As an additional control, a stream of nitrogen was swept over a flask containing 600 μL of tributylstannane and AIBN (2 mg) heated at 190 °C. After 7 h no precipitate was observed in the lead acetate solution.

Control Reactions for the Above Model Reaction (Scheme II). (A) Treatment of 3-(4-Methoxyphenyl)-5-methyl-2-oxazolidinone (11) with Tributylstannane and AIBN. The methyl derivative **11** (87 mg, 0.42 mmol), tributylstannane (159 μL, 0.59 mmol), and AIBN (2 mg) were heated at 190 °C for 7 h. Thin-layer chromatography of the reaction mixture showed only unreacted starting material and some polar decomposition products at the base line. The major product was isolated by silica gel chromatography (20 × 1.5 cm; 1:1 EtOAc/*n*-hexane) and was shown to be starting material by comparison of the thin-layer chromatography R_f values and NMR spectra to those of a sample of **11** prepared as described above.

(B) Heating of 8 at 190 °C. Chloride **8** (200 mg, 0.83 mmol) was heated at 190 °C for 7 h. Thin-layer chromatography (1:1 EtOAc/*n*-hexane) showed that the major product was unchanged starting material contaminated with a small amount of polar decomposition products. The starting material was isolated by silica gel chromatography (20 × 1.5 cm; 1:1 EtOAc/*n*-hexane) to yield 194 mg (97%) of **8** as a white crystalline solid. The identity of this product was confirmed by comparison of the thin-layer chromatography R_f values and NMR spectra to those of a sample of **8** prepared as described above.

Reaction of 5-[(Mesyloxy)methyl]-3-(4-methoxyphenyl)-2-oxazolidinone with Sodium Iodide and Zinc Metal (Scheme III). Mesylate **12** was prepared from the corresponding alcohol[36] by the method of Crossland and Servis.[39] A mixture of **12** (40 mg, 0.13 mmol), NaI (99 mg, 0.66 mmol), and powdered zinc metal (85 mg, 1.3 mmol) was stirred in refluxing dimethoxyethane for 1 h. The reaction mixture was filtered to remove the excess zinc powder, diluted with water (30 mL), and extracted with chloroform (3 × 20 mL). The combined organic layer was dried ($MgSO_4$), filtered, and rotary evaporated to yield a light yellow oil, which was purified by silica gel chromatography (20 × 1.5 cm; 1:1 EtOAc/*n*-hexane), giving 20 mg (96%) of a light yellow oil (**10**). The identity of this product was confirmed by comparison of the thin-layer chromatography R_f values and NMR spectra to those of a sample of **10** prepared as described above.

(39) Crossland, R. K.; Servis, K. L. *J. Org. Chem.* **1970**, *35*, 3195–3196.

Model Reaction for Carbanionic Pathway b (Scheme IV). (A) Grignard Reaction. Magnesium turnings (7 mg) were added to a solution of bromide **13** (50 mg, 0.17 mmol) in dry THF (2 mL). The solution was brought to reflux, and a small chip of iodine was added. After 1.5 h the mixture was cooled and then diluted with 5% HCl (5 mL), neutralized, and extracted with ethyl acetate. The combined organic layers were dried ($MgSO_4$) and filtered, and the solvent was evaporated to yield 26 mg (95%) of *N*-allylanisidine (**10**). The identity of the product was confirmed by comparison of the thin-layer chromatography R_f values and NMR spectra to those of a sample of **10** prepared as described above.

(B) Lithium Anion. A solution of bromide **13** (40 mg, 0.14 mmol) in dry THF (5 mL) was cooled to −78 °C, and then 113 μL of 1.6 M *n*-butyllithium was slowly added. The solution was allowed to warm slowly to 5 °C, and then 5 mL of saturated ammonium chloride was added. The solution was neutralized, diluted with 25 mL of water, and extracted with ethyl acetate. Two products were isolated by silica gel chromatography (20 × 1.5 cm; 1:1 EtOAc/*n*-hexane): **13** (6 mg, 15%) and *N*-allylanisidine (**10**) (19 mg, 85%). Products were identified by comparison of the thin-layer chromatography R_f values and NMR spectra to those of samples of **13** and **10** prepared as described above.

Release of $^{14}CO_2$ from Inactivation of Monoamine Oxidase by [*carbonyl*-^{14}C]-1. MAO (0.73 μM) was incubated for 29 h with [*carbonyl*-^{14}C]-**1** (200 μM) in a serum-capped vial with a center well (Kontes Catalog No. 882320-0000) containing 100 μL of 8 N KOH. The progress of the inactivation was followed by assaying a control reaction containing unlabeled inactivator. The enzyme reaction was quenched with 100 μL of 2 N H_2SO_4; after 30 min, the center well was placed in scintillation fluid, and the radioactivity in the center well was determined by scintillation counting. A nonenzymatic control reaction was run in parallel, and the contents of the center well were used for background radioactivity.

Acknowledgment. We thank Dr. Philippe Dostert for information regarding the synthesis of **1**. The National Institutes of Health (GM32634) is gratefully acknowledged for financial support of this work.

Registry No. 8, 121082-79-9; **10**, 71954-46-6; **11**, 121485-50-5; **12**, 121082-76-6; **13**, 121082-86-8; MAO, 9001-66-5; MD 780236, 84269-97-6; tributylstannane, 688-73-3.

(*E*)-4-(α-Halo-*p*-tolyl)-2-oxo-3-butenoic Acids Inhibit Yeast Pyruvate Decarboxylase by a Diversity of Mechanisms: Multiple Fate for the Thiamin-Bound Enamine Intermediate

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Abstract: (*E*)-4-(*p*-Tolyl)-2-oxo-3-butenoic acid and its α-(bromomethyl) and α-(chloromethyl) derivatives have been synthesized, and their interaction with brewers' yeast pyruvate decarboxylase was evaluated. The *p*-tolyl compound was found to be a slow substrate. The bromomethyl analogue led to partial time-dependent inactivation of the enzyme, but full activity was regained eventually. This analogue was shown to lose bromide ion in an enzyme-catalyzed, and time-dependent, fashion, and in its enzyme-catalyzed reaction it was converted quantitatively to *p*-methylcinnamic acid. The chloromethyl compound led to time-dependent inactivation of the enzyme; activity was not regained even after overnight incubation. This analogue released chloride ion in a time-dependent and enzyme-catalyzed reaction and produced *p*-(chloromethyl)cinnamaldehdye and *p*-methylcinnamic acid in a ratio of 4:6. All results are consistent with decarboxylation of the compounds followed by diverse fates for the central enamine intermediate: (1) the methyl derivative undergoes normal turnover; (2) the enamine derived from the decarboxylation of the *p*-(bromomethyl) derivative undergoes halide elimination, leading to a quinone methide that tautomerizes to a 2-acylthiamin diphosphate, which upon hydrolysis regenerates active enzyme [this behavior is analogous to that in a recent report on the decarboxylation of [*p*-(bromomethyl)benzoyl]formic acid by benzoylformate decarboxylase (Reynolds, L. J.; Garcia, G. A.; Kozarich, J. W.; Kenyon, G. L. *Biochemistry* **1988**, *27*, 5530)]; (3) the enamine derived from the chloromethyl compound is partitioned between chloride elimination and turnover and, most importantly, also leads to irreversible inactivation as reported earlier for some aromatic ring substituted (*E*)-4-phenyl-2-oxo-3-butenoic acids (Kuo, D. J.; Jordan, F. *Biochemistry* **1983**, *22*, 3735).

It was demonstrated during the past few years in this laboratory that some pyruvic acid analogues that have ring-substituted styrenes in place of a methyl group are processed by the enzyme pyruvate decarboxylase (PDC, EC 4.1.1.1) and have two very useful attributes. On the one hand these compounds are slow substrates, and some among them act as mechanism-based inactivators that are useful probes of the active center environment and functionalities.[1] In addition, the enamine (or 2α-carbanion)

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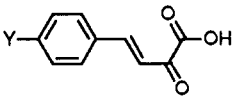
intermediate when derived from such inhibitors is detectable by virtue of its vis absorption centered near 430–440 nm.[2] In an earlier report Hubner showed that fluoropyruvate, while decarboxylated by PDC from brewers' yeast, undergoes fluoride elimination and concomitant production of acetate, thereby diverting the normal course of the enzymatic reaction.[3] Similar observations were recently reported on the enzyme isolated from wheat germ by Kluger's group.[4] Such a pathway had earlier been observed by Leung and Frey in the interaction of fluoropyruvate with the pyruvate dehydrogenase multienzyme complex.[5] In the mean time Kozarich, Kenyon, and their co-workers reported that the enzyme benzoylformate decarboxylase [the mechanism of which presumably is analogous to that of pyruvate decarboxylase since both enzymes perform thiamin diphosphate (TDP) dependent nonoxidative decarboxylations of α-keto acids to aldehydes] is inactivated by [*p*-(bromomethyl)benzoyl]formates, and this compound releases Br^- in an enzyme–TDP- and time-dependent fashion.[6] However, within a few hours enzyme activity was fully regained. The Br^- release was interpreted to mean that a quinone methide intermediate was formed that upon tautomerization formed a 2-acyl-TDP derivative, which in turn was slowly hydrolyzed to the active enzyme. Inspired in part by these reports, and continuing our search for more effective PDC inhibitors, we synthesized three compounds (**Ia–c**) and tested them as potential



Ia: Y = CH_3
b: Y = CH_2Cl
c: Y = CH_2Br

inhibitors of PDC. On the basis of a variety of measurements that included the kinetics of halide release, inactivation, and turnover, we can now report that the three compounds greatly differ in their interactions with the enzyme and serve as useful probes of the properties of the central enamine intermediate. Compound **Ia** is a substrate: **Ib** exhibits a complex behavior that has multiple pathways for inhibition: one reversible, the other not. And finally, **Ic** gives rise to inhibition accompanied by concomitant Br^- release and eventual recovery of activity. The enamine intermediate, the immediate product of decarboxylation, therefore undergoes three different reactions depending on the methyl substituent.

Materials and Methods

Synthesis. **(*E*)-4-(*p*-Tolyl)-2-oxo-3-butenoic acid**[7] **(Ia)** was synthesized by condensing *p*-tolualdehyde with pyruvic acid. Solid KOH (2.52 g, 0.045 mol) was dissolved in 20 mL of methanol and was added dropwise over 20 min to a stirred mixture of 3.10 g (0.03 mol) of *p*-tolualdehyde and 0.03 mol of pyruvic acid dissolved in 30 mL of methanol. Stirring was continued for a further 6 h. The resulting bright yellow solid was cooled in an ice bath; it was then filtered and washed with cold methanol. The resulting potassium salt was dissolved in water and acidified to pH 2.5 by the addition of 2 N H_2SO_4 and then extracted with 3 × 75-mL portions of ether. The combined ether layers were dried over Na_2SO_4, and the ether was removed with a rotary evaporator. The acid form was recrystallized from benzene or toluene and dried in vacuo, yielding 75% product (all-trans or *E* isomer): mp 128–131 °C; UV (CH_3OH) λ_{max} = 305 nm (ϵ_{305} = 22 800); ^1H NMR (CD_3CN/TMS) δ 7.32–8.21 (m, 6 H), 2.30 (s, 3 H). Anal. Calcd for $C_{11}H_{10}O_3$: C, 69.48; H, 5.26. Found: C, 69.65; H 5.32.

(*E*)-4-(α-Chloro-*p*-tolyl)-2-oxo-3-butenoic acid (Ib) was synthesized by treating 3.8 g (0.02 mol) of **Ia** with a 10 molar excess of SO_2Cl_2 under UV light (275 W). After 30 min the starting material was completely dissolved. The progress of the reaction was monitored by ^1H NMR, by observing the decrease of the methyl peak at 2.3 ppm and the buildup of the chloromethylene peak at 4.7 ppm. The reaction was terminated after 55 min, when a resonance at 6.7 ppm corresponding to the dichloromethyl derivative started to grow. The sulfuryl chloride was removed under vacuum to leave an orange oil. Attempts to purify the crude **Ib** did not succeed. Therefore, the acid was next converted to the *tert*-butyl ester by a modification of a procedure of Anderson and Callahan.[8] The crude acid was dissolved in 120 mL of CH_2Cl_2 containing four drops of concentrated H_2SO_4. 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% Na_2CO_3. The aqueous layer was back-extracted with CH_2Cl_2 (2 × 50 mL), and the combined organic layers were dried over Na_2SO_4. The filtrate was concentrated in vacuo. The *tert*-butyl ester products were flash chromatographed[9] on silica gel (5% ethyl acetate in hexane). The *tert*-butyl ester was converted to the free acid by cleavage in trifluoroacetic acid.[10] After 60 min at room temperature, the trifluoroacetic acid was removed in vacuo, and the product was recrystallized from 5% ethyl acetate in hexane. Pale yellowish brown crystals were obtained (0.76 g, 20% all-trans isomer; yield based on **Ia**): mp 90–94 °C; UV (CH_3OH) λ_{max} = 309 nm (ϵ_{309} = 23 800); ^1H NMR (CD_3CN/TMS) δ 7.21–8.03 (m, 6 H), 4.72 (s, 2 H). Anal. Calcd for $C_{11}H_9O_3Cl$: C, 58.81; H, 4.01; Cl, 15.79. Found: C, 58.92; H, 3.94, Cl 15.65.

(*E*)-4-(α-Bromo-*p*-tolyl)-2-oxo-3-butenoic Acid (Ic). To a homogeneous solution of 3.8 g (0.02 mol) of **Ia** dissolved in 150 mL of CCl_4 was added 3.92 g (0.022 mol) of *N*-bromosuccinimide and 5 mg of benzoyl peroxide. The reaction mixture was held at reflux and irradiated with a 275-W sun lamp for 1 h. The greenish yellow reaction mixture was filtered, and the CCl_4 was removed at a rotary evaporator. The residue was dissolved in benzene and refrigerated to accelerate crystallization. The pale yellow crystals were washed with cold benzene and then with a copious amount of water until the resonance at 2.3 ppm due to starting material disappeared yielding 2.3 g (42% all-trans isomer) of **Ic**: mp 79–84 °C; UV (CH_3OH) λ_{max} = 310 nm (ϵ_{310} = 24 200); ^1H NMR (CD_3CN/TMS) δ 7.13–8.24 ppm (m, 6 H), 4.61 (s, 2 H). Anal. Calcd for $C_{11}H_9O_3Br$: C, 49.1; H, 3.37; Br, 29.71. Found: C, 48.94; H, 3.35; Br, 28.99.

***p*-Methylcinnamaldehyde.** To a mixture of *p*-methylbenzaldehyde (3.6 g, 0.03 mol) and acetaldehyde (1.32 g, 0.03 mol) at 10 °C was added dropwise KOH (2.54 g, 0.04 mol) in 20 mL of methanol. The reaction mixture was stirred for an additional 4 h, and the yellowish brown oil was extracted into ether and dried ($MgSO_4$). The ether was removed under vacuo, and the resulting oil was purified on a silica gel column with hexane–ether (5:1): UV (CH_3OH) λ_{max} = 280 nm (ϵ_{280} = 18 950); ^1H NMR (acetone-d_6/TMS, 200 MHz) δ 9.53 (s, 1 H), 7.23–7.94 (m, 6 H), 2.3 (s, 3 H).

***p*-(Bromomethyl)cinnamaldehyde.** *p*-Methylcinnamaldehyde (0.5 g, 2.2 mmol) was dissolved in 50 mL of CCl_4, and *N*-bromosuccinimide (0.4 g, 2.2 mmol) was added. The reaction mixture was irradiated with a 275-W sun lamp while being stirred for 30 min. The mixture was filtered, and the CCl_4 was removed with a rotary evaporator. The resulting yellowish brown oil was chromatographed on silica gel (hexane–CH_2Cl_2, 2:1): UV (CH_3OH) λ_{max} = 288 nm (ϵ_{288} = 19 140); ^1H NMR (acetone-d_6/TMS, 200 MHz) δ 9.60 (s, 1 H), 7.31–8.06 (m, 6 H), 4.62 (s, 2 H).

Enzyme Purification and Assays. The purification protocol adopted in this laboratory has been reported elsewhere.[1a,b] PDC was assayed routinely with the pH-stat assay,[11] the release of hydroxide ion on enamine protonation being measured. Enzyme inhibition studies were performed at 30 °C. The typical incubation mixture contained 1 mM TDP, 1 mM $MgCl_2$ or $MgSO_4$, 1 mM EDTA in 0.1 M citrate buffer, pH 6.0, and the indicated concentration of inhibitor. At the indicated time intervals 10-μL aliquots were removed from the incubation mixtures and assayed for PDC activity at 30 °C by the pH-stat method. One unit of PDC converts 1 μmol of pyruvate to acetaldehyde per minute at 30 °C.

Detection of Halide Elimination. For the determination of bromide concentration, an Orion Model 811 pH meter equipped with an Orion 94-35 bromide-specific electrode, a Model 90-01 reference electrode, and a 91-70-02 temperature compensation probe was used. Standard solu-

(1) (a) Kuo, D. J.; Jordan, F. *Biochemistry* **1983**, *22*, 3735. (b) Kuo, D. J.; Dikdan, G.; Jordan, F. *J. Biol. Chem.* **1986**, *261*, 3316. (c) Jordan, F.; Akinoyosoye, O.; Dikdan, G.; Kudzin, Z.; Kuo, D. J. In *Thiamine Pyrophosphate Biochemistry*; Schellenberger, A.; Schowen, R. L., Eds.; CRC Press: Boca Raton, FL, 1988; Vol. 1, p 79.
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(11) Schellenberger, A.; Hubner, G.; Lehmann, H. *Angew. Chem., Int. Ed. Engl.* **1968**, *11*, 886.

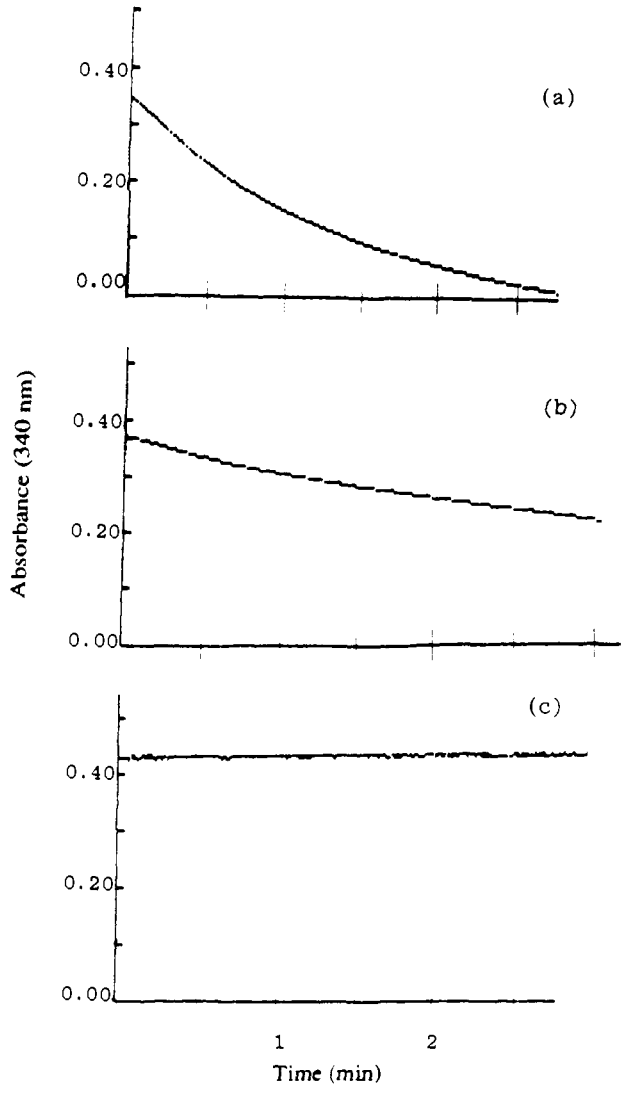

Figure 1. Coupled assay to determine aldehyde production from **Ia–c**. The reaction mixture contained 10 units of PDC, 17 units of horse liver alcohol dehydrogenase, 0.5 mM NADH, 1 mM **Ia** (a), **Ib** (b), or **Ic** (c), and 1 mM each of TDP, $MgCl_2$, and EDTA in 0.1 M citrate buffer, pH 6.0 at 30 °C.

tions of bromide were employed to generate linear plots from which the concentration of bromide in the various reaction mixtures could be interpolated. The enzyme solutions contained 1 mM TDP, 1 mM EDTA, 1 mM $MgSO_4$, 0.1 M sodium citrate, 20 units of PDC, and 0.77 mM **Ic**. To all reaction mixtures, 1% (v/v) ionic strength adjustor (20 mL of 5 M $NaNO_3$ added to 100 mL of water) was added to maintain a constant background ionic strength. Since chloride ions interfere with the bromide determinations, chloride salts had to be excluded from the assay mixture. Control experiments were performed in the absence of either PDC or compound **Ic**. Due to the limited water solubility of **Ic**, and to avoid spontaneous displacement of bromide, fresh stock solutions of 10 mM **Ic** were made up each day in methanol.

To measure chloride release from **Ib**, an Orion 96-17B combination chloride electrode was used. Orion chloride standard solutions were used to construct plots from which the chloride concentrations of various reaction mixtures could be interpolated. Typical solutions contained 0.15 M citrate, pH 6.0, 0.3 mM TDP, 0.3 mM EDTA, 0.03 mM $MgCl_2$, 1% (v/v) low-level ionic strength adjustor, and 20 units of PDC. The small concentration of $MgCl_2$ (in place of $MgSO_4$) gave much more stable background readings. The time-dependent concentration readings were corrected for this background. Stock solutions of 10 mM **Ib** in methanol were prepared fresh each day.

UV Absorbance Studies. UV–vis studies were performed at 30 °C on a Cary 219 or Varian DMS 300 spectrophotometer equipped with a thermostated cell compartment.

The coupled enzyme assay was run in 1-mL cuvettes containing 0.1 M citrate, pH 6.0, along with 1 mM TDP, 0.3 mM NADH, 17 units of horse liver alcohol dehydrogenase, and 2–10 units of PDC along with variable concentrations of compounds **Ia–c**.

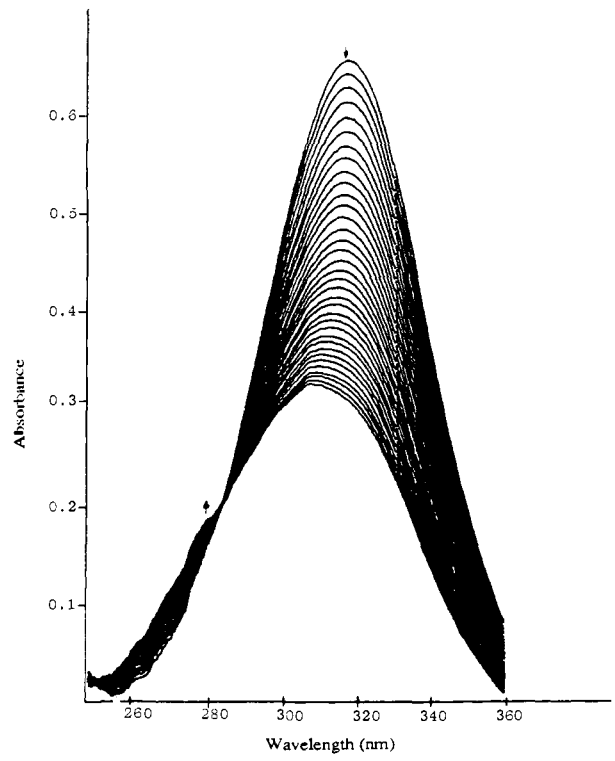

Figure 2. Repetitive-scan UV spectra (2-min intervals) of compound **Ia** in the presence of PDC. The reaction mixture contained 20 units of PDC, 0.1 mM **Ia**, 0.1 mM TDP, 1.0 mM $MgCl_2$, and 1.0 mM EDTA in 0.1 M citrate buffer, pH 6.0 at 30 °C.

The reactions of **Ia–c** would lead either to the product of normal enzymatic turnover, i.e., para-substituted cinnamaldehydes, or to *p*-methylcinnamic acid concomitant with halide elimination. Formation of the former could be monitored at 280 nm, while formation of the latter at 273 nm. These experiments were performed on reaction mixtures containing 0.1 M citrate, pH 6.0, 0.1 mM TDP, 1 mM EDTA, 1 mM $MgCl_2$, and 20 units/mL PDC along with 0.1 mM **Ia–c**. The concentration of TDP in these experiments was lower than usual, since the coenzyme also absorbs in the same wavelength region. Controls in the absence of PDC showed no time-dependent changes.

Repetitive scan spectra were run between 230 and 350 nm to enable quantification of the conversion of substrate to product(s).

Time-Dependent Inactivation Studies. Experiments testing for the time-dependent inhibition of PDC by **Ic** were performed at inhibitor concentrations ranging from 0.05 to 1.0 mM. The reaction mixture contained 1 mM TDP, 1 mM $MgCl_2$ or $MgSO_4$, 1 mM EDTA, and 0.1 M citrate buffer, pH 6.0 at 30 °C. Due to the very limited water solubility of the inhibitors, 10 mM stock solutions of inhibitors were prepared in methanol. Extensive controls on PDC in the presence of varying amounts of methanol (up to 10%, the maximum ever used) indicated that the enzyme activity was not diminished by methanol. At pH 6.0 there was significant time-dependent loss of bromide due to nucleophilic displacement by the buffer components, complicating the kinetic picture. Volumes of 1.0 mL of the above mix were incubated with varying amounts of inhibitor in polypropylene tubes at 30 °C. At the indicated times 10-μL aliquots were removed and assayed for the remaining activity.

Experiments employing **Ib** as inhibitor were performed in a similar manner. Nucleophilic displacement of chloride ion by the buffer components was nearly negligible.

Results

Interaction of PDC with Compound Ia. Compound **Ia** even at 1 or 2 mM concentration did not give rise to either time-dependent or time-independent inactivation. According to the alcohol dehydrogenase coupled assay, it was a substrate for PDC (Figure 1). Repetitive scan spectra also confirmed the formation of *p*-methylcinnamaldehyde (Figure 2). Authentic *p*-methylcinnamaldehyde was shown to be a substrate for the alcohol dehydrogenase. The K_M and k_{cat} values for **Ia** are 0.027 mM and 1.85×10^{-3} s^{-1}, respectively.

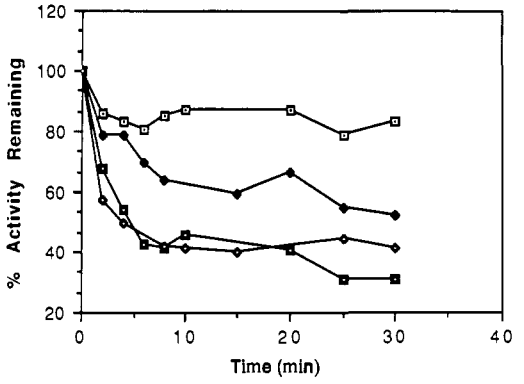

Figure 3. Time course of inactivation of PDC with compound **Ic**. 30 units of PDC was incubated with 0.1, 0.2, 0.3, and 0.4 mM inhibitor (indicated with the increasingly faster initial rates of inactivation) and 1 mM each of TDP, $MgCl_2$, and EDTA at 30 °C in 0.1 M citrate buffer, pH 6.0. At the times indicated 10-μL aliquots were removed, and the enzyme activity was measured against pyruvate, employing the pH-stat assay. The lines are drawn to connect related data.

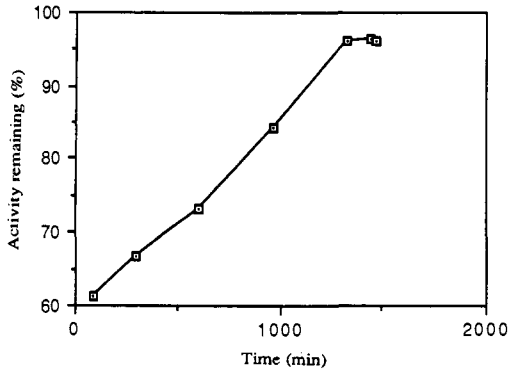

Figure 4. Recovery of activity of PDC partially inactivated by **Ic**. The reaction mixture contained 30 units of PDC, 0.3 mM **Ic**, and 1.0 mM each of TDP, EDTA, and $MgCl_2$, in 0.1 M citrate, pH 6.0 at 30 °C.

Interaction of PDC with Compound Ic. The time course of inactivation of PDC with **Ic** is illustrated in Figure 3. While from 0.1 to 0.4 mM inhibitor concentration the initial rate of inactivation increased with increasing concentration of inhibitor, at higher concentrations the extent of inhibition diminished. This behavior may be due to aggregation of this inhibitor at higher concentration. It is well-known that bromine promotes aromatic stacking-type interactions. We tested this possibility and found that the nuclear magnetic resonances corresponding to the aromatic hydrogens of **Ic** experienced an upfield chemical shift when the concentration was increased from 0.1 to 0.5 to 1 mM in 15% CD_3OD/D_2O. Compound **Ic** at 0.3 mM concentration at 30 °C provided 50% inactivation in ca. 2 min. Very importantly, upon 19-h incubation with this compound the initial activity of the enzyme was fully regained (Figure 4).

Concomitant with inactivation, there was significant and continuous Br^- release (see Figure 5). While this experiment is somewhat complicated by the presence of a background due to nonenzymic Br^- release, as Figure 5 illustrates, there clearly is an enzyme-catalyzed component. Also, the rate of bromide release was proportional to the initial concentration of added PDC (data not shown). Repetitive-scan UV spectra recorded concomitant with inactivation and bromide release (Figure 6) show a gradual diminution of the absorbance at 309 nm (corresponding to starting material) and a concomitant increase at 272 nm corresponding to *p*-methylcinnamic acid.

No evidence for aldehyde production was obtained (as monitored by the alcohol dehydrogenase coupled assay, Figure 1). In an essential control to this experiment, authentic *p*-(bromomethyl)cinnamaldehyde was synthesized and demonstrated to be a substrate for alcohol dehydrogenase (data not shown). *p*-Methylcinnamic acid appears to be the sole product of the reaction

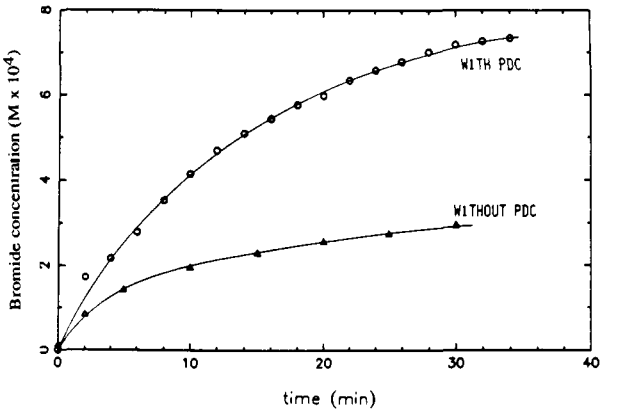

Figure 5. Time course of Br^- release from compound **Ic** in the presence and absence of PDC. The incubation mixture contained 20 units of PDC 1.0 mM each of TDP, $MgCl_2$, and EDTA, and 0.77 mM **Ic**, in 0.1 M citrate buffer, pH 6.0 at 30 °C.

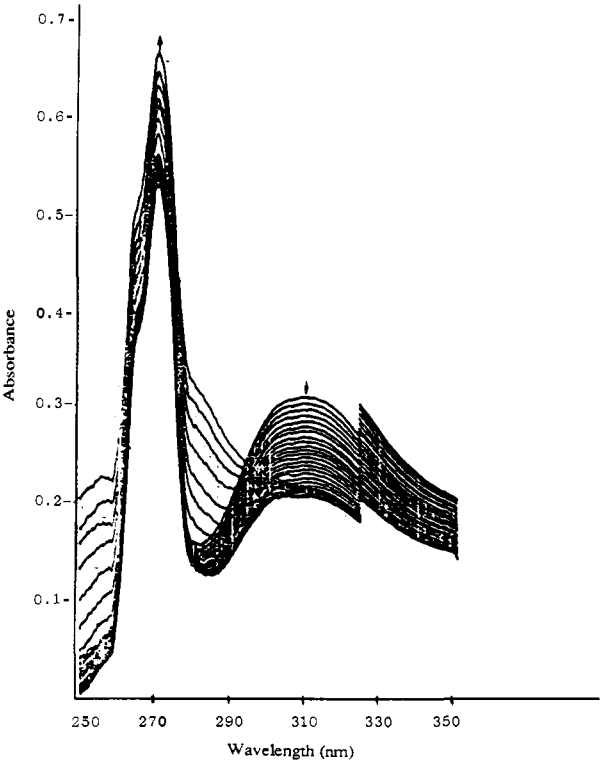

Figure 6. Repetitive-scan UV spectra of the reaction mixture containing 20 units/mL PDC, 0.1 mM **Ic**, 0.1 mM TDP, and 1 mM each of $MgCl_2$ and EDTA, in 0.1 M citrate buffer, pH 6.0 at 30 °C. Two-minute time intervals are shown. The decrease at 310 nm and the increase at 272 nm correspond to consumption of **Ic** and production of *p*-methylcinnamic acid, respectively.

between **Ic** and PDC. As further affirmation of the formation of 2-acyl-TDP as an intermediate, a diagnostic tool developed in this laboratory was also applied. It was shown earlier that the enamine produced by PDC can be trapped by the linear disulfide 4,4′-dithiodipyridine yielding 2-acyl-TDP that is deacylated by the nitrogen end of the reduced ambident nucleophile 4-thiopyridone, producing *N*-acyl-4-thiopyridone that has a characteristic absorption at 380 nm.[12a,b] Accordingly, if the 2-acyl-TDP is an intermediate on release of bromide, as hypothesized, addition of the reduced form 4-thiopyridone should generate an absorption centered at 380 nm. When compound **Ic** was mixed with PDC in the presence of 1 mM 4-thiopyridone, there was a diminution

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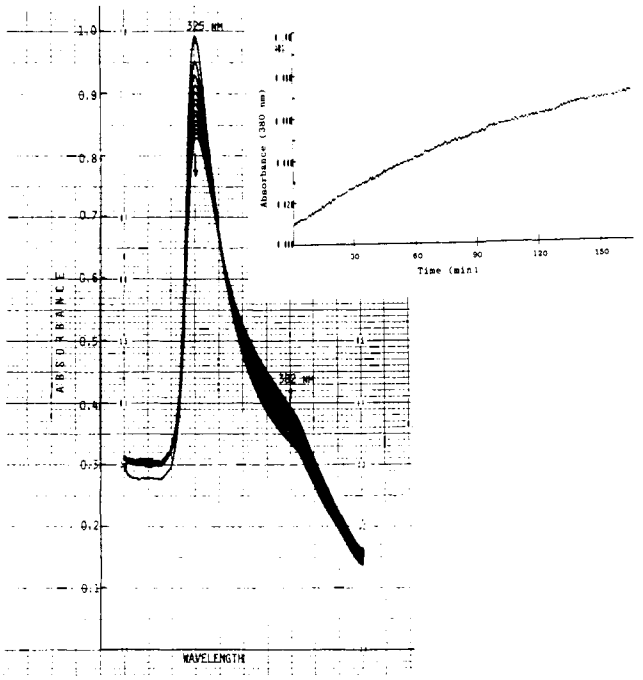


Figure 7. Repetitive-scan UV–vis spectra of the reaction of **Ic** with PDC in the presence of 4-thiopyridone. Both cells contained 1 mM each of TDP, $MgCl_2$, and EDTA, 15 units of PDC, and 1 mM 4-thiopyridone in 0.1 M citrate buffer, pH 6.0 at 4 °C, before the addition of 0.1 mM **Ic** to the sample cell. Scans were repeated every 2 min between 430 and 300 nm. The inset shows the time course of such a reaction at 380 nm.

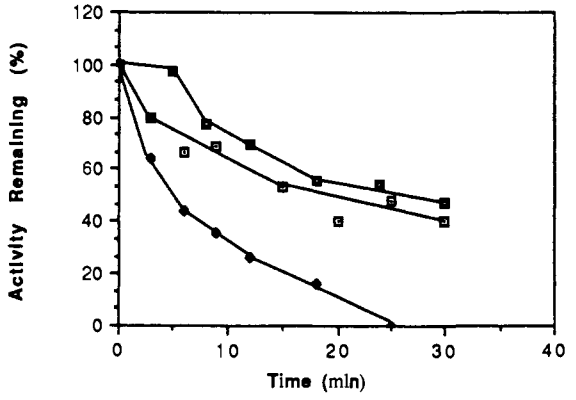


Figure 8. Inactivation of PDC by compound **Ib**. The incubation mixture contained 20 units of PDC, at 0.3, 0.5 and 1.0 mM **Ib**, leading to increased initial rates of inactivation, at 30 °C, in 0.1 M citrate buffer, pH 6.0. At the indicated times 10-μL aliquots were withdrawn, and the activity was measured against pyruvate by the pH-stat method. The lines are only drawn to connect related data points.

of the absorbance below 325 nm (reflecting consumption of starting material) concomitant with the formation of a new absorbance centered at 382 nm (Figure 7). Controls employing compound **Ia** or **Ib** under the same conditions showed no such new absorbance being formed at 380 nm. During the time shown at 4 °C ca. 13% of the starting material was trapped in this fashion (ϵ_{380} = 72200). No evidence for the formation of *p*-methylcinnamate was obtained under these conditions at 273 nm, indicating that the trapping was quantitative. The reason for monitoring this trapping reaction at 4 °C, rather than at 30 °C, the temperature employed for the other experiments, is the hydrolytic lability of the *N*-acyl-4-thiopyridone.

Interaction of PDC with Compound Ib. When incubated with PDC, **Ib** caused time-dependent inactivation (Figure 8). Unlike the behavior observed for **Ic**, the activity was not regained even after overnight dialysis against standard buffer. During the course of the inactivation there was also release of Cl^- (Figure 9), clearly in a PDC-dependent manner. Concomitant with inactivation and

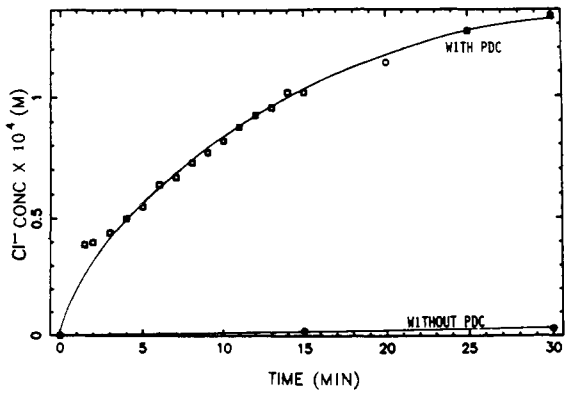


Figure 9. Time course of Cl^- release from compound **Ib** in the absence and presence of PDC. The reaction mixture contained 20 units of PDC, 1 mM **Ib**, 0.3 mM TDP, and 0.03 mM $MgCl_2$ in 0.1 M citrate buffer, pH 6.0 at ca. 25 °C. The chloride concentrations represent difference readings between the background and the actual reading.

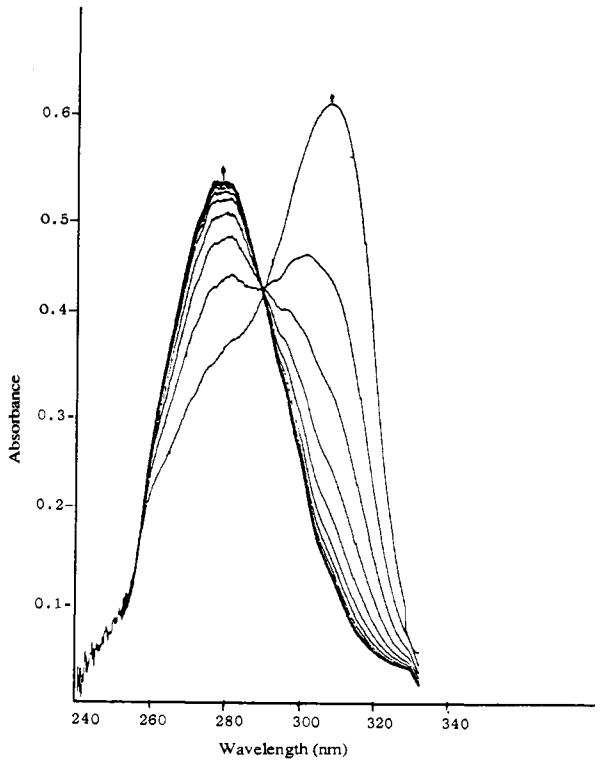


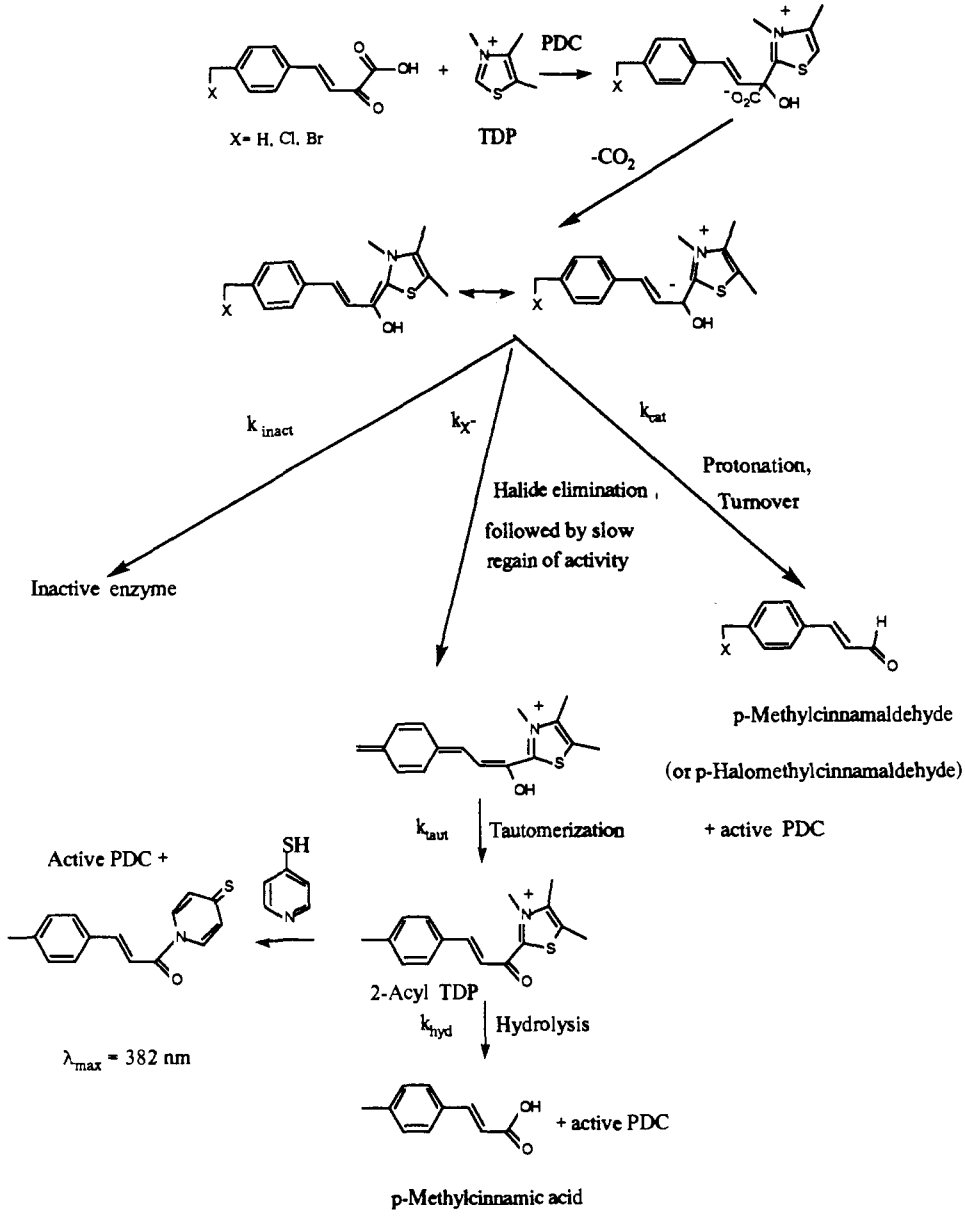
Figure 10. Repetitive-scan UV spectra (2-min intervals) of compound **Ib** in the presence of PDC. The reaction mixture contained 20 units of PDC, 0.1 mM **Ib**, 0.1 mM TDP, and 1.0 mM each of $MgCl_2$ and EDTA, in 0.1 M citrate buffer, pH 6.0 at 30 °C.

chloride ion release, *p*-methylcinnamic acid was being produced according to repetitive-scan UV spectra (Figure 10). Interestingly, according to the alcohol dehydrogenase assay, there was also *p*-(chloromethyl)cinnamaldehyde being produced (Figure 1). This substrate therefore produced two different products.

Discussion

All observations, including all products, can be accounted for as in Scheme I. Compound **Ia** is a slow substrate (K_m = 0.027 mM, k_{cat} = 1.85 × 10^{-3} s^{-1}; compared to 2 mM and 300 s^{-1} for pyruvate, respectively[3]). Compound **Ic** is an inhibitor that undergoes turnover and ties up the coenzyme at the active center. The facts that (1) bromide ion is released in a quantitative fashion, (2) there is only *p*-methylcinnamic acid produced (i.e., no turnover to the cinnamaldehyde product), and (3) total activity is regained with longer incubations are totally consistent with Scheme **I**. The behavior is also analogous to the observations by Dermeier et al. on the behavior of [*p*-(bromomethyl)benzoyl]formic acid vis-à-vis benzoylformate decarboxylase.[6] Presumably, in both cases there

Scheme I



is a quinone methide intermediate being produced that tautomerizes to a 2-acylthiamin diphosphate derivative, which in turn is hydrolyzed to the cinnamic acid and regenerates active coenzyme. While the intermediacy of quinone methide is implied, its existence is not yet demonstrated. The fact that there is temporary and partial inactivation of PDC is consistent with rate-limiting hydrolysis of the 2-acyl-TDP intermediate. The hydrolysis of the temporarily covalently modified coenzyme regenerates active enzyme. The experiment in which 4-thiopyridone was used to trap the acyl group also strongly supports the intermediacy of the 2-acyl-TDP. The curious kinetic plots for inactivation by this compound are due to a number of reasons including, and not exclusively, stacking at higher concentrations (as evidenced by the ring current induced upfield ^{1}H NMR shifts), continuous depletion of inhibitor due to buffer-catalyzed hydrolysis, and perhaps a second-order depletion of inhibitor due to reaction of the carboxy terminus of one molecule at the benzylic position of a second one thereby rendering both molecules ineffective as inhibitors.

Perhaps the most intriguing finding is the complex behavior of compound **Ib**. This compound undergoes decarboxylation to the enamine, which in turn is partitioned between turnover and Cl^- elimination in a ratio of 4:6. The central role of the enamine intermediate in all three processes is clear. Presumably, elimination of bromide is much faster than that of chloride, thereby driving the reaction of halide elimination to completion in the case of **Ic**. The enamine possessing the *p*-$ClCH_2$ substituent is longer lived; it has a choice between being protonated, eliminating halide, and in a very few events being attacked by an enzymic nucleophile and inactivating PDC, in a manner analogous to what had been reported from this laboratory on other highly conjugated pyruvic acid analogues.[1] The fact that the *p*-methyl compound does not inhibit the enzyme tells us that stabilization of the conjugated enamine intermediate by electron withdrawal is essential for irreversible inactivation to take place, as observed earlier. The results from Schellenberger's and Kluger's laboratories demonstrate that electron withdrawal per se is insufficient to render the enamine electrophilic enough to be attacked by an enzymic nucleophile (i.e., fluoropyruvate does not inhibit the enzyme yet proceeds along the pathway outlined for halide elimination in Scheme I). Also, benzoylformic acids, even with electron-withdrawing substituents, are substrates not inhibitors of PDC. Therefore, the inhibition observed with some benzylidene pyruvic acid structures is, in part, a result of the additional double bond, i.e., styrene in place of methyl in pyruvate.

There are several conclusions one can draw when comparing PDC to benzoylformate decarboxylase. PDC has a very spacious and nonselective active center; it accepts very large α-keto acids.

It also must have a rather reactive nucleophile at its active center (probably a cysteine[1c]). These two factors in combination explain why efforts to employ such conjugated analogues have only succeeded at inactivating PDC, but failed to inactivate pyruvate oxidase, pyruvate dehydrogenase, pyruvate-ferredoxin oxidoreductase, or benzoylformate decarboxylase. Furthermore, addition of excess TDP had no effect on our observations, whereas it enhanced the regain of activity by benzoylformate decarboxylase when treated with [p-(bromomethyl)benzoyl]formic acid.[6] This reflects the stronger binding of the coenzyme by PDC compared to benzoylformate decarboxylase.

These conjugated pyruvic acid analogues have become useful in metabolic studies as well. In a recent report (E)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid proved to be the most efficient reagent to demonstrate the participation of pyruvic acid in the formation of N^6-acetyl-N^6-hydroxylysine.[13]

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Voltammetric Studies of the Interaction of Metal Chelates with DNA. 2. Tris-Chelated Complexes of Cobalt(III) and Iron(II) with 1,10-Phenanthroline and 2,2′-Bipyridine

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Abstract: Voltammetric methods were used to probe the interaction (electrostatic or intercalative) of metal complexes, $ML_3^{3+/2+}$ (M = Fe, Co; L = 1,10-phenanthroline, 2,2′-bipyridine), with calf thymus DNA. Binding constants (K_{n+}) and binding site sizes (s) were determined from voltammetric data, i.e., shifts in potential and changes in limiting current with addition of DNA. The exact magnitude for the parameters depends on whether the $ML_3^{3+/2+}$/DNA reaction is assumed to be static (S) or mobile (M) within the characteristic time of a voltammetric experiment. $Co(phen)_3^{3+/2+}$ binds via intercalation with K_{3+} = 1.6 (±0.2) × 10^4 M^{-1} (S, s = 6 bp) to 2.6 (±0.4) × 10^4 M^{-1} (M, s = 5 bp). The 2+ ion interacts more favorably via hydrophobic interaction with the nucleotide bases than does the 3+ ion. Both forms of the $Fe(phen)_3^{2+/3+}$ couple bind with approximately the same affinity, K_{2+} = 7.1 (±0.2) × 10^3 M^{-1} (S, s = 5 bp) and 1.47 (±0.04) × 10^4 M^{-1} (M, s = 4 bp). $Co(bpy)_3^{3+/2+}$ shows appreciable electrostatic binding in 50 mM NaCl solution [K_{3+} = 9.4 (±1.5) × 10^3 M^{-1} (S) to 1.4 (±0.3) × 10^4 M^{-1} (M), s = 3 bp in each case], whereas $Fe(bpy)_3^{2+/3+}$ does not bind at these ionic strengths. At lower ionic strength (10 mM NaCl, 10 mM Tris, pH 7.1), binding of $Fe(bpy)_3^{2+/3+}$ is enhanced [K_{2+} = 1.1 (±0.6) × 10^3 M^{-1} nS, s = 4 bp) to 1.4 (±0.1) × 10^3 M^{-1} (M, s = 3 bp)].

We describe here voltammetric studies of the interaction of the coordination complexes $Co(bpy)_3^{3+}$, $Fe(bpy)_3^{2+}$ (bpy = 2,2′-bipyridyl), $Co(phen)_3^{3+}$, and $Fe(phen)_3^{2+}$ (phen = 1,10-phenanthroline) with calf thymus DNA. We extend our previously reported studies of $Co(phen)_3^{3+}$–DNA interactions via electrochemical methods[1] and describe the dependence of the electrochemical behavior on the nature of the ligands coordinated to the metal center.

A number of metal chelates have been used as probes of DNA structure, in solution,[2] as agents for mediation of strand scission of duplex DNA,[3] and as chemotherapeutic agents.[4] Ruthenium(II) complexes with phen and related ligands have been studied extensively as structural probes[5] and mediators of DNA cleavage reactions.[6] Enantioselective interactions of phen and bpy complexes of iron(II) have also been used as structural probes,[7] and FenII) chelated by EDTA[8] and other complexing agents,[9] tethered

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