Kinetics and Mechanism of Benzoylformate Decarboxylase Using ^{13}C and Solvent Deuterium Isotope Effects on Benzoylformate and Benzoylformate Analogues†

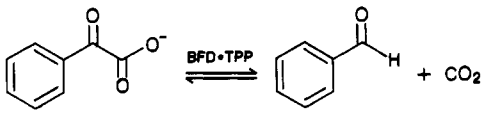
Paul M. Weiss,‡ George A. Garcia,§,‖ George L. Kenyon,*,§ W. W. Cleland,‡ and Paul F. Cook*,⊥

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706, Department of Biochemistry, North Texas State University/Texas College of Osteopathic Medicine, Denton, Texas 76203, and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143

Received August 31, 1987; Revised Manuscript Received November 3, 1987

ABSTRACT: Benzoylformate decarboxylase (benzoylformate carboxy-lyase, BFD; EC 4.1.1.7) from *Pseudomonas putida* is a thiamine pyrophosphate (TPP) dependent enzyme which converts benzoylformate to benzaldehyde and carbon dioxide. The kinetics and mechanism of the benzoylformate decarboxylase reaction were studied by solvent deuterium and ^{13}C kinetic isotope effects with benzoylformate and a series of substituted benzoylformates (*p*CH_3O, *p*CH_3, *p*Cl, and *m*F). The reaction was found to have two partially rate-determining steps: initial tetrahedral adduct formation (D_2O sensitive) and decarboxylation (^{13}C sensitive). Solvent deuterium and ^{13}C isotope effects indicate that electron-withdrawing substituents (*p*Cl and *m*F) reduce the rate dependence upon decarboxylation such that decreased $^{13}(V/K)$ effects are observed. Conversely, electron-donating substituents increase the rate dependence upon decarboxylation such that a larger $^{13}(V/K)$ is seen while the D_2O effects on V and V/K are not dramatically different from those for benzoylformate. All of the data are consistent with substituent stabilization or destabilization of the carbanionic intermediate (or carbanion-like transition state) formed during decarboxylation. Additional information regarding the mechanism of the enzymic reaction was obtained from pH studies on the reaction of benzoylformate and the binding of competitive inhibitors. These studies suggest that two enzymic bases are required to be in the correct protonation state (one protonated and one unprotonated) for optimal binding of substrate (or inhibitors).

Benzoylformate decarboxylase (benzoylformate carboxy-lyase, BFD,[1] EC 4.1.1.7) is a thiamine pyrophosphate utilizing enzyme that catalyzes the conversion of benzoylformate to benzaldehyde and carbon dioxide:



BFD is part of the degradative pathway for mandelic acid in *Pseudomonas putida* (Gunsalus et al., 1958). It has been purified to homogeneity and some of its physical, chemical, and kinetic properties have been characterized (Hegeman, 1970). Kinetically only the K_m values for benzoylformate (1 mM) and TPP (1 μM) have been reported. Only benzoylformate or para-substituted benzoylformates have been shown to serve as substrates for the decarboxylase. Virtually nothing has been reported about the details of the enzymic mechanism.

In this paper we report studies performed on the BFD-catalyzed decarboxylation of benzoylformate and a series of substituted benzoylformates. These substituents vary from being electron withdrawing to being electron donating with respect to hydrogen. Solvent deuterium and ^{13}C kinetic isotope effects indicate that the rate-determining steps in the benzoylformate decarboxylase reaction change with the changes in electronic nature of the substituent. Those analogues containing electron-donating substituents exhibit an increased rate dependence upon decarboxylation as evidenced by a larger ^{13}C kinetic isotope effect upon V/K. Those analogues whose substituents are electron-withdrawing show a decreased rate dependence upon decarboxylation. All of the analogues show a partial rate dependence upon a solvent deuterium sensitive step.

Information regarding the chemical mechanism and rate-determining steps of this reaction has also been obtained from pH studies of the enzymic reaction with benzoylformate and with competitive inhibitors. A chemical mechanism is postulated that is consistent with all of the data presented. This mechanism involves the presence of two enzymic bases in the active site of the decarboxylase and multiple partially rate-determining steps in the kinetic scheme.

Materials and Methods

General. Benzoylformic acid, thiamine pyrophosphate, bovine serum albumin, phenylpyruvate, *trans*-cinnamic acid, benzoic acid, phenoxyacetic acid, equine liver alcohol de-

† This work was supported by NIH grants to P.F.C. (GM36799), W.W.C. (GM18938), and G.L.K. (AR17323 and CA37655), a grant from Bristol-Myers Co. to G.L.K., and a grant from the Robert A. Welch Foundation (B-1031) to P.F.C. P.F.C. is the recipient of NIH Research Career Development Award AM01155. G.A.G. is the American Foundation for Pharmaceutical Education H. A. B. Dunning Memorial Fellow, 1985–1987.

‡ University of Wisconsin.

§ University of California.

‖ Present address: Department of Chemistry, Imperial College of Science and Technology, London SW7 2AY, U.K.

⊥ North Texas State University/Texas College of Osteopathic Medicine.

[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; BFD, benzoylformate decarboxylase; BF, benzoylformate; TPP, thiamine pyrophosphate; BSA, bovine serum albumin; LADH, equine liver alcohol dehydrogenase; APADH, 3-acetylpyridine adenine dinucleotide (reduced form); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; mp, melting point; ppm, parts per million.

0006-2960/88/0427-2197$01.50/0

hydrogenase, carbonic anhydrase, NADH, and acetylpyridine adenine dinucleotide (APADH) were purchased from Sigma Chemical Co. Phthalic acid, 2-carboxybenzaldehyde (phthalaldehydic acid), (*R*)-mandelic acid, (*S*)-mandelic acid, phthalimide, phenylacetic acid, sodium azide, and deuterium oxide (100 atom % D) were purchased from Aldrich Chemical Co. The buffers CHES, HEPES, MES, and TAPS were obtained from Research Organics Co.

Benzoylformate Decarboxylase. Benzoylformate decarboxylase was isolated to near homogeneity after the method of Hegeman (1970). The enzyme has a molecular weight of 57 500 as estimated from SDS–PAGE and a specific activity of 60 units/mg [Note: these values disagree with those determined by Hegeman (1970); however, they are very similar to those found by Barrowman and Fewson (1985) for the corresponding decarboxylase from *Acinetobacter calcoaceticus*]. During the course of this study, it was found that the decarboxylase rapidly lost activity upon dilution. This loss of activity was prevented by the addition of 1 mg/mL BSA and 1 mg/mL sodium azide (NaN_3). The enzyme was stable for at least 2 months when stored in 50 mM phosphate buffer, pH 6, with 300 mM NaCl, 1 mg/mL BSA, and 1 mg/mL NaN_3.

Chemical Syntheses. The (*p*-chlorobenzoyl)- and (*p*-methylbenzoyl)formates were prepared in a manner identical with that of Barnish et al. (1981). The (*p*-methoxybenzoyl)- and (*m*-fluorobenzoyl)formates were prepared from the corresponding acetophenones in a manner identical with that used for the synthesis of (*p*-chlorobenzoyl)formate.

(p-Methylbenzoyl)formate. The synthesis yielded 3.5 g (28%) of white crystals, mp 98.5–100 °C. Mass spectrometric analysis was consistent with the structure of (*p*-methylbenzoyl)formate. Elemental analysis was as follows. Calcd for $C_9H_8O_3$: C, 65.85; H, 4.91%. Found: C, 65.68; H, 4.76. ^1H NMR ($CDCl_3$) revealed the following resonances: δ 7.84 (2 H, d), 7.31 (2 H, d), 2.37 (3 H, s).

(p-Chlorobenzoyl)formate. The resulting orange residue was recrystallized from toluene/diethyl ether (80/20) at room temperature. (Note: heating the residue in toluene caused decomposition of the product to the corresponding benzoic acid.) The synthesis yielded 3.0 g (11%) of yellow crystals, mp 59–63 °C. Mass spectrometric analysis was consistent with the structure of (*p*-chlorobenzoyl)formate. Elemental analysis was as follows. Calcd for $C_8H_5O_3Cl{\cdot}H_2O$: C, 47.43; H, 3.48. Found: C, 48.14; H, 3.40. ^1H NMR ($CDCl_3$) revealed the following resonances: δ 7.95 (2 H, d), 7.59 (2 H, d).

(m-Fluorobenzoyl)formate. *m*-Fluoroacetophenone (5 g) and 4.4 g of selenium dioxide were added to 30 mL of dry pyridine. The solution was stirred and heated at 95 °C overnight. The solution was filtered to remove a black precipitate. The pyridine was removed in vacuo. The residue was taken up in approximately 60 mL of H_2O and extracted with diethyl ether. The water layer was treated with decolorizing carbon and filtered. The water portion was then acidified with 5 N HCl and extracted with diethyl ether. The aqueous layer was discarded, and the ether was removed in vacuo. The residue was recrystallized from CCl_4, yielding 1.8 g (30%) of white crystals, mp 77–78 °C. Mass spectrometric analysis was consistent with the structure of (*m*-fluorobenzoyl)formate. Elemental analysis was as follows. Calcd for $C_8H_5O_3F$: C, 57.15; H, 3.00. Found: C, 56.96; H, 2.86. ^1H NMR ($CDCl_3$) revealed the following resonances: δ 8.15 (2 H, mult), 7.50 (2 H, complex mult).

(p-Methoxybenzoyl)formate. *p*-Methoxyacetophenone (5 g) and 3.7 g of selenium dioxide were added to 30 mL of dry pyridine and were treated in exactly the same manner as for *m*-fluoroacetophenone. The resulting pale yellow precipitate was recrystallized from water/EtOH (80/20) yielding 1.9 g (32%) of white crystals, mp 87–88.5 °C. Mass spectrometric analysis was consistent with the structure of (*p*-methoxybenzoyl)formate. Elemental analysis was as follows. Calcd for $C_9H_8O_4{\cdot}H_2O$: C, 54.55; H, 5.09. Found: C, 54.61; H, 4.81. ^1H NMR ($CDCl_3$) revealed the following resonances: δ 8.44 (2 H, d), 7.00 (2 H, d), 3.92 (3 H, s).

2-Carboxybenzaldehyde-d_1. 2-Carboxybenzaldehyde-d_1 was prepared by carrying out the synthesis of Gardner and Naylar (1943) in D_2O. That the resulting compound contained a deuterium rather than a hydrogen specifically in the aldehydic group was established by ^1H NMR spectroscopy.

Analytical Methods. ^1H NMR spectra were obtained on a multinuclear 240-MHz NMR spectrometer equipped with a Nicolet 1180 computer and a 293-B pulse programmer. Mass spectra were obtained by direct insertion probe analysis on a Kratos MS-25 spectrometer. Elemental analyses were performed by the Microanalytical Lab, University of California, Berkeley.

Nomenclature. The nomenclature used is that of Northrop (1977) in which isotope effects on kinetic or thermodynamic parameters are defined by leading superscripts. Since all deuterium isotope effects determined in this study were solvent deuterium (D_2O) effects, the superscript D refers to such effects. For example, $^{13}(V/K)_D$ is the ^{13}C isotope effect in D_2O, and $^d(V/K)$ is the solvent deuterium isotope effect. For a further discussion of nomenclature, see Cook and Cleland (1981).

Initial Velocity Studies. Initial velocity data were obtained on a Gilford 250 spectrophotometer with a strip chart recorder. Data were collected at 25 °C maintained with a water bath with the capacity to heat and to cool the thermospacers in the cell compartment. All initial velocities were obtained by using the equine liver alcohol dehydrogenase reaction to couple away benzaldehyde produced by the benzoylformate decarboxylase reaction. Thus, the decrease in absorbance at 340 nm due to the loss of NADH was used to monitor the reaction continuously. Reduced 3-acetylpyridine adenine dinucleotide (APADH, λ_{max} = 363 nm) was used instead of NADH in some of the initial velocity studies in order to eliminate interference in the coupled assay by benzoylformate analogues that have a significant extinction coefficient at 340 nm (Table I). Under all conditions used in these studied, including the presence of inhibitors, the velocity was directly proportional to benzoylformate decarboxylase concentration, showing that the coupled assay reflected the true rate of BFD catalysis.

Another continuous assay for the decarboxylase monitors the absorbance decrease of benzoylformate at 334 nm (Hegeman, 1970). The extinction coefficient in this case is 81 M^{-1} cm^{-1}, which makes this assay 75-fold less sensitive than the coupled assay. However, similar specific activities were obtained with both assays. A typical coupled assay contained 100 mM buffer [see pH(D) Studies], 0.1 mM TPP, 0.3 mM NADH (0.2 mM APADH), 1 unit of LADH, variable

Table I: UV Absorbance Parameters for Benzoylformate and Analogues

compound	ϵ_{340} (M^{-1} cm^{-1})	ϵ_{363} (M^{-1} cm^{-1})
(*p*-methoxybenzoyl)formate (pCH_3O)	250	72.5
(*p*-methylbenzoyl)formate (pCH_3)	104	58.6
benzoylformate (*p*H)	74	51.2
(*p*-chlorobenzoyl)formate (*p*Cl)	197	15.2
(*m*-fluorobenzoyl)formate (*m*F)	72	58.4

amounts of benzoylformate (or analogue), and approximately 0.02 unit of BFD.

Once it was established that the inhibitory analogues of benzoylformate were competitive with respect to benzoylformate, all inhibition data were obtained from Dixon plots at a fixed level of benzoylformate with the concentration of inhibitor varied.

pH(D) Studies. The concentration of benzoylformate was varied to determine V and V/K for benzoylformate. K_i values for inhibitors competitive with respect to benzoylformate were obtained from Dixon plots. The pH of buffers at the final concentration of 100 mM that was used to obtain the pH profiles in H_2O was as follows: acetate, pH 4.5–5.5; MES, pH 5.5–6.8; HEPES, pH 6.5–8.0; TAPS, pH 8.0–9.0; CHES, pH 9.0–10.0. The same buffers were used in D_2O but at a range 0.5 pH unit higher than that in H_2O to correct for the equilibrium isotope effect on the acid dissociation constants of the buffers (Schowen, 1977). The pH value of each reaction mixture was obtained immediately after temperature equilibration (5 min in the water bath) and also after initial velocity data were collected. The pD values were obtained by addition of 0.4 to the pH meter reading (Schowen, 1977). In all cases, the pH change during the reaction was not significant. Below pH 6.5, both NADH and coupling enzyme were added just prior to addition of enzyme.

The decarboxylase is stable over the time course of the assay as judged by the linearity of the absorbance decrease at all pH values tested. Slow structural changes in the benzoylformate decarboxylase in D_2O were also ruled out by use of linearity of the absorbance decrease at all pD values tested.

Solvent Deuterium Isotope Effects. The solvent deuterium isotope effects were determined by the coupled assay with equine liver alcohol dehydrogenase to measure initial velocities at various substrate concentrations. Stock solutions were made up in both H_2O and D_2O. The reactions were carried out in 100 mM HEPES, pH 7.2 (pD 6.8), 1 mM TPP, 1 unit of LADH, 200 mM APADH, and 0.015 unit of BFD.

^{13}C Isotope Effects. The technique employed for the determination of ^{13}C isotope effects is that of O'Leary (1980) in which the natural abundance of ^{13}C in the C-1 position of benzoylformate is used. Both high-conversion (100% reaction) and low-conversion (~10% reaction) samples are collected. The $^{12}C/^{13}C$ isotope ratios in the CO_2 produced in the reactions are determined for both samples. From these ratios, the relative rates of reaction for ^{12}C versus ^{13}C, and thus the ^{13}C isotope effect, are calculated. Use of this natural abundance method minimizes the errors caused by atmospheric CO_2 contamination. Reaction mixtures for the low-conversion reactions contained the following in 20 mL: 30 mM benzoylformate, 0.1 mM TPP, and 100 mM buffer. The high-conversion samples were identical with the low-conversion ones except that the substrate concentration was decreased to 3 mM. The pH was adjusted with saturated KOH (or NaOD) after degassing with CO_2-free, dry N_2 for 2 h. This was followed by additional degassing for 1 h. The reaction was then initiated by addition of benzoylformate decarboxylase (30 units added to high-conversion samples and 12 units added to low-conversion samples).

Low-conversion samples were quenched with 1.5 mL of concentrated sulfuric acid at the appropriate time for ~10% reaction. The high-conversion samples were allowed to proceed overnight prior to addition of sulfuric acid and isolation of CO_2. Isolation and analysis of all samples were carried out on the same day the CO_2 was generated. All CO_2 analyses were carried out with a Finnigan Delta-E isotope ratio mass spectrometer. All ratios were corrected for ^{17}O according to Craig (1957).

Each concentration of benzoylformate (or substrate analogue) at $t = 0$ and after reactions were quenched was determined in duplicate by the LADH coupled assay. Conditions used were identical with those for the coupled assays described above, except that 1 unit of decarboxylase was used per determination.

Normalization of Deuterium Oxide. The deuterium oxide as purchased from Aldrich was significantly enriched in ^{18}O (and thus also presumably in ^{17}O) with a content of 0.28 atom % ^{18}O (natural abundance of ^{18}O is 0.20 atom %). In order to avoid enrichment of the CO_2 resulting from the decarboxylase reactions with ^{18}O and ^{17}O (thus necessitating a larger correction for ^{17}O and therefore a less accurate isotope effect determination), the D_2O was normalized prior to use.[2] Normalization was carried out by bubbling dry CO_2 through the D_2O in the presence of 0.5 g/L carbonic anhydrase. To follow the normalization process, samples of D_2O were removed, freed from CO_2 by sparging with CO_2-free, dry N_2, and equilibrated with natural abundance CO_2 such that the D_2O was in large excess. This CO_2 was isolated and analyzed for its ^{18}O content. After 12 h of normalization, the ^{18}O content of the D_2O had decreased from 0.28 atom % to 0.21 atom % (corresponding to a loss of 90% of the original excess over natural abundance). The deuterium content remained unchanged from the original 99.8 atom % D as determined by ^1H NMR and density measurements. Separate high-conversion samples were run in the "normalized" D_2O.

Data Processing. Reciprocal initial velocities were plotted versus the reciprocal of substrate concentration, and all plots were linear. Data were fit to the appropriate rate equations with the FORTRAN programs of Cleland (1979). Saturation curves obtained at each pH(D) were fit to eq 1. Data for the pH(D) dependence of V and V/K for benzoylformate and the pH dependence of $10K_i$ for (R)-mandelate were fit to eq 2. The data obtained for the pH dependence of $1/K_i$ for phthalate were fit to eq 3. Data for competitive inhibition were fit to eq 4. Dixon plots were fit to the equation for a straight line. The true K_i was then calculated by dividing the x-intercept by (1 + [benzoylformate]/$K_{\text{benzoylformate}}$).

$$v = \frac{VA}{K + A} \tag{1}$$

$$\log y = \log \frac{C}{1 + H/K_1 + K_2/H} \tag{2}$$

$$\log y = \log \frac{C}{1 + K_3/H} \tag{3}$$

$$v = \frac{VA}{K(1 + I/K_i) + A} \tag{4}$$

In eq 1 and 4, K is the K_m for benzoylformate, V is V_{max}, K_i is the inhibition constant for inhibitory analogues competitive versus benzoylformate, and v is the initial rate. A, H, and I are benzoylformate, H^+, and competitive inhibitor concentrations, respectively. In eq 2 and 3, y is the observed value of either V, V/K, or $1/K_i$; C is the pH independent plateau value of this parameter. In eq 2, K_1 and K_2 are acid dissociation constants for groups on the enzyme. In eq 3, K_3

[2] Alternatively, one can exchange the isolated CO_2 with H_2O and reisolate the CO_2 prior to mass spectroscopic analysis in order to remove the ^{17}O and ^{18}O excess. This method was used by O'Leary et al. (1981) in the determination of ^{13}C isotope effects in both H_2O and D_2O for glutamate decarboxylase.

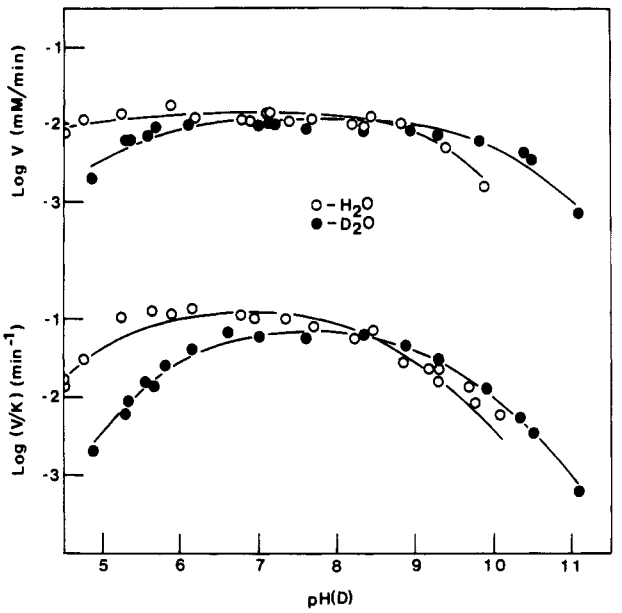


FIGURE 1: pH(D) dependence of the kinetic parameters for the benzoylformate decarboxylation reaction with benzoylformate in H_2O (open circles) and D_2O (closed circles). Data for V and V/K were fit to eq 2. In all cases, the points are experimental, and the curves are generated from the fits.

is the acid dissociation constant for a group on the inhibitor.

Initial rate data for benzoylformate and substrate analogues in H_2O and in D_2O were fit with algorithms that assume there are either different D_2O effects on V and V/K, equal D_2O effects on V and V/K, no D_2O effect on V, or no D_2O effect on V/K. The $^D(V)$ and/or $^D(V/K)$ effects were taken as the best fit for each set of data.

The $^{13}(V/K)$ isotope effects were computed from eq 5 (O'Leary, 1977), where R_0 and R_p are the $^{13}C/^{12}C$ isotope

$$^{13}(V/K) = \frac{\log (1 - f)}{\log (1 - fR_p/R_0)} \quad (5)$$

ratios for initial substrate (measured as product at $f = 1.0$) and product at fractional conversion f. Replicates from the same experiment were averaged to give the mean $^{13}(V/K)$ value.

RESULTS

pH(D) Dependence of Kinetic Parameters for Benzoylformate. The pH(D) dependence of V and V/K for benzoylformate is shown in Figure 1. Estimates of pK values for V/K are 5.2 ± 0.1 and 8.55 ± 0.09 in H_2O and 6.26 ± 0.09 and 9.14 ± 0.09 in D_2O. Those for V are 4.2 ± 0.3 and 9.17 ± 0.08 in H_2O and 5.2 ± 0.2 and 10.1 ± 0.1 in D_2O. The pH-independent solvent deuterium isotope effects are calculated from the ratio of the corresponding C values of eq 2 in H_2O to those in D_2O. $^D(V/K)$ is thus (0.12 ± 0.01)/(0.08 ± 0.01) or 1.5 ± 0.2, and $^D(V)$ is (0.0129 ± 0.0008)/(0.010 ± 0.001) or 1.3 ± 0.2.

pH(D) Dependence of ^{13}C Isotope Effect for Benzoylformate. The ^{13}C isotope effect on the decarboxylation of benzoylformate was obtained as a function of pH(D) (Table II). The isotope effect appears to be pH(D)-independent with average values for $^{13}(V/K)_H$ of 1.0081 ± 0.0007 and $^{13}(V/K)_D$ of 1.0054 ± 0.0009.

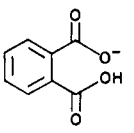
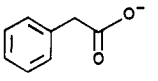
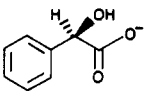
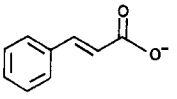
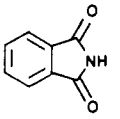
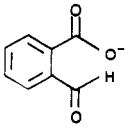
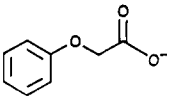
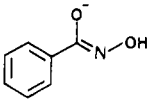
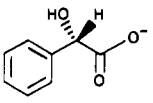
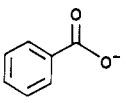
pH(D) Dependence of K_i for Competitive Inhibitors. A number of aromatic carboxylic acids were tested for substrate and inhibitory activity. None of the analogues tested including phenylpyruvate (data not shown) gave detectable substrate activity, but most were inhibitory. Inhibition constants and inhibitor chemical structures are shown in Table III.

Table II: pH(D) Dependence of $^{13}(V/K_{BF})^a$

pH	$^{13}(V/K_{BF})_H$	pD	$^{13}(V/K_{BF})_D$
6.0	1.0073	5.2	1.0054
	1.0078		1.0053
	mean 1.0076 ± 0.0003		mean 1.00535 ± 0.00005
7.5	1.0089	7.2	1.0058
	1.0086		1.0053
	mean 1.0088 ± 0.0002		mean 1.0056 ± 0.0003
9.0	1.0075	8.0	1.0050
	1.0075		1.0063
	1.0081		mean 1.0057 ± 0.0007
	mean 1.0077 ± 0.0003		
		9.6	1.0051
			1.0053
			mean 1.0052 ± 0.0001
	av 1.0080 ± 0.0005		av 1.0054 ± 0.0004

[a] At least two high-conversion $^{12}C/^{13}C$ ratios were averaged for each $^{13}(V/K)$ determination.

Table III: Dissociation Constants for Competitive Inhibitors of BFD[a]

	K_i (mM)		
 phthalate	0.098 ± 0.013[b]	6.8 ± 0.4	 phenylacetate
 (*R*)-mandelate	1.38 ± 0.09[b]	7 ± 2	 *trans*-cinnamate
 phthalimide	2.9 ± 0.1	7.4 ± 0.3	 2-carboxy-benzaldehyde
 phenoxyacetate	5.5 ± 2.7	no inhibition	 benzoylhydroxamate
 (*S*)-mandelate	6.7 ± 0.4	no inhibition	 benzoate

[a] All assays were carried out at pH 6.1, 100 mM MES, and 25 °C. The K_m for benzoylformate is 0.08 mM. [b] pH independent value of K_i from pK_i profile.

To test the possibility that an adduct was formed with the aldehyde group of 2-carboxybenzaldehyde, the inhibition constant was obtained with the unlabeled compound and with 2-carboxybenzaldehyde-d_1. At the three pH values tested (5, 6.95, and 9.75) K_i values of 13.3 ± 0.2 mM, 7.4 ± 0.3 mM, and 47.6 ± 13 mM were obtained for the unlabeled compound. At pH 6.95 and 9.75, K_i values of 7.22 ± 0.55 mM and 32.6 ± 9.4 mM were obtained for the deuteriated compound. The deuterium isotope effect on the K_i is calculated as 1.02 ± 0.09 and 1.46 ± 0.58, respectively, at pH 6.95 and 9.75.

The K_i values for (*R*)-mandelate and phthalate were obtained as a function of pH (Figure 2). The affinity for (*R*)-mandelate decreases below a pK of 5.3 ± 0.1 and above a pK of 8.2 ± 0.2, while the affinity for phthalate decreases

Table IV: Kinetic Parameters (in H_2O)

analogue	σ_p[a]	V_{max} (μM min^{-1})	V_{max}/K_m (min^{-1})	K_m (μM)	V_{max}(rel)[b]	V_{max}/K_m(rel)[b]
pCH$_3$O	−0.27	5.0 (±0.1)	0.090 (±0.009)	56 (±6)	0.23	0.24
pCH$_3$	−0.17	24 (±2)	0.128 (±0.009)	190 (±30)	1.1	0.36
pH	0	22 (±0.4)	0.38 (±0.02)	59 (±4)	1.0	1.0
pCl	0.23	11.4 (±0.7)	0.064 (±0.006)	180 (±27)	0.52	0.17
mF	0.34[c]	9.4 (±0.07)	0.41 (±0.02)	23 (±1)	0.43	1.08

[a] Taken from Hansch et al. (1973). [b] Relative values are calculated as the value for the analogue divided by the value for pH (benzoylformate). [c] σ_m.

Table V: Isotope Effects Summary

analogue	σ_p[a]	$^D(V)$	$^D(V/K)$	$^{13}(V/K)_H$	$^{13}(V/K)_D$
pCH$_3$O	−0.27	1.42 (±0.06)	1.42 (±0.06)	1.0197 (±0.0001)	1.0111 (±0.0001)
pCH$_3$	−0.17	2.47 (±0.2)	2.47 (±0.2)	1.0159 (±0.0002)	1.0081 (±0.0002)
pH	0	1.83 (±0.04)[b]	1.83 (±0.04)[b]	1.0080 (±0.0005)[c]	1.0054 (±0.0004)[c]
pCl	0.23	1	1.7 (± 0.1)	1.0061 (±0.0003)	1.0033 (±0.0002)
mF	0.34[d]	1.08 (±0.03)	3.1 (±0.2)	1.0021 (±0.001)	1.0027 (±0.0009)

[a] Taken from Hansch et al. (1973). [b] The values for pH (benzoylformate) were determined at a single pH along with the other analogues, as described under Materials and Methods. These values are slightly different from the pH-independent values discussed under Results. [c] Average values from Table I. [d] σ_m.

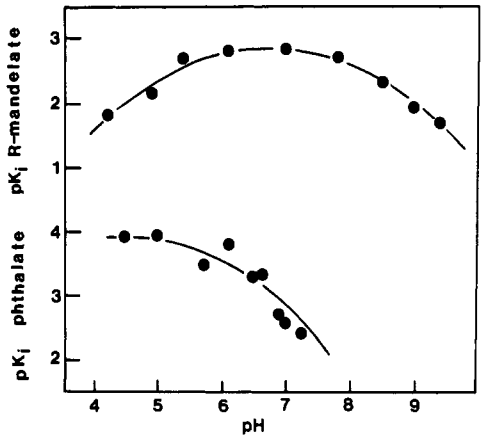


FIGURE 2: pH dependence of $1/K_i$ for (R)-mandelate and phthalate inhibition of benzoylformate decarboxylase. The K_i value for (R)-mandelate obtained at pH 6.8 is from a full inhibition pattern varying benzoylformate concentration at several fixed concentrations of (R)-mandelate. The remainder of the data are from Dixon plots correcting the apparent K_i for the substrate concentration as discussed under Materials and Methods. The (R)-mandelate data were fit to eq 2 while the phthalate data were fit to eq 3. In all cases, the points are experimental values while the curves are generated from the fits.

above a pK of 5.9 ± 0.2. The pH-independent values of K_i for both inhibitors are given in Table III.

Kinetic Parameters for Substrate Analogues. The kinetic parameters for benzoylformate and the substrate analogues in H_2O are given in Table IV. All of the analogues are good substrates for the decarboxylase. The "poorest" substrate analogue is less than 1 order of magnitude worse than benzoylformate. There appears to be a qualitative correlation between the size of the substituent and the K_m for the analogue. Analogues with substituents larger than hydrogen (pCH$_3$ and pCl) have K_m values larger than that of benzoylformate. An exception is the pCH$_3$O analogue, which may have additional interactions with the enzyme through the methoxy oxygen that may account for the analogue's lower K_m value. Those analogues whose σ values are closest to zero (pCH$_3$ and pCl) have V_{max} values that are not much different from that for benzoylformate, implying that, while the rate-determining steps may change with the change in electron-withdrawing/donating character of the substituent (as will be discussed later), the overall rate of chemical reaction on the enzyme is not perturbed greatly until a larger displacement of σ from zero is encountered.

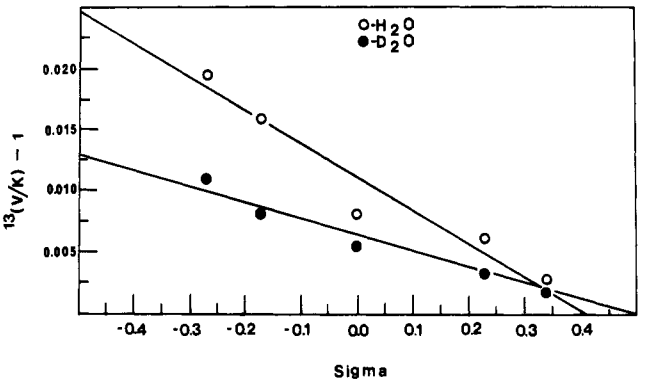


FIGURE 3: Plot of linear relationship between $^{13}(V/K) - 1$ versus σ for values in H_2O (open circles) and D_2O (closed circles). Each set of data was fit to the equation for a straight line (R = 0.97 in both cases). The points are the experimental data while the lines are from the fit.

Solvent Deuterium and ^{13}C Effects for Substrate Analogues. The solvent deuterium and ^{13}C isotope effects for benzoylformate and substrate analogues are given in Table V. Benzoylformate, the pCH$_3$O analogue, and the pCH$_3$ analogue all have equal D_2O effects on V and V/K. The pCl and mF analogues have different D_2O effects on V and V/K. The $^{13}(V/K)_D$ values were slightly less than the corresponding $^{13}(V/K)_H$ values for each substrate except (m-fluorobenzoyl)formate. The values for this latter case, however, are really too small to allow such differentiation. The ^{13}C effects minus one, in both H_2O and D_2O, decreased linearly versus the σ value for the substituent on the substrate (Figure 3).

Discussion

pH Dependence for Benzoylformate and Inhibitors. The pH(D) dependence of V/K for benzoylformate should reflect the pK values for groups on free enzyme and free substrate required for catalysis and/or binding. Benzoylformate has no pK's in the pH range 4–10; therefore, both of the groups whose pK values are observed in the V/K profile in H_2O and D_2O must be enzymic residues. Thus, the benzoylformate decarboxylase reaction requires one group protonated and the other group unprotonated for reaction. The pK's that are observed in the V profile, in both H_2O and D_2O, are similar to those observed in the V/K profile, in both H_2O and D_2O. The pK's in the V profile are, however, perturbed to lower and higher values by ~1 pH unit from the V/K values. Since

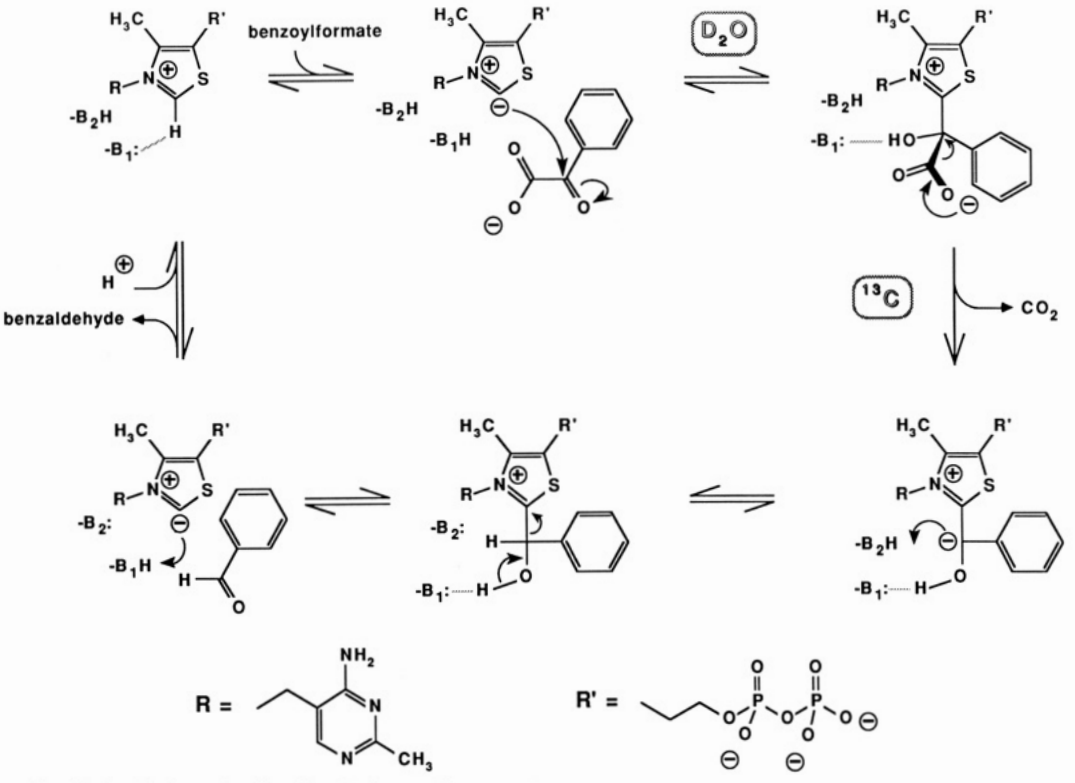


FIGURE 4: Postulated chemical mechanism for the benzoylformate decarboxylase reaction.

benzoylformate is a relatively fast substrate and we are looking at the direction of faster maximal velocity, this displacement in both pK's means one of two things: either some step(s) of the reaction mechanism after the V/K portion (decarboxylation), such as either benzaldehyde release or collapse of the second tetrahedral intermediate, has (have) become rate limiting (Cleland, 1977), or that substrate binding perturbs the pK_a's of the enzymic residues (the true pK_a's of which are seen in the V/K profile) to lower and higher values. Studies of the pH dependence of the reaction of the decarboxylase with (R)-mandelate and the fact that $^{D}(V)$ equals $^{D}(V/K)$ suggest the latter possibility.

Additional qualitative information is also obtained from studies of the pH dependence of the reaction of the decarboxylase with (R)-mandelate, which binds selectively to the enzyme when both groups are correctly protonated. The pK values in the pK_i profile for (R)-mandelate appear to be equal, within experimental error, to those observed in the V/K profile for benzoylformate. Since true pK's are obtained from the pH dependence of the K_i for a competitive inhibitor (Cleland, 1977), true pK's are also observed in the V/K profile, and therefore benzoylformate is not significantly sticky; that is, it dissociates from the enzyme faster than it reacts to give products.[3]

The pH–rate profile for phthalic acid exhibits a single pK of 5.8. This is somewhat higher than the enzymic pK of 5.2 and may correspond to the second pK of phthalic acid (5.4). Thus, the enzyme may only bind the monoanionic form of the dicarboxylic acid without a required protonation state for the enzymic group with pK of 5.2. The binding of phthalate will be discussed further below.

Interpretation of Inhibitor Data. Benzoylformate decarboxylase appears to be much more specific than pyruvate decarboxylase. The only compounds found thus far that are utilized as substrates are meta- and para-substituted benzoylformates (Hegeman, 1970; Reynolds et al., 1988; this study) while pyruvate decarboxylase will decarboxylate a number of aliphatic α-keto acids as well as para-substituted benzoylformates (Lehmann et al., 1973).

Benzoylformate decarboxylase is also quite stringent in its requirements for optimum binding. The K_m for benzoylformate is also its K_d since $^{D}(V)$ equals $^{D}(V/K)$ (Klinman & Matthews, 1985). The pH-independent value of the K_d for the substrate is 76 μM. Replacement of the α-keto group with a methylene, as in phenylacetate, decreases the affinity for the substrate by almost 100-fold, indicating an important hydrogen-bonding interaction of the α-keto group as would be expected mechanistically. Lengthening the alkyl chain, either as in phenoxyacetate or with a fixed geometry as in *trans*-cinnamate, gives the same decreased affinity which may be either a result of the absence of the α-keto group, steric problems, or a combination of both effects. Shortening the alkyl chain, as in benzoate, results in no detectable affinity. Benzoylhydroxamic acid, in which the carboxyl group is replaced by –NHOH, also gave no inhibition.

Inhibitors with the same chain length as benzoylformate, such as mandelate, also do not bind very tightly. Mandelate can be viewed as an analogue of the tetrahedral intermediate formed between benzoylformate and TPP. Consequently, the 5-fold discrimination in favor of the R isomer over the S isomer suggests that the tetrahedral intermediate may have the R absolute configuration. Even the R isomer, however, has a 20-fold lower affinity compared to benzoylformate, consistent with the hydrogen-bond acceptor role of the α-keto group postulated above. This difference is further evidence that benzoylformate forms a covalent adduct as shown in Figure 4.

An intriguing set of inhibitors is phthalate and its analogues. The phthalate monoanion appears to be an excellent analogue of benzoylformate with a K_d about the same as that of the

[3] A substrate is defined as "sticky" when partitioning of the E·S complex favors product formation over breakdown of the E·S complex to free E and S.

substrate. However, it may actually be a better analogue of the tetrahedral intermediate formed between substrate and TPP. The hydroxyl of the protonated carboxyl could hydrogen bond in place of the hydroxyl of the intermediate with the second carboxylate of the phthalate taking the place of the carboxylate of the substrate. That the hydroxyl of the protonated carboxyl plays an important role in binding is confirmed by comparison of phthalate with 2-carboxybenzaldehyde. Elimination of the hydroxyl present in the monoanion of phthalate decreases the affinity about 76-fold, consistent with a hydrogen-bonding interaction. Within experimental error, there is no deuterium isotope effect upon K_i for 2-carboxybenzaldehyde. Since an inverse deuterium isotope effect upon K_i would be expected if a covalent adduct were formed, we therefore conclude that this analogue does not form such an adduct with TPP and is only a competitive inhibitor. Phthalimide is 2-fold better than 2-carboxybenzaldehyde, perhaps suggesting that the NH can replace the OH of the phthalate monoanion to some extent, but optimum affinity is not attained in the absence of the negative charge.

Thus, for optimal binding, a phenyl ring (pyruvate was neither a substrate nor an inhibitor; data not shown) attached to the α-carbon of the substrate is required. In addition, the α-carbon should have a hydrogen-bond acceptor and a negatively charged group attached.

Solvent Deuterium and ^{13}C Isotope Effects. The pH-independent solvent deuterium isotope effect on V/K for benzoylformate is 1.5, while that on V is 1.3. In both cases, the pK values in the pH profiles are perturbed to higher pH values in D_2O as expected (Schowen, 1977). The increase in pK predicted for a nitrogen or oxygen acid–base catalyst in going from H_2O to D_2O is 0.5–0.6 pH unit. The perturbation in the basic-side pK for benzoylformate for both V (+0.93 pH unit) and V/K (+0.6 pH unit) is probably not significantly outside this range, and we can tentatively assign the group with this pK to be a protonated nitrogen or oxygen base. The perturbation in the acid-side pK for both V (+1.0 pH unit) and V/K (+1.1 pH units), however, does appear to lie outside this range. This large displacement is not due to the observation of nonmicroscopic pK's in H_2O since the pK values from the pK_i versus pH profile for (*R*)-mandelate and those from the V/K versus pH profile for benzoylformate are identical within experimental error. While displacements of many pK's in the neutral pH range in D_2O are in the range 0.4–0.5 pH unit, a number of polybasic acids show increasing displacements as the overall negative charge increases. For example, the third pK of tricarballylate is perturbed 0.8 pH unit (Laughton & Robertson, 1969). Dibasic acids in which the fractionation factor of the second proton decreases as a result of a strong hydrogen bonding after ionization of the first proton have higher displacements, such as 0.6 pH unit for pK_1 of maleic acid and 0.8 pH unit for pK_1 of 2,2′-dihydroxybiphenyl (Laughton & Robertson, 1969). The usual explanation given for the size of the effects is that the fractionation factors of protons prior to ionization are near unity relative to H_2O, while the three protons of H_3O^+ after ionization have fractionation factors of 0.69. Since $-3(\log 0.69) = 0.48$, this rationalization appears to account for the observed effect. However, the agreement of this calculation with experiment in most cases is probably due to the fortuitous cancellation of the effects on the fractionation factors of the protons in the hdyration shells of the group prior to ionization, and of the group after ionization. We cannot say why the pK's on the low side of both V and V/K pD profiles for benzoylformate are so greatly shifted in D_2O, but it is clear that a number of protons in addition to the three that occur in H_3O^+ have higher fractionation factors prior to ionization than afterward.

Table VI: Experimental and Calculated $^D(V/K)$ Values for Benzoylformate and Analogues

analogue	$^D(V/K)_{exp}$	$^D(V/K)_{calc}$[a]	difference[b]
pCH_3O	1.42 ± 0.06	1.78 ± 0.02	−0.36 ± 0.06
pCH_3	2.5 ± 0.2	1.96 ± 0.05	0.54 ± 0.2
*p*H	1.83 ± 0.04	1.95 ± 0.1	0.3 ± 0.1
*p*Cl	1.7 ± 0.1	1.9 ± 0.1	−0.2 ± 0.1
*m*F	3.1 ± 0.2	ND[c]	ND[c]

[a] Calculated values are from eq 7 where $^D(K_{eq}) = 1$. [b] The difference is the experimental value minus the calculated value. [c] ND, value not calculated due to reasons stated under Discussion.

The benzoylformate decarboxylase reaction pathway under V/K conditions is comprised of a minimum of three steps on the basis of the probable chemical course of a TPP-catalyzed decarboxylation as shown in eq 6, where k_3 and k_4 represent

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{·adduct} \xrightarrow{k_5} EX + CO_2 \qquad (6)$$

formation and decomposition of the tetrahedral adduct formed between benzoylformate and TPP. k_5 is the decarboxylation step, and EX is the resulting eneamine.

The ^{13}C isotope effect for benzoylformate determined in H_2O [$^{13}(V/K)_H$] appears to be pH-independent, and the value of 1.0080 certainly suggests that decarboxylation is not fully rate limiting [a good estimate of the intrinsic ^{13}C isotope effect of 1.051 was determined by Jordan et al. (1978) for the nonenzymatic decarboxylation of 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride, which is an excellent analogue of the tetrahedral adduct formed during the enzyme-catalyzed reaction]. ^{13}C isotope effects for an analogous reaction catalyzed by pyruvate decarboxylase from yeast have been reported. O'Leary (1976) determined that $^{13}(V/K)$ for pyruvate was 1.0083 at pH 6.8 and 25 °C. Jordan et al. (1978) found that $^{13}(V/K)$ for pyruvate varied with pH from 1.011 at pH 5 to 1.002 at pH 7.5 and 30 °C. Interestingly, Melzer and Schmidt (1987) determined that the ^{13}C isotope effect at C-1 of pyruvate was 1.0093 (*Escherichia coli*, pH 7.6 and 36 °C) and 1.024 (yeast, pH 7.8 and 36 °C) for the overall reaction catalyzed by the pyruvate dehydrogenase multienzyme complex.

The ^{13}C isotope effect for benzoylformate decarboxylase decreased from 1.0080 in H_2O to 1.0054 in D_2O. A similar trend of decreasing $^{13}(V/K)$ values is apparent for the other analogues used as substrates for the decarboxylase, except (*m*-fluorobenzoyl)formate. If the solvent deuterium isotope effect is on the decarboxylation step, or some step that accompanies decarboxylation, then the ^{13}C isotope effect should increase when determined in D_2O; while if it were on a step preceding decarboxylation, then the effect should decrease. The three experimentally determined isotope effects can be related by eq 7 for the situation we have here, where the ^{13}C

$$\frac{^{13}(V/K)_H - 1}{^{13}(V/K)_D - 1} = \frac{^D(V/K)}{^D(K_{eq})} \qquad (7)$$

isotope effect decreased when determined in D_2O (Hermes et al., 1982), where $^D(K_{eq})$ is the solvent deuterium isotope effect upon the equilibrium constant for the reaction shown in eq 6. If one determines $^D(K_{eq})$ from the isotope effect data in Table V for all but (*m*-fluorobenzoyl)formate, a weighted mean for $^D(K_{eq})$ of 1.1 ± 0.2 is calculated. The data for (*m*-fluorobenzoyl)formate were not included in this calculation

since the errors in the ^{13}C isotope effects are too large relative to this size of the effect. The value of $^{D}(K_{eq})$ has also been estimated from the fractionation factor for the C-2 proton of TPP to be 0.97 ± 0.05 (Alverez, 1985). Since neither of the values give a value significantly different from unity, we will assume $^{D}(K_{eq}) = 1$. On this basis, we have used eq 7 to calculate $^{D}(V/K)$ for all the analogues, except (*m*-fluorobenzoyl)formate, in an effort to determine how well the data fit the multiple isotope effect model represented by eq 7. The results are presented in Table VI. Since the differences appear to be random in that two of the calculated values are higher than the experimental values and two of the calculated values are lower than the experimental values, we conclude, on the basis of the observed decrease in the ^{13}C isotope effects in D_2O versus H_2O, that the solvent deuterium isotope sensitive step does precede the ^{13}C isotope sensitive step (i.e., decarboxylation) and that benzoylformate and the substituted analogues are decarboxylated in a stepwise fashion.

There are a number of qualitative observations that can be made from the isotope effects for the various benzoylformate analogues used as substrates for the decarboxylase. Electron-withdrawing substituents (*mF* and *pCl*) show diminished ^{13}C isotope effects suggesting that the substituents are stabilizing a carbanion-like transition state in the decarboxylation step (Figure 4). In fact, it appears that for (*m*-fluorobenzoyl)formate the solvent-sensitive step has become nearly entirely rate limiting and the $^{D}(V/K)$ value of 3.1 may represent the intrinsic solvent deuterium isotope effect for this substrate. Electron-donating substituents (p-CH_3 and p-CH_3O) have increased rate dependences upon decarboxylation as indicated by the larger $^{13}(V/K)$ values (Table V). This suggests a destabilizing effect upon the carbanion-like transition state in the decarboxylation step. Alternatively, or additionally, these electron-donating analogues may be stabilizing the initial tetrahedral intermediate (Figure 4), thereby significantly slowing the decarboxylation step. The lowest $^{D}(V/K)$ value observed corresponds to the most electron-donating substituent, which reinforces this latter point of an increased rate for the deuterium-sensitive step and decreased rate for the ^{13}C-sensitive step.

Chemical Mechanism. The basic chemistry of TPP-dependent decarboxylation reactions has been suggested by others (Breslow, 1958) and is used here as a framework to present the benzoylformate decarboxylase mechanism as shown in Figure 4. The free enzyme requires at least two bases for catalysis and/or binding, one protonated and one unprotonated, as suggested by the bell-shaped pH dependence of V/K. Although no role is evident for the protonated base (B_2) under V/K conditions, the substrate requires that both B_1 and B_2 be in the correct protonation state for optimum binding. The degree to which the pK values of these residues are perturbed on substrate binding is dependent upon the extent to which the substrate prefers this correctly protonated enzyme form. The unprotonated base (B_1) is most likely in close proximity to C-2 of TPP and may actually be hydrogen bonded to the proton at this position. Precedence for C-2 hydrogen of TPP being hydrogen bonded is provided by recent X-ray studies (Aoki & Yamazaki, 1985) that have shown that the C-2 hydrogen of thiamine monophosphate is intramolecularly hydrogen bonded to phosphate in the crystal lattice. Benzoylformate is bound in its ionized form; the enzymic base abstracts the C-2 proton of TPP and shuttles it to the keto oxygen of the substrate as the resulting ylide of TPP attacks the carbonyl carbon to give the first tetrahedral intermediate. This intermediate probably has the *R* absolute configuration on the basis of our studies of (*R*)- versus (*S*)-mandelate. If one pictures the monoanion of phthalate in the site, the hydroxyl of the protonated carboxyl could hydrogen bond to the enzymic base with the carbonyl oxygen in close proximity to the C-2 hydrogen of TPP, while the ionized carboxylate takes the place of the substrate carboxylate. Thus, as described above, phthalate may be an analogue of the intermediate. Decarboxylation can then occur as electrons are delocalized from the carboxylate group into the thiazolium ring and CO_2 is released. The resulting enolamine (or carbanion intermediate) is then protonated by the second enzymic base (B_2) to form the second tetrahedral intermediate (stereochemistry unknown). This intermediate then breaks down to produce benzaldehyde and regenerate the TPP ylide. Benzaldehyde is then released, the thiazolium ring of TPP is reprotonated, and the enzymic acid is regenerated.

ACKNOWLEDGMENTS

We gratefully acknowledge the UCSF Biomedical Mass Spectrometry Resource (NIH-RR01614), Dr. A. L. Burlingame, Director. A major portion of the funding for the isotope ratio mass spectrometer was provided by a grant from the NSF (PCM-8218027).

REFERENCES

Alvarez, F. J. (1985) Ph.D. Dissertation, Department of Chemistry, University of Kansas.

Aoki, K., & Yamazaki, H. (1985) *J. Am. Chem. Soc. 107*, 6242.

Barnish, I. T., Cross, P. E., Danilewicz, J. C., Dickinson, R. P., & Stopher, D. A. (1981) *J. Med. Chem. 24*, 399.

Barrowman, M. M., & Fewson, C. A. (1985) *Curr. Microbiol. 12*, 235.

Breslow, R. J. (1958) *J. Am. Chem. Soc. 80*, 3719.

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 297.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 1790.

Craig, H. (1957) *Geochim. Cosmochim. Acta 12*, 133.

Gardner, J. H., & Naylor, C. A., Jr. (1943) *Org. Synth. 2*, 67.

Gunsalus, I. C., Gunsalus, F. C., & Stanier, R. Y. (1958) *J. Bacteriol. 66*, 538.

Hansch, C., Leo, A., Unger, S. H., Kim, K. N., Nitkaitani, D., & Lien, E. J. (1973) *J. Med. Chem. 16(11)*, 1207.

Hegeman, G. D. (1970) *Methods Enzymol. 17a*, 674.

Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106.

Jordan, F., Kuo, D. J., & Monse, E. U. (1978) *J. Am. Chem. Soc. 100*, 3872.

Klinman, J. P., & Matthews, R. G. (1985) *J. Am. Chem. Soc. 107*, 1058.

Laughton, P. M., & Robertson, R. E. (1969) in *Solute-Solvent* Interactions (Coetzee, J. F., & Ritchie, C. D., Eds.) p 400, Decker, New York.

Lehmann, H., Fischer, G., Hubner, G., Kohnert, K.-D., & Schellenberger, A. (1973) *Eur. J. Biochem. 32*, 83.

Melzer, E., & Schmidt, H. L. (1987) *J. Biol. Chem. 262*, 8159.

Northrop, D. B. (1977) in *Isotope Effects in Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore, MD.

O'Leary, M. H. (1976) *Biochem. Biophys. Res. Commun. 73*, 614.

O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83.

O'Leary, M. H., Yamada, H., & Yapp, C. J. (1981) *Biochemistry 20*, 1476.
Reynolds, L. J., Garcia, G. A., Kozarich, J. W., & Kenyon, G. L. (1988) *Biochemistry* (in press).
Schowen, R. L. (1977) in *Isotope Effects in Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 64, University Park Press, Baltimore, MD.

Activation of a Calmodulin-Dependent Phosphatase by a Ca^{2+}-Dependent Protease†

E. Ann Tallant, Lynn M. Brumley, and Robert W. Wallace*
Department of Pharmacology, University of Alabama at Birmingham, Birmingham, Alabama 35294
Received September 1, 1987; Revised Manuscript Received November 12, 1987

ABSTRACT: A calmodulin-dependent protein phosphatase (calcineurin) was converted to an active, calmodulin-independent form by a Ca^{2+}-dependent protease (calpain I). Proteolysis could be blocked by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, leupeptin, or *N*-ethylmaleimide, but other protease inhibitors such as phenylmethanesulfonyl fluoride, aprotinin, benzamidine, diisopropyl fluorophosphate, and trypsin inhibitor were ineffective. Phosphatase proteolyzed in the absence of calmodulin was insensitive to Ca^{2+} or Ca^{2+}/calmodulin; the activity of the proteolyzed enzyme was greater than the Ca^{2+}/calmodulin-stimulated activity of the unproteolyzed enzyme. Proteolysis of the phosphatase in the presence of calmodulin proceeded at a more rapid rate than in its absence, and the proteolyzed enzyme retained a small degree of sensitivity to Ca^{2+}/calmodulin, being further stimulated some 15–20%. Proteolytic stimulation of phosphatase activity was accompanied by degradation of the 60-kilodalton (kDa) subunit; the 19-kDa subunit was not degraded. In the absence of calmodulin, the 60-kDa subunit was sequentially degraded to 58- and 45-kDa fragments; the 45-kDa fragment was incapable of binding ^{125}I-calmodulin. In the presence of calmodulin, the 60-kDa subunit was proteolyzed to fragments of 58, 55 (2), and 48 kDa, all of which retained some ability to bind calmodulin. These data, coupled with our previous report that the human platelet calmodulin-binding proteins undergo Ca^{2+}-dependent proteolysis upon platelet activation [Wallace, R. W., Tallant, E. A., & McManus, M. C. (1987) *Biochemistry 26*, 2766–2773], suggest that the Ca^{2+}-dependent protease may have a role in the platelet as an irreversible activator of certain Ca^{2+}/calmodulin-dependent reactions.

A Ca^{2+}/calmodulin-dependent protein phosphatase, also known as calcineurin, was originally isolated as the major calmodulin-binding protein in bovine brain (Wang & Desai, 1976; Klee & Krinks, 1978; Wallace et al., 1978). It was subsequently identified as a protein phosphatase on the basis of its similarity to a Ca^{2+}/calmodulin-dependent phosphatase purified from rabbit skeletal muscle (protein phosphatase 2B) (Stewart et al., 1982; Yang et al., 1982). A similar enzyme has also been isolated from bovine heart (Krinks et al., 1983) and human platelets (Tallant & Wallace, 1985) and appears to be widely distributed in other tissues (Wallace et al., 1980a; Ingebritsen et al., 1983). The brain enzyme is a heterodimer (M_r 80000) composed of a 60-kilodalton (kDa)[1] subunit which binds calmodulin in a Ca^{2+}-dependent manner (Richman & Klee, 1978) and contains the catalytic site (Winkler et al., 1984) and a 19-kDa subunit which contains four high-affinity Ca^{2+}-binding sites (Klee et al., 1979). Although the phosphatase dephosphorylates a wide variety of substrates in vitro (Tallant & Cheung, 1986; King et al., 1984), it exhibits a high catalytic efficiency toward only a few phosphoproteins (King et al., 1984), suggesting a more limited specificity in situ.

In addition to its reversible activation by Ca^{2+}/calmodulin, the phosphatase can be irreversibly activated and made calmodulin-independent upon limited proteolysis by either trypsin (Manalan & Klee, 1983; Tallant & Cheung, 1984b; Tallant & Wallace, 1985) or chymotrypsin (Kincaid et al., 1986). Limited trypsinization of the phosphatase in the absence of calmodulin results in degradation of the 60-kDa subunit to a 43–45-kDa fragment which no longer binds calmodulin (Manalan & Klee, 1983; Tallant & Cheung, 1984b); proteolysis in the presence of calmodulin proceeds at an altered rate (Manalan & Klee, 1983; Tallant & Cheung, 1984b) and produces a series of proteolytic fragments of 57, 55, 54, 46, and 40 kDa, all of which are still capable of binding calmodulin (Manalan & Klee, 1983). Other calmodulin-dependent enzymes are also irreversibly activated and made calmodulin-independent by limited proteolysis including cyclic nucleotide phosphodiesterase (Lin & Cheung, 1980; Kincaid et al., 1985), skeletal muscle phosphorylase kinase (Depaoli-Roach et al., 1979), erythrocyte Ca^{2+}-ATPase (Niggli et al., 1981), myosin light-chain kinase (Walsh et al., 1982), NAD kinase (Meijer & Guerrier, 1982), and adenylate cyclase (Keller et al., 1980). However, proteolytic activation has not been considered to be an important physiological mechanism for activation of calmodulin-dependent enzymes, because no calmodulin-dependent enzyme has been shown to be activated either in vitro or in

† This research was supported by NIH NHLBI Grant HL 29766.
* Address correspondence to this author.

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; CaM, calmodulin; kDa, kilodalton(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CANP, calcium-activated neutral protease(s); EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TCA, trichloroacetic acid.

0006-2960/88/0427-2205$01.50/0