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Differential Reactivity in the Processing of [*p*-(Halomethyl)benzoyl]formates by Benzoylformate Decarboxylase, a Thiamin Pyrophosphate Dependent Enzyme†

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ABSTRACT: A series of [*p*-(halomethyl)benzoyl]formates have been investigated as substrates for benzoylformate decarboxylase. These analogues vary from acting as normal substrates to acting as potent competitive inhibitors. The fluoro analogue is a substrate with K_m (190 μM) and turnover number (20 s^{-1}) similar to those of benzoylformate (K_m = 340 μM; 81 s^{-1}). The bromo analogue is a competitive inhibitor (K_i = 0.3 μM) and exhibits processing to eliminate bromide and form (*p*-methylbenzoyl)thiamin pyrophosphate. This modified cofactor hydrolyzes to form the *p*-methylbenzoate in quantitative yield. The chloro analogue [K_m(app) = 21 μM] partitions between these two pathways such that 0.6% of the analogue ultimately forms *p*-methylbenzoate. These data are consistent with the interpretation that the leaving group potential of the halogen determines the enzymic fate of the analogue and that the potent inhibition observed for the bromo analogue is due to covalent modification of the cofactor.

Benzoylformate decarboxylase (EC 4.1.1.7; benzoylformate carboxy-lyase; BFD[1]) from *Pseudomonas putida* catalyzes the formation of benzaldehyde from benzoylformate, an α-keto acid. Aside from its characterization as part of the mandelate pathway group (Hegeman, 1966a,b,c), this enzyme has been little studied. Until recently (Weiss et al., 1988), virtually nothing had been reported about the details of the enzymatic mechanism except for its strict requirement for thiamin pyrophosphate as a cofactor.

The proposed mechanism for the reaction catalyzed by BFD (Figure 1) is analogous to that for the formation of acetaldehyde from pyruvate catalyzed by pyruvate decarboxylase. The mechanism involves the formation of a covalent substrate–cofactor intermediate capable of stabilizing the carbanion generated by decarboxylation.

The K_m for benzoylformate has been measured as 1 mM and 0.08 mM in separate studies at pH 6.2 (Hegman, 1970) and pH 6.1 (Weiss et al., 1988), respectively. The K_m for TPP has been reported as 1 μM (Hegman, 1970). Benzoylformate decarboxylase has a higher substrate specificity than pyruvate decarboxylase. Only benzoylformate and para-substituted benzoylformates have been shown to be substrates (Weiss et al., 1988; Hegman, 1970). Pyruvate, α-ketobutyrate, and α-ketoglutarate are not substrates.

Previously, we have reported the inhibition of BFD by [*p*-(bromomethyl)benzoyl]formate (BrMeBF) (Dirmaier et al., 1986). We established that inhibition was due to an unusual enzymatic processing of BrMeBF resulting in decarboxylation, bromide ion elimination, and tautomerization to form a covalently modified cofactor (*p*-methylbenzoyl-TPP). In this paper we present further studies on a series of [*p*-(halomethyl)benzoyl]formates. We demonstrate that these analogues vary from acting as substrates for the enzyme to acting as potent competitive inhibitors. We postulate a common intermediate for these analogues and propose that halogen leaving group potential determines the partitioning between inhibition and normal substrate turnover.

Materials and Methods

Horse liver alcohol dehydrogenase (HLADH), thiamin pyrophosphate chloride (TPP), NADH, Hepes, and Tris were

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[1] Abbreviations: BFD, benzoylformate decarboxylase; BF, benzoylformate; MeBF, (*p*-methylbenzoyl)formate; BrMeBF, [*p*-(bromomethyl)benzoyl]formate; ClMeBF, [*p*-(chloromethyl)benzoyl]formate; FMeBF, [*p*-(fluoromethyl)benzoyl]formate; HOMeBF, [*p*-(hydroxymethyl)benzoyl]formate; TPP, thiamin pyrophosphate; HLADH, horse liver alcohol dehydrogenase; NADH, nicotinamide adenine dinucleotide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Tris, tris(hydroxymethyl)aminomethane.

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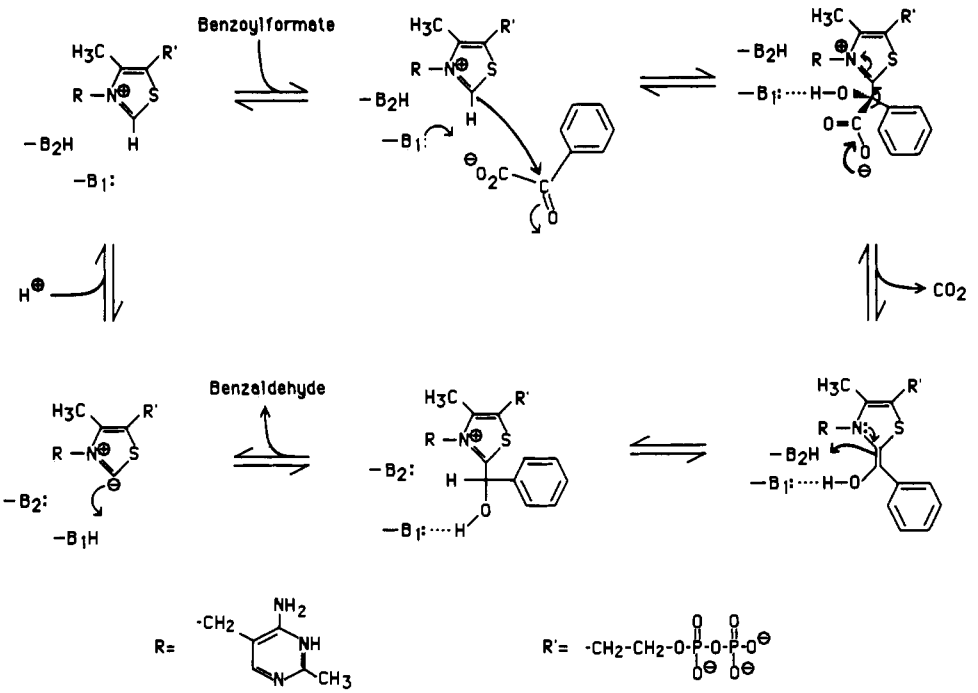

FIGURE 1: Mechanism for benzoylformate decarboxylase.

obtained from Sigma Chemical Co. *p*-(Bromomethyl)benzaldehyde was synthesized by Stephen's reduction of *p*-cyanobenzyl bromide (Aldrich) with $SnBr_2$ and HBr (Baker et al., 1956; Tanner et al., 1980). All other chemicals were of the highest purity available.

(p-Methylbenzoyl)formate and [p-(Hydroxymethyl)benzoyl]formate. (*p*-Methylbenzoyl)formate (MeBF) and [*p*-(hydroxymethyl)benzoyl]formate (HOMeBF) were synthesized as described by Barnish et al. (1981). Friedel-Crafts acylation of toluene with ethyloxalyl chloride gave ethyl (4-methylbenzoyl)formate. Hydrolysis of the ester in base yielded MeBF (mp 90–92 °C): ^{1}H NMR (acetone-d_6) δ 7.9 (2 H, d), 7.35 (2 H, d), 2.3 (3 H, s); UV (H_2O) λ_{max} 264 nm (ϵ 22950 M^{-1} cm^{-1}). Bromination of the ethyl ester with *N*-bromosuccinimide followed by reaction with silver acetate gave ethyl [4-(acetoxymethyl)benzoyl]formate. Hydrolysis in base yielded HOMeBF (mp 111–113 °C): ^{1}H NMR (acetone-d_6) δ 7.95 (2 H, d), 7.5 (2 H, d), 4.7 (2 H, s); UV (H_2O) λ_{max} 259 nm (ϵ 14800 M^{-1} cm^{-1}).

[p-(Bromomethyl)benzoyl]formate. (*p*-Methylbenzoyl)formic acid was treated with 1.1 equiv of *N*-bromosuccinimide in refluxing CCl_4 for 90 min in the presence of a trace of benzoyl peroxide and Pyrex-filtered UV light (275 W). The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Recrystallization from hot toluene/hexane afforded pale yellow crystals of BrMeBF (mp 68–70 °C; yield 54%): UV (H_2O) λ_{max} 265 nm (ϵ 22500 M^{-1} cm^{-1}); ^{1}H NMR (acetone-d_6) δ 7.5 (2 H, d), 7.15 (2 H, d), 4.2 (2 H, s). Anal. Theoretical for $C_9H_7O_3Br \cdot H_2O$: C, 41.41; H, 3.47. Found: C, 41.14; H, 3.20.

[p-(Chloromethyl)benzoyl]formate. (*p*-Methylbenzoyl)formate was treated with 6 equiv of sulfuryl chloride under UV light (275 W). After 20 min the starting material was completely dissolved. The progress of the reaction was monitored by ^{1}H NMR by observing the shift of the methyl peak (δ 2.6) to the chloromethylene (δ 4.7). The reaction was terminated after 45 min at about 80% completion when a peak at δ 6.8 corresponding to the dichloro compound began to appear. Removal of the sulfuryl chloride in vacuo left an orange oil which solidified on standing.

Attempts at purification of the crude ClMeBF were unsatisfactory so the acid was converted to its *tert*-butyl ester following a modification of a procedure by Anderson and Callahan (1960). One gram of crude acid was dissolved in 50 mL of methylene chloride containing 0.1 mL of concentrated sulfuric acid. The solution was cooled over ice, saturated with isobutylene, and then allowed to react overnight at room temperature. The reaction mixture was extracted with 5% sodium carbonate. The aqueous layer was reextracted with methylene chloride, and the combined organic layers were dried over sodium sulfate, filtered, and concentrated in vacuo. The *tert*-butyl ester product was chromatographed twice on a flash column (Still et al., 1978) containing 50 g of silica gel (5% ethyl acetate, 95% hexane) which yielded 400 mg of *tert*-butyl [*p*-(chloromethyl)benzoyl]formate: ^{1}H NMR ($CDCl_3$) δ 7.9 (2 H, d), 7.4 (2 H, d), 4.6 (2 H, s), 1.7 (9 H, s).

The *tert*-butyl ester was converted to the free acid by cleavage in neat trifluoroacetic acid (Cornforth et al., 1969). After 75 min at room temperature, the trifluoroacetic acid was removed in vacuo. Trituration of the remaining oil with ice-cold water resulted in the formation of white crystals (mp 43–45 °C): UV (H_2O) λ_{max} 259 nm (ϵ 23380 M^{-1} cm^{-1}); ^{1}H NMR (acetone-d_6) δ 8.07 (2 H, d), 7.69 (2 H, s), 4.83 (2 H, s). Anal. Theoretical for $C_9H_7O_3Cl \cdot 0.75H_2O$: C, 51.03; H, 4.03; Cl, 16.74. Found: C, 50.99; H, 4.05; Cl, 16.77.

[p-(Fluoromethyl)benzoyl]formate. *p*-(Fluoromethyl)-acetophenone was prepared from *p*-bromoacetophenone (Jarvis & Saukaitis, 1973) by an anion-exchange resin (Amberlite IRA-900; Fluoride) mediated fluorination (Colonna et al., 1979). Two hundred eighty milligrams (1.84 mmol) of *p*-(fluoromethyl)acetophenone was added to 15 mL of H_2O and stirred to form an emulsion. Potassium permanganate (2 equiv) and potassium hydroxide (2 equiv) were dissolved in 15 mL of H_2O, heated to 70 °C, and then added to the *p*-(fluoromethyl)acetophenone. After being stirred 30 min, the excess permanganate was quenched with sodium sulfite, and the brown manganese dioxide was removed by filtration. The filtrate was extracted with ether to remove starting material, acidified, and reextracted. This second extract was dried over

sodium sulfate, filtered, and concentrated in vacuo to yield 120 mg of impure FMeBF. This product was purified by preparative-scale HPLC on an Altex 5-μm ODS-ultrasphere reverse-phase column (10 mm × 25 cm). The column was eluted with a gradient of 0–60% methanol in water at 4 mL/min and monitored at 240 nm. A peak eluting at 5% methanol afforded FMeBF (yield off column = 20%): ^{1}H NMR (acetone-d_6) δ 8.06 (2 H, d), 7.62 (2 H, d), 5.55 (2 H, d, J = 49 Hz); UV (H_2O) λ_{max} 255 nm (ϵ 20 165 M^{-1} cm^{-1}).

Enzyme Isolation. Benzoylformate decarboxylase was isolated from *Pseudomonas putida* according to the method of Hegmen (1970). The final preparation contained 20.6 units of BFD/mL with 0.6 mg/mL to give a specific activity of 34 units/mg. The molecular mass of the enzyme, as estimated by SDS–PAGE, is 57 500 daltons. The molecular mass and specific activity are consistent with those found by Barrowman and Fewson (1985) for BFD from *Acinetobacter calcoaceticus*.

Coupled Enzyme Assay. The activity of BFD can be monitored by a coupled assay with HLADH. BFD catalyzes the conversion of benzoylformate to benzaldehyde and carbon dioxide. The benzaldehyde produced is reduced to benzyl alcohol by HLADH concomitant with oxidation of NADH. Thus, the activity of the decarboxylase can be followed by observing the loss of absorbance at 340 nm associated with the oxidation of NADH (ϵ = 6200). A typical assay contained 0.3 mM NADH, 1 mM TPP, 1.5 units of HLADH, 0.08 unit of BFD, and 0.2 mM benzoylformate in a total volume of 0.5 mL. The reactions were performed in either 0.01 M K-Hepes or 0.1 M potassium phosphate buffer at pH 7.0. One unit of enzyme was defined as the amount of enzyme catalyzing the decarboxylation of 1 μmol of 200 μM benzoylformate per minute under the above conditions.

UV Assay for [p-(Bromomethyl)benzoyl]formate Reaction. The reaction of BrMeBF with BFD can be monitored by observing the decrease in absorbance of the starting material at 265 nm and the increase in absorbance of the product, *p*-methylbenzoate, at 230 nm. The reaction was performed with 50 μM BrMeBF, 0.08 unit of BFD, and 0.2 mM TPP in 0.25 mL of 0.01 M K-Hepes (pH 7.0). The assay was performed in a 1-mm path-length cuvette with 200–360 nm spectra recorded at 5-min intervals. Since TPP has a significant absorbance in this range, this concentration of TPP was chosen as the minimum concentration necessary to achieve the full reaction rate (as determined from bromide release data).

Kinetics. Kinetic constants for BFD were determined by the coupled assay with substrate concentrations generally at or below K_m (benzoylformate, 75–200 μM; MeBF, 100–600 μM; FMeBF, 25–200 μM; ClMeBF, 12.5–100 μM). The concentration of enzyme used in the determination of turnover numbers was calculated from the following formula: μmol of BFD = no. of units × (1 mg/34 units) × (1 mM/57 500 mg) × (1000 μmol/mmol). The K_i for BrMeBF was determined with 75–180 μM benzoylformate as a substrate and 0.1–1 μM inhibitor. Evaluations of 4-(4-chlorophenyl)-2-oxo-3-butenic acid, *p*-(bromomethyl)acetophenone, and *p*-(bromomethyl)-benzoic acid as inhibitors were carried out at 1–200 μM compound in the presence of 200 μM benzoylformate. Due to their limited solubilities in aqueous solution, stock solutions of (bromomethyl)acetophenone, (bromomethyl)benzoic acid, and the benzaldehydes described below were prepared with dioxane as a solvent. The final concentration of dioxane in the assay solution was 2% and had no effect on the assay itself.

The kinetic constants for HLADH were determined with 0.0075 unit of HLADH, 0.3 mM NADH, and 0.1 M potassium phosphate (pH 7.0) in a total volume of 0.5 mL. Benzaldehyde, tolualdehyde, and (bromomethyl)benzaldehyde were evaluated as substrates in a concentration range of 25–100 μM.

Time-Dependent Inhibition. BrMeBF was tested for its ability to cause a time-dependent inhibition of BFD by incubating 10 μM BrMeBF with 3.2 units of enzyme in a 0.2-mL volume of 0.01 M Hepes (pH 7.0) at room temperature in both the presence and absence of 1 mM TPP. Identical incubations without BrMeBF served as controls. At different time points, the activity of BFD was determined with the coupled assay by adding 5 μL of the incubation mixture (0.08 unit) to a cuvette containing 0.3 mM NADH, 1.5 units of HLADH, 0.2 mM benzoylformate, and 1 mM TPP in a 1-mL volume with 0.01 M K-Hepes (pH 7.0). The activity of the incubate was also determined without the addition of TPP to the assay. The final concentration of BrMeBF in the assay was 0.05 μM, a concentration which did not cause a significant amount of competitive inhibition. Similar tests for time-dependent inhibition were performed with incubation mixtures containing 1 and 50 μM BrMeBF, 0.5 mM 4-(4-chlorophenyl)-2-oxo-3-butenoic acid, or 10 μM BrMeBF in 0.1 M Tris (pH 9.0).

Recovery from Inhibition. The recovery of BFD from inhibition by BrMeBF can be observed by a modification of the coupled assay. BrMeBF was incubated with NADH, TPP, HLADH, and BFD in 0.01 M potassium phosphate (pH 7.0). At varying time points 480 μL of this reaction mixture was removed and assayed for BFD activity by addition of 20 μL of 5 mM benzoylformate. The final concentration of compounds in the assay cuvette was 1 or 2 μM BrMeBF, 0.3 mM NADH, 1 mM TPP, 1.5 units of HLADH, 0.2 mM benzoylformate, and 0.08 unit of BFD. Controls were also run in the absence of BrMeBF, TPP, or BFD in the reaction mixture. In the latter case, BFD was added with benzoylformate at the initiation of the assay.

This recovery from inhibition was also studied under identical conditions to the bromide release experiments, which are described below. Again, BFD activity was measured at various time points by adding 20 μL of 5 mM benzoylformate to 480 μL of reaction mixture. The final concentrations in the assay cuvette were 1 mM TPP, 0.3 mM NADH, 0.1 mM sodium nitrate, 1.5 units of HLADH, 50 μM BrMeBF, 0.2 mM benzoylformate, and 0.25 unit of dialyzed BFD in 0.1 M potassium phosphate (pH 7.0).

Fluoride Elimination. The reaction of FMeBF with BFD was analyzed for possible fluoride elimination with an Orion Model 96-09 combination fluoride electrode with an Orion Model 811 pH/millivolt meter. The reaction solution contained 0.5 mM FMeBF, 2 mM TPP, and 0.16 unit of BFD in a 2-mL total volume of 0.1 potassium phosphate (pH 7.0).

Bromide Elimination. Bromide ion release was measured on an Orion Model 811/pH millivolt meter equipped with an Orion Model 94-35 bromide electrode, a Model 90-01 single-junction reference electrode, and a Model 91-70-02 automatic temperature compensation probe. The amount of bromide in solution was determined from a standard curve. Experiments were performed in 2 mL of assay solution containing 0.1 M potassium phosphate (pH 7.0) and 0.1 M sodium nitrate. The addition of sodium nitrate is recommended to stabilize electrode performance. Bromide elimination experiments were performed with dialyzed BFD to minimize the concentration of chloride in the assay which interferes with the electrode response.

For experiments with BrMeBF, reaction mixtures contained 50 μM BrMeBF, 1 unit of dialyzed BFD, and 1 mM TPP.

Control reactions were also studied in the absence of either BFD or TPP. Elimination of bromide was also observed with addition of either 5 mM benzoylformate or 5 mM carboxybenzaldehyde to the above reaction. The effect of TPP concentration on the rate of bromide release was observed with from 5 μM to 1 mM TPP. The effect of pH on the rate of bromide release was observed by performing the reaction in 0.1 M potassium phosphate (pH 6.0, 6.5, 7.0, 7.5, and 8.0).

The release of bromide ion from *p*-(bromomethyl)benzaldehyde was also studied in 0.1 M potassium phosphate (pH 7.0) with 0.1 M sodium nitrate in a 2-mL volume. Assay solutions contained 50 μM *p*-(bromomethyl)benzaldehyde, 1 mM TPP, and 2 units of dialyzed BFD. Due to its limited solubility in aqueous solutions, stock solutions of the benzaldehyde were prepared in ethanol. Control experiments were performed in the absence of either TPP or BFD.

HPLC Detection of Products. HPLC of reaction products was performed on a Rainin Microsorb Shortones C-18 reverse-phase column, 4.6 mm × 10 cm, with 3-μm particle diameter. The mobile phase was 5% acetic acid and 30% methanol in water at a rate of 1 mL/min. The effluent was monitored by a UV detector set between 245 and 260 nm. BrMeBF (0.2 mM) was incubated with 1 mM TPP and 1.2 units/mL dialyzed BFD. The reaction was monitored by the amount of bromide elimination and terminated at 24 h when the reaction was over half complete. In an effort to extract the *p*-methylbenzoate product, the reaction was acidified to pH 1.5 with sulfuric acid to protonate the acid and then heated to 80 °C for 20 min to hydrolyze any residual acyl-TPP complex. This solution was extracted with chloroform, evaporated to dryness in vacuo, and redissolved in ethanol prior to injection on HPLC. The chloro analogue was incubated and extracted in the same manner, except the reaction contained 0.4 unit/mL BFD. Peaks were identified by coelution with authentic [*p*-(halomethyl)benzoyl]formates and *p*-methylbenzoic acid.

In HPLC experiments examining the time course of product formation, 0.2 mM ClMeBF or BrMeBF was incubated with 1 mM TPP and 0.16 unit/mL BFD. Twenty-microliter aliquots of the reactions were injected directly into the HPLC at different time points without acid workup or extraction.

Product Analysis by GC/Mass Spectrometry. BrMeBF (50 μM) was incubated with 2.4 units of BFD in the presence of 1 mM TPP in 5 mL of 0.1 M potassium phosphate (pH 6.9) for 24 h. The solution was filtered through an ultrafiltration membrane (Amicon XM50) to remove protein. To hydrolyze any remaining *p*-methylbenzoyl-TPP, the filtrate was raised to pH 10 with 1 N NaOH, allowed to sit overnight, and then heated at reflux for 30 min. The basic solution was then acidified (pH 2) with 1 N HCl, and the water was removed by lyophilization. To aid in detection of the *p*-methylbenzoate by GC/mass spectrometry, the acid was first converted to its methyl ester by treating the residue with an ethereal solution of diazomethane until a yellow color persisted. The ether solution was concentrated and analyzed by GC/MS and quantitated by flame ionization detection GC (FID-GC) in comparison with an authentic sample of methyl *p*-methylbenzoate.

ClMeBF (500 μM) was incubated with 12.5 units of BFD in the presence of 1 mM TPP in 25 mL of 0.1 M potassium phosphate (pH 6.9) for 24 h. The solution was extracted with diethyl ether, acidified with HCl, and then extracted again with diethyl ether. The second ethereal extract was dried over magnesium sulfate and filtered, and then the solvent was removed in vacuo. The residue was treated with an ethereal solution of diazomethane until a yellow color persisted. The ether solution was concentrated and analyzed by GC/MS and FID-GC in comparison with an authentic sample of methyl *p*-methylbenzoate.

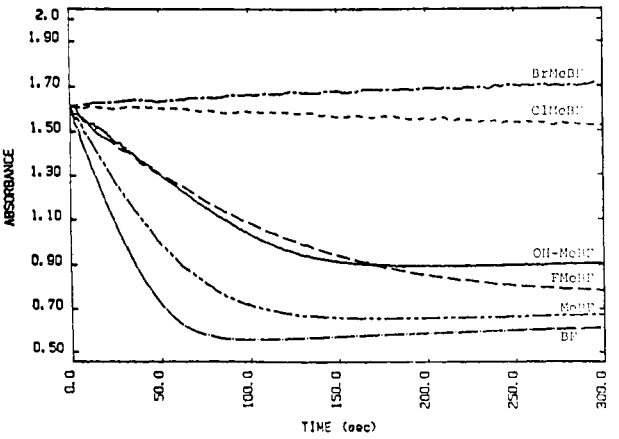

FIGURE 2: Behavior of [*p*-(halomethyl)benzoyl]formates in the coupled assay. Reactions contained 1 mM TPP, 0.3 mM NADH, 1.5 units of HLADH, and 0.08 unit of BFD in 0.5 mL of 0.01 M K-Hepes (pH 7.0) with 0.2 mM benzoylformate, (*p*-methylbenzoyl)formate, HOMeBF, FMeBF, ClMeBF, or BrMeBF. The absorbance was followed at 340 nm.

p-(Bromomethyl)benzaldehyde was incubated in the same fashion as ClMeBF with the exceptions that 50 μM analogue and 2.5 units of BFD were used. Workup and analysis were identical with those for the chloro analogue.

The GC/MS analysis was performed on a Kratos MS-25 mass spectrometer interfaced to a Varion 3700 capillary gas chromatograph equipped with a 30-m J&W Scientific DB-5 (dimethylsiloxane) capillary column. A gradient of 160–260 °C at 15 °C/min was run.

The GC quantitation was performed on an HP-5890A gas chromatograph with flame ionization detection. A gradient of 30–120 °C at 70 °C/min and then of 120–160 °C at 5 °C/min was used to generate the standard curve. A gradient of 30–90 °C at 70 °C/min and then of 90–150 °C at 5 °C/min was used to analyze the sample. Both the standards and the sample were quantitated against a methyl or ethyl benzoate internal standard (5 μL).

RESULTS

Behavior of [(Halomethyl)benzoyl]formates in Coupled Assay. The reaction of benzoylformate analogues in the coupled assay is shown in Figure 2. Under these conditions, the reactions of benzoylformate, MeBF, and FMeBF are complete within 5 min. FMeBF and HOMeBF appear to have nearly identical rates. Benzoylformate shows the fastest rate followed by MeBF. While the reaction of FMeBF is complete in about 3 min, ClMeBF takes more than 10 times longer, 38 min, to reach completion. BeMeBF shows no rate in the coupled assay.

Behavior in the Coupled Assay in the Absence of TPP. When the coupled assay was run at 200 μM benzoylformate without the addition of TPP, a reaction was still observed but at a rate 68% of that seen with 1 mM TPP present. This activity is due to the presence of TPP bound to BFD. When the same reactions were tried with dialyzed BFD, the decrease in activity without TPP in the assay was more marked. With 200 μM benzoylformate and 0.15 unit of dialyzed BFD, the rate constant with 1 mM TPP present was 31 s^{-1} and without TPP was 6 s^{-1}, a 5.2-fold difference. These numbers were the average of three determinations. FMeBF behaves similarly to benzoylformate in the absence of TPP. With 0.2 mM

Table I: Kinetic Data for Benzoylformate Decarboxylase[a]

compound	K_m (μM)	V_{max} (nM/min)	V/E (s^{-1})
benzoylformate	340	100	81
(*p*-methylbenzoyl)formate	580	65	52
[*p*-(fluoromethyl)benzoyl]formate	190	25	20
[*p*-(chloromethyl)benzoyl]formate	21[b]	3	2.3
[*p*-(bromomethyl)benzoyl]-formate	0.3 (K_I)		

[a] Rates were measured in the coupled assay which detected no production of *p*-(bromomethyl)benzaldehyde from BrMeBF. [b] Apparent K_m.

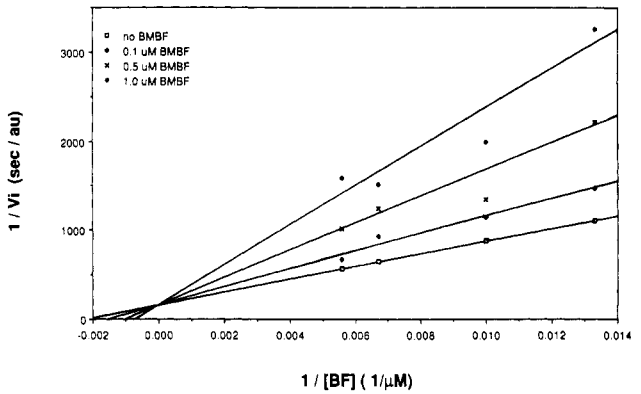

FIGURE 3: Competitive inhibition of BFD by [*p*-(bromomethyl)-benzoyl]formate. The rate of reaction of BFD with benzoylformate was measured in 0.5 mL of 0.01 M K-Hepes (pH 7.0) with 0.08 unit of BFD, 1.5 units of HLADH, 1 mM TPP, and 0.3 mM NADH in the presence of varying amounts of BrMeBF. Velocities are expressed as OD per second at 340 nm.

FMeBF and dialyzed BFD the rate constant with TPP is 11 s^{-1} and without TPP was 2 s^{-1}, a 5.5-fold difference (data not shown).

ClMeBF behaves differently than benzoylformate in the absence of TPP (data not shown). With 100 μM ClMeBF and 0.15 unit of dialyzed BFD the rate constant in the presence of 1 mM TPP is 3.5 s^{-1}. In the absence of TPP, the reaction displays a short initial burst followed by a slower phase. The rate constant during the initial burst is about 0.4 s^{-1} or 23 min^{-1} while the slower phase displays a rate of 1 min^{-1}, a 23-fold difference.

Kinetic Constants for Benzoylformate Decarboxylase. The kinetic constants for benzoylformate, MeBF, FMeBF, ClMeBF, and BrBeBF are shown in Table I. The K_i plot for BrMeBF is shown in Figure 3. ClMeBF has an apparent K_m which is almost 10-fold lower than the K_m for FMeBF but almost 10-fold higher than the K_i for BrMeBF.

p-(Bromomethyl)acetophenone, *p*-(bromomethyl)benzoic acid, and 4-(4-chlorophenyl)-2-oxo-3-butenoic acid did not demonstrate competitive inhibition of BFD.

Time-Dependent Inactivation. BrBeBF, from 1 to 50 μM, under various conditions described above, failed to cause any time-dependent inactivation of BFD. Incubation with 4-(4-chlorophenyl)-2-oxo-3-butenoic acid also showed no time-dependent inhibition.

Recovery from Inhibition by [p-(Bromomethyl)benzoyl]-formate. On prolonged incubation of BFD with BrMeBF, the magnitude of competitive inhibition decreases. This suggest that BrMeBF is consumed with time. Recovery takes about 25 min with 2 μM BrMeBF and 0.08 unit of BFD. With 1 μM BrMeBF the recovery takes only 10 min. In the absence of TPP, no detectable recovery was observed over 4 h. Controls also showed that in the absence of inhibitor the activity of the enzyme did not change for at least 75 min. When BFD was

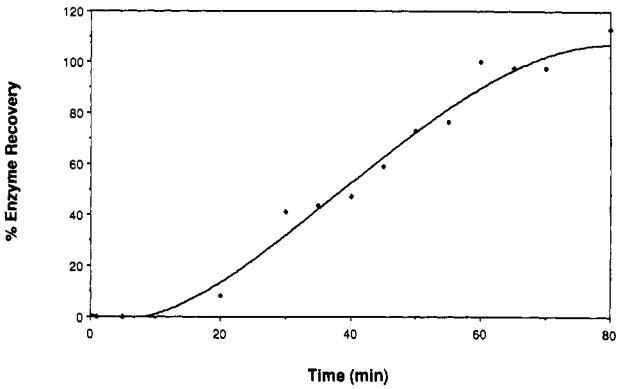

FIGURE 4: Recovery of benzoylformate decarboxylase from inhibition by 50 μM [*p*-(bromomethyl)benzoyl]formate. BrMeBF (50 μM) was incuated with 0.5 unit/mL dialyzed BFD, 1 mM TPP, 0.3 mM NADH, 0.1 mM sodium nitrate, and 1.5 units of HLADH in 0.1 M potassium phosphate (pH 7.0). Benzoylformate (0.2 mM) was added at different time points, and the rate of turnover of this compound was observed at 340 nm. This rate of benzoylformate turnover is expressed relative to the rate at 100 min.

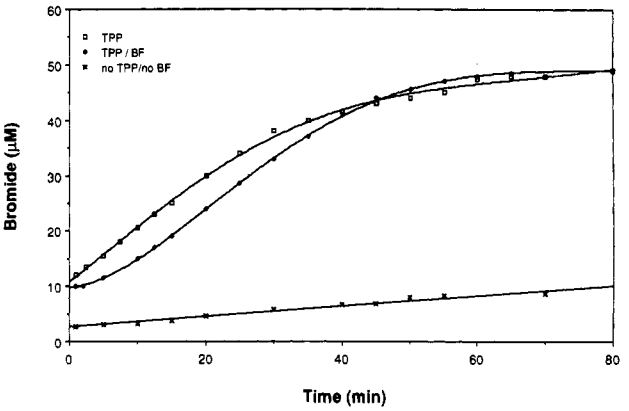

FIGURE 5: Benzoylformate decarboxylase catalyzed elimination of bromide from BrMeBF. Reaction mixtures (2 mL) contained 50 μM BrMeBF and 0.5 unit of dialyzed BFD in 0.1 M potassium phosphate (pH 7.0) in addition to 1 mM TPP (□), 1 mM TPP and 5 mM benzoylformate (●), or no TPP (×). The rate of spontaneous bromide released from 50 μM BrMeBF in buffer has been subtracted from the line representing bromide release in the absence of TPP.

not present during incubation but added just prior to assay, little or no recovery is seen. When the recovery from inhibition was observed under similar conditions to those of the bromide elimination experiments described below, the activity recovered in about 1 h following an initial lag phase (Figure 4).

Bromide Elimination with [p-(Bromomethyl)benzoyl]-formate. The elimination of bromide ion over time was observed with 50 μM BrMeBF and BFD (Figure 5).[2] Under these conditions, in the presence of TPP, bromide release is complete in about 1 h with an initial velocity of 0.93 μM bromide/min. The time required for 100% bromide release is the same time required for recovery from inhibition described above. Addition of 5 mM benzoylformate or 5 mM carbox-

[2] Figure 5 shows a variation in the apparent concentrations of bromide present at time zero, up to 10 μM, when the value should be zero. This anomaly is due to two characteristics of the bromide electrode. First, for measurements taken at bromide concentrations below 10 μM the electrode response becomes nonlinear and falls off rapidly. The second factor is chloride ion interference when the ratio of chloride to bromide is greater than 400:1. At the end point of these assays (50 μM bromide), the ratio of chloride to bromide is 20:1, well below the limit of interference. However, when the bromide concentration is ≤10 μM, chloride interferes. The source of chloride is the thiamin pyrophosphate which is purchased as the chloride salt.

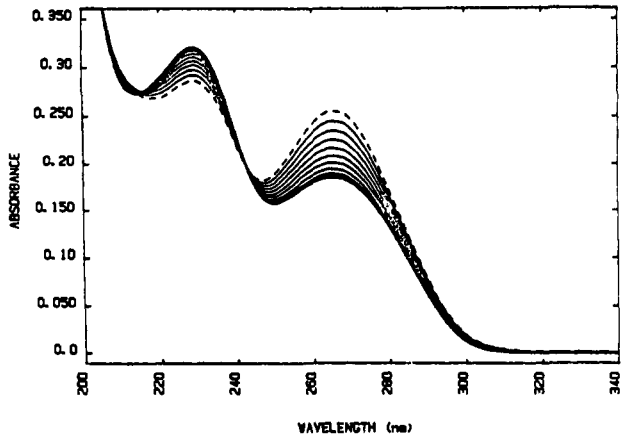

FIGURE 6: UV spectra of benzoylformate decarboxylase reaction with [*p*-(bromomethyl)benzoyl]formate. The reaction contained 50 μM BrMeBF, 0.2 mM TPP, and 0.08 unit of BFD in 0.01 M K-Hepes. Spectra were taken at 5-min intervals from time zero (--) to 75 min.

ybenzaldehyde to the incubation mixture caused a short lag in bromide elimination. The rate of bromide released from BrMeBF in the absence of BFD is 0.043 μM/min without TPP and slightly faster, 0.05 μM/min, with TPP present. When BrMeBF is incubated with BFD but in the absence of TPP, the rate of bromide release is faster than the background bromide release by 0.033 μM/min. The dependence of bromide release with BFD on the presence of TPP suggests that the release of bromide is due to enzymatic processing. Turnover under these conditions would correspond to 3.7 min^{-1} with TPP and 0.13 min^{-1} without TPP, a 28-fold difference.

The rate of bromide release is dependent on the concentration of TPP present. A maximum rate of bromide elimination is seen with 0.2 mM or greater TPP.

Test for Fluoride Elimination with [p-(Fluoromethyl)benzoyl]formate. No evidence of fluoride elimination was seen with the reaction of 0.5 mM FMeBF with BFD. Under the conditions of this experiment an elimination of 1% fluoride should have been detectable.

UV Assay of [p-(Bromomethyl)benzoyl]formate. One possible product of the reaction of BrMeBF with BFD is *p*-methylbenzoic acid. Unlike the benzaldehyde, this product absorbs in a distinctly different UV region, at 234 nm. Therefore, it is possible to observe the progress of this reaction directly by UV (Figure 6). The spectra show a decrease in absorbance over time at 265 nm and an increase at 230 nm. The reaction takes 1 h to reach completion.

Kinetic Constants for Horse Liver Alcohol Dehydrogenase. The kinetic constants for the reaction of benzaldehyde, *p*-methylbenzaldehyde, and *p*-(bromomethyl)benzaldehyde are nearly identical with turnover numbers of 26 ± 2 s^{-1}. The results show that *p*-(bromomethyl)benzaldehyde is a normal substrate for HLADH. This observation means that the failure of BrMeBF to show a rate in the coupled assay is not due to a failure of *p*-(bromomethyl)benzaldehyde to react with HLADH.

Bromide Elimination from p-(Bromomethyl)benzaldehyde. The ability of *p*-(bromomethyl)benzaldehyde to undergo a backreaction with benzoylformate decarboxylase was tested. When 50 μM *p*-(bromomethyl)benzaldehyde was incubated with BFD and TPP, an elimination of bromide ion at a rate of 0.07 μM/min was observed which reached 100% completion in about 2 h. *p*-(Bromomethyl)benzaldehyde alone in buffer released bromide at a rate of 0.048 μM/min and at a rate of 0.05 μM/min with the addition of TPP. *p*-(Bromomethyl)-benzaldehyde with BFD in the absence of TPP displayed a rate of bromide release of 0.084 μM/min.

HPLC of Benzoylformate Decarboxylase Reactions. The reactions of ClMeBF and BrMeBF with BFD were followed by HPLC to determine if *p*-methylbenzoic acid was a reaction product. Under conditions described under Materials and Methods, authentic ClMeBF eluted at 2.2 min, BrMeBF at 2.5 min, *p*-methylbenzoic acid at 17 min, and *p*-methylbenzaldehyde at 20 min. When 200 μM ClMeBF or BrMeBF was treated with BFD and TPP overnight and then workedup and extracted as described under Materials and Methods, HPLC for both reactions showed a peak which comigrated with authentic *p*-methylbenzoic acid.

When the reaction of BrMeBF was examined over time, a peak at 2.5 min corresponding to BrMeBF decreased with a concomitant increase in the peak corresponding to *p*-methylbenzoic acid. No further change was observed by HPLC for up to 20 h.

The time course for ClMeBF appeared different. The peak for starting material, eluting at 2.7 min, decreased with time while a new peak at 19 min developed. This new peak did not comigrate with *p*-methylbenzoic acid and reached maximum height within 70 min. Its elution time and the absorbance maximum at 257 nm suggested that it was *p*-(chloromethyl)benzaldehyde. A small peak at 16 min, which coelutes with *p*-methylbenzoic acid and has a λ_{max} less than 243 nm, began to appear after 2 h. After incubation overnight, the peak at 19 min disappeared completely while the peak at 16 min was larger.

The use of acetic acid in the mobile phase resulted in a high background absorbance which precluded detection of peaks below 245 nm. Since the λ_{max} of *p*-methylbenzoic acid is 234 nm, it was difficult to detect small amounts of this compound which would occur at early time points.

Product Analyses. GC/MS analysis of the reaction product of BrMeBF with BFD, after treatment with diazomethane, revealed a peak with molecular weight of 150 corresponding to the methyl ester of *p*-methylbenzoic acid. Quantitation by gas chromatography gave a recovery of 36 ± 3 μg or 240 ± 20 nmol of methyl *p*-methylbenzoate. Since 250 nmol of analogue was used, the results indicate that *p*-methylbenzoic acid was produced quantitatively from BrMeBF in the enzymatic reaction.

Similar analysis of the reaction product of ClMeBF afforded 11.4 μg of methyl *p*-methylbenzoate or 76 nmol. Since 12.5 μmol of analogue was incubated, this corresponds to a 0.6% yield. Analysis of the reaction product of the *p*-(bromomethyl)benzaldehyde incubation failed to detect any methyl *p*-methylbenzoate.

DISCUSSION

The three [*p*-(halomethyl)benzoyl]formates described display three distinct behaviors in the benzoylformate decarboxylase coupled assay (Figure 2). [*p*-(Fluoromethyl)-benzoyl]formate appears to behave as a normal substrate with a rate constant on the same order of magnitude as that of benzoylformate. [*p*-(Chloromethyl)benzoyl]formate demonstrates a reaction also, but with a rate constant an order of magnitude slower than that of FMeBF. [*p*-(Bromomethyl)-benzoyl]formate shows no rate in the coupled assay.

Initial studies on BrMeBF showed it to be a potent inhibitor of BFD with a K_i of 0.3 μM. The nature of this inhibition by BrMeBF is not due solely to the presence of a benzylic bromide, as demonstrated by the failure of *p*-(bromomethyl)benzoic acid and *p*-(bromomethyl)acetophenone to inhibit BFD. Further, experiments under a variety of conditions failed to demonstrate any time-dependent inhibition

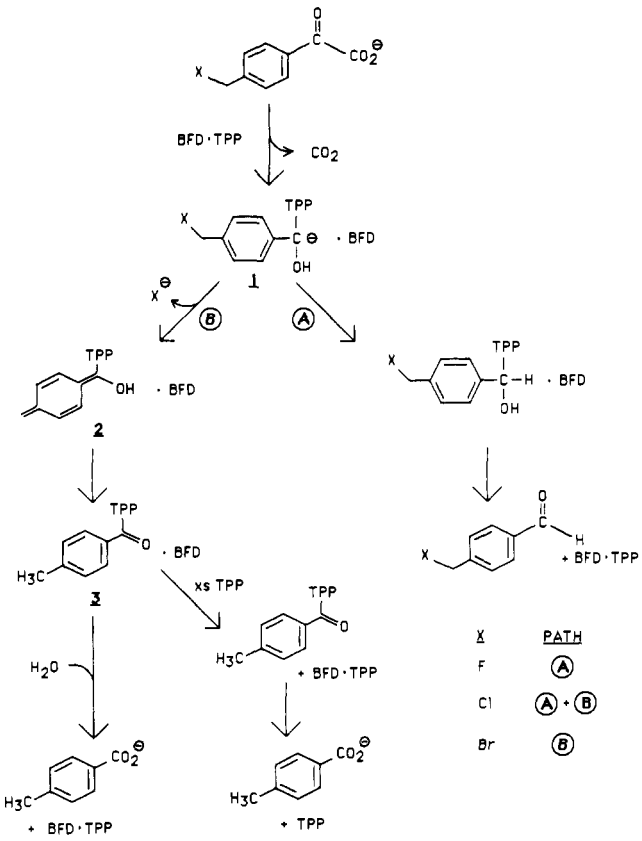

FIGURE 7: Scheme of benzoylformate decarboxylase reaction with [*p*-(halomethyl)benzoyl]formates.

by BrMeBF, showing that the inhibition is not due to covalent modification of the enzyme.

When BFD was incubated with BrMeBF and TPP, complete recovery from inhibition with time was observed (Figures 4 and 5). The transient inhibition suggests that BrMeBF is being processed to a noninhibitory species. The degree of inhibition was not decreased by incubation of BrMeBF alone in buffer, showing that recovery from inhibition is not due to chemical decomposition of the analogue. The dependence of recovery on the presence of TPP suggests that this phenomenon is due to an enzyme-mediated process. [*p*-(Bromomethyl)-benzaldehyde is a substrate for HLADH and, therefore, cannot be the product of the reaction.

A time plot of BFD recovery in the presence of 50 μM BrMeBF exhibited a lag phase. This concentration of inhibitor is over 100 times higher than K_i. The observation of a lag phase suggests that a significant proportion of the inhibitor must be processed before the enzyme demonstrates any recovery.

The release of bromide ion was quantitative and occurred in the same time frame as the recovery from inhibition. The addition of either benzoylformate or carboxybenzaldehyde, a competitive inhibitor found by Weiss et al. (1988), to the reaction caused an initial lag in bromide release indicative of active-site protection. The rate of bromide release was dependent on the concentration of TPP, although a slow enzyme-dependent release of bromide was also seen in the absence of TPP.

An early step in BFD catalysis is the decarboxylation of the benzoylformate–TPP adduct resulting in generation of a carbanion at the C-2 position. In the normal reaction (Figure 7, path A), the carbanion (**1**) becomes protonated yielding a hydroxybenzyl-TPP intermediate which decomposes to the benzaldehyde product. However, BrMeBF is able to undergo carbanionic elimination of the benzylic bromide (path B). The resulting quinone methide intermediate (**2**) is a potentially reactive Michael acceptor. The failure to demonstrate any time-dependent inhibition suggests that this intermediate undergoes a rapid tautomerization to give *p*-methylbenzoyl-TPP (**3**). The driving force for this tautomerization is rearomatization. This acyl-TPP, upon hydrolysis, would yield *p*-methylbenzoic acid. The detection of this product by HPLC and the quantitative isolation of methyl *p*-methylbenzoate by GC–mass spectrometry are consistent with this mechanism.

The formation of an acyl-TPP adduct by halide elimination has precedence in the literature. Leung and Frey (1978) reported the reaction of fluoropyruvate with pyruvate decarboxylase. They found that the decarboxylation of the substrate was followed by elimination of fluoride and tautomerization to give acetyl-TPP. This intermediate hydrolyzed to yield acetic acid as a product. The turnover of fluoropyruvate was found to be 10 times slower than that of the normal reaction.

The potent inhibition of benzoylformate decarboxylase by BrMeBF is most likely due to a slower hydrolysis of *p*-methylbenzoyl-TPP compared to the elimination of hydroxybenzyl-TPP found in the normal reaction. Thus, the basis of the BFD inhibition is a modification of its cofactor. The concept of inhibition of an enzyme by inhibition of its cofactor also has precedence in the literature. Rando (1977) and Rando and Bangerter (1977) described the modification of pyridoxal phosphate in the active site of γ-aminobutyric acid transaminase by gabculine. The driving force for this inhibition was also the aromatization of an inhibitor–cofactor adduct via an enzyme-catalyzed tautomerization.

The hypothesis that a slow hydrolysis of *p*-methylbenzoyl-TPP is responsible for the inhibition seen by BrMeBF is supported by the rates of bromide elimination observed in the presence and absence of excess TPP. In the absence of added TPP, a slow enzyme-dependent rate of bromide elimination is seen corresponding to a turnover of 0.13 min^{-1}. This enzymatic activity must be due to enzyme-bound TPP which remains after dialysis of the enzyme. Under these conditions, the rate constant of 0.13 min^{-1} appears to be indicative of this process.

In the presence of excess TPP, the rate of bromide release afforded a turnover of 3.7 min^{-1}, 28 times faster than that in the absence of TPP. This faster turnover in the presence of excess TPP suggests that an unmodified TPP from solution is able to "rescue" the enzyme by exchange with *p*-methylbenzoyl-TPP at the active site. The observed rate in this case should be indicative of the dissociation rate of *p*-methylbenzoyl-TPP from the enzyme or of an acyl exchange between enzyme-bound and free TPP.

The unusual mechanism of reaction of BrMeBF by BFD yields a product, *p*-methylbenzoate, which is not a substrate for HLADH. This explains the failure of BrMeBF to demonstrate a rate in the coupled assay. However, the generation of this product makes it possible to observe the reaction directly by UV due to a shift in absorbance to shorter λ_{max} (Figure 6).

FMeBF appears to behave as a normal substrate in the coupled assay with kinetic constants comparable to those of other substrates. With dialyzed enzyme, FMeBF behaves similarly to benzoylformate with both compounds showing a 5–6-fold decrease in rate in the absence of added TPP. The failure to detect fluoride elimination further confirms that FMeBF is a normal substrate for BFD.

While BrMeBF is an inhibitor and FMeBF is a substrate, ClMeBF appears to occupy a position between these two ex-

tremes. ClMeBF does show a rate in the HLADH assay, indicating that there is conversion to *p*-(chloromethyl)benzaldehyde. The reaction appears to go nearly to completion, but the difficulty in quantitating the end points of the coupled assay prevents an accurate determination of the extent of this reaction. Since *p*-(bromomethyl)benzaldehyde was shown to be a good substrate with HLADH, one can presume that the 10-fold slower rate of ClMeBF is not due to a slow reduction of *p*-(chloromethyl)benzaldehyde by HLADH.

The apparent K_m for ClMeBF of 32 μM lies between the K_m of FMeBF (200 μM) and the K_i for BrMeBF (0.3 μM). This observation, along with the slow reaction rate, suggests that ClMeBF may be partitioning between the normal and the elimination pathway. The hypothesis is not easily tested by observing chloride elimination since the chloride ion electrodes available are not sensitive enough in the required concentration range. Examination of the reaction product by GC analysis has shown that ClMeBF partitions 0.6% or 1:170 to *p*-methylbenzoate.

The behavior of ClMeBF with dialyzed BFD is also consistent with this substrate partitioning. In the absence of added TPP, ClMeBF exhibits an initial burst of activity in the coupled assay followed by a rapid turnover of substrate via the normal reaction pathway. Eventually, because of partitioning to the elimination pathway, most of the BFD becomes bound with *p*-methylbenzoyl-TPP. Thus, a slower rate is observed for the remainder of the reaction in which substrate can be processed in the normal reaction.

During studies on the time course of ClMeBF reaction by HPLC, it was observed that an initial product peak corresponding to the *p*-methylbenzoic acid peak increased. This result suggests that (chloromethyl)benzaldehyde may be able to backreact with BFD to produce *p*-methylbenzoic acid.

The ability of thiamine-dependent decarboxylases to catalyze a partial reaction in the reverse direction has been demonstrated previously by Chen and Jordan (1984) using pyruvate decarboxylase. The normal product of the pyruvate decarboxylase reaction with pyruvic acid is acetaldehyde, but a side product, acetoin, is also formed in 3–4% yield. Chen and Jordan demonstrated that incubation of acetaldehyde with the enzyme resulted in production of acetoin. Thus, acetaldehyde was able to recombine with TPP on the enzyme to form the hydroxyethyl-TPP intermediate. Abstraction of a proton from this adduct generated the TPP-stabilized carbanion that is normally found in the forward direction after the decarboxylation step. Acetoin formation occurs when this carbanion condenses with a second molecule of acetaldehyde from solution. The rate-limiting step of this reaction was shown to be the deprotonation step. This step generates the TPP-stabilized carbanion from acetaldehyde at a rate 80 times slower than that of its production from pyruvate.

The ability of benzoylformate to catalyze a backreaction on the enzyme was studied by observing its reaction with *p*-(bromomethyl)benzaldehyde. *p*-(Bromomethyl)benzaldehyde showed a quantitative loss of bromide in 2 h on incubation with BFD and TPP. This enzyme-dependent rate of bromide release was approximately half of that seen by BrMeBF in the forward direction. As with the BrMeBF reaction, the rate of bromide release was dependent of the presence of cofactor, showing a 20-fold slower rate in the absence of TPP. However, the failure to detect any *p*-methylbenzoate requires that the BFD-catalyzed bromide ion release from *p*-(bromomethyl)benzaldehyde results in a different and as yet uncharacterized product. Evaluation of the rate of backreaction by measuring enzyme-catalyzed exchange of the aldehydic proton of benzaldehyde with solvent deuterium by ^{1}H NMR suggested that this process may be substantially slower than the rate of bromide release from *p*-(bromomethyl)benzaldehyde (data not shown). This raises the possibility that bromide release may occur on the enzyme from the neutral TPP adduct by solvolysis, a process leading to the formation of *p*-(hydroxymethyl)benzaldehyde and not *p*-methylbenzoate. This point is under investigation.

These results are consistent with the interpretation that the leaving group potential of the halogen determines whether the hydroxybenzyl-TPP carbanion intermediate eliminates halide, ultimately forming *p*-methylbenzoate, or is protonated, ultimately forming *p*-(halomethyl)benzaldehyde. Apparently, the energy barrier due to loss of aromaticity serves to modulate the partitioning for a given halide. The relative leaving group potentials (bromide:chloride:fluoride 1.0:0.02:0.0001; Kosower, 1968) are consistent with BrMeBF eliminating entirely, with ClMeBF partitioning, and with FMeBF producing only the benzaldehyde product.

The quinone methide intermediate (Figure 7) bears a structural resemblence to the tautomer of 7-deoxydaunomycinone which has been directly observed by the chemical elimination of daunosamine from daunomycin hydroquinone (Kleyer & Koch, 1983). This tautomer may be trapped by electrophilic reagents (e.g., proton, benzaldehyde) (Kleyer & Koch, 1983, 1984) or, in the case of 7,11-dideoxydaunomycinone, by nucleophiles (Ramakrishnan & Fisher, 1983). Our results suggest that the quinone methide generated from BrMeBF, under our conditions, exhibits only electrophilic trapping. More significantly, its formation occurs from a cofactor-stabilized carbanionic enzyme intermediate and is modulated by the reactivity of the leaving group.

ACKNOWLEDGMENTS

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Registry No. BFD, 9025-00-7; BF, 611-73-4; HLADH, 9031-72-5; BrMeBF, 102047-01-8; MeBF, 7163-50-0; ClMeBF, 114996-73-5; FMeBF, 114996-72-4; *p*-methylbenzoyl-TPP, 102047-02-9; *tert*-butyl [*p*-(chloromethyl)benzoyl]formate, 114996-75-7; *p*-(fluoromethyl)-acetophenone, 114996-74-6; *p*-(bromomethyl)benzaldehyde, 51359-78-5.

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Catalysis of Angiotensin I Hydrolysis by Human Angiotensin-Converting Enzyme: Effect of Chloride and pH†

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ABSTRACT: The catalysis of the hydrolysis of angiotensin I, an important natural substrate, by human angiotensin-converting enzyme (ACE) was examined in detail as a function of chloride and hydrogen ion concentration. Chloride was found to be a nonessential activator over the pH range 5.0–10.0, with the chloride dependence increasing with increasing pH: the velocity enhancement at optimal [Cl^-] increased from 1.6- to 42-fold; the chloride optimum and K_a' increased from 20 to 520 mM and from 0.22 to 120 mM, respectively; and activity in the absence of chloride decreased from 60.9 to 2.4% (relative to maximal activation). Kinetic analyses at pH 6.0, 7.5, and 9.0 confirmed the nonessential activator mechanism. At all pH values tested chloride was found to be inhibitory (relative to maximal activation) at supraoptimal chloride levels. Depending on the [Cl^-] range, both apparent uncompetitive and competitive modes were demonstrated. From pH 6.0 to 9.0 K_is varied between 110 and 1140 mM (apparent). In all cases $K_i' \gg K_a'$. We suggest that at high [Cl^-] chloride binds to low-affinity inhibitory sites on the free enzyme and on the ES and EP complexes. The pH–rate profile demonstrated a chloride-dependent alkaline shift, with the pH optimum increasing from 7.1 at zero chloride to 7.6 at 400 mM NaCl. At [S] $\gg K_m$ a plot of log v vs pH revealed pKs of 5.9 and 9.4 in the ES complex in the absence of chloride, while at maximally activating [Cl^-] only one ionization at pK = 6.3 was observed. Thus, binding of chloride appeared to suppress the ionization of a basic group. A pK of 9.4 would be consistent with a lysyl residue in a positively charged, hydrophobic microenvironment, and a critical lysine has been implicated in chloride binding [Shapiro, R., & Riordan, J. F. (1983) *Biochemistry 22*, 5315–5321]. Our data indicate that human ACE is likely to be maximally activated for its natural substrate angiotensin I in most anatomic loci.

The striking activation of angiotensin-converting enzyme (ACE;[1] dipeptidyl carboxypeptidase, EC 3.4.15.1) by chloride and other monovalent anions is an unusual characteristic first noted by the enzyme's original discoverers (Skeggs et al., 1954) and since shown to be complex, dependent on substrate and pH, with both essential and nonessential activator mechanisms having been demonstrated (Cheung et al., 1980; Rohrbach et al., 1981; Bünning & Riordan, 1983; Shapiro et al., 1983). Furthermore, it is not unlikely that species differences in ACE may introduce an additional complicating factor in evaluating the effect of chloride on the hydrolysis of a particular substrate, since, for example, it has been shown that the activity for Hip-His-Leu differs for the rabbit and canine enzymes (Conroy et al., 1978).

Most of the kinetic work on the anion activation of ACE has been performed on the rabbit lung enzyme with synthetic N-blocked tripeptide substrates (Cheung et al., 1980; Bünning & Riordan, 1983; Shapiro et al., 1983). The information gained from these studies cannot necessarily be extrapolated to the human enzyme and its most important physiological substrate angiotensin I (AI). Work on human ACE has, until recently, been hampered by tedious purification procedures (Ehlers et al., 1986), while kinetic studies with AI were discouraged by its high cost and the lack of a continuous assay for this substrate. Thus, while the pathophysiological importance of the human enzyme in the conversion of AI to AII

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[1] Abbreviations: ACE, angiotensin-converting enzyme; AI, angiotensin I; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; His-Leu, L-histidyl-L-leucine; HSA, human serum albumin; ES, enzyme–substrate; EP, enzyme–product; EAS, enzyme–activator–substrate; EAP, enzyme–activator–product; Fa, 2-furanacryloyl; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; P_1' and P_2', penultimate and ultimate amino acid residues, respectively, of the substrate [terminology as described by Schechter and Berger (1967)].

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