# Inhibition of *p*-Hydroxyphenylpyruvate Dioxygenase by the Diketonitrile of Isoxaflutole: A Case of Half-Site Reactivity†

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ABSTRACT: *p*-Hydroxyphenylpyruvate dioxygenase (HPPD) catalyzes the formation of homogentisate from *p*-hydroxyphenylpyruvate and molecular oxygen. In plants, this enzyme is the molecular target of new families of very active bleaching herbicides. In the study presented here, we report for the first time on the purification to homogeneity of a plant enzyme, as obtained from recombinant *Escherichia coli* cells expressing a cDNA encoding carrot HPPD. The purified enzyme allowed us to carry out a detailed characterization of the inhibitory properties of a diketonitile (DKN), the active inhibitor formed from the benzoylisoxazole herbicide isoxaflutole. Inhibition kinetic analyses confirmed that DKN exerts a slow and tight-binding inhibition of HPPD, competitive with respect to the *p*-hydroxyphenylpyruvate substrate. The stoichiometry of DKN binding to HPPD determined by kinetic analyses or by direct binding of [$^{14}$C]-DKN revealed a half-site reactivity of DKN.

*p*-Hydroxyphenylpyruvate dioxygenase (HPPD,[1] EC 1.13.11.27) catalyzes the formation of homogentisate (HGA, 2,5-dihydroxyphenylacetate) from *p*-hydroxyphenylpyruvate (*p*-HPP) and molecular oxygen (Figure 1). This enzyme is a non-heme, α-keto acid-dependent enzyme where the α-keto acid is not a cofactor but part of the *p*-HPP substrate (*1*). It is proposed that the reaction proceeds through an oxidative decarboxylation of the 2-oxoacid chain of *p*-HPP accompanied by an intramolecular hydroxylation of the aromatic ring where two molecules of oxygen are incorporated into homogentisate, and an ortho migration of the carboxymethyl chain (*1*−*3*). In most organisms, this enzymatic activity is involved in the catabolism of tyrosine. A deficiency in HPPD activity is responsible for a hereditary disease in humans, called hypertyrosinaemia (*4*). Therefore, a thorough study of mammalian and bacterial HPPDs was engaged, and this enzyme activity was purified from many different species, including rat (*5*), avian (*6*), pig (*7*, *8*), human (*9*), and *Pseudomonas* (*10*). All mammalian HPPDs behave as homodimers of 43−49 kDa subunits, whereas the *Pseudomonas* enzyme is a homotetramer with 41 kDa subunits. In photosynthetic organisms, besides this role in tyrosine catabolism, HPPD is also involved in the biosynthesis of prenylquinones. This is because homogentisate is the aromatic precursor of tocopherols and plastoquinones, which are antioxidant compounds and elements of the photosynthetic electron-transfer chain, respectively. Despite this major role, only until recently have studies on plant HPPD been carried out. The interest in the plant enzyme was brought about by the recent demonstration that HPPD is the target of new bleaching herbicide families, the triketones (*11*−*13*) and the benzoylisoxazoles (*14*). Among

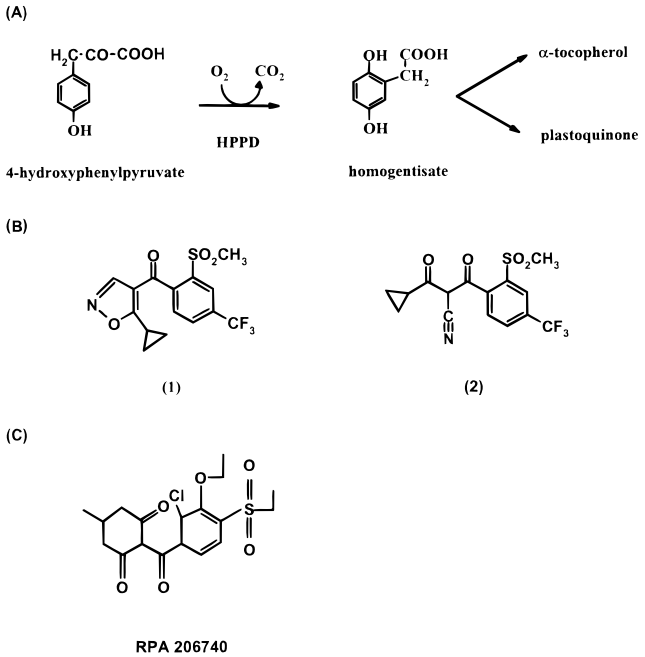


FIGURE 1: Reaction catalyzed by HPPD and inhibitors. (A) Schematic representation of the reaction catalyzed by HPPD. (B) Structure of isoxaflutole (1) and of its corresponding diketonitrile (2). (C) Structure of the triketone molecule RPA 206740 {2-[2-chloro-3-ethoxy-4-(ethylsulfonyl) benzoyl]-5-methyl-1,3-cyclohexanedione}.

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[1] Abbreviations: DKN, diketonitrile of isoxaflutole; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HGA, homogentisate; HPLC, high-performance liquid chromatography; *p*-HPP, *p*-hydroxyphenylpyruvate; HPPD, *p*-hydroxyphenylpyruvate dioxygenase; PCR, polymerase chain reaction; RPA 206740, 2-[2-chloro-3-ethoxy-4-(ethylsulfonyl)benzoyl]-5-methyl-1,3-cyclohexanedione; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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the latter, isoxaflutole [5-cyclopropylisoxazol-4-yl 2-mesyl-4-(trifluoromethyl)phenyl ketone] is a new pre- and early post-emergence herbicide, with selectivity in maize (*14*). In plants and soils, isoxaflutole is quickly and nonenzymatically hydrolyzed into its corresponding diketonitrile (DKN, Figure 1), which is the active herbicide principle of isoxaflutole (*14*). The susceptibility of plants toward DKN depends on their capacity to degrade this compound to an inactive benzoic acid derivative (*14*). The bleaching effect of these herbicides in susceptible weeds, which ultimately leads to plant death, is associated with a depletion of carotenoid and chlorophyll contents and an accumulation of the carotenoid precursor, phytoene. This results from an indirect inhibition of phytoene desaturase activity due to the depletion of its plastoquinone cofactor pool (*14*−*16*).

To improve our knowledge of this novel herbicide target, we recently characterized the HPPD from carrot (*Daucus carota*) cultured cells (*17*) and isolated cDNAs encoding the enzyme from carrot (*18*), and *Arabidopsis thaliana* (*19*). In many respects, the carrot and *Arabidopsis* enzymes resemble the mammalian HPPDs; they behave as homodimers with 48 and 49 kDa subunits, respectively, and are strongly inhibited by both triketones and the DKN of isoxaflutole. Recent work carried out with protein extracts from rat liver and carrot cells suggested that triketones and DKN act as tight-binding inhibitors, competitive versus the *p*-HPP substrate (*20*−*22*).

In this paper, we report on the purification of a recombinant carrot HPPD. This is to our knowledge the first report on the purification to homogeneity of a plant HPPD. This purification allowed us to carry out a detailed biochemical characterization of the carrot HPPD, in particular, concerning the inhibitory properties of benzoylisoxazole compounds. The results presented here demonstrate that DKN binds to HPPD with a half-site reactivity.

## MATERIALS AND METHODS

*Cloning of the Carrot HPPD Sequence into the Expression Vector pTrc 99A.* The p*Trc* 99A-HPPD Car13 plasmid encoding the carrot HPPD protein was constructed via site-directed mutagenesis using PCR amplification of the entire coding sequence of carrot HPPD cDNA (*18*). The following oligonucleotides were used: P1 (5′-GCGTGGAAACCAAACCATGGGGAAAAAACAATCGG-3′), which introduces a *Nco*I restriction site containing the ATG translation−initiation codon (underlined); and P2 (5′-GGAATTTTTTTTGTCGACTGGACTCTC-3′), which is complementary to the 3′-end of the cDNA coding region and introduces a *Sal*I restriction site (underlined) downstream of the TGA stop codon. PCR was performed for 30 cycles, including denaturation for 1 min at 95 °C, annealing for 1 min at 58 °C, and DNA elongation for 2 min at 72 °C driven by the Pwo DNA polymerase (Boehringer). The PCR-amplified DNA fragment was cloned into the p*Trc* 99A vector (Pharmacia) digested by *Nco*I−*Sal*I restriction enzymes. This oriented cloning put the carrot HPPD cDNA under the control of the *lacUV*5 promoter. The DNA insert was sequenced on both strands to ensure that no mutation had been introduced during the course of PCR amplification. The plasmid was termed p*Trc* 99A-HPPD Car13.

*Overproduction and Purification of the Recombinant Carrot HPPD. E. coli* JM 105 cells were transformed by the p*Trc* 99A Car13 plasmid and grown at 37 °C in 2 L of Luria-Bertani medium supplemented with 100 $\mu$g/mL carbenicillin. When the cell growth was equivalent to an $A_{600}$ of 0.6, 1 mM isopropyl $\beta$-D-thiogalactoside was added in the culture medium to initiate the synthesis of the recombinant protein. The cells were further grown for 16 h at 28 °C. After being harvested, they were centrifuged for 20 min at 40000*g*. The pellet was resuspended in buffer A containing 50 mM Na/Hepes (pH 7.5), 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 1 mM benzamidine HCl, and 5 mM aminocaproic acid and sonicated with a Vibra-cell disruptor (Sonics and Materials, Danbury, CT) (100 pulses every 3 s on power setting 5). The crude extract that was obtained was centrifuged for 20 min at 40000*g*. The cell-free supernatant was submitted to a streptomycin sulfate precipitation [0.1% (w/v)] and centrifuged for 20 min at 40000*g*. The remaining supernatant (1.9 g of total protein) was used for the purification of carrot HPPD, which was performed at 4 °C, without freezing intermediate fractions. The soluble protein extract containing the recombinant carrot HPPD was applied onto a EMD DEAE 650 (M) column (2.6 cm × 35 cm, Merck) previously equilibrated with buffer A. Elution was performed with a 450 mL gradient of 0 to 350 mM NaCl in buffer A (flow rate of 2 mL/min, fraction size of 2.5 mL). Chromatographic fractions containing the recombinant protein were pooled, concentrated by centrifugation at 5000*g* with a Macrosep 30K (Filtron) device, and desalted on a PD10 Sephadex G25 (M) column (Pharmacia) equilibrated in buffer A. The extract that was obtained (127 mg of protein) was applied onto a Source 15-Q column (1.5 cm × 10 cm, Pharmacia) previously equilibrated with buffer A. Elution was performed with a 200 mL gradient of 0 to 500 mM NaCl in buffer A (flow rate of 1 mL/min, fraction size of 2.5 mL). Chromatographic fractions containing the recombinant protein were pooled, concentrated as described above, and desalted on a PD10 Sephadex G25 (M) column equilibrated in buffer B consisting of 20 mM potassium phosphate (pH 6.8), 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine HCl, and 5 mM aminocaproic acid. The extract that was obtained (39 mg of protein) was applied onto a 1.6 cm × 7 cm column packed with 4 g of hydroxyapatite (Bio-Rad) previously equilibrated in buffer B. The column was washed with 30 mL of equilibrating buffer, and elution was carried out by four successive washes with increasing concentrations of potassium phosphate (40, 60, 80, and 100 mM) (flow rate of 0.5 mL/min, fraction size of 2 mL). Pure enzyme (4.5 mg) was concentrated with a Macrosep 30 K device, desalted onto a PD10 Sephadex G25 (M) column equilibrated in 20 mM Na/Hepes (pH 7.5), and concentrated again with a Macrosep 30 K device. This preparation (16 mg of HPPD/mL) was frozen in liquid nitrogen and could be stored at −80 °C for months.

*Protein Concentration.* The total protein concentration was determined with the Bio-Rad protein assay using $\gamma$-globulin as the standard (*23*). The concentration of the pure HPPD was also determined by measuring the absorbance at 205 nm (*24*) and at 280 nm ($\epsilon_{280}^{HPPD} = 69\,019\ M^{-1}\ cm^{-1}$, as calculated on the basis of the tyrosine and tryptophan content of the recombinant HPPD after denaturation in 0.1 M NaOH).

*Electrophoretic Analyses of Proteins.* Polypeptides were separated by SDS−PAGE containing 12% (w/v) acrylamide as described in detail by Chua (*25*). PAGE under nondena-

Table 1: Purification of Recombinant Carrot HPPD

| purification stage | total protein (mg) | total activity (units)[a] | specific activity (units/mg) | recovery (%) |
|---|---|---|---|---|
| total extract | 1890 | 296.4 | 0.16 | 100 |
| EMD DEAE 650 (M) | 126.7 | 116.7 | 0.92 | 39 |
| Source 15-Q | 38.5 | 74.5 | 1.93 | 25 |
| hydroxyapatite | 4.5 | 10.5 | 2.33 | 3.5 |

[a] One unit corresponds to 1 $\mu$mol of $O_2$ consumed in 1 min under the standard conditions described in Materials and Methods.

turing conditions was carried out at equilibrium in the absence of any denaturing agent as described by Lasky (*26*) on a linear acrylamide gradient (3.5 to 27%) with a 3.5% acrylamide stacking gel.

*Assay for HPPD Activity.* The activity of the purified recombinant carrot HPPD was monitored by following the consumption of $O_2$ during the formation of homogentisate from *p*-HPP (*21*, *27*), with a Clark-type $O_2$ electrode (Hansatech, King's Lynn, Norfolk, U.K.). Under standard conditions, the reaction medium [composed of 0.1 M Tris-acetate (pH 6.0), 0.5 mM sodium ascorbate (Fluka), 123 $\mu$M ammonium ferrous sulfate (Fluka), and 89 $\mu$M *p*-HPP (Sigma) in a total volume of 500 $\mu$L] was incubated for 10 min at 30 °C. Then, the reaction was initiated by the addition of 192 pmol on a dimeric basis of purified carrot HPPD, and the rate of $O_2$ consumption was recorded versus time at 30 °C. For the determination of the apparent $K_m$ value for *p*-HPP, a range of substrate concentrations from 4 to 200 $\mu$M was used. Enzyme activity was also assayed by HPLC determination of the amount of homogentisate formed during the reaction, as described in ref *19*.

*Kinetic Studies of Inhibition.* DKN and RPA 206740 {2-[2-chloro-3-ethoxy-4-(ethylsulfonyl)benzoyl]-5-methyl-1,3-cyclohexanedione, Figure 1} with a minimum purity of 98% were supplied by Rhône-Poulenc Agriculture Limited. Analyses of the stoichiometry of inhibitor binding to the enzyme were carried out in the following way. A fixed concentration of DKN (150 nM) or of RPA 206740 (240 nM) was incubated with increasing concentrations of enzyme for 10 min at 30 °C before starting the enzymatic reaction by addition of the *p*-HPP substrate (89 $\mu$M). Time courses of inhibition of the enzyme (192 pmol on a dimer basis) by DKN concentrations from 0.5 to 5 $\mu$M were analyzed by monitoring $O_2$ consumption in the simultaneous presence of the *p*-HPP substrate (89 $\mu$M) and the inhibitor, as described in detail by Tian and Tsou (*28*).

*DKN Binding to HPPD.* [$^{14}$C]DKN ($54 \times 10^6$ cpm/$\mu$mol), labeled uniformly in the aromatic ring, was supplied by Rhône-Poulenc Agriculture Limited. In the standard assay, the reaction medium [composed of 0.1 M Tris-acetate (pH 6.0), 10 mM sodium ascorbate, 1.4 mM ammonium ferrous sulfate, 192 pmol on a dimer basis of purified carrot HPPD, and [$^{14}$C]DKN in a total volume of 25 $\mu$L] was incubated for 10 min at 30 °C. [$^{14}$C]DKN bound to HPPD and free [$^{14}$C]DKN were separated by passing the reaction medium through a Sephadex G-25 column (0.5 cm × 6 cm). Elution was performed with 625 $\mu$L of 0.1 M Tris-acetate buffer (pH 6.0) (fraction size of 25 $\mu$L). Radioactivity in the eluted fractions was determined with a Beckman LS-1801 liquid scintillation spectrometer. The stoichiometry of inhibitor binding to the enzyme was determined by incubating a fixed amount of HPPD (192 pmol on a dimer basis) with increasing amounts of [$^{14}$C]DKN (96, 192, 384, and 576 pmol) for 10 min at 30 °C before bound and free [$^{14}$C]DKN were separated by gel filtration as described above. The dissociation rate constant for dissociation of the enzyme−DKN complex was estimated by incubating the enzyme for 5 min at 30 °C with 15.4 $\mu$M [$^{14}$C]DKN under the standard assay conditions. Then, 4.6 mM cold DKN (representing the [$^{14}$C]DKN concentration × 300) was added. At various time points, bound and free [$^{14}$C]DKN were separated by passing them through a Sephadex G-25 column as described above.

*Data Analysis.* Kinetic and binding data were fitted to the appropriate theoretical equations by using the Kaleidagraph program (Abelbeck Software).

## RESULTS

*Purification of the Recombinant Carrot HPPD.* Carrot HPPD overexpressed in *E. coli* JM105 cells was purified to homogeneity by a sequence of chromatographic steps, including two ion-exchange chromatographies [EMD DEAE 650 (M) and Source 15-Q] and a chromatography on hydroxyapatite as described in Materials and Methods. We note that during the process of purification the recombinant HPPD rapidly lost its activity. EPR analysis of the pure enzyme suggested that this could arise because of a loss of enzyme-bound iron (data not shown). In agreement with this finding, this loss in enzyme activity could be fully restored by addition of ferrous iron to the reaction medium. Such a strict requirement of iron in its ferrous state is in good agreement with the proposed reaction mechanism for HPPD as the initial step of the reaction is proposed to be the coordination of the α-keto acid moiety of the substrate to ferrous iron (*3*). Hence, enzymatic activity was measured in the presence of an excess of ammonium ferrous sulfate (123 $\mu$M) and sodium ascorbate (0.5 mM) as a reductant to maintain the iron in its ferrous form. The purification procedure resulted in a 15-fold purification of enzyme activity. In this way, 4.5 mg of pure HPPD could be obtained after starting from 1.9 g of *E. coli* total soluble protein, which corresponds to a yield of enzyme activity of about 3.5% (Table 1). This procedure, although very drastic, yielded enough protein that was highly pure and had a high specific activity ($2.0 \pm 0.3$ $\mu$mol min$^{-1}$ mg$^{-1}$) to carry out a thorough biochemical characterization of the carrot HPPD.

Proteins at different steps of the purification procedure are shown in Figure 2A. On the basis of SDS−PAGE analysis, the recombinant HPPD was purified to homogeneity. The preparation of the pure recombinant carrot HPPD exhibited a unique 48 kDa polypeptide by SDS−PAGE (Figure 2A), and behaved as an ~100 kDa homodimer under nondenaturing PAGE conditions (Figure 2B).

*Characterization of the Recombinant Carrot HPPD.* The purified enzyme displayed Michaelis−Menten behavior when steady-state rates were measured as a function of *p*-HPP substrate concentrations. The apparent Michaelis constant,

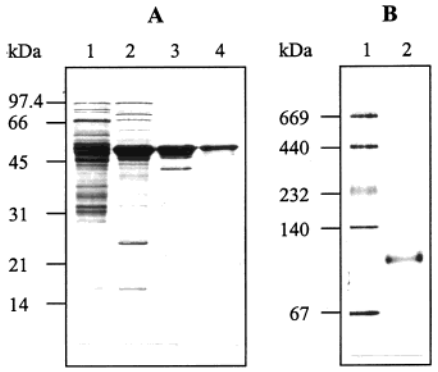


FIGURE 2: Documentation of the purification of the recombinant carrot HPPD. (A) SDS−PAGE analysis of protein fractions. Proteins were separated on a 12% (w/v) polyacrylamide gel under denaturing conditions and stained with Coomassie brillant blue R 250: lane 1, total soluble proteins of the crude extract of *E. coli* JM105 overproducing the carrot HPPD, 20 $\mu$g; lane 2, EMD DEAE 650 (M) pool, 20 $\mu$g; lane 3, Source 15-Q pool, 10 $\mu$g; and lane 4, hydroxyapatite pool, 5 $\mu$g. Molecular masses are indicated on the left in kilodaltons. (B) Analysis of recombinant carrot HPPD by PAGE under nondenaturing conditions. Purified recombinant HPPD (10 $\mu$g) was analyzed by native PAGE using a linear gradient of 3 to 27% (w/v) acrylamide and stained with Coomassie brillant blue R 250: lane 1, molecular mass markers (molecular masses are indicated on the left in kilodaltons); and lane 2, recombinant purified HPPD.

$K_m$, for *p*-HPP and the apparent specific activity, $V_m$, were on the order of 7.5 ± 2.5 $\mu$M and 2.0 ± 0.3 $\mu$mol of $O_2$ $min^{-1}$ (mg of purified HPPD)$^{-1}$, respectively. The high specific activity determined was in the range of specific activities observed for purified HPPD from other organisms (*6*−*9*). Moreover, the $K_m$ value for *p*-HPP corresponds well with that determined for plant HPPDs (*19*, *20*, *29*). The apparent $K_m$ for *p*-HPP determined for purified mammalian or bacterial HPPDs (*9*, *10*) is higher, being in the range of 30 $\mu$M.

Previous results obtained with a crude carrot HPPD preparation suggested that DKN behaves as a slow-binding inhibitor of the plant HPPD (*20*). This behavior was also seen with the pure recombinant enzyme. Thus, when the enzyme reaction was monitored upon simultaneous addition of substrate and inhibitor, nonlinear time courses of $O_2$ consumption were observed. Furthermore, at low *p*-HPP substrate concentrations, the inhibition was complete, suggesting that DKN behaved as a nearly irreversible inhibitor. A kinetic analysis according to Tian and Tsou (*28*) and Liu and Tsou (*30*) revealed that DKN binding is competitive with respect to *p*-HPP substrate and proceeds in a single step. This analysis also allowed determination of the bimolecular rate of association of DKN with HPPD, $k_{on}$, which was on the order of $1.5 \times 10^4$ $M^{-1}$ $s^{-1}$. This value is several orders of magnitude smaller than that expected for diffusion-controlled bimolecular reactions ($10^8$−$10^{10}$ $M^{-1}$ $s^{-1}$), confirming the slow-binding behavior of DKN. We conclude that the highly purified carrot HPPD exhibits the known enzyme characteristics previously evidenced for mammalian or plant HPPDs.

*Stoichiometry of DKN Binding to the Enzyme.* The availability of a highly purified enzyme allowed us to determine the stoichiometry of DKN binding to the plant HPPD. To address this question, increasing amounts of enzyme were incubated for 10 min at 30 °C in the presence

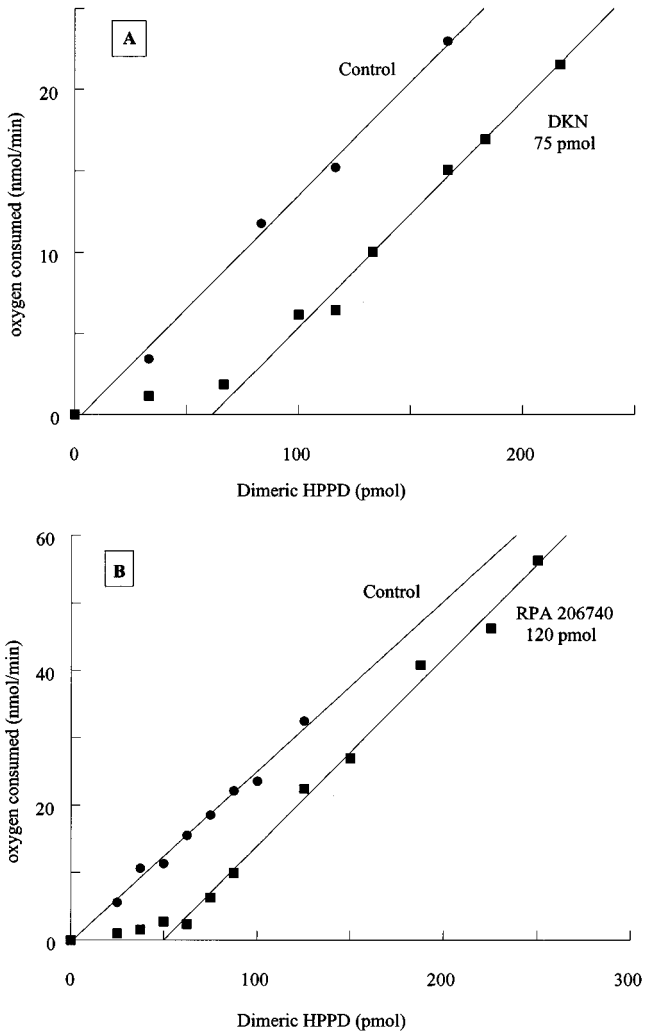


FIGURE 3: Stoichiometry of HPPD−inhibitor binding with DKN and the triketone RPA 206740. DKN (A) [75 pmol (■)] or RPA 206740 (B) [120 pmol (■)] were preincubated with increasing quantities of enzyme (0−250 pmol on a dimeric basis) for 10 min at 30 °C. The reaction medium contained 0.1 M Tris-acetate (pH 6.0), 0.5 mM sodium ascorbate, and 123 $\mu$M ammonium ferrous sulfate, in a total volume of 500 $\mu$L. The enzymatic reaction was started by addition of *p*-HPP (89 $\mu$M). (●) Control reactions in the absence of inhibitors.

of a fixed amount of DKN (75 pmol). Figure 3A shows that the enzymatic activity was completely abolished in the low-enzyme concentration range. Then, the activity increased further upon increasing the HPPD concentration. This increase in HPPD activity paralleled that of the control reaction carried out under the same conditions but without DKN. This behavior is typical of tight-binding inhibition (*31*). The horizontal axis intercept of the plot of activity versus enzyme concentration gives the stoichiometry of DKN binding to HPPD. Data in Figure 3A revealed that in the presence of 75 pmol of DKN this intercept corresponded to approximately 70 pmol of HPPD on a dimeric basis. Thus, a molar ratio of inhibitor/dimeric enzyme of 1/1 was obtained. This means that modification of only one active site of the homodimeric enzyme by DKN was sufficient to provide a complete inhibition of the enzymatic activity. This suggested a half-site reactivity of the enzyme upon DKN binding. To determine if this behavior is a common feature of tight-binding inhibitors of HPPD, similar experiments were

Table 2: Effect of Ascorbate, Iron, Other Metal Ions, and Combinations of These Compounds on the Binding of DKN to HPPD[a]

| compounds | DKN bound (pmol)[b] |
|---|---|
| HPPD/DKN/ascorbate/$Fe^{2+}$ | 191 |
| HPPD/DKN | 8 |
| HPPD/DKN/ascrobate | 18 |
| HPPD/DKN/$Fe^{2+}$ | 185 |
| HPPD/DKN/$Fe^{3+}$ | 8 |
| HPPD/DKN/ascorbate/$Fe^{3+}$ | 191 |
| HPPD/DKN/ascorbate/$Cu^{2+}$ | 21 |
| HPPD/DKN/ascorbate/$Zn^{2+}$ | 38 |

[a] Dimeric HPPD (192 pmol) and [$^{14}$C]DKN (576 pmol) were incubated for 10 min at 30 °C in 0.1 M Tris-acetate (pH 6.0) in the presence or absence of ascorbate (10 mM) and various metal ions (1.4 mM), in a total volume of 25 $\mu$L. [b] [$^{14}$C]DKN bound to HPPD was separated from free [$^{14}$C]DKN by passing the reaction assays onto a Sephadex G-25 column.

carried out with the triketone molecule RPA 206740 (see Figure 1C). As for DKN, a plot characteristic of tight-binding inhibition was obtained with this latter compound (Figure 3B). However, in this case, in the presence of 120 pmol of inhibitor, the horizontal axis intercept corresponded to 50 pmol of HPPD on a dimeric basis, which corresponds approximately to 1.2 pmol of inhibitor per picomole of HPPD subunit. This is typical of stoichiometric binding of this compound to the carrot HPPD. Thus, in marked contrast with the behavior observed with DKN, two molecules of the triketone molecule RPA 206740 were needed to fully inactivate the dimeric plant enzyme. It appears, therefore, that although DKN and RPA 206740 both behave as tight-binding inhibitors of the plant enzyme, they exhibit different reactivities toward this enzyme. These experiments were repeated with three independent sets of purified enzyme, yielding reproducible results.

*Interactions between DKN and HPPD.* The mechanism of interaction between DKN and HPPD was directly assessed by monitoring the binding of [$^{14}$C]DKN to the purified enzyme. Purified HPPD and [$^{14}$C]DKN were incubated together as described in Materials and Methods. Bound and free [$^{14}$C]DKN were separated by passing the reaction medium through a Sephadex G-25 column. Indeed, the tight binding characteristic of the inhibition of plant HPPD by DKN (Figure 3A) made possible the separation of bound and free [$^{14}$C]DKN by elution on a gel filtration column.

In a first approach, we examined the requirements for DKN binding to the plant enzyme. For this purpose, we have taken advantage of the fact that the purified HPPD was nearly completely devoid of iron as evidenced by EPR analyses (results not shown). We analyzed the capacity of [$^{14}$C]DKN to bind purified carrot HPPD in the presence or absence of iron (ferrous and ferric forms), ascorbate, various metallic ions, and combinations of these compounds. Results are summarized in Table 2. We found that DKN cannot bind HPPD in the absence of iron. Furthermore, the oxidation state of iron was a major determinant for an efficient binding of DKN to the enzyme. Only with ferrous iron could the binding be evidenced. Iron substitution by other metallic ions only allowed a very weak DKN binding.

On the basis of these results, the stoichiometry of DKN binding to the enzyme was then directly measured by monitoring the amounts of radioactive ligand bound to the

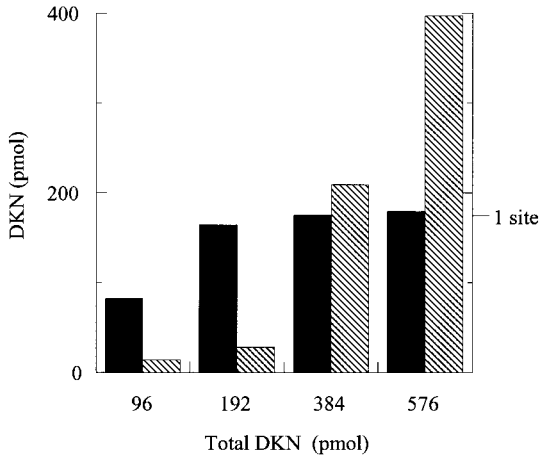


FIGURE 4: Stoichiometry of inhibitor binding with [$^{14}$C]DKN. HPPD (192 pmol on a dimeric basis) was incubated for 10 min at 30 °C in a total volume of 25 $\mu$L with various amounts of [$^{14}$C]-DKN in the presence of 0.1 M Tris-acetate (pH 6.0), 10 mM sodium ascorbate, and 1.4 mM ammonium ferrous sulfate. Bound (black) and free [$^{14}$C]DKN (cross-hatched) were separated by passing the reaction assays through a Sephadex G-25 gel filtration column.

enzyme in the presence of ascorbate and ferrous iron. Carrot HPPD was incubated with increasing [$^{14}$C]DKN amounts that corresponded to amounts 0.5, 1, 2, and 3 times greater than the enzyme amount on a dimeric structure basis. When the [$^{14}$C]DKN amount was lower than that of HPPD on a dimeric basis, all the radioactivity was recovered in the bound fraction, and free [$^{14}$C]DKN remained at background levels. When the [$^{14}$C]DKN concentration surpassed that of the enzyme, the amount of bound DKN still corresponded exactly to the concentration of the enzyme on a dimer structure basis, whatever the concentration of total [$^{14}$C]DKN that was used (Figure 4). Again, these direct binding experiments confirmed the half-site reactivity of enzyme upon DKN binding, since only one molecule of DKN was capable of binding to the dimeric HPPD. Similar experiments were carried out with purified HPPD from *Pseudomonas fluorescens* that behaves as a homotetramer (*10*). Again, these experiments disclosed a half-site reactivity with two molecules of DKN bound per enzyme molecule on a tetrameric structure basis (results not shown).

A possible explanation of the unexpected stoichiometry observed upon DKN binding to the plant HPPD could be that the enzyme DKN complex can dissociate to some extent during the gel filtration experiments. This point was investigated by conducting a chase experiment in which preformed HPPD−[$^{14}$C]DKN complex was incubated with a 300-fold excess of cold DKN. Then, the bound [$^{14}$C]DKN fraction was estimated at different times following the addition of the cold DKN. As shown in Figure 5, this fraction decayed in an exponential manner with a half-life of about 2 h. Since during the gel filtration experiment the enzyme−DKN complex was eluted within 5 min of loading the columns, we conclude that dissociation of the enzyme−DKN complex was not the cause of the abnormal stoichiometry observed upon DKN binding to the plant HPPD. From the data in Figure 5, the dissociation rate constant for dissociation of the HPPD−DKN complex, $k_{off}$, was on the order of $9 \times 10^{-5}$ $s^{-1}$. From the rate of dissociation ($k_{off}$) and the bimolecular rate constant of association ($k_{on}$) determined

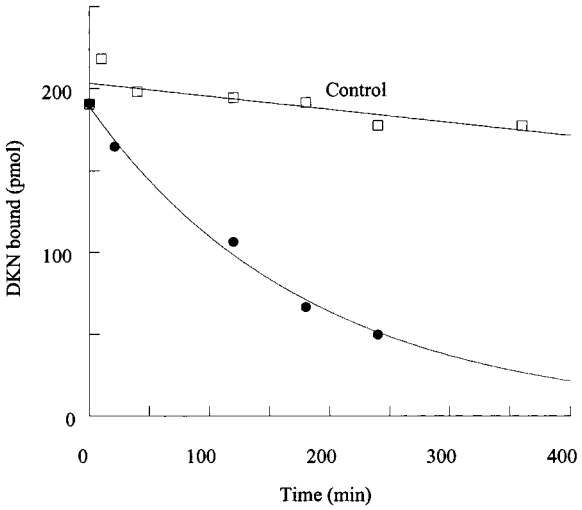


FIGURE 5: Time course dissociation of the HPPD−DKN complex. HPPD (192 pmol on a dimeric basis) was incubated for 5 min at 30 °C with 384 pmol of [$^{14}$C]DKN in a total volume of 25 $\mu$L in the presence of 0.1 M Tris-acetate (pH 6.0), 10 mM sodium ascorbate, and 1.4 mM ammonium ferrous sulfate prior to the addition of 4.6 mM DKN (●) or water (□). Bound [$^{14}$C]DKN was separated from free [$^{14}$C]DKN at different times by elution through a Sephadex G-25 gel filtration column. Data were analyzed by nonlinear regression using the equation $DKN_b = DKN_0\, e^{-k_{off}t}$, where $DKN_b$ is the quantity of [$^{14}$C]DKN bound to HPPD at time $t$, $DKN_0$ the quantity of [$^{14}$C]DKN bound to HPPD at time zero, and $k_{off}$ the first-order rate constant for dissociation of the enzyme−DKN complex.

above, a $K_d$ of 6 nM could be calculated, testifying to the high affinity of the plant HPPD for DKN.

Since oxygen is the second substrate of HPPD, we have investigated if its presence was required for the binding of DKN to HPPD. For that purpose, binding experiments were performed in an atmosphere depleted of oxygen by bubbling all the buffers and the gel filtration column with argon. Under these conditions, the absence of oxygen did not prevent the binding of DKN to HPPD (data not shown).

## DISCUSSION

DKN and triketones are new and very active families of bleaching herbicides targeting HPPD and that act at low doses (*11*−*14*). Previous studies carried out with protein extracts of rat liver (*21*, *22*) or carrot cells (*20*) have revealed that these herbicides behave as tight-binding inhibitors of HPPD. However, the utilization of crude preparations of enzyme precluded a detailed analysis of the interactions between the enzyme and inhibitors. In the study presented here, carrot HPPD was overproduced in *E. coli* JM105 and purified to homogeneity. The mechanism of interactions between DKN and HPPD was examined by kinetic and binding approaches.

Inhibition kinetic analysis confirmed previous findings that DKN behaved as a nearly irreversible inhibitor of the plant enzyme, competitive with respect to *p*-HPP substrate and that binds in a single step to the enzyme (*20*). The formation of the enzyme−inhibitor complex was slow, the rate constant of association of DKN with HPPD being on the order of $1.5 \times 10^4$ $M^{-1}$ $s^{-1}$. Nevertheless, this rather low rate of association does not hamper the herbicidal activity of DKN since this compound is able to control susceptible weeds at low application rates (*14*). One possible explanation would be that *in planta* the *p*-HPP substrate never accumulates in a sufficient amount in DKN-treated plants to prevent the binding of DKN to its HPPD target.

The mechanism of interaction between enzyme and DKN was directly assessed by monitoring the binding of [$^{14}$C]-DKN to HPPD. The data clearly revealed that DKN was unable to bind the apoenzyme, or the holoenzyme when iron was in a ferric state. These results demonstrated that ferrous iron, which was required for catalytic activity, was also indispensable for stabilization of the binding of DKN, presumably via the $\beta$-(1,3)-diketone moiety of DKN. This binding presumably mimics that of the substrate *p*-HPP via its α-keto acid moiety, as evidenced by the competition for enzyme binding between DKN and the *p*-HPP substrate. It is possible that the coordination environment of iron in the active site does not allow ferric iron to coordinate the α-keto acid moiety of the substrate or the $\beta$-(1,3)-diketone moiety of DKN. Binding experiments carried out in the absence of oxygen revealed that oxygen was not required for the binding of DKN to HPPD. These results are in good agreement with the steady-state kinetic study carried out on human liver HPPD, which revealed an ordered bi-bi kinetic mechanism where the *p*-HPP substrate binds prior to oxygen (*32*).

Although DKN proved to act as a slow-binding inhibitor of plant HPPD, it was not possible in our manual mixing experiments to directly determine the rate constant of association of DKN with the enzyme. Indeed, under the conditions of our binding assay and from the value of $k_{on}$ determined from enzyme inhibition kinetics, a $t_{1/2}$ of about 6 s could be estimated for the formation of the binary enzyme−inhibitor complex, in the presence of 7.7 $\mu$M DKN. The possibility that the binding of DKN was reversible to some extent was investigated by a competitive substitution of bound [$^{14}$C]DKN with a 300-fold excess of cold DKN. The time-dependent decay of bound [$^{14}$C]DKN revealed that the dissociation of the enzyme−DKN complex was effective but very slow, the dissociation rate constant $k_{off}$ being on the order of $9 \times 10^{-5}$ $s^{-1}$. This value corresponds to a half-life of the enzyme−DKN complex of approximately 2 h at 30 °C, meaning that DKN behaved as a nearly irreversible inhibitor. This low dissociation rate is most probably a key determinant in accounting for the herbicidal potency of DKN. Its $K_d$ of 6 nM is a good illustration of that potency. A similar behavior was previously observed with other inhibitors of HPPD that belong to the triketone family (*21*).

One remarkable feature of DKN−enzyme interaction was that the association of DKN with the enzyme obeys a half-site reactivity. This was evidenced by both enzymatic activity (Figure 3) and binding (Figure 4) measurements. Only one molecule of DKN was bound per dimeric HPPD and proved to be sufficient to fully inhibit the enzyme activity. Furthermore, binding experiments carried out with purified *Pseudomonas* HPPD also revealed a half-site reactivity of this enzyme upon DKN binding (data not shown). As a whole, these data demonstrate that the binding of DKN actually occurred though a half-site reactivity. There are different explanations for this phenomenon (*33*, *34*). First, binding sites (one per subunit of the homodimeric enzyme) are near the symmetry axes of the enzyme dimer. They overlap or are too close to each other so that the binding of the ligand at one site prevents its binding at the other site because of steric or electrostatic constraints. However, the three-dimensional

structure of the *Pseudomonas* HPPD (*35*), which also exhibits a half-site reactivity upon DKN inhibition, rules out this hypothesis. An examination of this structure clearly reveals that actives sites, as evidenced by the presence of the bound iron, are far from each other. In a second possibility, called pre-existing asymmetry (*33*, *34*), the subunit dimerization creates a conformational change in one of the two sites that becomes unfunctional. For example, this explanation has been proposed to account for the half-site reactivity of active cysteine residues of glyceraldehyde-3-phosphate dehydrogenase (*36*). Finally, in the case of the induced asymmetry model (*33*, *34*), binding of the ligand to one site induces a conformational change of the second binding site that becomes inaccessible. A relevant example of such behavior is the case of 6-diazo-5-oxonorleucine binding to cystidine triphosphate synthetase (*33*). The fact that in marked contrast with DKN, the triketone RPA 206740 binds in a stoichiometric manner to the plant enzyme strongly suggests that the half-site reactivity encountered with DKN originates from an induced asymmetry and not from a pre-existing asymmetry. Indeed, it was proposed (*37*) that in the case of induced asymmetry, the magnitude of the half-site reactivity varies in the function of the ligand while it does not in the case of pre-existing asymmetry. Crystallographic studies of recombinant carrot HPPD in the presence and absence of DKN currently in progress in our laboratory will provide more insight into the mechanism responsible of the half-site reactivity.

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## REFERENCES

1. Que, L., Jr., and Ho, R. Y. N. (1996) *Chem. Rev. 96*, 2607−2624.
2. Lindblad, B., Lindstedt, G., and Lindstedt, S. (1970) *J. Am. Chem. Soc. 92*, 7446−7449.
3. Crouch, N. P., Adlington, R. M., Baldwin, J. E., Lee, M.-H., and MacKinnon, C. H. (1997) *Tetrahedron 53*, 6993−7010.
4. Endo, F., Kitano, A., Uehara, I., Nagata, N., Matsuda, I., Shinka, T., Kuhara, T., and Matsumoto, I. (1983) *Pediatr. Res. 17*, 92−96.
5. Lin, K.-T., and Crawhall, J. C. (1976) *Can. J. Biochem. 54*, 423−431.
6. Wada, G. H., Fellman, J. H., Fujita, T. S., and Roth, E. S. (1975) *J. Biol. Chem. 250*, 6720−6726.
7. Buckthal, D. J., Roche, P. A., Moorehead, T. J., Forbes, B. J. R., and Hamilton, G. A. (1987) *Methods Enzymol. 142*, 132−138.
8. Roche, P. A., Moorehead, T. J., and Hamilton, G. A. (1982) *Arch. Biochem. Biophys. 216*, 62−73.
9. Lindblad, B., Lindstedt, G., Lindstedt, S., and Rundgren, M. (1977) *J. Biol. Chem. 252*, 5073−5084.
10. Lindstedt, S., and Odelhög, B. (1987) *Methods Enzymol. 142*, 143−148.
11. Schulz, A., Ort, O., Beyer, P., and Kleinig, H. (1993) *FEBS Lett. 318*, 162−166.
12. Secor, J. (1994) *Plant Physiol. 106*, 1429−1433.
13. Prisbylla, M. P., Onisko, B. C., Shribbs, J. M., Adams, D. O., Liu, Y., Ellis, M. K., Hawkes, T. R., and Mutter, L. C. (1993) *Brighton Crop Prot. Conf.−Weeds 2*, 731−738.
14. Pallett, K. E., Little, J. P., Sheekey, M., and Veerasekaran, P. (1998) *Pestic. Biochem. Physiol. 62*, 113−124.
15. Mayer, M. P., Beyer, P., and Kleinig, H. (1990) *Eur. J. Biochem. 191*, 359−363.
16. Norris, S. R., Barrette, T. R., and DellaPenna, D. (1995) *Plant Cell 7*, 2139−2149.
17. Lenne, C., Matringe, M., Rolland, A., Pallett, K. E., and Douce, R. (1995) in *Photosynthesis: from light to Biosphere* (Mathis, P., Ed.) Vol. V, pp 285−288, Kluwer Academic Publishers.
18. Garcia, I., Rodgers, M., Lenne, C., Rolland, A., Sailland, A., and Matringe, M. (1997) *Biochem. J. 325*, 761−769.
19. Garcia, I., Rodgers, M., Pepin, R., Hsieh, T.-F., and Matringe, M. (1999) *Plant Physiol. 119*, 1507−1516.
20. Viviani, F., Little, J. P., and Pallett, K. E. (1998) *Pestic. Biochem. Physiol. 62*, 125−134.
21. Ellis, M. K., Whitfield, A. C., Gowans, L. A., Auton, T. R., MacLean Provan, W., Lock, E. A., and Smith, L. L. (1995) *Toxicol. Appl. Pharmacol. 133*, 12−19.
22. Ellis, M. K., Whitfield, A. C., Gowans, L. A., Auton, T. R., MacLean Provan, W., Lock, E. A., Lee, D. L., and Smith, L. L. (1996) *Chem. Res. Toxicol. 9*, 24−27.
23. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
24. Scopes, R. K. (1974) *Anal. Biochem. 59*, 277−282.
25. Chua, N. H. (1980) *Methods Enzymol. 69*, 434−436.
26. Lasky, M. (1978) in *Electrophoresis* (Catsimpoolis, G., Ed.) pp 195−205, Elsevier North-Holland, New York.
27. Coufalik, A. H., and Monder, C. (1980) *Arch. Biochem. Biophys. 199*, 67−75.
28. Tian, W.-X., and Tsou, C.-L. (1982) *Biochemistry 21*, 1028−1032.
29. Barta, I. Cs., and Böger, P. (1996) *Pestic. Sci. 48*, 109−116.
30. Liu, W., and Tsou, C.-L. (1986) *Biochim. Biophys. Acta 870*, 185−190.
31. Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177−2185.
32. Rundgren, M. (1977) *J. Biol. Chem. 252*, 5094−5099.
33. Levitzki, A., Stallcup, W. B., and Koshland, D. E., Jr. (1971) *Biochemistry 10*, 3371−3378.
34. Seydoux, F., Malhotra, O. P., and Bernhard, S. A. (1974) *CRC Crit. Rev. Biochem. 2*, 227−257.
35. Serre, L., Sailland, A., Sy, D., Boudec, P., Rolland, A., Pebay-Peyroula, E., and Cohen-Addad, C. (1999) *Structure 7*, 977−988.
36. Bernhard, S. A., and MacQuarrie, R.-A. (1973) *J. Mol. Biol. 74*, 73−78.
37. Franzen, J. S., Ashcom, J., Marchetti, P., Cardamone, J. J., Jr., and Feingold, D. S. (1980) *Biochim. Biophys. Acta 614*, 242−255.

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