_4^{2-} ions in all three cases. The Crag herbicide sulfatase therefore acts by cleaving the O-S bond of the C-O-S ester linkage, in contrast with other alkylsulfatases acting on long-chain alkyl sulfates.

Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate (Crag herbicide; Fig. 1a), a herbicide first described by King and Lambrech (11) and currently marketed under the trade name Herbon, becomes active only after coming into contact with soil. Studies by Vlitos (20, 21) established that exposure of the herbicide to nonsterile soil at neutral pH led to its activation, whereas no activation was observed after exposure to sterile soil. A soil microorganism, identified as *Bacillus cereus* var. *mycoides*, was isolated, and cell-free filtrates of nutrient broth in which it had been grown were shown tc be capable of converting the herbicide to 2-(2,4-dichlorophenoxy)ethanol. It was concluded that some type of sulfatase must be responsible for this conversion, although attempts to detect arylsulfatase activity were unsuccessful. Other experiments established that 2-(2,4-dichlorophenoxy)acetic acid (2,4-D) was present in nonsterile soils that had been treated with 2-(2,4-dichlorophenoxy)ethanol, suggesting that the biological activity of Crag herbicide could be related to the removal of the ester sulfate group (possibly by a new type of sulfatase, an alkylsulfatase), followed by the enzymatic oxidation of the liberated alcohol to 2,4-D.

Since this early work, a number of bacterial alkylsulfatases have been discovered that act on either primary or secondary alkyl sulfate esters of chain lengths greater than C_5 (for reviews, see references 8 and 9). For example, *Pseudomonas* sp. strain C12B can produce a total of five such enzymes, none of which, however, exhibits activity toward Crag herbicide. This paper describes the isolation of a soil microorganism that can initiate the activation of the herbicide by removing the ester sulfate group and establishes that the responsible enzyme is a new type of alkylsulfatase.

MATERIALS AND METHODS

Sulfate esters. Crag herbicide was a sample kindly supplied several years ago by the Union Carbide

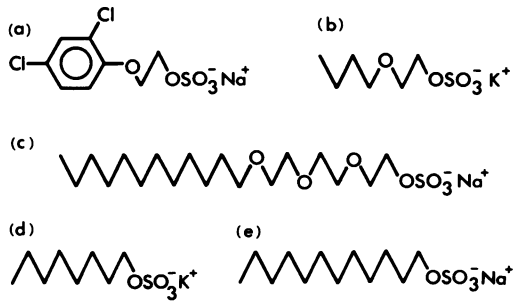

FIG. 1. Chemical structures of Crag herbicide and related compounds. (a) Crag herbicide; (b) potassium 2-butoxyethyl sulfate; (c) sodium dodecyltriethoxy sulfate; (d) potassium octyl sulfate; (e) SDS.

Corp., New York, N.Y. Before use, it was recrystallized from methanol-diethyl ether (60:40 [vol/vol]), and its authenticity was confirmed by infrared, UV, and nuclear magnetic resonance spectroscopy and by analysis of sodium and ester sulfate contents. "Specially pure" sodium dodecyl sulfate (SDS) was obtained from BDH, Poole, Dorset, United Kingdom, and sodium dodecyltriethoxy sulfate was provided by G. K. Watson of Unilever Research Laboratories, Port Sunlight, United Kingdom. The potassium salts of octyl sulfate and "DL-decan 5-yl sulfate" were prepared by the sulfuric acid method of Dodgson et al. (7). The latter ester is in quotation marks because the method of preparation is now known to lead to the production of other isomers (14), including decan 2-yl sulfate, but it does provide a useful substrate for detecting a variety of different secondary alkylsulfatases on gel zymograms (7, 15). The sulfate esters of D- and L-octan 2-ol, as well as 2-butoxyethanol, were prepared as potassium salts by the method of White et al. (23) and were characterized by analysis for potassium and ester sulfate contents and by infrared and nuclear magnetic resonance spectroscopy.

Other materials. DEAE-cellulose (DE-52; Whatman Biochemicals, Maidstone, Kent, United Kingdom), Sephacryl 200-S (Pharmacia [Great Britain], Ltd., Hounslow, Middlesex, United Kingdom), and butyl agarose (Miles Laboratories, Stoke Poges, Buckinghamshire, United Kingdom) were washed and equilibrated according to the instructions of the manufacturers. 2-(2,4-Dichlorophenoxy)ethanol was prepared by reduction of a solution of 2,4-D (Sigma Chemical Co., Poole, Dorset, United Kingdom) in diethyl ether with lithium aluminium hydride. The product was characterized by its melting point (56 to 57°C) and by infrared and nuclear magnetic resonance spectroscopy. Protein standards for molecular weight determinations were obtained from Boehringer Mannheim, Lewes, Sussex, United Kingdom, and $H_2{}^{18}O$, normalized at 71.3 atom % excess, was a product of the British Oxygen Co., Ltd., London, United Kingdom. Other chemicals were the purest available from BDH or Sigma.

Isolation of *Pseudomonas putida* FLA and detection of herbicide-desulfating activity. The bacterium, identified as a strain of *P. putida* (biotype A) at the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, United Kingdom, was isolated from garden soil collected in the Bristol Channel area of South Wales. Soils were percolated with a mineral salts medium containing the following: K_2HPO_4, 3.5 g; KH_2PO_4, 1.5 g; NH_4Cl, 0.5 g; NaCl, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.15 g; Crag herbicide, 5.0 g; distilled water, 1 liter. Effluents were collected and used to inoculate tubes of sterile medium containing the mineral salts described above, together with 0.1% Crag herbicide and either 0.15% yeast extract or 0.15% sodium acetate. No growth occurred if yeast extract or sodium acetate was omitted. Colony transfers were made onto nutrient agar plates, and representative colonies were separated and successively transferred back to the liquid medium and then to agar plates. Forty pure cultures were eventually obtained, each of which was tested for the ability to degrade the herbicide. Cultures were grown in 100 ml of the salts-herbicide-sodium acetate medium described above. After growth for 48 h at 30°C, the total contents of each culture flask were extracted twice with diethyl ether (25 ml each time). Extracts were concentrated almost to dryness and subjected to thin-layer chromatography on plastic sheets coated with silica gel (0.2-mm thickness, containing a fluorescent indicator) (E. Merck AG, Darmstadt, Germany) with methanol-chloroform (1:4 [vol/vol]) as the solvent. Developed plates were examined under UV light for material with an R_f equal to that exhibited by authentic 2-(2,4-dichlorophenoxy)ethanol. Control cultures were grown in the absence of the herbicide and treated in the same manner.

A number of the isolated organisms were able to desulfate the herbicide, but one, subsequently identified as *P. putida* (designated strain FLA), was clearly the most active as judged from thin-layer chromatography plates. No 2-(2,4-dichlorophenoxy)ethanol was ever detected in control experiments. In later (and larger-scale) experiments, the production of the alcohol by *P. putida* FLA was confirmed by melting point and mixed melting point measurements of the isolated alcohol and by gas-liquid chromatography.

Maintenance, growth, and harvesting. The isolate was maintained on agar slopes with and without Crag herbicide. Unless otherwise stated, the organism was grown (at 30°C in batch culture) on nutrient broth (Difco Laboratories, Detroit, Mich.) in the absence of the herbicide. For enzyme purification studies, the organism was grown at the University of Georgia in a 400-liter-capacity New Brunswick fermentor containing 350 liters of nutrient broth. The culture was aerated with 1.8 m^3 of air per h and agitated at 100 rpm. Cells were harvested at the late-stationary phase by Sharples centrifugation (flow rate, 90 liters/h), and the wet cells (815 g) were freeze-dried and stored at −10°C until required.

Cell extracts. Cells were ruptured by suspending them in 10 mM Tris-hydrochloride (pH 7.5) and passing them three times through a chilled French pressure cell at 126 MPa. Cell debris was removed by centrifugation, and the clear extracts were dialyzed against 10 mM Tris-hydrochloride (pH 7.5).

Measurement of sulfatase activity. The barium chloride-gelatin method (method B) of Dodgson (6) as modified by Thomas and Tudball (19) was used for the assay of sulfatase activity toward octyl sulfate and 2-butoxyethyl sulfate. However, this method could not be used when Crag herbicide was serving as substrate because preliminary experiments showed that appar-

ent recoveries of added SO_4^{2-} ions were always high in its presence. Attention was therefore turned to the possibility of measuring enzymatically liberated 2-(2,4-dichlorophenoxy)ethanol.

The method finally developed involved the incubation at 30°C of 100 μl of a solution (20 mM, unless otherwise stated) of Crag herbicide in 0.2 M buffer (acetate, Tris-hydrochloride, or Tris-maleate, depending on the circumstances) with an equal volume of suitably diluted enzyme solution. The enzyme reaction was stopped by adding 50 μl of 15% (wt/vol) trichloroacetic acid, and precipitated protein was separated by centrifugation. A portion (200 μl) of the clear supernatant was transferred to a clean tube containing 2.5 ml of chloroform, and the whole was thoroughly mixed. The mixture was separated into two layers by centrifugation (1 min on a bench centrifuge), after which the chloroform layer was carefully transferred to a clean tube containing a small amount of anhydrous Na_2SO_4 to remove any traces of water carried over from the extraction. The absorbance of the dried chloroform solution was measured spectrophotometrically (285 nm) in 2-cm cells against a chloroform blank. A calibration curve was prepared over a concentration range of 2-(2,4-dichlorophenoxy)ethanol that varied from 0 to 0.5 mM. Control determinations were done in a similar way, except that enzyme and substrate solutions were incubated separately and trichloroacetic acid was added to the substrate before adding enzyme. The method was tested under a variety of conditions, and particular care was taken to check that the herbicide was stable in the presence of the trichloroacetic acid over the period of contact, that good recoveries of the liberated alcohol were obtained, and that unreacted herbicide remained in the aqueous phase during the chloroform extraction. A unit of enzyme activity toward the herbicide is defined as the amount of enzyme that produces 1 μmol of 2-(2,4-dichlorophenoxy)ethanol per min.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was used for the detection of alkylsulfatase activity (gel zymography), for following enzyme purification, and for molecular weight determinations. When used for the first two purposes, gels were prepared and run, and primary and secondary alkylsulfatases were detected on gels, by the procedures of Payne et al. (15). SDS served as the detection substrate for primary alkylsulfatases, and "DL-decan 5-yl sulfate" served as the detection substrate for secondary alkylsulfatases. Sulfatase activity toward Crag herbicide and 2-butoxyethyl sulfate was revealed by incubating gels in 40 mM Tris-hydrochloride buffer (pH 7.8) containing the appropriate substrate (20 mM) and 20 mM barium acetate. Barium sulfate precipitated as a band of minute white spots at the site of the enzymatic release of SO_4^{2-} ions (4).

In purification studies, protein bands were detected on gels with Coomassie blue (5). The methods of Zwann (24) and Weber and Osborne (22) were used for molecular weight determinations under nondenaturing and denaturing conditions, respectively. For reasons that will later become apparent, the enzyme was boiled for 1 min before treatment overnight with mercaptoethanol and SDS when the latter method was used. Protein standards for the Weber and Osborne method were chymotrypsinogen, alcohol dehydrogenase, aldolase, catalase, bovine serum albumin monomer, and β-galactosidase. Standards for the Zwann method were bovine serum albumin monomer, lactate dehydrogenase, bovine serum albumin dimer, and ferritin.

Isotope incorporation experiments. Incorporation of ^{18}O from $H_2{}^{18}O$ into one or another of the products resulting from the action of the purified enzyme on Crag herbicide, octyl sulfate, or 2-butoxyethyl sulfate was investigated as follows. Incubation mixtures contained 20 μmol of the appropriate substrate in 50 μl of 0.2 M Tris-maleate buffer (pH 6.0), 0.25 ml of $H_2{}^{18}O$ (71.3 atom % excess), and 0.2 ml (72 μg) of the purified enzyme in 10 mM Tris-hydrochloride buffer (pH 7.5). Incubation was for 16 h with each substrate. Subsequently, the enzymatically liberated alcohol product was extracted twice with diethyl ether (2.5 ml each time) and analyzed for ^{18}O content by gas-liquid chromatography-mass spectrometry (2). The SO_4^{2-} ions remaining in the aqueous layer after ether extraction were then precipitated as $BaSO_4$ by the addition of 1 ml of 2 M HCl and 1 ml of 10% $BaCl_2$ solution. The precipitate was separated by centrifugation, washed twice successively with 3-ml portions of 2 M HCl, water, ethanol, and diethyl ether, and then mixed with 0.2 g of finely ground, infrared-grade KBr before being dried overnight at 110°C. The mixture was pressed into a disk and analyzed for ^{18}O by the method of Spencer (18). A control experiment in which relevant quantities of the products of hydrolysis of the herbicide were incubated with the sulfatase preparation showed that no isotope exchange occurred between $H_2{}^{18}O$ and the products.

Purification of Crag herbicide sulfatase. All purification procedures were carried out at 0 to 4°C, and enzyme samples were stored at 4°C. Centrifugations were performed at 26,000 × *g* unless stated otherwise. Nucleic acid (12) and protein (12, 13) contents of enzyme solutions were monitored by routine methods, except in the final stages, when a more sensitive assay for protein (16) was preferred.

(i) Stage 1. Freeze-dried cells (20 g) of *P. putida* FLA were suspended in 200 ml of 10 mM Tris-hydrochloride buffer (pH 7.5), and a cell extract was prepared and dialyzed as described previously.

(ii) Stage 2. Nucleic acid was precipitated from the stage 1 supernatant (170 ml) by the slow addition (20 min with gentle stirring) of 17 ml of a 28.2% (wt/vol) solution of streptomycin sulfate in 10 mM Tris-hydrochloride buffer (pH 7.5). After being stirred for an additional 30 min, precipitated nucleic acid was removed by centrifugation, and the clear supernatant was dialyzed overnight against several changes of buffer.

(iii) Stage 3. Portions (10 ml) of the stage 2 material contained in large boiling tubes (2 by 15 cm) were immersed in a hot water bath (82°C) for 30 s. After rapid cooling, the treated samples were pooled and centrifuged. The clear supernatant was dialyzed for 16 h against several changes of 50 mM Tris-hydrochloride buffer (pH 7.5).

(iv) Stage 4. Material from stage 3 (170 ml) was loaded onto a column (4.5 by 45.0 cm) of DEAE-cellulose (DE-52) that had been equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.5). The column was eluted with a linear gradient (0 to 0.2 M) of NaCl in a total volume of 1.5 liters of 50 mM Tris-hydrochloride buffer (pH 7.5), and 15-ml fractions were collect-

ed. The appropriate fractions (66 to 70; Fig. 2a) were pooled.

(v) **Stage 5.** The pooled stage 4 material (total volume, 55 ml) was concentrated to about 22 ml (immersible CX-18 ultrafiltration unit; Millipore Corp., Bedford, Mass.) before being loaded onto a Sephacryl 200-S column (3.5 by 72 cm) that had been equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.5). The column was eluted with the same buffer, fractions (10 ml) were collected, and appropriate fractions (14 to 16; Fig. 2b) were pooled and dialyzed against several changes of 10 mM Tris-hydrochloride buffer (pH 7.5).

(vi) **Stage 6.** Stage 5 material (26 ml) was loaded onto a butyl agarose column (2.5 by 40 cm, previously equilibrated with 10 mM Tris-hydrochloride buffer [pH 7.5]), and the enzyme which was retained on the column was eluted with a linear gradient (0 to 0.12 M) of NaCl in a total volume of 1 liter of 10 mM Tris-hydrochloride buffer (pH 7.5) (Fig. 2c). Fractions (ca. 8 ml) were stored separately at 0 to 4°C, and the final preparation was stable for at least 2 months. Care was taken in all subsequent work to ensure that experiments conducted at pHs other than 7.5 were sufficiently buffered and checked to the pH required.

RESULTS

Preliminary experiments. *P. putida* FLA grew well on nutrient broth, pyruvate (1%), and succinate (1%), and less well on acetate (1%). Ability to release 2-(2,4-dichlorophenoxy)ethanol into the growth medium was present in all cases if the Crag herbicide was also present. Visual assessment of the thin-layer chromatography plates suggested that the greatest activity was present in broth cultures, followed by pyruvate cultures. No evidence for the production of the corresponding acid, 2,4-D, was ever detected. Subsequent experiments showed that the ability of cell extracts to desulfate the herbicide was roughly the same whether or not the herbicide was present in the growth medium. In broth cultures, the ability to desulfate Crag herbicide began to appear in cells in the late-logarithmic growth phase and reached a maximum at about 10 h into the stationary phase.

Gel zymography. Extracts of broth-grown cells of the bacteria, when subjected to gel zymography, gave six distinct bands of alkylsulfatase activity. Three of these bands, designated FP1, FP2, and FP3, (Fig. 3a), were detected when extruded gels were incubated with SDS, indicating the presence of three primary alkylsulfatase enzymes. Three different bands, designated FS1, FS2, and FS3 (Fig. 3c), were detected on gels when "DL-decan 5-yl sulfate" was substituted for SDS, indicating secondary alkylsulfatase activity. One of the bands (FS2) was of very low intensity.

When gels were incubated with Crag herbicide (barium acetate detection method), a single and somewhat diffuse band corresponding roughly to

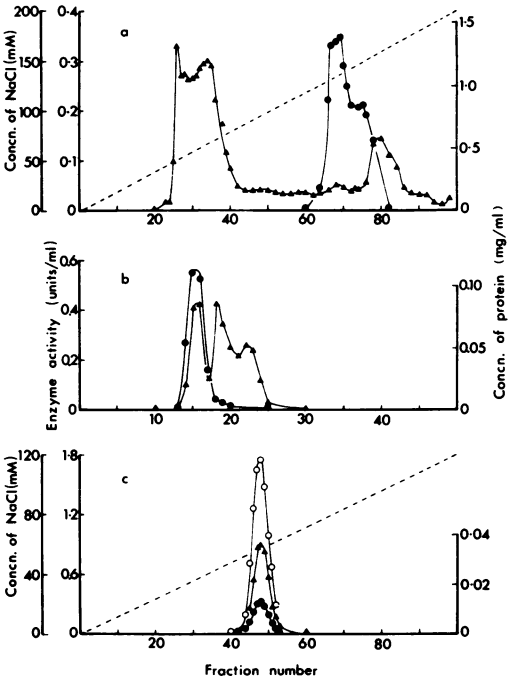

FIG. 2. Purification of Crag herbicide sulfatase by column chromatography. (a) Elution from DEAE-cellulose; (b) elution from Sephacryl 200-S; (c) elution from butyl agarose. Symbols: ▲, protein concentration; ●, Crag herbicide sulfatase activity. In (c), activity was also measured toward octyl sulfate (○). Broken lines represent NaCl concentration gradients. See the text for further details.

FP1 was obtained (Fig. 3b). This result raised the possibility that Crag herbicide was desulfated by a primary alkylsulfatase exhibiting multiple specificity.

Extracts prepared from cells grown in basal salts medium containing 1% sodium pyruvate (carbon and energy source) and 1.6 mM Crag herbicide (sulfur source) exhibited all bands except FP2. Substitution of 1.6 mM sodium sulfate for the herbicide produced cell extracts that gave only four of the bands (FP1, FP3, FS1, and FS2). In these extracts also, the activity towards Crag herbicide coincided with the FP1 alkylsulfatase band.

Preliminary experiments on Crag herbicide sulfatase. A number of experiments were carried out to establish some properties of Crag herbicide sulfatase that might be relevant to the purification of the enzyme. The cell extract exhibited activity toward the herbicide over a broad pH range, with maximum activity occurring at a substrate concentration of 10 mM and at pH 6.0 in 0.1 M sodium acetate-acetic acid buffer over an incubation period of 5 min. However, the enzyme activity-time relationship,

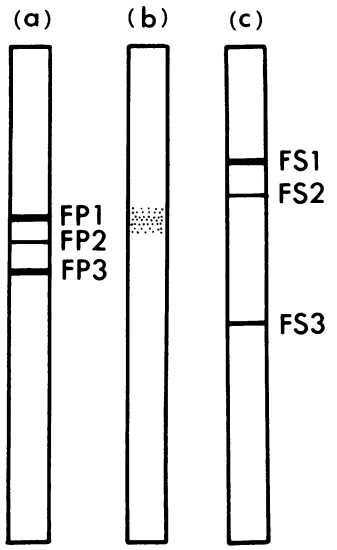

FIG. 3. Diagrammatic representation of gel zymograms of alkylsulfatases and Crag herbicide sulfatase present in cell extracts of broth-grown *P. putida* FLA. (a) Primary alkylsulfatases stained with SDS; (b) Crag herbicide sulfatase stained with Crag herbicide-barium acetate; (c) secondary alkylsulfatases stained with "DL-decan 5-yl sulfate." See the text for further details.

even over such a short incubation period (less than 10% hydrolysis), was not linear. Furthermore, the relationship between enzyme activity and enzyme concentration was also nonlinear. In monitoring the progress of the purification of the enzyme, attempts were made to ensure that roughly the same enzyme concentration and the same incubation time were always used in the assay procedure.

Enzyme purification. The purification sequence and results are summarized in Table 1. The final product migrated as a single protein band on polyacrylamide gel electrophoresis (20-μg protein load), representing a 2,670-fold purification from the crude cell extracts with a recovery of almost 50% of the total activity. Gel electrophoresis at each purification stage, coupled with staining for alkylsulfatase activities, indicated that all of the sulfatase bands were still present at the end of stage 2. The secondary alkylsulfatase FS3 disappeared at stage 3, and the FP2 and FP3 primary and FS1 and FS2 secondary alkylsulfatase bands could no longer be detected after stage 4. Only the FP1 band was present at the final two stages. This band, which coincided with the single protein band, could be visualized with either SDS or Crag herbicide as the substrate. Enzyme assays confirmed that the final preparation was active toward primary alkyl sulfates (with octyl sulfate used as a substrate) and Crag herbicide, as well as 2-butoxyethyl sulfate. Dodecyltriethoxy sulfate was not hydrolyzed.

These results reinforced the possibility, raised by the gel experiments with crude extracts, that a single enzyme with multiple specificities was present. This possibility was further strengthened in subsequent purifications when Crag herbicide and octyl sulfate were used to monitor the final stage. The activity profiles from the column were similar.

Molecular weight. The molecular weight of the enzyme under nondenaturing conditions was ca. 295,000, whereas under denaturing conditions a value of 67,610 was obtained, suggesting a tetrameric structure for the intact enzyme. In addition to the major band, a very faint band with a slightly greater relative mobility was present on gels run under denaturing conditions. Because of the activity of the enzyme toward SDS, a property known to cause problems with SDS gel electrophoresis of primary alkylsulfatases (4), it was necessary to inactivate the enzyme before the addition of SDS, as described above.

Enzyme activity toward Crag herbicide. The purified enzyme exhibited maximum activity (10-min incubation period) at a herbicide concentration of 10 mM in the presence of 0.1 M Tris-maleate buffer at the optimum pH (6.0). There was some inhibition by excess substrate. Under these conditions, the relationship between enzyme activity and time of incubation diverged slightly from linearity (compare crude extracts), despite the fact that less than 10% of the substrate was hydrolyzed. The deviation from linearity increased markedly after 10 to 15 min, so that only 80% of the substrate was hydrolyzed after 24 h. Similarly, the activity-enzyme concentration curve also deviated slightly from linearity. The K_m and V_{max} values calculated from the Lineweaver-Burk double-reciprocal plot were 1.0 mM and 10.9 μmol/min per mg of protein, respectively. Corresponding K_m and V_{max} values calculated from direct linear plots (10) were 0.7 mM and 10.7 μmol/min per mg of protein, respectively.

Enzyme activity toward octyl sulfate. Maximum enzyme activity (5-min incubation period) was obtained at a 10 mM concentration of octyl sulfate in the presence of 0.1 M Tris-maleate buffer at the optimum pH of 6.0. There was some inhibition by excess substrate. Similar results were obtained over a 10-min incubation period, the relationship between enzyme activity and time of incubation being linear during that time. About 85% of the substrate was hydrolyzed when incubation was prolonged for 24 h. A linear relationship between enzyme concentration and activity was obtained provided that no more than 12% of the substrate was hydrolyzed.

TABLE 1. Summary of the purification procedure for Crag herbicide sulfatase from *P. putida* FLA

Stage	Procedure	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)	Purification (fold)
1	Cell extraction	170	6,460	21.8	0.0034	100	1.0
2	Streptomycin sulfate[a]	180	2,916	39.6	0.0136	182	4.0
3	Heat treatment	170	1,156	47.6	0.0412	218	12.2
4	DEAE-cellulose	55	4.73	16.5	3.49	75.6	1,036
5	Sephacryl 200-S	26	1.92	12.0	6.23	54.9	1,849
6	Butyl agarose	40	1.20	10.8	9.00	49.5	2,670

[a] This treatment resulted in the reduction of nucleic acid content from 12.6 to 2.7% of the UV-absorbing material.

K_m and V_{max} values were 2.6 mM and 75.0 μmol/min per mg, respectively, by the Lineweaver-Burk method, and 2.7 mM and 81.7 μmol/min per mg, respectively, from direct linear plots.

Enzyme activity toward 2-butoxyethyl sulfate. Because limited amounts of 2-butoxyethyl sulfate were available, it was necessary to restrict assays to substrate concentrations from 1 to 10 mM. Under these conditions, the release of inorganic sulfate was linear with time up to at least 8% hydrolysis. K_m and V_{max} values were, respectively, 34 mM and 11.5 μmol/min per mg (Lineweaver-Burk method) and 28 mM and 9.6 μmol/min per mg (direct linear plot). These values must be interpreted with caution because the substrate concentration used was well below the estimates of K_m.

^{18}O incorporation experiments. ^{18}O incorporation studies with all three substrates yielded identical results. In all cases, the ^{18}O component of $H_2{}^{18}O$ was incorporated quantitatively into the liberated inorganic sulfate, and no ^{18}O in excess of natural abundance appeared in the respective alcohols. These findings clearly establish that enzymatic fission of the O-S bond of the C-O-S ester sulfate linkage had occurred, a surprising result in light of the fact that of four microbial alkylsulfatases (one primary and three secondary) that had previously been examined (2, 3, 17), all were shown to rupture the C-O bond of the ester linkage of their substrates. However, the results were taken as confirmation that a single enzyme was responsible for the activity toward the three different substrates.

DISCUSSION

Despite the inability of *P. putida* FLA to grow on Crag herbicide as the sole source of carbon, energy, and sulfur, or to convert 2-(2,4-dichlorophenoxy)ethanol to 2,4-D, the bacteria exhibited a high capacity for desulfating the herbicide to give the parent alcohol. Thus, although other soil organisms must be involved in generating 2,4-D, *P. putida* FLA could have an important role to play in initiating the activation and ultimate biodegradation of the herbicide. The single, constitutive enzyme responsible for the desulfation is one of six alkylsulfatases produced when the organism is grown on broth. Alkylsulfatase activity may be present or inducible in many different bacteria inhabiting soil and fresh and salt water (for reviews, see references 8 and 9), and complex patterns of activity exist in the few organisms in which an adequate search has been made. The reasons for such complexity may reflect the different types of alkyl sulfate substrates that occur naturally or are introduced into the environment by humans (9). The surfactant-degrading organism *Pseudomonas* sp. strain C12B provides a good example of alkylsulfatase complexity in that it produces five distinct enzymes under suitable growth and inducer conditions. Two of the enzymes attack primary alkyl sulfates, and the remainder attack different types of secondary alkyl sulfates (8, 9). Crag herbicide cannot serve as a substrate for any of them. *P. putida* FLA appears to produce an even more complex alkylsulfatase pattern, even when grown on broth alone without added inducers.

The FP1 alkylsulfatase of *P. putida* FLA presents two features of considerable interest. First, it exhibits multiple specificity. Not only can it hydrolyze simple alkyl sulphates such as octyl sulfate and SDS, but also two ethoxy sulfate esters of quite different chemical structures (Fig. 1). Surprisingly, the enzyme exhibits no detectable activity towards dodecyltriethoxy sulfate (Fig. 1). Second, the mechanism of action of the enzyme as an O-S bond splitter must be very different from those exhibited by the other alkylsulfatases so far examined (the P1, S1, and S3 enzymes of *Pseudomonas* sp. strain C12B and the CS2 enzyme of *Comamonas terrigena*), all of which operate by fission of the C-O bond (8, 9). The optimum pH of the FP1 enzyme is considerably more acidic than those of the C-O bond-splitting enzymes (9), and this too may reflect differences in the mechanism of action. Studies on the C-O bond-splitting enzymes have revealed that increasing the length of the alkyl chain exerts considerable influence in determining the affinity of substrate for the enzyme (1, 4,

17). It will be surprising if similar considerations apply to the FP1 enzyme, the binding site of which is apparently capable of accepting a ring structure as well as alkyl and short-chain alkylethoxylate chains but not the long-chain dodecyltriethoxy sulfate. Another intriguing kinetic feature of the FP1 enzyme is that 2-(2,4-dichlorophenoxy)ethyl sulfate and 2-butoxyethyl sulfate, although bound with markedly different affinities, are hydrolyzed with the same V_{max}, and moreover, this V_{max} is much lower than that for the octyl sulfate. Clearly, it will now be important to investigate the mechanism of action of that enzyme in detail and to carry the specificity studies further.

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