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Stereochemical Studies on a Plasmid-Coded Fluoroacetate Halidohydrolase

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The stereochemical course of action of haloacetate halidohydrolase H-1 from *Pseudomonas* sp., strain A, which catalyzes the dehalogenation of fluoroacetate to glycolate, has been determined by enzymatic analysis of products from incubations with both enantiomers of 2-fluoropropionate, and by ^{1}H NMR analysis of the ester of (−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid with phenacyl [2-^{2}H$_1$]glycolate derived from the product of incubation with the (*S*)-monodeuterofluoroacetate. The results support a direct displacement mechanism for this enzyme, since they indicate that the reaction is catalyzed with inversion of configuration.

INTRODUCTION

Fluoroacetate, a naturally occurring compound with a rare carbon–fluorine linkage, is toxic because of metabolic processing to 2-fluorocitrate (*1*), specifically the (−)-erythro (2*R*, 3*R*) diastereomer (*2–4*), whose target has long been held to be aconitase (but see Ref. (*5*)). Microorganisms resistant to fluoroacetate have been detected. Some yeasts overproduce aconitase (*6*), the proposed target enzyme for fluorocitrate toxicity, while various bacteria induce haloacid halidohydrolases (dehalogenases), which detoxify 2-haloacids by conversion to 2-hydroxyacids. Goldman *et al.* (*7*) have previously noted that an impure enzyme from a pseudomonad will convert L-2-chloropropionate(2*S*) to D-lactate with net inversion of configuration. Very recently, Motosugi *et al.* (*8*) purified a haloacid dehalogenase from a pseudomonad grown on DL-2-chloropropionate, and found that the pure enzyme will convert both D- and L-2-chloropropionate to the corresponding L- and D-lactates, respectively. This enzyme will also work on chloroacetate, bromoacetate, and iodoacetate, but it shows no activity toward fluoroacetate or other 2-fluoroacids, perhaps because of the low reactivity of C–F bonds to S_N2 displacements (*9, 10*). On the other hand, Kawasaki and colleagues (*11, 12*) have found pseudomonads with constitutive resistance to fluoroacetate by virtue of harboring a plasmid encoding a fluoroacetate-specific halidohydrolase, H-1, which they have purified and crystallized, making it the enzyme of choice to analyze how enzymic cleavage of the strong C–F bond by the weak nucleophile H_2O (or hydroxide ion, enzyme's pH optimum = 9) is effected in fluoroacetate detoxification (See Scheme 1). Kawasaki *et al.* have shown that the H-1 halidohydrolase is

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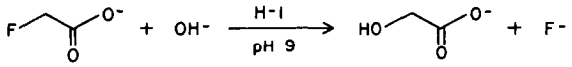


SCHEME 1. Reaction catalyzed by haloacetate halidohydrolase H-1.

inactivated by sulfhydryl-blocking reagents, raising the possibility that an active-site cysteine could be involved in covalent catalysis, e.g., by way of an *S*-carboxymethyl enzyme intermediate, as proposed initially by Goldman (*13*). This possibility was also raised by the very recent observation by Weightman *et al.* (*14*) that certain bacteria contain two halidohydolase activities separable at a crude extract stage by gel electrophoresis. One activity converts chiral chloropropionate to lactate with inversion, the other with retention of configuration at carbon two; this latter one shows a markedly higher sensitivity to sulfhydryl-blocking reagents.

We have now investigated the stereochemical course of action of purified haloacetate halidohydrolase from *Pseudomonas* sp., strain A, with both enantiomers of 2-fluoropropionate and the (*S*) enantiomer of monodeuterated fluoroacetate to analyze stereochemical outcome on a 2-fluoroacid substrate for the first time.

EXPERIMENTAL PROCEDURES

Methods. Haloacetate halidohydrolase activity was assayed with an Orion fluoride ion electrode by monitoring fluoride ion production from incubations carried out according to the procedure of Kawasaki *et al.* (*11*). Standards containing known amounts of fluoride ion in a background solution made up of the components of the quenched incubation mixtures (except enzyme) were used to prepare calibration curves for the fluoride electrode. D-Lactate was measured by the procedure of Gawehn and Bergmeyer (*15*). L-Lactate was measured by the procedure of Gutmann and Wahlefeld (*16*). ^{1}H NMR spectra were recorded with a JEOL FX-90Q, Bruker WM250 or Bruker WM270 spectrometer. Chemical shifts are reported in ppm on the δ scale relative to internal standards (tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate). Abbreviations used to present NMR data are the following: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets; dq, doublet of quarters; *J*, coupling constant. The relaxation delay was set to 4.0 s for deuterated compounds. ^{19}F NMR spectra were recorded with a JEOL FX-90Q spectrometer. UV measurements were made with a Perkin–Elmer 554 or Lambda 5 spectrophotometer.

Materials. Haloacetate halidohydrolase H-1 was purified from *Pseudomonas* sp., strain A (kindly provided by Dr. H. Kawasaki, College of Agriculture, University of Osaka) by the procedure of Kawasaki *et al.* (*11*), with the following modifications. (a) A DE52 column (2.5 × 34 cm), which was run twice, was substituted for the DEAE-cellulose column, using the same elution buffer as in Ref. (*11*); (b) After purification on hydroxylapatite, the enzyme was concentrated by ultrafiltration (Amicon PM 10 membrane), frozen in liquid nitrogen, and stored

at −70°C. The specific activity of the enzyme was 32.2 μmol min^{-1} mg^{-1} at 30°C (lit. value, 38.4 U/mg for crystalline enzyme (*11*)).

L-Lactic dehydrogenase (LDH) from rabbit muscle was purchased from Boehringer–Mannheim. Hydroxylapatite (HTP) and AG50W-X8 (50–100 mesh) were from Bio-Rad. DE52 was from Whatman. D-Lactic dehydrogenase from *Lactobacillus leichmanii,* L-alanine aminotransferase (glutamic pyruvic transaminase) from porcine heart, L-alanine, D-alanine, glycine-d_5, NAD, NADH, and glycolic acid were purchased from Sigma. (*S*)-[2-$^2H_1$]Glycolic acid was prepared by the method of Massey *et al.* (*17*) by reduction of dimethyloxalate with Mg in D_2O to deuterated glyoxylic acid followed by reduction of the glyoxylate with L-LDH and NADH. Hydrogen fluoride–pyridine, (−)-MTPA [(*S*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid] and (*R*)-(−)-mandelic acid were from Aldrich. Analytical and preparative TLC were performed on E. Merck precoated silica gel 60F-254 plates. Flash column chromatography was carried out on E. Merck silica gel 60 (230–400 mesh, ASTM). All other chemicals were of reagent grade and were used without further purification, unless otherwise noted.

Substrates. The (*R*) and (*S*) isomers of 2-fluoropropionic acid were prepared from D- and L-alanine, respectively, by the procedure of Olah *et al.* (*18*) for the synthesis of 2-fluorobutanoic acid, except that D- or L-alanine was substituted for 2-aminobutanoic acid. The products were purified by distillation under reduced pressure. ^{1}H NMR (250 MHz) ($CDCl_3$) δ 9.9 (1 H, broad), 5.08 (1 H, dq, J_{HH} = 6.9 Hz, J_{HF} = 48 Hz), 1.64 (3 H, dd, J_{HH} = 6.9 Hz, J_{HF} = 24 Hz). The ^{19}F NMR (D_2O) showed a doublet of quartets (J = 24 Hz, 48 Hz).

(*S*)-2-Chloropropionic acid was prepared from L-alanine by the method of Fu *et al.* (*19*). ^{1}H NMR (250 MHz) ($CDCl_3$) δ 10.1 (1 H, broad), 4.46 (1 H, q, J = 6.9 Hz), 1.74 (3 H, d, J = 6.8 Hz).

The concentration of the 2-halopropionic acids in aqueous solution was determined by titration with a standard solution of NaOH.

(*S*)-[2-$^2H_1$]Fluoroacetic acid was prepared from (*S*)-[2-$^2H_1$]glycine by the procedure of Keck (*20*), which was essentially analogous to the method used to prepare fluoropropionic acid, except that continuous extraction was used to extract the product from the quenched reaction mixture. ^{1}H NMR (90 MHz) ($NaOD/D_2O$) δ 4.7 (1 H, dt, J_{HD} = 2 Hz, J_{HF} = 46 Hz). The ^{19}F NMR (D_2O) showed a doublet of triplets (J_{HF} = 46 Hz, J_{DF} = 7.3 Hz), plus a smaller signal due to dideuterated fluoroacetate (m, J_{DF} = 7.3 Hz) and a tiny signal due to nondeuterated fluoroacetate (t, J_{HF} = 48 Hz). (*S*)-[2-$^2H_1$]Glycine was prepared from glycine-d_5 by the following procedure, based on that of Keck (*20*). Glycine-d_5 (1.05 g) was placed in a flask with 0.106 g pyridoxal 5′-phosphate, and 200 ml 10 m*M* KP_i buffer, pH 6.5. The pellet from centrifugation of 1000 U (10 mg) L-alanine aminotransferase was resuspended in 10 ml of the buffer, and added to the reaction mixture at 37°C. After incubation for 48 hr, the reaction was quenched with trichloroacetic acid and then neutralized with KOH. The resulting solution was applied to an AG50W-X8 (H^+ form) 50–100 mesh column (2.5 × 35 cm), washed with 2 liters of deionized-distilled water, and then eluted with 700 ml 2 N NH_4OH. The NH_4OH eluate was concentrated to dryness under reduced pressure, and the residue was recrystallized in ethanol/water to give 0.78 g product. ^{1}H NMR (D_2O) analysis of the

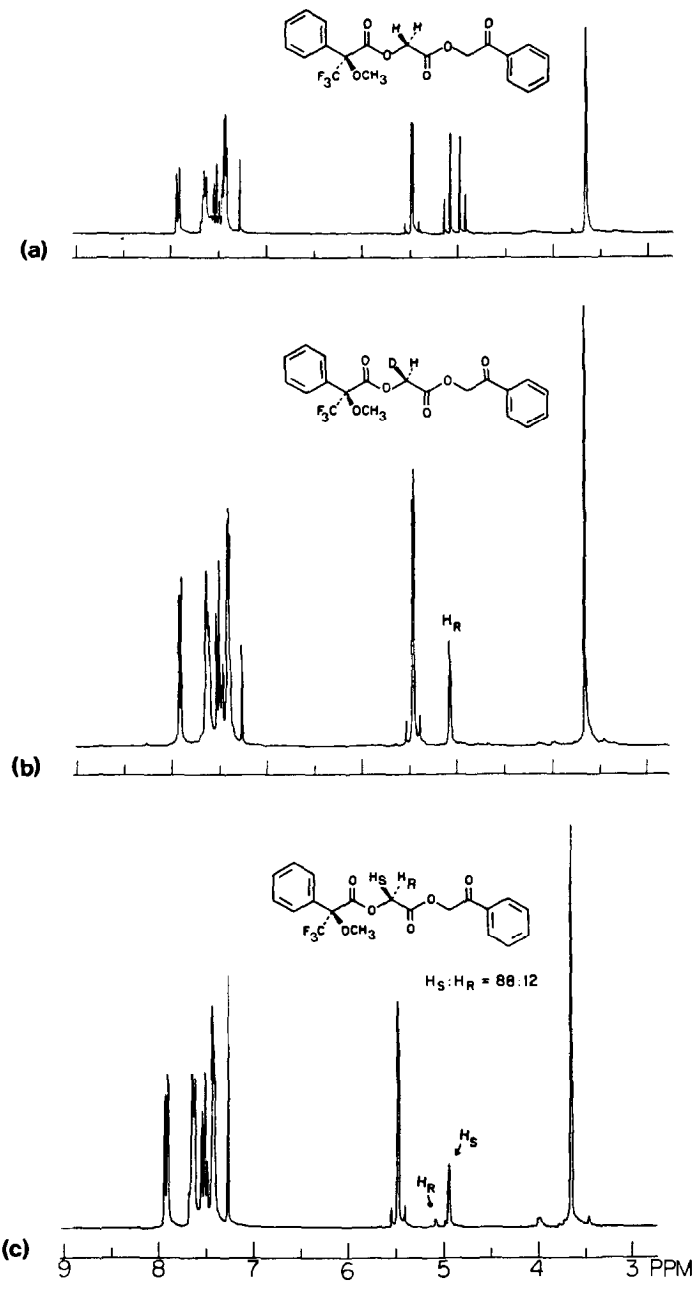


FIG. 1. ^1H NMR (250 MHz) spectra ($CDCl_3$): (a) (*S*)-(−)-MTPA, phenacyl glycolate ester; (b) (*S*)-(−)-MTPA esterified with the phenacyl ester of authentic (*S*)-[2-$^2H_1$]glycolate; and (c) (*S*)-(−)-MTPA esterified with the phenacyl ester of the incubation product of (*S*)-[2-$^2H_1$]-fluoroacetate with halidohydrolase H-1.

methyl ester of the product prepared by the method of Rachele (*21*) showed that 25–30% of the glycine was still dideuterated at C-2.

Derivatives of substrates and incubation products. The esters of 2-haloacids with methyl (*R*)-mandelate and the esters of (−)-MTPA with deuterated and nondeuterated phenacyl glycolate were prepared by the procedure of Neises and Steglich at 0°C (*22*), with the amounts of reagents scaled down appropriately for the amount of haloacid or alcohol to be esterified. Typically 0.1–1.0 mmol of the limiting acid or alcohol was used. The products were purified by flash column chromatography on silica gel with ethyl acetate/hexane (typically 25–35% ethyl acetate) as the solvent. Diastereomeric products eluted together in this solvent system. (*R*)-2-Fluoropropionic acid esterified with methyl (*R*)-mandelate: 250-MHz ^{1}H NMR ($CDCl_3$) δ 7.48–7.39 (5 H, aromatic), 6.02 (1 H, s), 5.14 (1 H, dq, J_{HH} = 6.9 Hz, J_{HF} = 48 Hz; this signal was partially overlapped by a small dq downfield), 3.74 (3 H, s), 1.71 (2.4 H, dd, J_{HH} = 6.9 Hz, J_{HF} = 24 Hz), 1.63 (0.6 H, dd, J_{HH} = 6.8 Hz, J_{HF} = 24 Hz). (*S*)-2-Fluoropropionic acid esterified with methyl (*R*)-mandelate: 250-MHz ^{1}H NMR ($CDCl_3$) δ 7.49–7.38 (5 H, aromatic), 6.03 (1 H, s), 5.18 (1 H, dq, J_{HH} = 6.9 Hz, J_{HF} = 48 Hz; this signal was partially overlapped by a small dq upfield), 3.74 (3 H, s), 1.71 (0.48 H, dd, J_{HH} = 6.8 Hz, J_{HF} = 24 Hz), 1.63 (2.52 H, dd, J_{HH} = 6.9 Hz, J_{HF} = 23 Hz). (*R*)-2-Chloropropionic acid esterified with methyl (*R*)-mandelate: 250-MHz ^{1}H NMR ($CDCl_3$) δ 7.49–7.39 (5 H, aromatic), 5.98 (1 H, s), 4.56 (1 H, q, J = 7.0 Hz), 3.74 (3 H, s), 1.79 (3 H, d, J = 7.0 Hz). (*S*)-2-Chloropropionic acid esterified with methyl (*R*)-mandelate: 250-MHz ^{1}H NMR ($CDCl_3$) δ 7.50–7.39 (5 H, aromatic), 5.98 (1 H, s), 4.54 (1 H, q, J = 7.2 Hz), 3.74 (3 H, s), 1.78 (0.2 H, d, J = 7.2 Hz), 1.75 (2.8 H, J = 7.2 Hz). Fluoroacetic acid esterified with methyl (*R*)-mandelate: 270-MHz ^{1}H NMR ($CDCl_3$) δ 7.48–7.39 (5 H, aromatic), 6.07 (1 H, s), 5.04 (1 H, dd, J_{HH} = 15.3 Hz, J_{HF} = 47 Hz), 4.95 (1 H, dd, J_{HH} = 15.3 Hz, J_{HF} = 47 Hz), 3.74 (3 H, s). (*S*)-[2-$^2H_1$]Fluoroacetic acid esterified with methyl (*R*)-mandelate: 250-MHz ^{1}H NMR ($CDCl_3$) δ 7.45–7.39 (5 H, aromatic), 6.07 (1 H, s), 5.02 (0.7 H, dt, triplet unresolved, J_{HF} = 47 Hz), 4.96 (0.08 H, dt, triplet unresolved, J_{HF} = 47 Hz; some small unresolved peaks, presumably due to nondeuterated compound are also present), 3.75 (3 H, s). (−)-MTPA esterified with phenacyl glycolate: 250-MHz ^{1}H NMR ($CDCl_3$) δ 7.93–7.41 (10 H, aromatic), 5.51 (1 H, d, J = 16.3 Hz), 5.43 (1 H, d, J = 16.3 Hz), 5.10 (1 H, d, J = 15.8 Hz), 4.94 (1 H, d, J = 15.7 Hz), 3.65 (3 H, broad singlet). (−)-MTPA esterified with phenacyl (*S*)-[2-$^2H_1$]glycolate: 250-MHz ^{1}H NMR ($CDCl_3$) δ 7.91–7.39 (10 H, aromatic), 5.50 (1 H, d, J = 16.2 Hz), 5.42 (1 H, d, J = 16.2 Hz), 5.07 (1 H, unresolved triplet), 3.64 (3 H, broad singlet). (−)-MTPA esterified with phenacyl [2-$^2H_1$]glycolate derived from H-1 incubation with (*S*)-[2-$^2H_1$]fluoroacetate: 250-MHz ^{1}H NMR ($CDCl_3$) δ 7.92–7.40 (10 H, aromatic), 5.50 (1 H, d, J = 16.3 Hz), 5.43 (1 H, d, J = 16.4 Hz), 5.08 (0.09 H, unresolved triplet), 4.93 (0.63 H, unresolved triplet), 3.65 (3 H, broad singlet); three small peaks due to the nondeuterated species were observed at 5.13, 5.07, and 4.97 ppm (a fourth peak was obscured by the signal due to the monodeutero species).

The methyl ester of (*R*)-(−)-mandelic acid was prepared according to the procedure of Rachele (*21*). The product, recrystallized from petroleum ether and derivatized with (−)-MTPA by the procedure of Ref. (*22*), was shown to be enan-

tiomerically pure by comparison of its ^1H NMR spectrum ($CDCl_3$) with that of the ester of (−)-MTPA with racemic methyl mandelate.

Phenacyl esters of glycolate and fluoroacetate were prepared by the method of Clark and Miller (*23*), using commercially available α-bromoacetophenone, which was recrystallized from petroleum ether before use.

RESULTS AND DISCUSSION

Stereochemical Studies with (R)- and (S)-2-Fluoropropionate

The procedure of Olah *et al.* (*18*), employing HF–pyridine and sodium nitrite, was used to convert D-alanine (2*R*) and L-alanine (2*S*) to (*R*)-2-fluoropropionate and (*S*)-2-fluoropropionate, respectively. Keck and Retey (*24*) have reported that this reagent mix produces retention of configuration for several amino acids, although Lowe and Potter (*25*), in its use in the preparation of 2-fluorosuccinates, present evidence that partial racemization may occur.

In the incubation of the synthesized (*S*)-2-fluoropropionate sample with halidohydrolase H-1, the initial rate of fluoride ion production, assayed by fluoride ion electrode, was found to be about 9% of the V_{max} observed for fluoroacetate conversion. When the (*R*)-2-fluoropropionate sample was tested as a substrate, an even lower rate of fluoride ion production (about 5% of the V_{max} with fluoroacetate) was measured. We suspected that this fluoride ion formation might derive from enzymatic processing of contaminating (*S*)-2-fluoropropionate in the (*R*)-2-fluoropropionate sample generated in a stereoselective but not stereospecific synthetic route. We therefore assayed the chiral purity of the (*R*)- and (*S*)-2-fluoropropionate samples by NMR analysis of their esters with methyl (*R*)-(−)-mandelate. The diastereomeric composition of these derivatives as determined by 250-MHz ^1H NMR suggested that the "(*R*)" sample contained about 20% (*S*) isomer and the "(*S*)" sample contained about 16% (*R*) isomer. Apparently the Olah procedure in the present case yielded a mixture (about 4 : 1 in favor of retention) of 2-fluoropropionates from D- and L-alanine.

In order to further define the stereochemical course of this enzymatic reaction, large-scale incubations of the "(*R*)"- and "(*S*)"-2-fluoropropionate samples (10 μmol each) were conducted for 30 min with 0.35 mg enzyme each (sp act, 32.2 U/mg) in duplicate samples and blanks, and the amounts of D-lactate, L-lactate, and fluoride ion product were analyzed. From the "(*R*)"-2-fluoropropionate, 1.8 ± 0.1 μmol D-lactate was produced, a nominal 18% conversion, while 6.9 ± 0.2 μmol D-lactate was produced from the "(*S*)"-2-fluoropropionate, a 69% conversion. No significant L-lactate was detected in either sample. Similar results were obtained by fluoride ion electrode assay, since the "(*R*)" sample yielded 1.7 ± 0.0 μmol F^- ion, while the "(*S*)" sample yielded 6.6 ± 0.2 μmol F^- ion. As reported previously by Goldman (*7*), partially purified haloacid degrading enzyme used only the (*S*) isomer of 2-chloropropionate. Incubation of (*S*)-2-chloropropionate (9.3 μmol) with purified enzyme (0.86 mg) for 1 hr, did lead to conversion of 84% of the substrate (7.8 μmol) to D-lactate, with no significant production of L-lactate. The

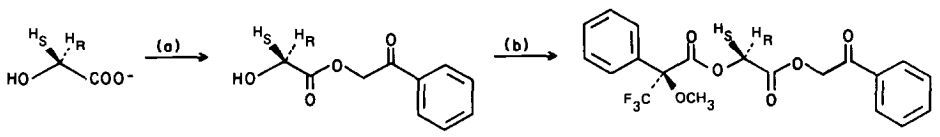
enantiomeric purity of the (*S*)-2-chloropropionate was estimated to be at least 93% by 250-MHz ^{1}H NMR analysis of its ester with methyl (*R*)-(−)-mandelate. These results indicate that the H-1 halidohydrolase processes (*S*)-2-fluoropropionate with inversion, and that any D-lactate produced from the "(*R*)" enantiomer is explicable by the approximately 20% contamination of (*S*) enantiomer present.

Chiral Fluoroacetate Processing

Because the H-1 halidohydrolase apparently recognizes only one enantiomer of 2-fluoropropionate, and even then at about 1/10 the V_{max} rate with fluoroacetate, we decided to examine the stereochemistry of processing of the preferred substrate fluoroacetate, which would be free of these constraints. For this purpose we needed a chiral fluoroacetate sample and an assay for the chirality of the enzymatic product, glycolate. Keck *et al.* (*26*) have reported the preparation of chiral monodeuterofluoroacetates by the Olah procedure. Enzymatic conversion should yield chiral monodeuteroglycolate.

(*S*)-[2-^{2}H$_1$]Glycine was prepared by enzymatic exchange at the ^2H_R position of glycine-d_5 in H_2O with L-alanine aminotransferase. The ^{1}H NMR of the corresponding methyl ester prepared by the method of Rachele (*21*) indicated that about 0.70–0.75 proton equivalent was incorporated at C-2 of glycine. Therefore, about 25% of the glycine product was still dideuterated at C-2. Olah's procedure was then used to generate (*S*)-[2-^{2}H$_1$]fluoroacetate. The chiral purity at C-2 of the synthesized monodeuterated fluoroacetate sample was determined by 250-MHz ^{1}H NMR of its ester with methyl (*R*)-(−)-mandelate. (The NMR spectrum of nondeuterated fluoroacetate esterified with methyl (*R*)-mandelate showed sufficient resolution of the diastereotopic C_2-protons.) As expected, about 25% of the fluoroacetate molecules were still dideuterated at C-2, 70–75% were monodeuterated, and a small amount was the diprotio species. The exact amount of diprotio species was not measurable because of insufficient resolution of its ^{1}H NMR signal from that due to the monodeutero species. The monodeutero species (plus diprotio contributors at H_R and H_S) showed a net H_R/H_S ratio of 90/10. The presence of the dideutero species should not complicate the ^{1}H NMR analysis of enzyme product chirality, since the dideutero species should make no contribution to the H_R and H_S signals of either the derivatized substrate or derivatized product.

The synthetic (*S*)-[2-^{2}H$_1$]fluoroacetate sample (50 mg) was incubated with 87 units enzyme for 40 min at 30°C, and reaction progress was monitored by assay of removed aliquots for F^- production. The incubation mixture was brought to pH 1, and then continuously extracted with 25 vol of ether for 3 days. The collected ether extract was evaporated, and the residue was neutralized with 1 N LiOH. The water was then removed under reduced pressure, and the resulting [2-^{2}H$_1$]glycolate was converted to its phenacyl ester and separated from the phenacyl ester of unreacted fluoroacetate by preparative TLC (silica plate, 1% methanol/chloroform). TLC of the product of phenacyl esterification of a control sample (containing no enzyme) showed that no phenacyl glycolate was produced from the control. The recovered phenacyl [2-^{2}H$_1$]glycolate was recrystallized from ether/hexane,



SCHEME 2. Derivatization of glycolate for stereochemical analysis by NMR: (a) $PhCOCH_2Br/KF/DMF$, 25°C; and (b) (*S*)-(−)-MTPA/DCC/4-dimethylaminopyridine/CH_2Cl_2, 0°C.

further derivatized on its hydroxyl group with (−)-MTPA, and analyzed by ^1H NMR at 250 MHz (See Scheme 2).

The ^1H NMR spectra of two standards employed for reference and of the derivatized enzyme incubation product are shown in Figs. 1a, b, and c. As shown in Fig. 1a, a baseline-resolved AB quartet was observed for the now diastereotopic C-2 methylene hydrogens of (−)-MTPA esterified with nondeuterated phenacyl glycolate. Assignment of the low-field and high-field doublets as the H_R and H_S resonances, respectively, was based on the NMR spectrum of (−)-MTPA esterified with the phenacyl ester of authentic (*S*)-[2-$^2H_1$]glycolate, prepared by reduction of [2-$^2H_1$]glyoxylate with NADH and L-LDH. Integration of the corresponding C-2 methylene peaks in Fig. 1c showed an 88/12 ratio of protons at the H_S position to protons at the H_R position. Therefore, the [2-$^2H_1$]glycolate incubation product was predominantly the (*R*) enantiomer. Since, within experimental error, the (*S*)-[2-$^2H_1$]fluoroacetate sample (H_R/H_S = 90/10) was converted stereospecifically to predominantly (*R*)-[2-$^2H_1$]glycolate (H_R/H_S = 12/88), the enzymatic reaction catalyzed by halidohydrolase H-1 must proceed with inversion of configuration at C-2 of fluoroacetate.

These combined stereochemical results render unlikely a double-displacement mechanism, involving a covalent enzyme–substrate intermediate (unless one step goes with inversion and the other with retention), and favor the direct displacement process for this enzyme. Since this enzyme is the only one purified to homogeneity which carries out the decomposition of fluoroacetate to glycolate, it is the prime candidate for subsequent mechanistic studies on the cleavage mechanism of the strong C–F bond. Because the enzyme is encoded by a stable multicopy plasmid with constitutive expression, it is available in large quantities, and recombinant methodology for enzyme structure analysis will be possible. This will ultimately be instructive in determining how organisms carry out C–F cleavage and detoxification (*27*) of this prototypic fluoroacid involved in the celebrated "lethal synthesis" metabolic reactions (*1*).

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