Ascorbic Acid-Dependent Turnover and Reactivation of 2,4-Dichlorophenoxyacetic Acid/α-Ketoglutarate Dioxygenase Using Thiophenoxyacetic Acid†

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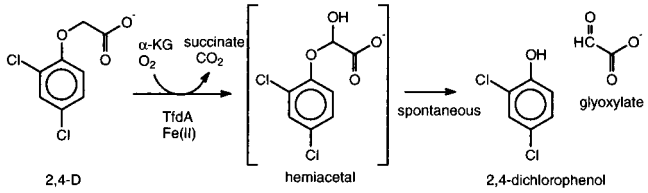
ABSTRACT: The first step in catabolism of the broadleaf herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is catalyzed by 2,4-D/α-ketoglutarate (α-KG)-dioxygenase (TfdA) in *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134. This oxygen- and ferrous-ion-dependent enzyme couples the oxidative decarboxylation of α-KG (yielding CO_2 and succinate) with the oxidation of 2,4-D to produce 2,4-dichlorophenol and glyoxylate. TfdA was shown to utilize thiophenoxyacetic acid (TPAA) to produce thiophenol, allowing the development of a continuous spectrophotometric assay for the enzyme using the thiol-reactive reagent 4,4′-dithiodipyridine. In contrast to the reaction with 2,4-D, however, the kinetics of TPAA oxidation were nonlinear and ascorbic acid was found to be required for and consumed during TPAA oxidation. The ascorbic acid was needed to reduce a reversibly oxidized inactive state that was formed by reaction of the ferrous enzyme with oxygen, either in the absence of substrate or in the presence of TPAA. The dependency on this reductant was not due to an uncoupling of α-KG decarboxylation from substrate hydroxylation, as has been reported for several other α-KG-dependent hydroxylases. Significantly, the rate of formation of this reversibly oxidized species was much lower when the enzyme was turning over 2,4-D. Evidence also was obtained for the generation of an inactive enzyme species that could not be reversed by ascorbate. The latter species, not associated with protein fragmentation, arose from an oxidative reaction that is likely to involve hydroxyl radical reactions. On the basis of initial rate studies, the k_{cat} and K_m values for TPAA were estimated to be 20-fold lower and 80-fold higher than the corresponding values for 2,4-D. The results are incorporated into a model of TfdA reactivity involving both catalytic and inactivating events.

2,4-Dichlorophenoxyacetic acid (2,4-D)[1] is a broad leaf herbicide that is rapidly degraded in the environment. The soil isolate *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*; *1*) JMP134 carries the pJP4 plasmid which encodes a series of enzymes in the pathway for 2,4-D mineralization (*2*). The first enzyme in the biodegradative pathway is 2,4-D/α-ketoglutarate (α-KG) dioxygenase (*3*, *4*) or TfdA (from the *tfdA* gene designation; *5*). This ferrous-ion-dependent enzyme catalyzes the oxidation of 2,4-D to form 2,4-dichlorophenol and glyoxylic acid coupled with oxidative decarboxylation of α-KG to form CO_2 and succinate, as illustrated in Scheme 1. The reaction is thought to proceed via hydroxylation at the 2,4-D methylene group followed by spontaneous breakdown of the resulting hemiacetal.

Mechanistic details of the TfdA-catalyzed reaction are unknown, although there is information regarding the order

Scheme 1



of substrate and cofactor binding, the identity of some of the metallocenter ligands, and the identity of additional amino acids required for activity. Electron paramagnetic resonance and electron spin−echo envelope modulation studies of inactive copper-substituted TfdA provide evidence for the binding of α-KG prior to 2,4-D (*6*). These spectroscopic studies also demonstrate the presence of two histidyl groups binding the metallocenter. Additional, nonligand histidines are likely to be essential for catalysis based on enzyme inactivation studies with the histidine-reactive reagent diethylpyrocarbonate that revealed protection only by the simultaneous presence of Fe(II), α-KG, and 2,4-D (*4*). Cysteinyl residues do not participate in catalysis since thiol-reactive reagents do not inhibit TfdA. On the basis of the chemical modification studies, a model of the active site has been proposed involving histidine residues coordinating the iron atom and stabilizing binding of substrate and cofactor (*4*).

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[1] Abbreviations: α-KG, α-ketoglutaric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; DTDP, 4,4′-dithiodipyridine; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; TfdA, 2,4-D/α-KG dioxygenase; TPAA, thiophenoxyacetic acid.

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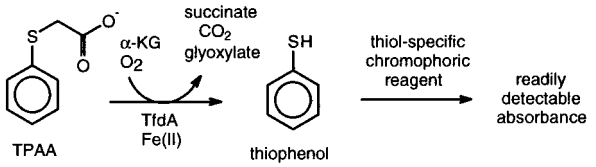
Scheme 2



The experiments reported here focus on the interaction of TfdA with thiophenoxyacetic acid (TPAA). These studies were undertaken in an attempt to develop an alternate assay for TfdA and as a means to explore specific aspects of the enzyme mechanism. Assuming that TPAA is a substrate for TfdA, the released thiophenol could be reacted with any of a number of thiol-specific chemical reagents to provide a simple, continuous, colorimetric assay for the enzyme (Scheme 2). Such an assay may be more useful than the commonly used discontinuous colorimetric (*7*) and HPLC (*3*) assays and may complement the recently devised continuous spectrophotometric assay for TfdA based on 4-nitrophenoxyacetic acid (*8*). The latter assay is limited in usefulness because many TfdAs, including that from *R. eutropha*, exhibit a high K_M for this substrate, and it is not utilized by all TfdA-like activities. In addition to allowing the possible development of a new assay, we reasoned that studies of thioether-substituted substrates may provide insight into the mechanism of substrate binding and hydroxylation. For example, a postulated interaction between the ether heteroatom and the metal center (*4*) may be more readily detected by selected spectroscopic methods when using the large, soft, thioether group of TPAA than for the hard, small, oxygen ether linkage found in 2,4-D. Additionally, TPAA may be a useful mechanism-based inhibitor of TfdA if the thiophenol generated by turnover were to interact tightly with the metallocenter. Finally, the regiospecificity of hydroxylation for TfdA could be examined by using the thioether substrate; i.e., in addition to hydroxylation at the methylene carbon, the enzyme could reasonably oxidize the sulfur heteroatom to the sulfone or sulfoxide. Precedent for such a change in regiospecificity is available in peptidylglycine α-amidating monooxygenase, for which substitution of a thioether for the oxyether in the substrate [(4-nitrobenzyl)-oxy]acetic acid changes the reaction from O-dealkylation to sulfoxidation (*9*).

We report that TPAA is a substrate for TfdA, that the thiol released from this substrate can be used in a qualitative assay for TfdA, and that the reaction mechanism observed with TPAA is distinct from that seen with 2,4-D in its requirement for ascorbic acid. Studies to characterize the differences in mechanism for the different substrates provide important new insight into the mechanism and inactivation reactions of the enzyme.

EXPERIMENTAL PROCEDURES

Chemicals. Phenoxy and thiophenoxy compounds were obtained from Aldrich. α-[1-^{14}C]KG was obtained from DuPont NEN. Bovine liver catalase and bovine erythrocyte superoxide dismutase were purchased from Sigma. Other chemicals were reagent grade.

Purification of TfdA. TfdA was purified from *Escherichia coli* DH5α (pUS311), carrying the *tfdA* gene of *R. eutrophus* JMP134, as previously described (*4*). The protein was stable for months when stored at 4 °C in the buffer used in the final chromatography step (20 mM Tris, 1 mM EDTA, ∼60 mM NaCl, pH 7.2). In indicated cases, the EDTA concentration was reduced by overnight dialysis of the protein (12000−14000 molecular weight cutoff, Spectra/Por, VWR Scientific) against 10 mM imidazole buffer, pH 6.75. Protein concentrations were determined by using the Bio-Rad protein assay with bovine serum albumin as the standard.

Assays of TfdA Activity. Typical assay mixes contained 50 μM $(NH_4)_2Fe(SO_4)_2$, 50 μM ascorbic acid, 1 mM α-KG, 10 mM imidazole (pH 6.75), the indicated amount of thiophenoxyacetic or phenoxyacetic acid substrate, and TfdA. Reactions were performed at 30 °C. For 2,4-D colorimetric assays, reactions were quenched with EDTA (5 mM final concentration) and the levels of 2,4-dichlorophenol were determined by using the 4-aminoantipyrene method (*7*) based on a measured extinction coefficient of 15.7 mM^{-1} cm^{-1}. For TPAA spectroscopic assays, thiophenol levels were determined by reaction with 4,4′-dithiodipyridine (DTDP) (200 μM final concentration) to release thiopyridine which was monitored at 324 nm, using a measured extinction coefficient of 21.2 mM^{-1} cm^{-1}. The assay was used both for fixed time assays, in which the DTDP was added after terminating TfdA turnover by adding EDTA, and for continuous assays, in which the DTDP was added before starting turnover. In the former case, it is essential to correct for a slow, time-dependent rise in absorbance at 324 that likely arises from ascorbic acid-dependent reduction of DTDP. Alternatively, TPAA and thiophenol concentrations were determined by HPLC using a RP-18 LiChrosorb column (E. M. Separations) with a mobile phase of phosphoric acid (0.1% in water) and methanol in varying proportions, with detection at 250 nm. TfdA activity was also measured by release of $^{14}CO_2$ from α-[1-^{14}C]KG (specific activity of 6000 cpm/nmol, concentration of 1 mM). One milliliter reactions in sealed vials were terminated by addition of 0.25 mL of 50% trichloroacetic acid, and the released CO_2 was trapped in plastic wells (Kontes) with 0.2 mL 1 N NaOH and counted (Beckman LS 7500). These data were corrected for 70% recovery of counts.

In selected studies, TfdA assay reactions were terminated with an equal volume of stop solution [metaphosphoric acid (10%, w/v), thiourea (1 mM), and EDTA (1 mM)] and analyzed by HPLC for remaining ascorbic acid concentrations. Samples were chromatographed on an RP-18 column (E. M. Separations or Waters Delta Pak) with a mobile phase of 50 mM potassium phosphate buffer (pH 3), and the eluent was monitored by electrochemical (Model 5200 Coulochem II, ESA Inc.) or 266 nm detection (Series 1050, Hewlett-Packard).

To assess the effect of anaerobic conditions on enzyme stability, incubation mixes (1 mL in serum bottles with 9.7 mL capacity) were prepared in an anaerobic chamber and sealed with silicon/PTFE septa and aluminum crimp caps (Sun International Trading). To some of the samples, 3 mL of oxygen was added by syringe, and the bottles were shaken 15 min to equilibrate. TfdA (160 μg, EDTA removed) was added by syringe, and aliquots were removed at various times for enzyme assay using 2,4-D. TfdA can adsorb to the septa; thus, the bottles were not inverted while removing the TfdA samples.

To assess the effect of superoxide dismutase and catalase, various amount of these enzymes were added to TPAA turnover mixes (prior to TfdA addition). Superoxide dismutase activity was measured by inhibition of hematoxylin autoxidation (*10*). Catalase activity was determined by following the disappearance of hydrogen peroxide at 240 nm, as described (*11*), except that imidazole buffer (pH 6.75, 10 mM) was used rather than phosphate buffer for the reaction.

Synthesis of Dehydroascorbic Acid. Dehydroascorbic acid was prepared by oxidation of 20 mM ascorbic acid in water with bromine, followed by purging with nitrogen gas (*12*). The concentration of dehydroascorbic acid was verified by reaction of an aliquot (~0.1 mM final concentration) with dithiothreitol (10 mM) in phosphate buffer (pH 7, 25 mM) and observing the absorbance at 266 nm, which arises from production of ascorbic acid.

Synthesis of Phenylsulfinylacetic Acid and Phenylsulfonylacetic Acid. Phenylsulfinylacetic acid was synthesized by oxidation of TPAA with a slight molar excess of aqueous sodium metaperiodate at 0 °C and recrystallized from ethyl acetate (*13*). Phenylsulfonylacetic acid was synthesized by oxidation of TPAA with hydrogen peroxide at 50 °C (*14*). Product identities were confirmed by ^{1}H NMR in $CDCl_3$. TPAA: δ 3.7 (s, 2H); δ 7.2−7.5 (m, 5H); δ 11.3 (s, 1H). Phenylsulfinylacetic acid: δ 3.8 (s, 2H); δ 7.4−7.7 (m, 5H); δ 8.5 (s, 1H). Phenylsulfonylacetic acid: δ 3.8 (d, 1 H) δ 4.0 (d, 1 H) δ 7.5−7.7 (m, 5 H), δ 13.2 (broad, s, 1 H)

Analysis for Proteolysis. TfdA (150 μg) was incubated at 30 °C for 18 min with TPAA (10 mM) in assay mix containing 400 μM ascorbic acid. Aliquots (0.6 μg of protein) were compared to samples incubated at 4 or 30 °C with imidazole buffer (10 mM) by analysis on a 12% denaturing SDS−polyacrylamide gel (*15*).

Numerical Computations. Michaelis constants were calculated by the method of Cornish-Bowden (*16*) or by nonlinear regression using KaleidaGraph (Synergy Software), using values based on initial rates (V_o). For enzyme samples that lost activity over time, the rate of product formation with TPAA as a substrate appeared to be consistent with a first-order rate inactivation process, as shown in eq 1, where k_{app} is the apparent rate constant of inactivation, P_t is the product at time t, and V_t is the rate of product formation at time t.

$$dP_t/dt = V_t = V_o\, e^{-k_{app}t} \quad (1)$$

Integration of this equation, given the boundary condition that there is no product present at the start of the reactions, yields eq 2:

$$P_t = V_o\,(1 - e^{-k_{app}t})\, k_{app}^{-1} \quad (2)$$

Progress curves of thiophenol production versus time were fitted to eq 2 by nonlinear regression using KaleidaGraph.

RESULTS

Demonstration That TPAA Is A Substrate for TfdA. Using standard assay conditions that were optimized for 2,4-D degradation (*4*), TfdA was shown by HPLC and spectrophotometric methods to form thiophenol from TPAA. As illustrated in Figure 1, however, the concentrations of thiophenol generated from various initial concentrations of

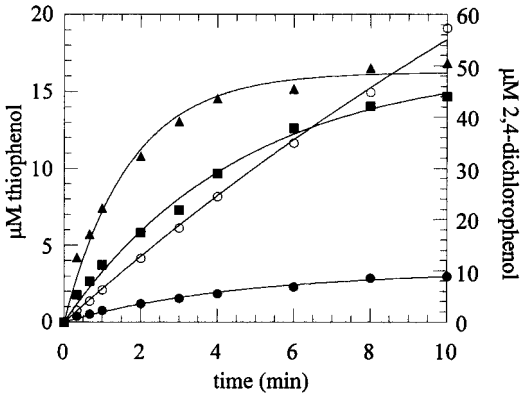


FIGURE 1: TfdA-catalyzed production of thiophenol from TPAA. Thiophenol production from TPAA by TfdA (21 μg/mL) was examined in the presence of 0.1 (●), 1 (■), or 10 mM (▲) substrate using standard assay buffer conditions as described in the text. The reactions were stopped at the indicated times with EDTA, and the amounts of product thiophenol were determined after reaction with DTDP. The data were fit to eq 2 as described in the Experimental Procedures and gave apparent inactivation rate constants of 0.22 ± 0.02 min^{-1}, 0.22 ± 0.02 min^{-1}, and 0.59 ± 0.02 min^{-1}, respectively. For comparison, 2,4-dichlorophenol production from TfdA (0.42 μg/mL) was examined in the presence of 0.3 mM 2,4-D (○). The reactions were stopped at the indicated times with EDTA, and the levels of product 2,4-dichlorophenol were determined after reaction with 4-aminoantipyrene. Each point represents the average of three determinations.

TPAA leveled off by approximately 10 min and accounted for, at most, 3% of the added substrate. The decreases in rates of thiophenol formation as a function of time could be fit by assuming a first-order inactivation process, with inactivation rate constants of 0.22 min^{-1} for samples containing 0.1 or 1 mM TPAA and 0.59 min^{-1} for samples containing 10 mM TPAA (however, see further discussion below). These results contrast with the linear production of 2,4-dichlorophenol from 2,4-D over this time span.

The inactivation observed during thiophenol production was not due to product inhibition. For example, no significant inhibition of TPAA decomposition was observed when the reaction was examined in the presence of 30 μM thiophenol. Furthermore, the possibility was discounted that alternate oxidation products, such as the phenylsulfinylacetic acid or phenylsulfonylacetic acid, were formed and subsequently acted as inhibitors. In particular, alternative reaction products with absorbance at 250 nm were not detected during HPLC analyses, and authentic samples of the sulfur-oxidized compounds failed to act as effective inhibitors of the reaction (data not shown).

Thiophenol Production from TPAA Requires and Consumes Ascorbic Acid. Since the linearity of 2,4-D degradation by TfdA previously was shown to be somewhat dependent on the presence of ascorbic acid (*4*), the ascorbic acid dependency of TPAA utilization was examined. As illustrated in Figure 2, TfdA-catalyzed formation of thiophenol from TPAA exhibited an absolute dependence on the presence of ascorbic acid. This result contrasts with conversion of 2,4-D to 2,4-dichlorophenol where ascorbic acid is not required for activity. Thiophenol formation by TfdA exhibited an optimal ascorbate concentration of ~200 μM, well above the 50 μM concentration used in the routine assay for 2,4-D. For all ascorbic acid concentrations, however, the activity decayed in a pseudo-first-order process (Figure

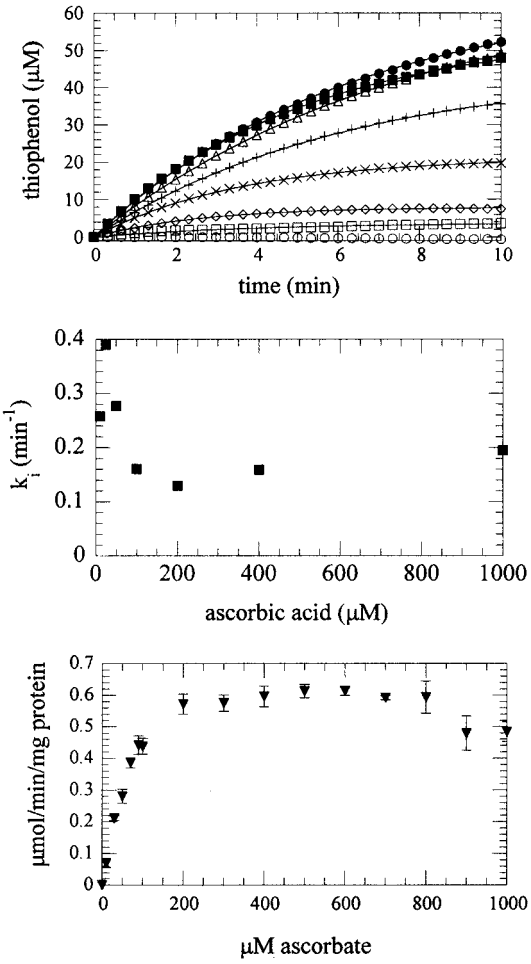


FIGURE 2: Effect of ascorbic acid concentration on the production of thiophenol from TPAA by TfdA. (A) Progress curves for thiophenol production for varied ascorbic acid levels. TfdA (36 μg/mL) was incubated in the presence of 10 mM TPAA and assay buffer containing ascorbic acid at 0 (○), 10 (□), 25 (◇), 50 (×), 100 (+), 200 (△), 400 (●) and 1000 μM (■) concentrations. Thiophenol production was monitored continuously at 324 nm and fit to eq 2. (B) Effect of ascorbic acid concentration on the apparent inactivation rates of TfdA during turnover of TPAA. (C) Effect of ascorbic acid concentration on the initial rate of thiophenol production from TPAA (10 mM) by TfdA (20 μg mL^{-1}). The data represent the average of two determinations.

2B). At ascorbate concentrations below ~100 μM, the apparent inactivation rate was elevated and variable, while higher concentrations yielded more uniform values of approximately 0.16 min^{-1}. Initial rates of thiophenol production appeared to reach a plateau between 200 and 800 μM ascorbic acid, with half-maximal rates observed at about 50 μM levels (Figure 2C).

HPLC methods were used to demonstrate that ascorbic acid was consumed during the TfdA-catalyzed reaction with TPAA. For example, comparison of ascorbic acid consumption with thiophenol production at selected timepoints during reactions using a wide range of reductant concentrations indicated that 1.55 ± 0.09 mol of ascorbic acid was used per mole of product generated (Figure 3). Under TPAA turnover conditions [i.e., the presence of enzyme, Fe(II), α-KG, and this aromatic substrate], over 60% of the reductant (initially 200 μM) was lost during a 10 min time interval (Table 1). For comparison, a decrease of only about 10% of the ascorbic acid was observed under conditions in which

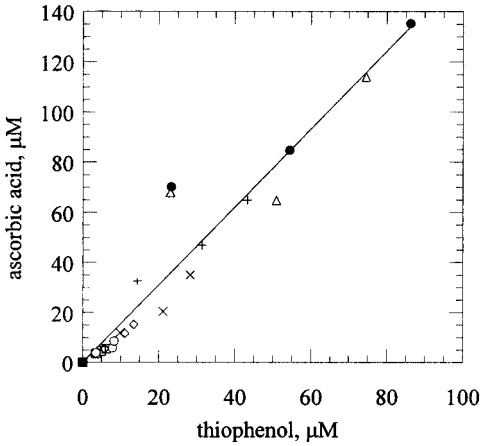


FIGURE 3: Stoichiometry of thiophenol production and ascorbate consumption during TPAA turnover. TfdA (40 μg/mL) was incubated in 10 mM TPAA assay mix with 10 (□), 15 (○), 25 (◇), 50 (×), 100 (+), 200 (△), or 400 (●) μM ascorbic acid, and the reactions were monitored continuously at 324 nm. Replicate aliquots were stopped at various times and the levels of ascorbate consumed were calculated based on the concentrations of ascorbic acid remaining as assayed by HPLC. The Fe(II) concentration was lowered to 10 μM to reduce background consumption of ascorbic acid, and the data were corrected to account for nonenzymatic losses.

Table 1: Consumption of Ascorbic Acid by TfdA

reaction components[a]	ascorbic acid consumption[b] μM	%
blank	−16 ± 25	−8
Fe	63 ± 8	32
Fe + α-KG	57 ± 15	28
Fe + TPAA	60 ± 14	30
Fe + α-KG + TPAA (turnover)	127 ± 10	64
Fe + α-KG + 2,4-D (turnover)	21 ± 16	10

[a] All mixes had imidazole (10 mM, pH 6.75) and 200 μM ascorbic acid. When present, the other concentrations were Fe(II), 50 μM; TPAA, 10 mM; α-KG, 1 mM; 2,4-D, 1 mM, TfdA 36 μg/mL. [b] The levels of ascorbic acid remaining after 10 min reaction were measured by HPLC. The differences from the initial concentration were calculated and corrected for the losses in the absence of TfdA, which averaged 7 μM. The values shown represent mean ± standard deviation for triplicate samples.

2,4-D was used as a substrate. The extent of ascorbic acid loss during 2,4-D turnover was less than that observed when the enzyme was incubated in the presence of Fe(II) alone, Fe(II) plus α-KG, or Fe(II) plus TPAA (~30% consumption in each case).

In light of the demonstration that ascorbate is consumed during the reaction, portions of the data shown in Figures 1 and 2 may be reinterpreted. The apparent inactivation rates calculated for the 10 mM TPAA sample in Figure 1 and the <100 μM ascorbate data in Figure 2B may represent a summation of the actual inactivation rate (0.12−0.22 min^{-1}) and activity loss due to ascorbate depletion. Restated, it was reasonable to suspect that low concentrations of ascorbic acid may limit the extent of the reaction. Evidence supporting this conjecture was derived from continuous spectrophotometric assay supplementation studies. Using a variation of the experiment shown in Figure 2A, TfdA was reacted with TPAA in the presence of Fe(II), α-KG, and buffer containing 50 or 400 μM ascorbic acid, and at selected timepoints the mixtures were supplemented with 400 μM additional ascorbic

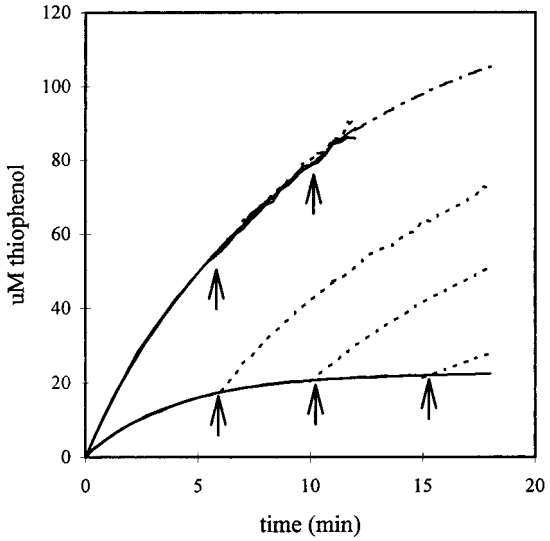


FIGURE 4: Effect of supplemental ascorbic acid on progress curves for the TfdA-catalyzed decomposition of TPAA. TfdA (40 μg/mL) was reacted with 10 mM TPAA in the presence of 50 μM (lower curves) or 400 μM (upper curves) ascorbic acid initially, and at the times indicated by the arrows, the reaction mixtures were supplemented with an additional 400 μM ascorbic acid (dashed lines). The reactions were monitored continuously at 324 nm. Three independent time courses were averaged for each experiment and normalized to their rate at 5 min. The curve for 400 μM ascorbic acid was extrapolated from 13 to 18 min (dot−dash line) because the absorbance readings were above the linear range.

acid (Figure 4). The supplementation led to a burst of product formation at each time point in samples that originally contained 50 μM ascorbic acid; however, no effect was noted for samples that initially possessed 400 μM reductant. The rates observed immediately after adding supplemental ascorbate to the 50 μM samples appeared to parallel the rates at that timepoint for sample to which 400 μM reductant was added at the start of the reaction. Further studies were performed to distinguish whether the burst of activity upon supplementation was due to reactivation of TfdA or to some effect of ascorbate that was specific to TPAA turnover; i.e., the effect of ascorbic acid supplementation was examined using the substrate 2,4-D. Aliquots of enzyme from the TPAA turnover mix with 50 μM ascorbic acid were assayed for their activity toward 2,4-D mix immediately prior to or after supplementation of the TPAA mix by 400 μM ascorbic acid. The observed ~3-fold enhancement in activity toward 2,4-D upon adding supplemental ascorbic acid (data not shown) was consistent with the presence of an ascorbate-reversible inactivation process.

Because ascorbic acid was consumed in the TfdA-catalyzed decomposition of TPAA, it was important to test whether the ascorbate-derived product had an effect on this reaction. Ascorbic acid is known to be oxidized to ascorbyl radical, which disproportionates to ascorbate and dehydroascorbic acid (*17*). On the basis of its ability to react with thiols such as glutathione (*18*), dehydroascorbic acid could reasonably react with thiophenol to interfere with the spectrophotometric assay. This concern was negated by the demonstration that addition of authentic dehydroascorbic acid in concentrations up to 400 μM did not affect the thiophenol standard curve at the pH of the assay. Another potential complication from dehydroascorbic acid is that it might react with arginine residues in the protein, since α,β-diketones such as glyoxal and 2,3-butanediones are known to form a complex with this amino acid residue (*19*). Interactions between dehydroascorbic acid and TfdA do not appear to be a major source of inactivation, since dehydroascorbic acid (400 μM) was only slightly inhibitory to TfdA turnover of TPAA (7 ± 1% rate decrease).

α-KG Decomposition Is Not Uncoupled from Substrate Hydroxylation. The demonstration that ascorbic acid was consumed during the TfdA-catalyzed hydroxylation of TPAA raised the possibility that α-KG decomposition may be partially uncoupled from substrate hydroxylation. Such a situation is known to occur for several other α-KG-dependent dioxygenases, especially when using poor substrates. For example, in addition to carrying out its normal reaction prolyl hydroxylase catalyzes the oxidative conversion of α-KG to CO_2 plus succinate in the absence of substrate hydroxylation (*20*−*24*). This uncoupled reaction in prolyl hydroxylase leaves the enzyme metallocenter in an oxidized state that requires the presence of ascorbic acid to restore the catalytically active species. The consumption of ascorbic acid in the reaction of TfdA with TPAA could plausibly arise from a similar uncoupling process.

To assess the extent of uncoupling of α-KG decomposition from substrate hydroxylation in the TfdA-catalyzed reaction with TPAA, the levels of CO_2 produced or α-KG consumed were compared to the amount of thiophenol generated. Using ^{14}C-labeled cofactor in the assay and monitoring the formation of both [^{14}C]CO_2 and thiophenol, the ratio of CO_2 released to thiophenol produced by TfdA (54 μg/mL) was shown to be 1.1 ± 0.3 for a range of ascorbic acid concentrations (10−400 μM). To compare product formation with α-KG consumption, reactions were run with limiting concentrations of α-KG and plentiful ascorbic acid (400 μM) while measuring the total amount of thiol released; a ratio of 1.07 ± 0.03 mol of α-KG consumed per mole of thiophenol produced was established. These data are inconsistent with the presence of extensive uncoupling of α-KG decomposition from TPAA hydroxylation.

Oxidative Inactivation of TfdA. The nonlinear reaction kinetics observed when using TPAA led us to carry out a detailed analysis of TfdA inactivation rates under a variety of conditions. When the enzyme was incubated in assay buffer (including 400 μM ascorbate) that lacked TPAA, the enzyme was found to lose activity with pseudo-first-order kinetics at a rate of 0.50 ± 0.03 min^{-1} (determined by measuring initial rates of thiophenol production upon addition of TPAA to the incubation mixtures at varying times). Analysis of the rate of inactivation for another aliquot of the same TfdA sample incubated in the same buffer conditions, but with 10 mM TPAA included at the start of the reaction (i.e., during turnover conditions), yielded a value of 0.42 ± 0.03 min^{-1}. (The exact inactivation rates were found to depend on the TfdA preparation, with values for other protein samples ranging 0.2−0.4 min^{-1} during TPAA turnover.) Thus, TPAA offered little to no protection from enzyme inactivation, and increasing the TPAA concentration to as high as 32 mM did not offer improved protection from inactivation. By contrast, inclusion of 1 mM 2,4-D in the buffer gave rise to negligible rates of enzyme inactivation (Figure 1; $k_i = 0.04 \pm 0.01$ min^{-1} when the 2,4-D data were fit to eq 2). These data provide evidence for the presence of an apparently irreversible enzyme inactivation process for

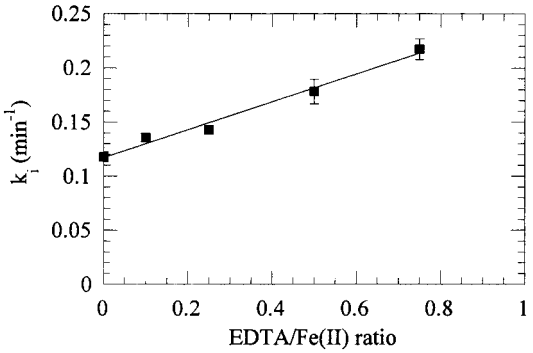


FIGURE 5: Effect of the EDTA to Fe(II) ratio on the TfdA inactivation rate. TfdA (20 μg/mL) was reacted with 10 mM TPAA in assay buffer containing 400 μM ascorbic acid and various concentrations of EDTA and Fe(II). The reactions were monitored continuously at 324 nm, and the enzyme inactivation rates were calculated using eq 2. At EDTA/Fe(II) ratios greater than 1, the enzyme was inactive. The data represent studies using Fe(II) concentrations ranging from 10 to 300 μM.

incubation mixtures containing enzyme or enzyme plus TPAA, but not for enzyme plus 2,4-D. Further results demonstrated that oxygen was involved in the inactivation process. Specifically, TfdA inactivation rates were compared for samples that were incubated in anaerobic and aerobic reaction mixes and assayed for activity toward 2,4-D. After a 20 min incubation period, oxygenated samples containing and lacking TPAA possessed only 4 and 21%, respectively, of the activity of the corresponding samples incubated anaerobically.

The oxidative inactivation process was more fully characterized to examine the identity of the oxygen species that was responsible. Inclusion of catalase at 200 μg mL^{-1} in the assay (capable of removing 90 μmol H_2O_2/min) failed to protect the enzyme against inactivation during TPAA turnover (data not shown). These results suggested that free hydrogen peroxide was unlikely to be the agent responsible for inactivation, although addition of 30 mM H_2O_2 to the assay mixture abolished TfdA activity. Inclusion of superoxide dismutase (100 or 370 units/mL) also failed to protect the enzyme against inactivation. An alternative active oxygen species that could play a role in TfdA inactivation was the hydroxyl radical. Addition of hydroxyl radical scavengers (DMSO, formate, glucose, mannitol, thiourea; 0.1−10 mM) failed to abolish or significantly diminish the inactivation process (data not shown). The results preclude the involvement of free hydroxyl radicals, but do not rule out the possibility of a protein-associated species.

Because hydroxyl radicals are known to be formed from Fe−EDTA complexes in the presence of ascorbic acid (*25*), we tested whether the EDTA concentration in the assay mixture had an effect on the inactivation rate. Enzyme samples were rigorously purified of the trace levels of EDTA present from enzyme storage, and the inactivation rates were determined for varied concentrations of Fe(II) and EDTA (Figure 5). The rates of inactivation increased from a value of 0.1 min^{-1} in the absence of EDTA to double this value as the EDTA concentration approached that of Fe(II). When the EDTA concentration exceeded the concentration of Fe(II), no activity was detected due to the complete sequestration of the metal ion. The enzyme was stable under high EDTA conditions, as shown by restoration of activity after removal of EDTA. These results are consistent with the participation of hydroxyl radicals during EDTA-dependent inactivation, perhaps involving a TfdA·Fe(II)·EDTA complex.

Table 2: Kinetics Values for TfdA Substrates[a]

substrate	K_m (mM)	k_{cat} (min^{-1})
TPAA	2.4 ± 0.2	29 ± 1
4-chlorothiophenoxyacetic acid	1.1 ± 0.3	45 ± 2
phenoxyacetic acid	0.74 ± 0.10	647 ± 42
4-chlorophenoxyacetic acid[b]	0.117 ± 0.006	595 ± 15
2,4-D	0.030 ± 0.003	587 ± 23

[a] Ascorbate concentrations were 400 μM for thioether substrates and 50 μM for ether substrates. Values for the thioether substrates were based on initial rates that were calculated using eq 2. [b] Values were previously reported (*4*).

Protein fragmentation has been associated with oxidative inactivation of other enzymes such as aminocyclopropane carboxylate oxidase (*26*); thus, the stability of TfdA during inactivation was assessed. Following 18 min of incubation under conditions of TPAA turnover, or control experiments, TfdA samples were examined for fragmentation by denaturing polyacrylamide gel electrophoresis. The turnover conditions were conservatively estimated to lead to at least 80% loss of activity toward TPAA; however, no significant decreases in the band on the gel for TfdA and no significant increases in other bands were observed (data not shown).

Kinetic Constants for TfdA Acting on Thiophenoxyacetic Acids and Phenoxyacetic Acids. Using 400 μM ascorbic acid in the reaction assays and examining only the initial rates of product formation, kinetic constants were assessed for TPAA, 4-chlorothiophenoxyacetic acid, 2,4-D, 4-chlorophenoxyacetic acid, and phenoxyacetic acid (Table 2; 2,4-dichlorothiophenoxyacetic acid is not commercially available and was not tested). The K_M values for the thioether compounds were 3- and 9-fold greater than those of the corresponding oxy-ether forms. In both sets of compounds, TfdA exhibited greater affinity for the more highly chlorinated species. Substitution of the ether oxygen in the phenoxyacetates by a sulfur atom reduced the k_{cat} by 13- and 22-fold relative to the corresponding oxy-ether forms. Thus, the catalytic efficiency of TfdA toward the thiophenoxyacetic acids was greatly reduced (700−2400-fold) compared to the benchmark substrate, 2,4-D. Both thiophenoxyacetic acids tested exhibited the rapid inactivation reaction as detailed for TPAA, while the three phenoxyacetic acids resulted in linear kinetics at the concentrations and over the time courses examined.

DISCUSSION

TPAA-Based Assay for TfdA. TPAA is a substrate for TfdA and is converted to thiophenol as the sole aromatic product. The release of this thiol allows for use of TPAA in a qualitative, short-term continuous assay for the enzyme. We have chosen to utilize DTDP to monitor released thiol in this assay because the released thiopyridine chromophore exhibits a pH-independent spectrum near pH 7 (*27*); however, dithiodinitrobenzoate (Ellman's reagent) or other thiol-specific reagents can also be used. It is important to include high concentrations of ascorbic acid in the assay, and the assay is not suitable for quantitative measurements because of the observed rapid inactivation of the enzyme.

Model To Explain Ascorbic Acid Dependency and Enzyme Inactivation during TPAA Utilization. Results from studies

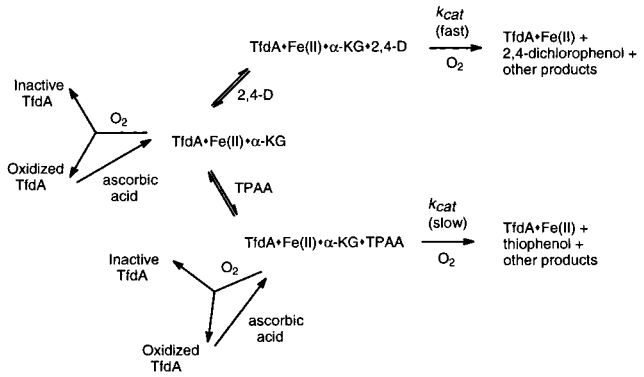


FIGURE 6: Model of TfdA reactivity with 2,4-D and TPAA. The active form of the enzyme is shown as the species with bound Fe(II) and α-KG. Spectroscopic studies have provided evidence consistent with the existence of this species (*6*). This species is suggested to be inactivated in the presence of oxygen via a mechanism that forms an irreversibly inactive species and an ascorbic acid-reversible form of the protein. TfdA binds 2,4-D with high affinity and rapidly catalyzes the decomposition of the substrate. The TfdA·Fe(II)·α-KG·2,4-D species is proposed to be protected from the oxidative inactivation processes. TfdA binds TPAA with lower affinity and catalyzes its decomposition at a reduced rate compared to 2,4-D. The TfdA·Fe(II)·α-KG·TPAA state of the enzyme is capable of undergoing both types of oxidative inactivation reactions.

to characterize the dependence of ascorbic acid on TPAA degradation and to elucidate the features associated with TfdA inactivation have been integrated into the model illustrated in Figure 6. Significantly, this model is distinct from that involving an uncoupling of α-KG decomposition from substrate hydroxylation, which has been used to explain the requirement for ascorbic acid in several other α-KG-dependent dioxygenases (e.g., *20*, *28*). In TfdA, oxidative decarboxylation of α-KG is tightly coupled to hydroxylation of substrate. Our model suggests that selected states of TfdA can undergo two distinct oxidative inactivation processes, one of which is reversed by ascorbic acid.

In the absence of any aromatic substrate, the TfdA·Fe(II)·α-KG complex is proposed to react with oxygen resulting in one inactive species that is reversible by ascorbic acid and another species that is irreversibly inactivated. In the presence of 1 mM 2,4-D (a substrate possessing a very low K_M), the enzyme is primarily in the form of the TfdA·Fe(II)·α-KG·2,4-D complex, which is stable against the oxidative inactivation reactions, and is rapidly and efficiently converted to products. Ascorbic acid consumption is greatly reduced in the presence of 2,4-D, compared to the enzyme-lacking substrate. By contrast, the rates of ascorbic acid consumption in the presence of TPAA, a poor substrate with a high K_m and low k_{cat} relative to 2,4-D, are twice that of the substrate-free enzyme, suggesting that the TfdA·Fe(II)·α-KG·TPAA complex may be especially susceptible to oxidation. As for substrate-free enzymes, the oxidative reaction partitions between an irreversible state and an inactive state that can be reversed by ascorbate. In the absence of ascorbic acid and the presence of TPAA, the enzyme should be competent to catalyze very limited substrate hydroxylation, but it rapidly undergoes both inactivation processes. The net effect is that TfdA catalyzes very few turnovers before it is completely inactivated. Given the low concentration of enzyme in the assay mixtures, the level of product generated before losing all activity is too low to be detected. A portion of the inactive TfdA can be restored to activity by subsequent addition of ascorbate.

Several aspects of this model are consistent with known iron chemistry. For example, the ascorbate-reversible inactive state(s) of the enzyme may represent Fe(III) species arising from one-electron transfer to oxygen; Fe(III) is readily reduced to Fe(II) by ascorbic acid (*29*). The irreversibly inactivated enzyme species may arise from reaction of the protein with hydroxyl radicals (*30*) generated via the Fenton reaction:

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(III)} + \mathrm{HO^-} + \mathrm{HO^\bullet} \qquad (3)$$

Hydrogen peroxide may be formed in the TfdA assay mix by the autoxidation of ascorbic acid (*31*) or by the dismutation of superoxide formed by reduction of molecular oxygen by Fe(II) (*32*). We note, however, that catalase, superoxide dismutase, or hydroxyl radical scavengers failed to protect the enzyme from inactivation, indicating the absence of free H_2O_2, $O_2^{\bullet-}$, and $HO^\bullet$. Rather, the inactivating species is likely to be protein associated.

The presence of EDTA is known to enhance the production of hydroxyl radicals or species with similar chemical and kinetic properties by Fenton-type reactions (reviewed in ref *32*). Consistent with this, increasing the EDTA/Fe(II) ratio present in the assay (up to a 1:1 ratio) roughly doubled the rate of enzyme inactivation (Figure 5), possibly due to formation of a TfdA·Fe(II)·EDTA complex. In a typical TPAA assay, the amount of EDTA present from enzyme storage corresponded to a ratio of 0.2 EDTA/Fe, so by comparison with Figure 5, less than 20% of the inactivation observed in the typical assays is an artifact due to the presence of EDTA. The postulated protein damage arising from hydroxyl radicals does not appear to give rise to significant protein fragmentation.

What Accounts for the Differences in TfdA Reactivity with 2,4-D and TPAA? Substitution of the ether oxygen of phenoxyacetates with a thioether atom found in the thiophenoxyacetates results in significant changes in TfdA reactivity toward these compounds (Table 2). The enzyme exhibits 3-fold higher affinity for phenoxyacetic acid over TPAA and nearly 10-fold greater affinity for 4-chlorophenoxyacetic acid over 4-chlorothiophenoxyacetic acid, demonstrating that the heteroatom identity influences binding of the substrate. Additionally, the presence of a thioether adjacent to the methylene C−H bond undergoing oxidation reduces the catalytic rate in the range of 13−22-fold. The decreased rate is unlikely to arise from changes in the C−H bond strength. For example, the C−H bond strengths in the model compounds methanol and thiomethane are within a few percent of each other (401.9 ± 0.6 and 393 ± 8 kJ mol^{-1}, respectively; *33*). Rather, the rate effects are likely to arise from subtle changes due to sulfur being larger and more polarizable than oxygen. For example, the precise docking of substrate to enzyme will be affected by the heteroatom identity (especially if this heteroatom interacts with the enzyme metallocenter as speculated earlier; *4*), thus affecting the position and geometry of the C−H bond undergoing oxidative chemistry relative to the metal-bound oxygen atom that is inserted. A positional shift such as this may also explain the high reactivity of the TfdA·Fe(II)·α-KG·TPAA complex toward the oxidative inactivation reactions described

above. Alternative explanations for the reduced reactivity of TfdA for thiophenoxyacetic acids versus phenoxyacetic acids could include stabilization of an intermediate carbon-centered radical by the adjacent sulfur atom or influences on oxidative addition by carbon-heteroatom bond polarization.

The studies reported here may provide useful insights for catalytic mechanisms and inactivation reactions of other α-ketoglutarate-dependent dioxygenases. Ascorbic acid requirement for turnover, especially turnover of poor substrates, should not automatically be attributed to uncoupling of the reaction. It is ironic that the very things that assist in TfdA turnover, iron, oxygen, and ascorbic acid, can also contribute to the generation of reactive oxygen species that lead to enzyme inactivation.

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