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Interaction of Protocatechuate-3,4-dioxygenase with Fluoro-Substituted Hydroxybenzoic Acids and Related Compounds†

Sheldon W. May,*,‡ Robert S. Phillips, and Charlie D. Oldham

ABSTRACT: The substrate analogues 3-fluoro-4-hydroxybenzoic acid (3-FHB) and 4-fluoro-3-hydroxybenzoic acid (4-FHB) were synthesized and examined as active site probes for protocatechuate-3,4-dioxygenase (PCD). Upon incubation of 3-FHB with PCD, a complex is formed which causes alterations in both the visible and CD spectra of the enzyme, and these changes can be reversed by dialysis or by displacement with other ligands. Upon prolonged incubation of 3-FHB with PCD, neither oxidation of the fluoro compound nor irreversible inactivation of the enzyme occurs. Steady-state kinetic measurements established that 3-FHB acts as a simple competitive inhibitor of PCD. Inhibition constants were determined in the pH range 6.0 to 9.4, with maximal potency at pH 7.0 where 3-FHB is the most potent PCD inhibitor known ($K_I = 0.3\ \mu M$). 3-FHB is an ideal active site titrant for PCD, and titration data indicate that 8.0 mol of ligand bind per mol of enzyme. Mathematical analysis of the titration data gave dissociation constants in excellent agreement with the kinetically determined K_I values at several different pHs. In contrast, the isomeric 4-FHB was found to be a very weak PCD inhibitor with a K_I approximately 1000-fold greater than that of 3-FHB. The contrasting behavior of the isomeric fluorohydroxybenzoates was found to be mirrored in the behavior of the corresponding defluoro ligands, 3- and 4-hydroxybenzoic acid (3-HB and 4-HB, respectively), but the presence of the fluorine substituent in 3-FHB increases the ligand's affinity for the enzyme approximately 500-fold. Analysis of the pH dependencies of inhibition constants indicated preferential binding of the protonated forms of 3-FHB and 4-HB. That the efficient binding of 3-FHB at the active site cannot be attributed solely to simple chelation of the iron atom is supported by spectral examination of Fe(III) complexation by 3-FHB and related compounds. Displacement studies were carried out using stopped-flow techniques in order to examine the kinetics of dissociation of the complexes of PCD with 3-FHB and other inhibitors. The utility of fluoro substituted ligands as active site probes for analysis of binding interactions and for evaluation of catalytic mechanisms for dioxygenases such as PCD is discussed.

Protocatechuate-3,4-dioxygenase (PCD)[1] catalyzes the cleavage of the aromatic ring of protocatechuic acid with the concomitant insertion of oxygen to give β-carboxy-*cis,cis*-muconic acid (Stanier & Ingraham, 1954). It has been identified and isolated from a number of microbial sources, but the enzyme from *Pseudomonas aeruginosa* is particularly suitable for study, due to the ease with which it can be isolated and crystallized, and also to its unusually high stability (Fujisawa & Hayaishi, 1968). The strict specificity of the enzyme is remarkable, with protocatechuic acid and its homologues, catechol, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxyphenylpropionic acid, being among the few known substrates. Other substrate analogues such as 4-hydroxybenzoic acid, protocatechualdehyde, and 4-nitrocatechol are all nonmetabolizable competitive inhibitors of varying potency (Fujisawa et al., 1972a,b; Tyson, 1975; Zaborsky et al., 1975).

In order to facilitate investigations of the mechanisms and binding parameters operative at the active sites of oxygenases, we have been designing various fluorine-substituted substrate analogues (May & Phillips, 1977). The fluorine atom, if properly positioned, would be expected to present minimal steric interference with normal binding at the active site (see examples in Barnett, 1972), and an examination of the catalytic consequences of the replacement of hydroxyl groups or hydrogens by fluorine provides valuable mechanistic information regarding the enzymatic oxygen insertion process. Also, a fluorine substituent perturbs the pK_as of adjacent ionizable groups such as amines or phenols and thus can be of considerable value in studies on the pH dependencies of inhibitor binding and catalytic activity. Furthermore, fluoro analogues are exceedingly useful in NMR studies (Dwek, 1972) on the

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[1] Abbreviations used: PCD, protocatechuate-3,4-dioxygenase; 4-NC, 4-nitrocatechol; 3-FHB, 3-fluoro-4-hydroxybenzoic acid; 4-FHB, 4-fluoro-3-hydroxybenzoic acid; 4-HB, 4-hydroxybenzoic acid; 3-HB, 3-hydroxybenzoic acid.

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relationship between the substrate binding site and the metal ion—presumably the locus of oxygen binding and activation. We report herein on the interaction of PCD with two of these compounds—3-FHB and the isomeric 4-FHB—and compare the results with those obtained using the corresponding hydrogen analogues, 4-HB and 3-HB. The results demonstrate that the unique properties of fluorinated compounds may render them exceedingly potent and useful active site probes for dioxygenases.

Materials and Methods

All commercial materials were the highest grades obtainable. Protocatechuic acid, 4-nitrocatechol, 3-HB, and 4-HB were recrystallized from water. *3-FHB was synthesized as follows:* 30 mL of tetrachloroethane, 2.98 g of *o*-fluoroanisole, and 2.81 g of acetic anhydride were combined and cooled to 5 °C, 4.9 g of anhydrous $AlCl_3$ was added, and the mixture was stirred for 1.5 h. Gentle heating (33 °C) was then applied for 2 h, and the reaction finally quenched by pouring on to ice. The aqueous and organic layers were separated, and the organic layer washed twice with water, twice with dilute (0.01 N) NaOH, and once again with water. After drying over $MgSO_4$, the solvent was removed by distillation, and the residue vacuum distilled. The fraction collected at 150–165 °C was recrystallized from ethanol to yield 0.902 g (23%) of 3-fluoro-4-methoxyacetophenone, mp 92–92.5 °C [lit. 92 °C (English et al., 1940)].

3-Fluoro-4-methoxyacetophenone (1.8 g) was added to 80 mL of 4–6% NaOCl. Heat was then applied and the reaction driven to completion by distillation of chloroform from the reaction mixture. The reaction was terminated by the addition of solid $NaHSO_3$ until the reaction mixture no longer turned starch–KI test paper blue. Upon acidification with concentrated HCl and cooling in ice, the product precipitated and was collected by filtration. Recrystallization from water gave 1.3 g of 3-fluoro-4-methoxybenzoic acid (72%), mp 206–210 °C [lit. 208–210 °C (Minor & Vanderwerf, 1952)].

The above product (0.25 g), 3 mL of 57% HI, 0.71 g of red phosphorus, and 3 mL of acetic anhydride were combined, and the mixture was refluxed for 3 h (Deulofeu & Guerrero, 1955). The pH of the reaction mixture was adjusted to 8.5 with 2 N NaOH and the solid red phosphorus removed by filtration. The filtrate was then acidified with HCl and the solution reduced in vacuo until a considerable amount of salt had precipitated. The aqueous layer was extracted with ether and the solid washed with ether. The ether extracts were combined and dried over $MgSO_4$, and the ether was removed to yield a brownish crystalline solid. Sublimation of the product, followed by recrystallization from water gave 0.113 g of 3-FHB (50%), mp 163–163.5 °C [lit. 163 °C (Minor & Vanderwerf, 1952)]. Anal. Calcd for $C_7H_5FO_3$: C, 53.85; H, 3.23. Found: C, 53.56; H, 3.31. Mass spectral analysis showed the expected parent ion peak at *m/e* 156, and analysis of the fragmentation pattern showed the expected major peaks at *m/e* 139 (loss of OH) and *m/e* 111 (loss of CO_2H). The NMR and IR spectra were fully consistent with the structure.

The synthesis of 4-FHB was carried out as follows. Five grams of *p*-fluorobenzoic acid was added to a stirred mixture of 15 mL of concentrated nitric acid and 15 mL of concentrated sulfuric acid. The mixture was heated to 50 °C for 30 min and then poured over 30 mL of 1:1 ice–water. Filtration and washing with water gave 4.53 g (69%) of 4-fluoro-3-nitrobenzoic acid, mp 120–121.5 °C [lit. 121.5 °C (Rouche 1921)]. Three grams of the above was dissolved in 100 mL of methanol and subjected to low-pressure hydrogenation in the presence of 300 mg of 10% palladium on charcoal to give 2.40 g (95%) of crude 3-amino-4-fluorobenzoic acid, which was recrystallized from methanol, mp 183–184 °C [lit. 182–183 °C (dec) (Dunker et al., 1939)]. The above product (0.82 g) was dissolved in a solution of 3.5 g of 50% fluoboric acid and 3 mL of water. The solution was cooled to below 10 °C and a sodium nitrite solution (0.35 g in 0.7 mL of water) was added dropwise with vigorous stirring. The precipitate was collected by filtration and washed successively with 5% fluoboric acid, methanol, and ether to give 0.74 g (58%) of 5-carboxy-2-fluorobenzenediazonium tetrafluoroborate. Oxidation of the salt was accomplished by a procedure based on the new method of Cohen et al. (1977). The diazonium salt (0.71 g) was added with stirring to a solution containing 400 g of cupric nitrate trihydrate in 325 mL of water. Stirring was increased and 0.27 g of cuprous oxide was added. After reaction, the solution was extracted with 250 mL of ether and then back extracted with 100 mL 0.1 M sodium hydroxide solution. Acidification followed by ether extraction and evaporation gave 0.35 g (79%) of crude product. Recrystallization from water gave 4-fluoro-3-hydroxybenzoic acid, mp 215.5–216.5 °C (dec). Anal. Calcd for $C_7H_5FO_3$: C, 53.85; H, 3.23. Found: C, 53.66; H, 3.25. Analysis of the mass spectrum showed it to completely confirm the structure, as did the NMR and IR spectra.

Growth of P. aeruginosa. P. aeruginosa cultures (ATCC 23975) were stored as lyophils in sealed glass ampoules. For large scale growth, 10 L of medium containing (per L) 3.0 g of sodium citrate, 3.0 g of $(NH_4)_2HPO_4$, 1.2 g of K_2HPO_4, 5 g of 4-hydroxybenzoic acid, 0.5 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of anhydrous $MgSO_4$, and 1 mL of poly(propylene glycol) as an antifoam was placed in a 14-L New Brunswick fermentor. A 200-mL inoculum was added, and the pH was monitored and maintained between 7.2 and 7.4 at 30 °C. Additional substrate (4-hydroxybenzoic acid) was added as required. Cells were harvested in the late log phase and, typically, 220 g of cells (wet weight) was obtained from a single fermentation. The cells were stored frozen at −20 °C.

Purification of Protocatechuate-3,4-dioxygenase. Protocatechuate-3,4-dioxygenase (PCD) was isolated by the method of Fujisawa & Hayaishi, (1968) with some modifications. After sonication and heat treatment the protamine step was deleted and the ammonium sulfate fraction was taken between 35% and 50% saturation, rather than 40–60%. Very little enzyme activity was detectable in the supernatant at above 50% saturation.

The 35–50% cut was resuspended in 0.05 M Tris-Cl, pH 8.5, and centrifuged to remove a yellow material which would not redissolve. It was then applied to a column of Sepharose 4B (2.5 × 78 cm) or to a column of Sepharose 4B (2.5 × 45 cm) followed by a column of Bio-Gel A-5m, 400 mesh (2.5 × 40 cm), and eluted with 0.05 M Tris-Cl, pH 8.5. The red fractions (total volume about 60 mL) were pooled and concentrated using an Amicon XM-100 A membrane, until the protein concentration was about 6%. 2-Mercaptoethanol was added to 5 mM and the enzyme solution was brought to 35% $(NH_4)_2SO_4$ saturation. After standing for 30 min, the solution was centrifuged. The deep red supernatant was allowed to stand with refrigeration, and the PCD precipitated within several hours. The precipitate was redissolved in 0.05 M Tris-Cl, pH 8.5, and buffer containing 90% saturated $(NH_4)_2SO_4$ added dropwise until faint turbidity appeared. Within 2 days of standing at 4 °C in the refrigerator, crystals appeared in the form of hexagonal columns capped with pyramids on both ends. Recrystallization was effected by redissolving in a minimal amount of buffer and adding 90% ammonium sulfate solution until turbidity appeared. Purity of the enzyme was established on the basis of specific activity (70

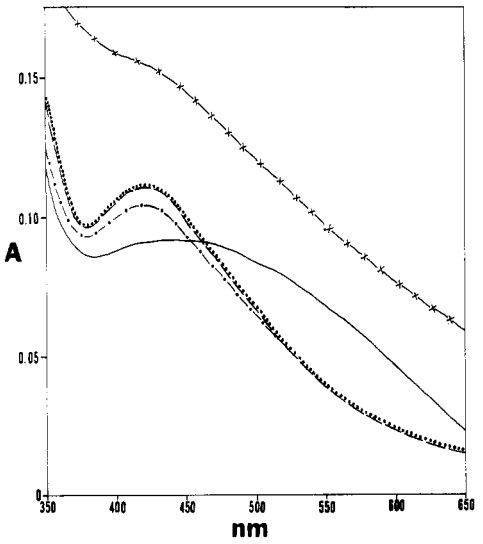


FIGURE 1: Visible spectra of PCD and complexes. (Curve 1) Native enzyme (1.97 mg) in 1.05 mL of 0.05 M Tris-Cl, pH 7.5 (—). (Curve 2) Spectrum obtained after adding 25 μL of 19.9 mM 3-FHB (0.498 μmol) to a solution identical with that used to run curve 1 (- - -). Under these conditions, the enzyme is completely complexed. (Curve 3) Sample prepared in a manner identical with that used to run curve 2, except prepared in a Thunberg cell and purged with purified nitrogen for an hour (····). (Curve 4) Spectrum obtained after addition of 50 μL of 25.2 mM 4-HB (1.26 μmol) to a solution identical with that used to run curve 1 (-·-·-). (Curve 5) Spectrum obtained after addition of 150 μL of 10.0 mM 4-FHB to a solution identical with that used to run curve 1 (×-×). All curves are corrected for dilution.

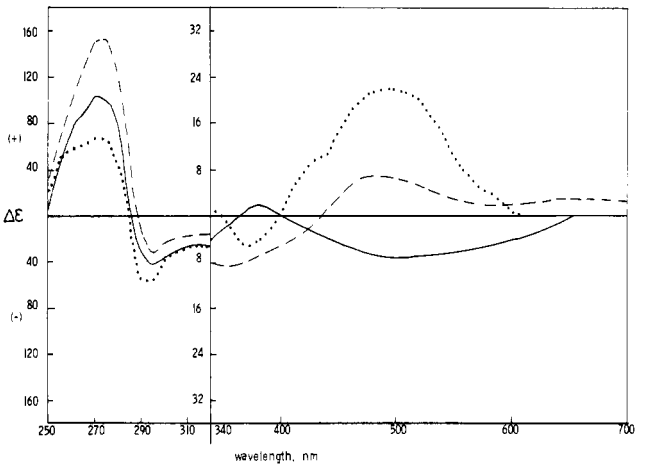


FIGURE 2: Circular dichroism of PCD and complexes. Visible: (curve 1 (—)) 1 mL of PCD solution (7.98 mg/mL); (curve 2 (- -)): 1 mL of PCD solution (7.98 mg/mL) to which 15 μL of 10.1 mM 3-FHB had been added; (curve 3(···)): 1 mL of PCD solution (7.98 mg/mL) to which 15 μL of 10.1 mM 4-NC had been added. UV: (curve 1 (—)): 1.2 mL of PCD solution (1.66 mg/mL); (curve 2 (- -)): 1.2 mL of PCD solution (1.66 mg/mL) to which 4 μL of 10.1 mM 3-FHB had been added; (curve 3 (···)): 1.2 mL of PCD solution (1.66 mg/mL) to which 4 μL of 10.1 mM 4-NC had been added.

U/mg) and spectral properties (see below).

General Procedures. Visible and ultraviolet spectra were obtained on either an Aminco DW-2 or a Beckman Acta MVI spectrophotometer, and CD spectra on a Jasco ORD-UV5. Enzyme activity was determined spectrophotometrically at 290 nm (Stanier & Ingraham, 1954) or by monitoring oxygen consumption on a YSI Model 53 dissolved oxygen monitor. The method of Lowry et al. (1951) was used to determine the protein content of crude extracts, and protein concentration of purified enzyme was determined spectrophotometrically, using $E_{280}^{1\%}$ = 13.2 (Fujisawa & Hayaishi, 1968) and assuming the molecular weight to be 700 000. Fluoride release was measured with an Orion combination fluoride-specific electrode, using NaF as a standard.

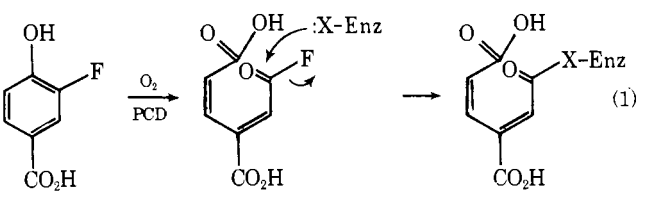
Kinetic experiments were performed on an Aminco DW-2 UV-visible spectrophotometer equipped with an Aminco-Morrow stopped-flow accessory. For the steady-state kinetic measurements, various concentrations of substrate in air-saturated buffer were mixed with PCD solutions using the stopped-flow accessory, and the reaction followed in dual-wavelength mode with the sample monochromator on 290 nm and the reference monochromator on 270 nm. For the displacement reaction, enzyme·inhibitor complexes were flowed against high concentrations of the displacing ligand, and the reaction followed by setting the monochromators to the appropriate wavelengths (see Figure 3).

Results

Spectral Studies with Hydroxybenzoic Acids. Upon addition of either 3-FHB or 4-HB to solutions of PCD, an immediate change in color from deep red to yellow is observed. In contrast, neither the isomeric 4-FHB nor 3-HB induces this type of color change when incubated with PCD under the same conditions. Figure 1 shows the spectra of the complexes of PCD with 3-FHB, 4-HB, and 4-FHB. It is evident that both 3-FHB and 4-HB cause a shift in the λ_{max} of PCD from about 450 nm to about 420 nm, along with a large decrease in the absorbance at around 550 nm. Neither 3-FHB nor 4-HB exhibit visible absorbance under these conditions, and thus these spectral changes are associated with alterations in the PCD chromophore upon complex formation. In order to ascertain whether formation of the PCD·3-FHB complex requires the presence of oxygen, a similar experiment was carried out under anaerobic conditions in a Thunberg cuvette. As shown in the figure, the complex exhibited identical spectral characteristics under these conditions.

Addition of 3-FHB to PCD also causes marked changes in the CD spectrum, as shown in Figure 2. It is interesting to compare our data with those reported by Zaborsky et al. (1975) for the complex of PCD with 4-HB. The PCD·3-FHB complex shows a band of negative ellipticity below 420 nm and the 4-HB complex also shows a similar minimum which does not, however, extend into the negative region. Both complexes cause an intensification of the positive band below 290 nm, and the creation of a new positive band at about 490–500 nm. Also shown in Figure 2 is the CD spectrum for the complex of PCD with the inhibitor 4-NC. This latter spectrum differs considerably from those for the 3-FHB and 4-HB complexes, but resembles the spectrum reported for the PCD·protocatechualdehyde complex (Zaborsky et al., 1975).

Control Experiments with Fluorohydroxybenzoic Acids. In contrast to the hydroxybenzoic acids, fluorohydroxybenzoic acids could, in principle, interact with PCD as either (a) substrates; (b) simple reversible ligands; or (c) irreversible chemical modification reagents through a mechanism such as that envisioned in eq 1.



(1)

In order to distinguish between these possibilities, the following experiments were carried out. (1) A solution of 3-FHB

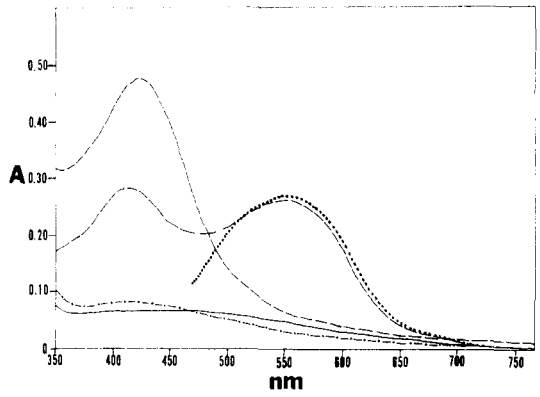


FIGURE 3: Equilibrium displacement reactions. (Curve 1) Spectrum of solution after addition of 75 μL of stock PCD (2.9 mg; 4.1 nmol) to 2.00 mL of 0.05 M Tris-Cl, pH 7.44 (—). (Curve 2) Spectrum after addition of 3 μL of 19.9 mM 3-FHB (59.7 nmol) (·····) to solution of curve 1. (Curve 3) Spectrum after addition of 3 μL of 20.1 mM 4-NC (60.3 nmol) to solution of curve 1 (lower - - - -). (Curve 4) Spectrum of solution used to run curve 2 after addition of 50 μL of 20.1 mM 4-NC (1.00 μmol) to both reference and sample cuvettes (····). (Curve 5) Spectrum of solution used to run curve 3 after addition of 200 μL of 19.9 mM 3-FHB (3.98 μmol) (upper - - - -). All curves are corrected for dilution.

(2.5×10^{-4} M in 0.05 M Tris-Cl, pH 7.5) was prepared and the spectrum recorded before and after the addition of a catalytic amount of PCD. No change in the 3-FHB spectrum was observed after more than 3 days of standing at room temperature. It should be noted that PCD would be expected to retain its catalytic activity upon standing under these conditions. (2) Crystalline PCD (1.5 mg; 2.1 nmol) was added to a 5-mL volumetric flask and diluted to the mark with 0.05 M Tris-Cl, pH 7.5. The activity was measured under standard assay conditions and then 5 μL of a 10.1 mM solution of 3-FHB was added (total of 50.5 nmol). Assay of aliquots (5 and 25 μL) of this solution immediately after addition of the fluoro compound gave activity identical with that of the starting enzyme. The mixture was incubated at 4 °C and periodically assayed in the same manner. After 2 weeks, more than 95% of the initial enzymatic activity remained, indicating that slow irreversible inhibition of PCD by the fluoro compound does not occur. It should be noted that, although 3-FHB is a reversible inhibitor of PCD (see below), aliquots of the incubation solution *in this experiment* did not reflect this effect due to dilution of 3-FHB and the large excess of substrate in the assay mixture. A similar experiment was performed with 4-FHB, and, again, no evidence for either enzymatic oxygenation or slow irreversible inactivation was obtained.

Another very sensitive test against slow irreversible inactivation was carried out during the course of steady-state kinetic measurements described in the following section. Enzyme stock solutions with and without 3-FHB ([PCD] = 3.4×10^{-9} M; $[3\text{-FHB}]_0 = 4.02 \times 10^{-6}$ M) were prepared, kinetic measurements for double-reciprocal plots of inhibited and uninhibited reactions performed (pH 9.0), and the two enzyme stock solutions allowed to stand overnight at room temperature. The following day, the kinetic measurements were repeated using the same stock solutions. The plots for the day-old enzyme solutions were essentially identical with those obtained in the initial series. This result could not have been obtained if irreversible inactivation of PCD by 3-FHB had occurred under these conditions.[2]

As a further test for reversibility of binding, both dialysis and equilibrium displacement experiments were performed. A solution of the PCD·3-FHB complex (2.9 μM) was dialyzed 1:200 against Tris buffer for 2 days with four changes of buffer.

TABLE I: Inhibition and Dissociation Constants for 3-FHB and 4-HB.

Kinetically determined K_I values				K_D values from titration data	
4-HB		3-FHB		3-FHB	
pH	$10^4 \times K_I$	pH	$10^6 \times K_I$	pH	$10^6 \times K_D$
5.95	1.54	5.90	1.02		
6.50	1.27	6.50	0.52	6.60	0.34
7.00	1.08	7.00	0.33		
7.50	0.98	7.60	0.65	7.60	0.75
8.40	0.87	8.40	0.86		
		8.60	1.01	8.50	1.14
9.00	1.17	9.00	1.66		
		9.20	3.07		
		9.40	4.53		

The recovered enzyme exhibited an A_{420}/A_{550} ratio of 1.23 as compared with 1.32 for the native enzyme and 2.04 for the PCD·3-FHB complex. Figure 3 illustrates the results of displacement experiments which establish the complete interconvertibility of the PCD·3-FHB and PCD·4-NC complexes. As shown in the figure, the characteristic 550-nm absorption of the PCD·4-NC complex is virtually identical starting from equal concentrations of either free enzyme or PCD·3-FHB complex. Conversely, addition of an excess of 3-FHB to PCD·4-NC complex liberates the 4-NC anion, and detailed analysis of the extinction of curve 5 established greater than 90% displacement of this ligand by 3-FHB. (Note that in the experiment of curve 4 the large excess of strongly absorbing 4-NC present in both samples and reference cells precluded scanning below about 450 nm). It should be noted that, due to its high affinity for PCD, 3-FHB is the only ligand with which total displacement of 4-NC is experimentally achievable.

Steady-State Kinetic Measurements. In order to evaluate the inhibitory potency of both 3-FHB and 4-FHB for comparison with the defluoro analogues, steady-state kinetic experiments in the presence and absence of these compounds were carried out at several different pH values. In all cases linear Lineweaver–Burke plots were obtained (least-squares analyses) and both 3-FHB and 4-HB exhibited pure competitive inhibition. In sharp contrast, both 4-FHB and its defluoro analogue 3-HB exhibited much lower inhibitory potency. Inhibition studies with these latter two compounds had to be carried out in the presence of large inhibitor concentrations which often made spectrophotometric assay at 290 nm difficult and necessitated use of the oxygen monitor. At pH 7.5, the K_I value for 4-FHB is approximately 2×10^{-4} M, while that for 3-HB must be several-fold greater, and thus this compound is a very weak inhibitor of PCD.

Table I lists kinetically determined K_I values for 3-FHB and 4-HB at a number of different pH values in the range 5.9 to 9.4. It is apparent that the presence of the 3-fluoro substituent causes a striking increase in binding affinity toward PCD, with 3-FHB binding approximately 400-fold more tightly than 4-HB at pH 7.0. It is interesting to note that, although 4-FHB is a poor inhibitor of PCD, in this compound as well the pres-

[2] Recent experiments with an ion-specific fluoride electrode and ^{19}F NMR studies indicate that inorganic fluoride is released very slowly, when 3-FHB and PCD in *high concentrations* are incubated for extended periods (i.e., *many weeks*). It is not known whether this is the result of oxidative ring cleavage, or occurs through some other mechanism. Fluoride release was not detected in the absence of PCD. Within the time scale of all of the experiments reported in this paper, 3-FHB acts as a reversible, nonmetabolized ligand for PCD.

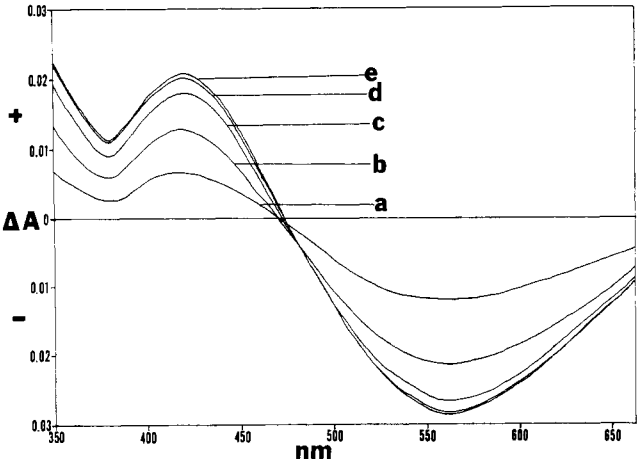


FIGURE 4: Titration of PCD with 3-FHB. One milliliter of PCD stock solution (2.47 mg/mL; 3.53 nmol) in 0.05 M Tris-Cl, pH 7.5, was added to both sample and reference cuvettes. The instrument baseline was adjusted to the center of the chart, and then aliquots of a 4.98×10^{-4} M 3-FHB solution were added to the sample cell, while equal volumes of buffer were added to the reference cell. (Curve a) After addition of 20 μL 3-FHB stock (9.96 nmol); (curve b) after addition of another 20 μL of 3-FHB stock (19.9 nmol); (curve c) after addition of another 20 μL of 3-FHB stock (29.9 nmol); (curve d) after addition of another 20 μL of 3-FHB stock (39.8 nmol); (curve e) after addition of a final 10 μL of 3-FHB stock (44.8 nmol). Actually, smaller aliquots (3–5 μL) of 3-FHB stock were added to obtain many more data points for the break and Scatchard plots (Figure 5) but in this figure intermediate curves have been omitted for the sake of clarity.

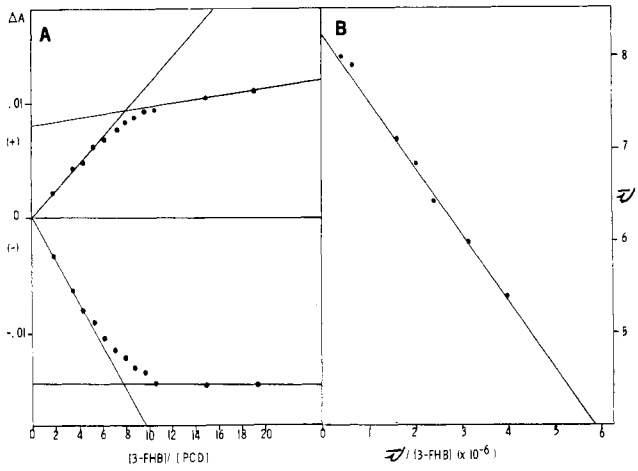


FIGURE 5: Break plot and scatchard plot. Data similar to those shown in Figure 4 were used to determine the titration end point and K_D for the PCD·3-FHB complex. (A) (Left side of graph) The plots of ΔA either at 420 nm (upper curve) or 560 nm (lower curve) against the ratio of moles of 3-FHB added to moles of PCD exhibit sharp breaks at 7.9–8.0 equiv. (B) (Right side of graph) Using a value of $n = 8$, as determined by the break plot, $\bar{\nu}$ was calculated from the relationship, $\bar{\nu} = [\text{PCD·3-FHB}] \cdot n/[\text{PCD}]_{\text{total}}$. [PCD·3-FHB] was calculated based on ΔA at 560 nm. The slope of the plot, as determined by least-squares analysis, gives a K_D of 7.5×10^{-7} M.

ence of the fluoro substituent substantially increases binding affinity when compared with its respective defluoro analogue, 3-HB.

The K_I value at pH 7.0 of 3×10^{-7} M for 3-FHB is approached only by 4-NC, which in our hands gives an apparent K_I of about 1×10^{-6} M at this pH. However, a number of complications were observed in inhibition studies with 4-NC; for example, inhibition is apparently noncompetitive using our standard DW-2 mixing technique, but the double-reciprocal plot pattern apparently alters when the order of inhibitor and substrate addition, or the preincubation time, is changed. Essentially identical observations have been reported by Wells (1972) in studies on the interaction of 4-NC with the *Thiobacillus* enzyme. In our view, these complications may be related to a very slow dissociation of the PCD·4-NC complex (see below).

From spectrophotometric titration data, we determined that the pK of 3-FHB is 7.9, while that of 4-HB is listed in the CRC Handbook of Chemistry and Physics as 9.32. Thus, the *p*-hydroxyl in 3-FHB is markedly altering its ionization state in the pH range of our kinetic studies, while that in 4-HB is protonated essentially throughout the pH range. It is evident from the data in Table I that the K_I of 3-FHB is much more profoundly pH dependent than that of 4-HB, binding diminishing as the pH is increased. Thus, these data imply that the hydroxybenzoates are preferentially bound when the *p*-OH is protonated. A detailed kinetic analysis of the effects of ionizations in PCD and its complexes on catalysis as well as on inhibitor and substrate binding is currently in progress.

Titration of PCD with 3-FHB. Because of the very tight binding of 3-FHB to PCD and the lack of spectral features of the ligand in the region of the enzyme chromophore, 3-FHB should be an ideal titrant for PCD. Typical difference spectra obtained during titration of PCD with 3-FHB are shown in Figure 4. Plots of absorbance (either at 420 or 560 nm) against the ratio moles of ligand per mole of enzyme (Figure 5) exhibit a sharp break at about 8 equiv.

Data similar to those shown in Figure 4 were used to determine apparent dissociation constants, K_{diss}, for the binding of 3-FHB to PCD at several pH values. A typical Scatchard plot (Scatchard et al., 1957) of the data at pH 7.5 is shown in Figure 5. The plot yields a dissociation constant of 0.75×10^{-6} M. This value is in excellent agreement with our kinetically determined K_I value of 0.65×10^{-6} M for 3-FHB at this pH. As shown in Table I, the values of K_D obtained by titration at pH 6.60 and 8.50 were also in excellent agreement with the corresponding kinetically determined K_I values. These results provide strong support for our conclusion that 3-FHB acts as a simple reversible ligand for PCD. Taken together, the unique spectral properties, binding characteristics, and simple kinetic behaviour of 3-FHB are ideal for obtaining quantitative titration and binding data.

Displacement Experiments. The displacement technique of Gutfreund (1972) was used to examine the kinetics of dissociation of PCD·inhibitor complexes. For the interaction of PCD with 3-FHB and 4-NC depicted in eq 2, it is theoretically possible to evaluate the dissociation constants for the complexes by appropriate choice of experimental conditions. Thus, if a solution of PCD·4-NC complex is flowed against a sufficient excess of 3-FHB, a simple first-order reaction will be observed when $k_{-1} \ll k_{+2}$ [3-FHB] and k_{+1} [4-NC] $\ll k_{+2}$ [3-FHB]. Since 3-FHB is so potent an inhibitor, our expectation was that such conditions should be readily achievable. As shown in Figure 6, simple first-order reactions were indeed observed upon displacement of 4-NC by 3-FHB at pH 6.0, 7.5, and 9.0. The reactions were followed by monitoring $A_{420} - A_{550}$ which corresponds to the formation of free 4-NC and disappearance of the PCD·4-NC complex (Figure 3). At pH 7.5 and 25 °C, a rate constant of 0.050 s^{-1} was obtained. As required by the kinetic scheme, identical values were obtained at various concentrations of PCD, 4-NC and 3-FHB, so long as 3-FHB was maintained in sufficient excess. At pH 6.0 and 9.0, dissociation rate constants of 0.043 and 0.015 s^{-1}, respectively, were obtained for the PCD·4-NC complex. Thus, the dissociation of 4-NC is slower at more alkaline pH and is surprisingly slow in all three cases. The possibility must be considered that this slow dissociation is reflected in some of the compli-

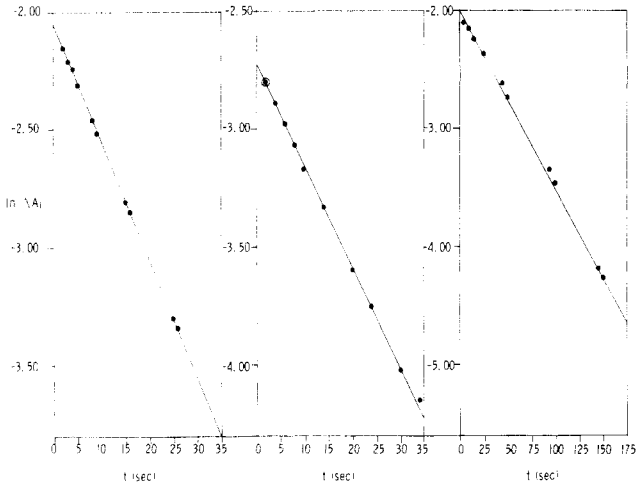
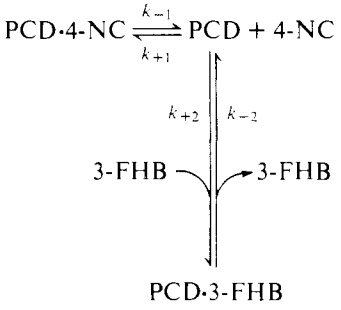


FIGURE 6: Displacement of 4-NC from PCD. Plot 1: (Left-hand side) A solution of PCD (1.2 mg/mL, 1.7 μM) in 0.05 M Tris-Cl, pH 7.44, containing 2.4×10^{-5} M 4-NC (1.8 equiv/binding site) was flowed against a solution of 3-FHB (1.87 mM; 78-fold excess over 4-NC) in the same buffer in the stopped-flow spectrophotometer. The reaction was followed in dual wavelength mode with the sample monochromator at 550 nm and the reference monochromator at 420 nm; the rate was such that the reaction could readily be followed on the X–Y chart recorder of the DW-2. The plot of $\ln |A_\infty - A_t|$ against t shows points obtained from two separate reactions under the same conditions; the slope of the plot, determined by a least-squares analysis, gives a k_{diss} of 0.050 s^{-1}. Plot 2. (Middle) All conditions identical with those used to obtain the data of plot 1, except the buffer was 0.05 M phosphate, pH 6.00. The k_{diss} at this pH is 0.043 s^{-1}. Plot 3: (Right-hand side) All conditions identical with those used to obtain the data of plot 1, except the buffer was 0.05 M Tris, pH 9.00. The k_{diss} under these conditions is 0.015 s^{-1}. The instrument was thermostated at 25 °C for all these experiments.

cations encountered in steady state kinetic measurements with 4-NC.



(2)

Upon reversing the experiment and displacing 3-FHB with excess 4-NC, biphasic kinetics were observed. In order to test whether this was a reflection of slow association of 4-NC, displacement of 3-FHB was also carried out using the substrate, protocatechuic acid, under anaerobic conditions, since association of substrate has been reported to be extremely rapid (Fujisawa et al., 1971, 1972b). However, biphasic kinetics were also observed in these experiments, as well as for the displacement of 4-HB by 4-NC. It should be noted that the rapid phase exhibited rate constants in the range 1–3 s^{-1} which is considerably faster than that noted for the dissociation of 4-NC.

Fe(III) Complexation Studies. The suggestion has been made that substrates of PCD chelate the ferric iron at the active site upon binding (Tyson, 1975). To test the possibility that the very effective binding of 3-FHB is attributable to strong chelation of the iron by the oxygen and fluorine on the ring, aqueous solutions of $Fe(NO_3)_3$ were added to solutions of protocatechuic acid, 4-HB, 3-FHB, 3-HB, and 4-(chloroacetyl)catechol. Among all these compounds, only substrate and 4-(chloroacetyl)catechol caused any major spectral changes in the visible region—new peaks are seen at 640 and 560 nm, respectively (Figure 7). These results are consistent with the conclusion that the ferric iron is chelated by the vicinal hydroxyls of these compounds rather than by either the carboxyl group itself or by the *m*-OH–carboxylate pair. On this basis, as well as on theoretical grounds, it appears that the effective binding of 3-FHB to PCD is not attributable solely to a chelation effect.

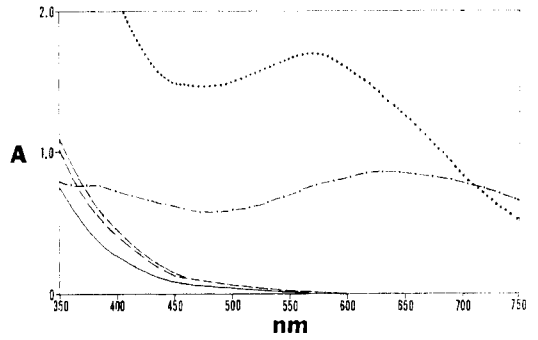
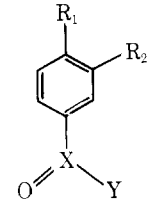


FIGURE 7: Iron complexation studies. (Curve 1) Spectrum obtained upon addition of 10 μL of 0.15 M $(Fe(NO_3)_3$ solution (1.5 μmol) to 3.02 mL of 1 mM protocatechuic acid (3.0 μmol) in 2 mM KOH (6.0 μmol of OH^-) (·····). (Curve 2) Spectrum obtained upon addition of 10 μL of 0.15 M $Fe(NO_3)_3$ solution to 3.02 mL of 1 mM 4-(chloroacetyl)catechol in 2 mM KOH (····). (Curve 3) Spectrum obtained upon addition of 10 μL of 0.15 M $Fe(NO_3)_3$ solution to 3.02 mL of 1 mM 3-HB in 2 mM KOH (—). (Curve 4) Spectrum obtained upon addition of 10 μL of 0.15 M $Fe(NO_3)_3$ solution to 3.02 mL of 1 mM 4-HB in 2 mM KOH (upper - - - - -). (Curve 5) Spectrum obtained upon addition of 10 μL of 0.15 M $Fe(NO_3)_3$ solution to 3.02 mL of 1 mM 3-FHB in 2 mM KOH (lower - - - - -).

Discussion

Protocatechuate-3,4-dioxygenase is remarkably selective in its choice of substrate. Minor variations in the skeleton of protocatechuic acid (I) invariably result in either a substrate with vastly decreased catalytic activity, or in a nonmetabolized competitive inhibitor. Compounds II, III, and IV are all inhibitors (simple competitive, except possibly III) even though the reactive vicinal hydroxyl groups of the substrate are still intact. In III, the carboxyl has been replaced by the isoelectronic nitro group, and this compound is a very potent inhibitor. However, II and IV are also quite capable of effective binding to PCD [$K_I = 1.4 \times 10^{-5}$ M (Fujisawa & Hayaishi, 1968) and 4×10^{-5} M (S. W. May & R. S. Phillips, unpublished observations), respectively]. As might have been expected, substitution directly at the catalytically sensitive bond in V and VII also prevents reaction. However, these latter two compounds differ greatly in their binding affinity for PCD, thus under-



I, $R_1 = R_2 =$ OH; X–Y = C–OH
II, $R_1 = R_2 =$ OH; X–Y = C–H
III, $R_1 = R_2 =$ OH; X–Y = N–O
IV, $R_1 = R_2 =$ OH; X–Y = C–CH_2Cl
V, $R_1 =$ OH; $R_2 =$ H; X–Y = C–OH
VI, $R_1 =$ OH; $R_2 =$ F; X–Y = C–OH
VII, $R_1 =$ H; $R_2 =$ OH; X–Y = C–OH
VIII, $R_1 =$ F; $R_2 =$ OH; X–Y = C–OH

scoring the positional specificity of the active site with respect to substitution in the aromatic ring.

Fluorine has been shown to occasionally substitute for oxygen with retention of biological activity, as in the case of α-D-glucopyranosyl fluoride, which is rapidly hydrolyzed by α-glucosidase (Barnett, 1972). In general, fluorine substitution in substrates or inhibitors often presents minimal steric interference and results in compounds capable of providing valuable mechanistic and structural information. In the case of fluorohydroxybenzoates and PCD, a number of possible consequences of fluorine substitution can be envisioned, ranging from altered binding potency to suicide inactivation through enzymatic generation of a reactive acyl fluoride at the active site.

The results presented here clearly provide an example of how judicious placement of a fluorine substituent can provide an excellent ligand for probing the active site of an enzyme. The data establish that 3-FHB binding to PCD is freely reversible, and this ligand is the most potent competitive inhibitor known to date. Perhaps most significantly, the spectral properties of 3-FHB and the PCD·3-FHB complex are such that this ligand is an ideal active site titrant. Free 3-FHB exhibits negligible spectral features in the visible region and it is thus possible to follow *directly* changes in the visible spectrum attributable to alterations of the ligand field at the active-site iron, thereby minimizing complications from "nonspecific" binding. In contrast, serious limitations are encountered in the use of other titrants proposed for this enzyme. Protocatechualdehyde exhibits both weak binding and visible absorption. The weak binding is overcome by the use of 4-nitrocatechol, but here one has the complication of strong absorption from both the free and complexed 4-NC moiety; sharp titration endpoints are not obtained, and calculations of K_D from titration data are much more difficult and less accurate. It is clear that 3-FHB is an ideal active site probe in optical techniques focusing on the charge transfer transitions of the iron, such as laser Raman spectroscopy. Our results also demonstrate that the K_D values obtained from spectral titrations of PCD with 3-FHB are essentially identical with the K_I values obtained from steady-state kinetic measurements at several different pH values, thus establishing *quantitatively* that no complicating processes occur upon binding of this ligand. Finally the titration endpoint of 8 mol of 3-FHB bound per mol of PCD is in agreement with information on the subunit composition and iron content of PCD and the stoichiometry of binding of other ligands (Fujisawa et al., 1972a; Tyson, 1975; Yoshida et al., 1976). However, it should be noted that there is a report that reconstituted PCD contains additional iron (Lipscomb et al., 1976), and differences between PCD preparations with respect to iron content have been noted (Fujiwara & Nozaki, 1973).

In a preliminary communication (May & Phillips, 1977), we reported a K_I value for 3-FHB at pH 7.5, and K_I values for 3-FHB and 4-FHB (pH not specified; no data given) are quoted in the text of a symposium presentation by Wood et al. (1977). The data reported in this paper firmly establish the spectral and kinetic behavior of fluorohydroxybenzoate inhibition and present a comparison of fluoro- and defluorohydroxybenzoate binding throughout the pH range. These comparative studies with the isomeric fluorohydroxybenzoates and the corresponding hydroxybenzoates lead to several important conclusions regarding binding interactions at the active site of PCD. In the first place, the comparative binding data support our conclusions from the data in Figure 7 that the effective binding cannot be attributed solely to a chelation of the active-site iron by the ortho oxygen and fluorine substituents. Among the factors which might be considered to account for the effective binding of 3-FHB is the formation of a strong H bond between the fluorine and a group at the active site, since the difference in binding free energy between 3-FHB (VI) and 4-HB (V) is approximately 3.5 kcal/mol. Indeed, the much lower binding of 4-FHB could then be attributable to a meta vs. para specificity of such an interaction. However, very recent studies with the corresponding chloro analogue, 3-chloro-4-hydroxybenzoate, have revealed a K_I for this compound of about 4 μM at pH 7.5 (R. S. Phillips, unpublished observations), and hydrogen bonding would not be expected to be important with this ligand. Thus, although hydrogen bonding may well account for the better binding of 3-FHB, there are clearly other factors which are also important.

We note that very similar spectral changes occur upon binding a series of ligands with a *p*-hydroxyl substituent to PCD, even though the inhibitory potency varies considerably within the series (Figure 1 plus unpublished data of R. S. Phillips with 3-chloro-4-hydroxybenzoate and 2,4-dihydroxybenzoate). Also the contrasting behavior of 4-HB and 3-HB, although much less pronounced than the difference between the corresponding fluoro compounds, reflects a positional specificity of binding interactions involving the hydroxyl substituent. Taken together, these results suggest that the *p*-hydroxyl is brought within the coordination sphere of the iron. In this regard it is interesting that the data in Table I for 3-FHB binding may reflect preferential binding of the protonated *p*-hydroxyl, and among the roles which could be envisioned for this proton is a promotion of ligand exchange or rehybridization at the iron atom. However, firm conclusions on this point must await a detailed study of the pH dependencies of both catalytic and binding parameters. A mechanism modeled after that proposed by Hamilton (1974) can be envisioned for PCD, where it is specifically the *m*-hydroxyl which plays an active role in catalysis through ketonization to supply electron density for para attack of oxygen. Such a mechanism is consistent with all of our binding data and with the fact that the fluoro compounds are not oxidatively cleaved, the inertness of 4-FHB being ascribed to a lack of proper orientation with respect to the iron atom. Further mechanistic studies with PCD are currently in progress.

In contrast to the implications of the 3-FHB data in Table I, the rate of displacement of 4-NC from PCD is much decreased at alkaline pH (Figure 6). In this regard we note that all of the substrates and effective inhibitors examined to date possess a single negative charge at neutral pH, and it is possible to make a case that the position of the negatively charged substituent with respect to the diphenolic OH groups critically determines whether a given compound will be a substrate or a nonmetabolized inhibitor. Thus, for example, compounds such as 4-NC with acidic *p*-hydroxyls might bind "backward" with respect to protocatechuic acid or 3-FHB. A second ionization, such as that of the *p*-hydroxyl of 3-FHB or substrate, would place substantial negative charge density at both ends of the molecule, perhaps leading to electrostatic repulsion and decreased binding.

We have thus demonstrated the value of fluorine substituents as active site probes for protocatechuate-3,4-dioxygenase. The enhancement of binding affinity brought about by fluorine substitution is position sensitive, thus allowing differentiation of the roles of the two phenolic hydroxyls of the substrate in binding or catalysis. Also, the perturbation of the pK_as of adjacent phenolic groups brought on by fluorine substitution is of great value in pH dependent kinetic studies. In addition, with the isomeric fluoro compounds in hand, ^{19}F NMR relaxation studies will help pinpoint the location of the iron atom with respect to the meta and para substituents of

enzyme-bound 3-FHB and 4-FHB, respectively.

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Plant DNA-Dependent RNA Polymerases: Subunit Structures and Enzymatic Properties of the Class II Enzymes from Quiescent and Proliferating Tissues†

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ABSTRACT: Class II DNA-dependent RNA polymerases were purified from soybean tissues of different physiological states: (1) from seed embryo tissue, representative of a quiescent, low metabolic state and (2) from auxin-treated hypocotyl tissue, representative of a highly proliferative and metabolically active state. Dodecyl sulfate, polyacrylamide gel electrophoresis indicates that RNA polymerase II from embryonic tissue consists largely (90–95%) of the form IIA enzyme, the largest subunit having a molecular weight of 215 000. RNA polymerase II from hypocotyl tissue is exclusively a form IIB enzyme, the largest subunit having a molecular weight of 180 000. Polypeptides common to RNA polymerases IIA and IIB have the following molecular weights: 138 000; 42 000; 27 000; 22 000; 19 000; 17 600; 17 000; 16 200; 16 100; and 14 000. Peptide mapping in the presence of dodecyl sulfate suggests that the 215 000 and 180 000 subunits possess similar peptide fragments. Plant embryo tissues do not contain protease activity capable of cleaving the 215 000 subunit to the 180 000 subunit, but proliferating plant tissues do contain such an activity. Mixing experiments indicate that appreciable amounts of RNA polymerase IIB are not being artifactually produced during protein purification.

DNA-dependent RNA polymerase II enzymes have been purified to homogeneity from a number of plant and animal tissues and apparently exist in two or three forms which differ from one another in the molecular weight of their largest subunits (for reviews, see: Roeder, 1976; Chambon, 1975). The two major types of class II RNA polymerase, IIA and IIB, possess largest subunits of approximately 215 000 and 180 000, respectively. It has been suggested that RNA polymerase IIA gives rise to IIB enzyme via proteolytic cleavage of the 215 000 polypeptide (Dezelée et al., 1976; Greenleaf et al., 1976;

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