J|A|C|S
ARTICLES
Published on Web 08/13/2005

Reaction Coordinate Analysis for β-Diketone Cleavage by the Non-Heme Fe^{2+}-Dependent Dioxygenase Dke1

Grit D. Straganz* and Bernd Nidetzky*

Contribution from the Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria

Received December 21, 2004; Revised Manuscript Received July 15, 2005; E-mail: grit.straganz@TUGraz.at; bernd.nidetzky@TUGraz.at

Abstract: Acetylacetone dioxygenase from *Acinetobacter johnsonii* (Dke1) utilizes a non-heme Fe^{2+} cofactor to promote dioxygen-dependent conversion of 2,4-pentanedione (PD) into methylglyoxal and acetate. An oxidative carbon−carbon bond cleavage by Dke1 is triggered from a C-3 peroxidate intermediate that performs an intramolecular nucleophilic attack on the adjacent carbonyl group. But how does Dke1 bring about the initial reduction of dioxygen? To answer this question, we report here a reaction coordinate analysis for the part of the Dke1 catalytic cycle that involves O_2 chemistry. A weak visible absorption band ($\epsilon \approx 0.2$ mM^{-1} cm^{-1}) that is characteristic of an enzyme-bound Fe^{2+}−β-keto−enolate complex served as spectroscopic probe of substrate binding and internal catalytic steps. Transient and steady-state kinetic studies reveal that O_2-dependent conversion of the chromogenic binary complex is rate-limiting for the overall reaction. Linear free-energy relationship analysis, in which apparent turnover numbers (k_{cat}^{app}) for enzymatic bond cleavage of a series of substituted β-dicarbonyl substrates were correlated with calculated energies for the highest occupied molecular orbitals of the corresponding β-keto−enolate structures, demonstrates unambiguously that k_{cat}^{app} is governed by the electron-donating ability of the substrate. The case of 2′-hydroxyacetophenone (2′HAP), a completely inactive β-dicarbonyl analogue that has the enol double bond delocalized into the aromatic ring, indicates that dioxygen reduction and C−O bond formation cannot be decoupled and therefore take place in one single kinetic step.

Introduction

Acetylacetone dioxygenase Dke1 from *Acinetobacter johnsonii* catalyzes the O_2-dependent conversion of 2,4-pentanedione (PD) into methylglyoxal and acetate.[1] Oxidative carbon−carbon bond cleavage in the β-dicarbonyl substrate proceeds with stoichiometric consumption of O_2. Results of ^{18}O labeling experiments support classification of Dke1 as a novel dioxygenase (EC 1.13.11.50), showing that one atom of ^{18}O is incorporated into each reaction product upon the enzymatic bond fission.[1a] Dke1 is a functional homotetramer composed of subunits of 153 amino acids with a molecular mass of 16 607 Da. It belongs to a group of mononuclear non-heme metal-dependent enzymes (NHMEs) that elegantly couples dioxygen chemistry with a wide range of substrate transformations, including C−C bond cleavage, decarboxylation, hydroxylation, and carbon−heteroatom bond formation.[2] Fe^{2+} appears to be central to the enzymatic mechanism because the turnover number of Dke1 is strictly correlated with the mole equivalent of bound Fe^{2+}. Only Fe^{2+} but not other divalent metal ions such as Zn^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, or Co^{2+} can be employed to reconstitute active Dke1 from the metal free apo-enzyme.[1b] Selective use of Fe^{2+} for catalysis links Dke1 to prominent NHME superfamilies such as the α-keto-acid-dependent dioxygenases and extradiol-catechol ring-cleaving dioxygenases. The all-histidine metal-binding site in Dke1, which is constituted by His-62, His-64, and His-104,[3] however, differs from the active site configuration in these NHMEs that use a facial triad of two histidines and one carboxylate to bind Fe^{2+}.[2e]

Dke1 can accept a range of β-dicarbonyl compounds that represent a wide variety of substituents at both the central C-3 and the methyl groups of the natural PD substrate.[1] The relaxed substrate specificity of Dke1 suggested the possibility of probing the enzymatic mechanism through quantitative structure−reactivity correlations of kinetic substituent effects. Considering possible pathways for enzymatic C−C bond cleavage, we rationalized that variation of the substrate structure could affect different points of the reaction coordinate, arguably leading to changes in the catalytic rate (this work) and the cleavage specificity of the enzyme.[1a] We showed in a recent article that the oxygenative cleavage of asymmetric β-dicarbonyl compounds is controlled by the electronic properties of the substituents and takes place at the carbon adjacent to the most electron-deficient carbonyl group. The linear free-energy relationship

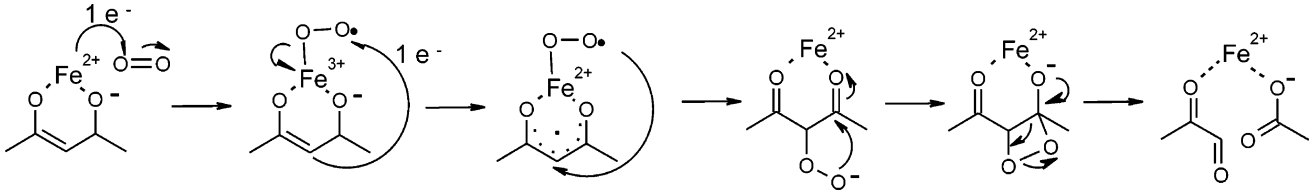
(1) (a) Straganz, G. D.; Hofer, H.; Steiner, W.; Nidetzky, B. *J. Am. Chem. Soc.* **2004**, *126*, 12202−12203. (b) Straganz, G. D.; Glieder, A.; Brecker, L.; Ribbons, D. W.; Steiner, W. *Biochem. J.* **2003**, *369*, 573−581.

(2) For a review, see: (a) Costas, M.; Mehn, M. P.; Jensen, M. P.; Que, L., Jr. *Chem. Rev.* **2004**, *104*, 939−986. (b) Bugg, T. D. *Curr. Opin. Chem. Biol.* **2001**, *5*, 550−555. (c) Solomon, E. I.; Brunold, T. C.; Davis, M. I.; Kemsley, J. N.; Lee, S. K.; Lehnert, N.; Neese, F.; Skulan, A. J.; Yang, Y. S.; Zhou, J. *Chem. Rev.* **2000**, *100*, 235−350. (d) Lange, S. J.; Que, L., Jr. *Curr. Opin. Chem. Biol.* **1998**, *2*, 159−172. (e) Hegg, E. L.; Que, L., Jr. *Eur. J. Biochem.* **1997**, *250*, 625−629. (f) Que, L., Jr.; Ho, R. Y. *Chem. Rev.* **1996**, *96*, 2607−2624.

(3) Stranzl, G. D. Ph.D. Thesis, Karl Franzens University, Graz, Austria, 2002.

10.1021/ja042313q CCC: $30.25

Scheme 1. Proposed Mechanism of Oxygenative C−C Bond Cleavage in PD Catalyzed by Dke1[a]



[a] The steps that involve dioxygen activation and reduction of O_2 are hypothetical and adapted from related non-heme Fe^{2+} dioxygenases.[2] Subsequent reactions triggered from a peroxidate intermediate are supported by results of Straganz et al.[1a]

analysis for the cleavage specificity of Dke1 suggested a mechanism of carbon−carbon bond fission that is summarized in Scheme 1. The reaction of O_2 with the PD substrate is proposed to yield a C-3 peroxidate intermediate that performs intramolecular nucleophilic attack on the juxtaposed carbonyl group to generate a dioxetane that then decomposes into the products. In this article we address the question of how Dke1 promotes the crucial first steps of the catalytic sequence that involve reduction of O_2 and formation of the peroxidate.

Reactions of singlet substrates and triplet dioxygen via concerted two-electron transfer resulting in a singlet product are formally spin-forbidden and therefore do not take place at appreciable rates in the absence of a catalyst that alters the spin state of dioxygen or substrate. The mechanism by which NHMEs turn on their O_2 reactions has thus aroused considerable interest among biochemists.[2,4] In consideration of the wide variety of enzymatic transformations performed on totally unrelated substrate structures and promoted by active sites harnessing different metal ions, catalytic factors and their relative contributions to rate acceleration are hardly uniform among the NHMEs. A classical example is provided by the group of Fe^{3+}-dependent catechol intradiol-cleaving dioxygenases which are thought to activate their substrate (not dioxygen) via the metal cofactor. NHMEs requiring ferrous iron for activity utilize a different catalytic mechanism. A chemically convincing scenario is that activation of dioxygen initiates the enzymatic reaction and takes place through one-electron reduction of O_2 mediated by Fe^{2+}, concomitant with formation of an Fe^{3+}−superoxide intermediate. For the first time, very recently an Fe^{3+}-bound superoxide intermediate could be spectroscopically characterized during the oxygenation reaction of a non-heme diiron(II) complex.[5]

A dioxygenase reaction that is promoted by the oxidation of enzyme-bound Fe^{2+} poses the question of how the enzyme prevents the conversion of Fe^{2+} to Fe^{3+}, uncoupled from the event of substrate transformation. A significant observation reported in several articles is that dioxygen reactivity increases in the substrate-bound enzyme by as much as $\sim 10^7$-fold, compared with the reference reaction of O_2 and the resting Fe^{2+} enzyme in the absence of substrate or ligand. A number of factors, summarized later in this article, have been suggested to contribute to this large reactivity enhancement toward dioxygen upon substrate binding.[2,4] The lack of an observable Fe^{3+} intermediate in NHMEs has called into question a distinctly stepwise conversion of dioxygen to peroxide via superoxide. Because one-electron reduction of dioxygen is thermodynamically unfavorable,[4b,6] it has been suggested that additional electrons from bound ligand could be used to pull the reaction forward, and this plausibly explains the increased reactivity of Fe^{2+} in the presence of substrate. Another possibility is that substrate binding lowers the redox potential of Fe^{2+}, thereby facilitating reduction of dioxygen through enhancement of the stability of Fe^{3+}. It consolidates the finding that inactivation of dihydroxy-2,3-biphenyl dioxygenase as a result of Fe^{2+} oxidation proceeds much more quickly during substrate turnover than in the resting state. Time-dependent formation of Fe^{3+} in this enzyme parallels the evolution of superoxide, suggesting that one-electron reduction of dioxygen by ferrous iron is partially decoupled from the subsequent catalytic cycle.[7] In an alternative scenario, the concept of substrate activation by the metal cofactor as has been reported for Fe^{3+}-dependent dioxygenases[8] and Cu^{2+}-containing quercetin dioxygenase[9] might also assist O_2 reduction in Fe^{2+}-dependent enzymes, as has been suggested for the enzyme mechanism of Fe^{2+}-dependent 2,3-dihydroxybiphenyl dioxygenase based on molecular orbital calculations of the active site.[4b] Finally, a conformational rearrangement of the active site, triggered by the event of substrate binding, may prime the enzymatic reaction in one of several ways. α-Ketoglutarate-dependent dioxygenases change the geometry of the Fe^{2+} cofactor from six-coordinate to five-coordinate upon formation of the ternary complex between enzyme, α-ketoglutarate cosubstrate, and substrate and thus generate an open binding position for dioxygen on the previously protected Fe^{2+}, which will consequently react with O_2.[4a] A more favorable orientation of the molecular orbitals of Fe^{2+} and O_2 might also be induced upon substrate binding, thereby bringing about the reaction.

Here we report a detailed reaction coordinate analysis for the Dke1-catalyzed conversion of PD into the C-3 peroxidate intermediate (Scheme 1) which decomposes into the products. The results provide insights into kinetics and chemistry of dioxygen reduction by Dke1.

Experimental Section

Chemicals and Enzyme. PD, 1,1,1-trifluoro-2,4-pentanedione (TFPD), 4,4-difluoro-1-phenyl-1,3-butanedione (DFPB), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TFPB), 1-phenyl-1,3-butanedione (PBD), and 2′-

(4) (a) Solomon, E. I.; Decker, A.; Lehnert, N. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3589−3594. (b) Davis, M. I.; Wasinger, E. C.; Decker, A.; Pau, M. Y. M.; Vaillancourt, F. H.; Bolin, J. T.; Eltis, L. D.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **2003**, *125*, 11214−11227.
(5) Shan, X.; Que, L., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5340−5345.
(6) (a) Bassan, A.; Borowski, T.; Siegbahn, P. E. M. *J. Chem. Soc., Dalton Trans.* **2004**, *20*, 3153−3162. (b) Sawyer, D. T. In *Oxygen Complexes and Oxygen Activation by Transition Metals*; Martell, A. E., Sawyer, D. T., Eds.; Plenum: New York, 1988; pp 131−148.
(7) Vaillancourt, F. H.; Labbe, G.; Drouin, N. M.; Fortin, P. D.; Eltis, L. D. *J. Biol. Chem.* **2002**, *277*, 2019−2027.
(8) (a) Que, L., Jr.; Lipscomb, J. D.; Munck, E.; Wood, J. M. *Biochim. Biophys. Acta* **1977**, *485*, 60−74. (b) Hamilton, G. A. In *Molecular Mechanisms of Oxygen Actiuation*; Hayaishi, O., Ed.; Academic Press: New York, 1974; pp 443−445.
(9) Steiner, R. A.; Kalk, K. H.; Dijkstra, B. W. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 16625−16630.

hydroxyacetophenone (2′HAP) and all other chemicals were from Sigma Aldrich (St. Louis, MO) except 1,1-difluoro-2,4-pentanedione (DFPD), which was from Matrix Scientific (Columbia, SC). They were all of the highest available quality and unless otherwise mentioned greater than 97% pure. Recombinant Dke1 harboring a C-terminal decapeptide fusion was produced and purified by affinity chromatography as described elsewhere.[10] The tagging has no effect on Dke1 activity and specificity. The molar concentration of purified Dke1 was determined from the absorption (at 280 nm) of the enzyme in the presence of 6 M guanidine HCl, using an extinction coefficient of 2.1 mM^{-1} cm^{-1}, which was calculated from the tagged polypeptide sequence. Protein-bound Fe^{2+} was measured using ferene S as metal chelator. The assay employed 500 μL of an appropriately diluted protein sample that was made anaerobically by repeated cycles of evacuation and nitrogen flushing. The protein solution was mixed with 500 μL of a nitrogen-purged solution of ferene S (40 mM; ~1000-fold molar excess to the concentration of Dke1 subunits) in 20 mM Tris/HCl, pH 7.5, and incubated at 25 °C. The increase in absorbance at 592 nm was monitored over time, typically up to 6 h, until it reached a constant value. Enzyme-bound Fe^{2+} concentrations were calculated from the difference of initial and final absorbance with an extinction coefficient of 35.5 mM^{-1} cm^{-1} for the stable complex between ferene S and Fe^{2+}.[11] The control used 20 mM ferene S in 20 mM Tris/HCl, pH 7.5, in the absence of Dke1.

Initial Rate Studies. Initial rates were recorded by measuring the depletion of substrate or dioxygen. It was proven that both methods yielded consistent results. Substrate conversion was monitored spectrophotometrically with a DU 800 UV−vis spectrophotometer (Beckmann Coulter, Inc., Fullerton, CA) at 25 °C. The wavelength of maximum absorption for the enolate of each β-diketone at pH 7.5 (λ_1) was determined in separate experiments, as described in the Supporting Information, and used to monitor the time course of the enzymatic reaction. Assays were performed in a total volume of 1.5 mL. Tris/HCl buffer, 20 mM pH 7.5, was filled into a sealed quartz cuvette and brought to a defined concentration of dissolved dioxygen (0−1200 μM) by flushing with mixtures of O_2 and N_2. Then Dke1 was added, and the resulting dioxygen concentration (c_{O_2}) was recorded. The enzymatic reactions were started by addition of substrate (3−30 μL) dissolved in the above Tris/HCl buffer using a Hamilton syringe. It will be shown in the Results section that Dke1 could not be saturated with dioxygen at the steady state. We thus define an apparent turnover number (k_{cat}^{app}) that is the mole of substrate cleaved per mole of enzyme-bound iron and second at the respective O_2 concentration. Initial rates of dioxygen consumption and all other c_{O_2} measurements were performed with a micro-optode O_2 sensor (Microtox TX3-AOT, PreSens, Regensburg, Germany) that was introduced directly into the sealed cuvette. The sensor used noninvasive fluorescence quenching as a method of detection.

Spectroscopic Characterization of Dke1-Substrate Complexes. An anaerobic solution of Dke1 (270 μM subunits and 75% Fe^{2+} content, giving 200 μM active sites) in 20 mM Tris buffer, pH 7.5, was prepared in an evacuable quartz cuvette sealed with a septum. Spectroscopic titrations were carried out by adding aliquots of 0.5−5 μL from an anaerobic stock solution of ligand with a Hamilton syringe. Absorbance wavelength scans in the range 300−700 nm were carried out on the free enzyme and after each addition of ligand, and the resulting spectra were compared after appropriate corrections for dilution. Controls were obtained by the same procedure without Dke1, and corrections for blank values at the corresponding ligand concentrations were made in all cases.

Equilibrium Binding Studies. Dissociation constants (K_d) of Dke1−substrate complexes were determined using data from absorbance or fluorescence titration. Among two new absorbance bands that appeared in the spectrum of substrate-bound Dke1 compared with the spectrum of free enzyme (see the Results section), the intensity of the band that was lowest in energy (λ_3) was chosen as reporter of the binding event. Aliquots of ligand solution were added as described above until the band intensity did not increase further. Because binding of most ligands was tight relative to the enzyme concentration used in the experiment, a significant portion of the total concentration of each binding partner is in the binary complex at equilibrium. Therefore, eq 1 was used to fit the data with the program Microcal Origin Pro 6.1 (OriginLab Corporation, Northampton, MA):

$$A_3 = \epsilon_3\{(K_d + c_{Dke1} + L) - [(K_d + c_{Dke1} + L)^2 - 4c_{Dke1}L]^{0.5}\}/2 \quad (1)$$

where A_3 is the absorbance at λ_3 with the corresponding absorption coefficient ϵ_3, c_{Dke1} is the concentration of enzyme active sites, and L is the ligand concentration.

The fluorescence titrations were performed in the same way just described except that c_{Dke1} was 0.2 μM (95% Fe^{2+} occupancy) and the temperature was 4 °C or 25 °C, as indicated. Experiments were performed with a Hitachi F-4500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) using excitation and emission wavelengths of 290 and 330 nm, respectively. Constant slit widths of 5 nm were used. The quenching of the intrinsic Dke1 tryptophan fluorescence was measured as a function of the ligand concentration. After corrections for dilution and blank readings, data were plotted as $(I_0 - I)/(I_0 - I)_{max}$ against L, where I_0 and I are fluorescence intensities in the absence and presence of ligand, respectively. Equation 2 was used to fit the data:

$$I_0 - I = (I_0 - I)_{max}L/(L + K_d) \quad (2)$$

where $(I_0 - I)_{max}$ is the difference between I_0 and I at saturating L.

Gel Filtration Analysis of the Dke1−PD Complex. A HiTrap desalting column (5 mL, Amersham Biosciences, Uppsala, Sweden) was used to fractionate high and low molecular weight complexes of Fe^{2+} and PD. The column was equilibrated with degassed Tris/HCl buffer (20 mM, pH 7.5) containing 5 mM PD which was added to prevent bleaching of the chromogenic Fe^{2+} complex in the presence of residual O_2. The anaerobic sample (0.5 mL) contained 1 mM Dke1 active sites dissolved in the buffer supplemented with PD, and 500 μL of it was applied to the column. It was eluted at a flow rate of 1 mL min^{-1} with automatic collection of 0.5-mL fractions. Color associated with certain fractions was clearly visible and confirmed by recording a UV−vis spectrum. Dke1-containing fractions were identified using the Bio-Rad protein dye binding assay (Bio-Rad, Hercules, CA).

Transient Kinetic Measurements. Stopped-flow measurements were carried out on an Applied Photophysics instrument (model SX.18 MV, Applied Photophysics LTD, Leatherhead, U.K.) equipped with a modular optical system. Data acquisition and analysis were done using Applied Photophysics software. Detection was by absorbance, using a wavelength characteristic of the free substrate enolate or the enzyme−substrate complex (A_3). Solutions of the reactants were prepared in air-saturated 20 mM Tris/HCl buffer, pH 7.5, the c_{O_2} being 260 μM if not otherwise stated. From two separate syringes, 100 μL of each purified recombinant enzyme (580 μM subunits, 75%, Fe^{2+} content) and substrate (400 μM) was shot into a 20-μL flow cell having a 1-cm path length. Alternatively, for slow reactions, a DU 800 UV−vis spectrophotometer was used to monitor transient kinetics under analogous conditions. All experiments were done at 25 °C in triplicate and analyzed, and the resulting k values were averaged. Appropriate controls were recorded in all cases to exclude the possibility of artifacts. The observed rate constants for formation (k_1) and decay (k_2) of the binary complex were obtained from nonlinear fits of the appropriate parts of the experimental absorbance trace at A_3 to eqs 3 and 4, respectively. In eqs 3 and 4, $A_{3,max}$ is the theoretical maximum

(10) Straganz, G. D.; Slavica, A.; Hofer, H.; Mandl, U.; Steiner, W.; Nidetzky, B. *Biocatal. Biotransform.*, in press.
(11) Johnson-Winters, K.; Purpero, V. M.; Kavana, M.; Nelson, T.; Moran, G. R. *Biochemistry* **2003**, *42*, 2072−2080.

Table 1. Steady-State and Transient Kinetic Analysis of the Reaction of Dke1 with a Series of β-Dicarbonyl Compounds and Corresponding Calculated Energies of Their Highest Occupied Molecular Orbital (ϵ_{HOMO}) for the Monoanionic Substrate Structures[a]

substrate	k_{cat}^{app} (s^{-1})	K_m (μM)	ϵ_{HOMO}[b] (eV)	k_1 (s^{-1})	k_2 (s^{-1})
PD	6.5	9.1 ± 1.5[c]	−3.20	225	6.6
DFPD	3.6×10^{-2}	2.7 ± 0.7	−3.78	9.5	4.2×10^{-2}
TFPD	4.9×10^{-3}	2.5 ± 0.5	−4.15	0.73	5.3×10^{-3}
DFPB	4.3×10^{-4} [d]	2.0 ± 1.0[d]	−4.03	20	4.1×10^{-4}
TFPB	n.d.[e]	n.d.[e]	−4.40	n.d.[e]	5.2×10^{-5}
2′HAP	0	−	−3.31	n.d.[e]	0

[a] All experiments were performed at 25 °C and $c_{O_2} = 260$ μM. k_{cat}^{app} and observed rate constants of enzyme−substrate complex formation (k_1) and decay (k_2) are within the experimental error of 10%. [b] The values of ϵ_{HOMO} were obtained with the program Spartan'02 (Wavefunction, Inc., CA) using semiempirical calculation with AM1 basis set. [c] Determined previously.[1b] [d] Assays with DFPB were performed under conditions of single turnover ($c_{Dke1} > c_{DFPB}$). [e] n.d. = not determined.

absorbance value under the conditions used. Because formation and breakdown of the enzyme−substrate complex occur in well-separated time domains ($k_1 > 10k_2$), it was possible to treat binding and reaction as independent processes.

$$A_3(\text{buildup}) = A_{3,\max}[1 - \exp(-k_1 t)] \quad (3)$$

$$A_3(\text{breakdown}) = A_{3,\max} \exp(-k_2 t) \quad (4)$$

Results

A recent study has reported electronic substituent effects on the specificity of carbon−carbon bond cleavage catalyzed by Dke1.[1a] The rate of β-diketone substrate conversion at the steady state, however, shows an even more pronounced substituent dependence, prompting us to perform a detailed kinetic analysis for the reaction of Dke1 with a range of substituted β-dicarbonyl compounds.

Substituent Effects on Steady-State Kinetic Parameters. Initial rates of Dke1-catalyzed cleavage of different β-diketones were recorded under conditions in which the substrate concentration was varied and the c_{O_2} was constant at 260 μM. Spectrophotometric detection of substrate conversion at its characteristic λ_1 was employed (see the Supporting Information). Kinetic parameters were obtained from nonlinear fits of a rectangular hyperbola to the data and are summarized in Table 1.

The apparent turnover number (k_{cat}^{app}) value changed more than 10^5-fold upon alteration of the substituents on the core β-diketone structure of PD. The apparent Michaelis constant K_m, however, showed very little variation between 2 and 9 μM, across the same series of substrate analogues, suggesting that the kinetic substituent effect is almost exclusively on the catalytic rate and not on binding. The catalytic efficiency (k_{cat}^{app}/K_m), which represents the extrapolation to a limiting substrate concentration, therefore shows a similar correlation as k_{cat}^{app}.

We investigated the influence of the O_2 concentration on kinetic parameters for the conversion of PD, DFPD, and TFPD. As shown in Figure 1 (panel B), the value of K_m did not change significantly (±20%) in response to an increase of c_{O_2} from 20 to 1200 μM. k_{cat}^{app} showed a linear dependence on c_{O_2} irrespective of the substrate used (Figure 1, panel A), revealing clearly that Dke1 cannot be saturated with dioxygen at the steady state.

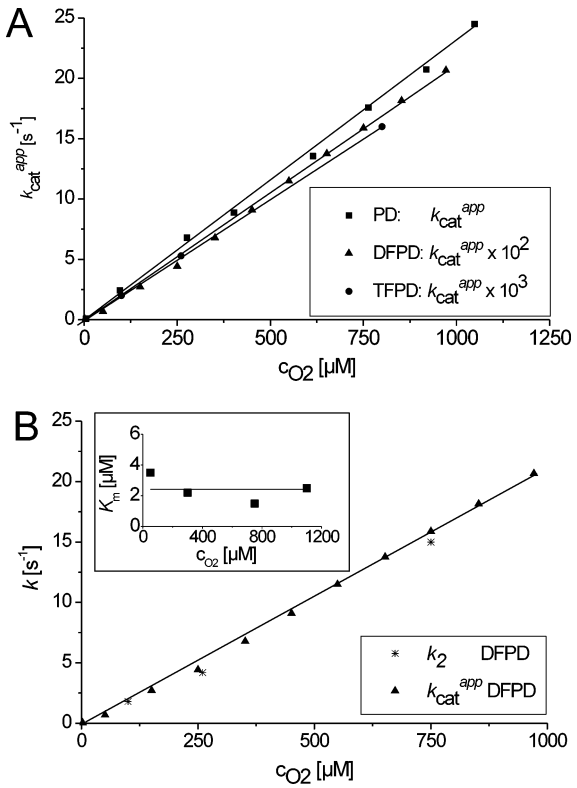


Figure 1. Apparent steady-state kinetic parameters of the Dke1-catalyzed conversion of β-diketones at varying dissolved dioxygen concentrations at 25 °C. (A) Correlation of k_{cat}^{app} and c_{O_2} for PD, DFPD, and TFPD. (B) Comparison of the dependences of k_{cat}^{app} and the transient reaction rate constant k_2 (see later) on c_{O_2} for the conversion of DFPD. The inset shows K_m values for DFPD determined at different levels of c_{O_2}.

Estimates of k_{cat}^{app}/K_m for O_2 are obtained from the slopes of the straight lines in Figure 1 (panel A).

These k_{cat}^{app}/K_m values are consistently lower than the corresponding second-order rate constants for the β-diketone substrates by a factor of about 50, recorded at a constant c_{O_2} of 260 μM. Figure 1 also shows that the transient reaction rate constant (k_2), obtained from single-turnover kinetic measurements described below, has exactly the same dependence on c_{O_2} as the steady-state turnover number. These results validate an experimental approach in which the reactivities of different substrates are compared at a precisely controlled and identical, however nonsaturating, concentration of dioxygen.

In search of an interpretable structure−reactivity correlation of the strong kinetic substituent effect on k_{cat}^{app}, we performed frontier molecular orbital calculations on the monoanions of the chosen β-diketones and used the thus-obtained energies of their respective highest occupied molecular orbital (ϵ_{HOMO}) as nucleophilic reactivity scale for the Dke1 substrates (Table 1). A plot of log k_{cat}^{app} against ϵ_{HOMO} (Figure 2) was linear with a coefficient of determination (r^2) of 0.97 and a slope of 4.4 eV^{-1}.

The mechanistic implication of this free-energy relationship is as follows. The absence of a break in the correlation in Figure 2 suggests that there is no transition between different rate-limiting steps across the series of homologous substrates, although it spans 5 orders of magnitude in enzymatic activity. The value of k_{cat}^{app} which then represents a common rate-limiting step is completely governed by the nucleophilic character of the monoanionic substrate or, in other words, its aptitude to donate electrons.

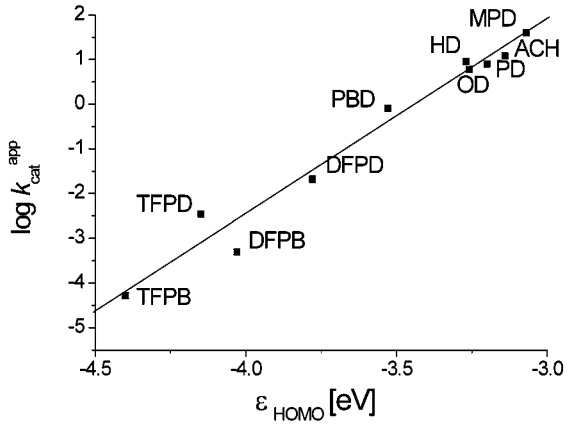


Figure 2. Linear free-energy relationship analysis for Dke1-catalyzed conversion of different β-diketones at 25 °C. k_{cat}^{app} values were determined in this study or, in the cases of 2-acetylcyclohexanone (ACH), 3,5-heptanedione (HD), 2,4-octanedione (OD), and 3-methylpentanedione (MPD), were reported previously.[1b] For TFPD the transient rate constant is given.

Spectral Properties of the Enzyme−Substrate Complex. Upon addition of PD or substituted β-dicarbonyl substrate analogues to a concentrated Dke1 solution under anaerobic conditions, two new bands (at λ_2 and λ_3) appeared in the UV−visible region of the protein absorbance spectrum. Because TFPB is turned over by the enzyme at a very slow rate, the spectrum of the anaerobic Dke1−TFPB complex could be compared with the spectrum of the same complex in an air-saturated buffer (Figure 3, panel A), and superimposable absorbance traces were obtained. If, however, Dke1−TFPB was incubated in the presence of dioxygen for long (>2 h), the pink color bleached gradually over time, as shown in Figure 3 (panel A). The time-dependent loss in band intensity at λ_2 and λ_3 paralleled the time course of substrate depletion recorded at λ_1 (Figure 3, panel B).

In concentrated solutions of fully active Dke1 ($\geq$100 μM, $Fe^{2+} \geq$ 90%) we found that a significant portion, up to 10% of the total Fe^{2+}, is dissociated, reflecting the binding equilibrium for the metal cofactor. The β-dicarbonyl substrates of Dke1 are reported to form stable complexes with Fe^{2+} in solution,[12] and the spectral properties thereof are comparable to those seen for the Dke1−substrate complexes. We did not detect formation of colored Fe^{2+}−diketone complexes in the concentration range relevant for our experiments ($\leq$10 μM free Fe^{2+}; $\leq$50 μM ligand). However, it was necessary to rigorously eliminate the possibility that a solution complex of Fe^{2+} interferes with the spectroscopic characterization of the enzyme−substrate adduct. Results of gel filtration analysis revealed that the color developed upon binding of PD or TFPB to fully active Dke1 eluted quantitatively in the protein-containing fractions, clearly indicating that it was completely associated with the enzyme. A control experiment used 200 μM Fe^{2+} in place of Dke1, and the metal complex with 5 mM PD was washed off the column wholly in the salt fraction. We conclude therefore that absorption transitions described in Figure 3 and Table 2 distinctly reflect noncovalent enzyme−substrate interactions at the level of the binary complex. Table 2 summarizes the values of λ_2 and λ_3 and the corresponding molar extinction coefficients for a series of complexes of Dke1 with substituted β-diketones.

Electron-withdrawing substituents lead to lower energy transitions, suggesting that the observed absorption bands can be assigned to metal-to-ligand charge transfer (MLCT) transitions in the Fe^{2+}−β-keto−enolate complex of Dke1 and PD (Scheme 1). The spectral properties of the Dke1 complexes apparently resemble those of α-ketoacid-dependent non-heme Fe^{2+} dioxygenases bound to a bidentate α-keto carboxylate ligand.[11,13] Evolution of band intensity of λ_3 in response to the presence of increasing amounts of dicarbonyl compound was used to titrate the enzyme, and nonlinear fits of the data to eq 1 yielded estimates for the respective dissociation constants (K_d). Dke1 bound to the inactive ligand 2′HAP also showed MLCT transitions, the K_d value of this complex being ~22 μM (see Table 2). Results of fluorescence titrations confirm the K_d value estimations that are based on measurements of MLCT band intensity (Table 2).

Single Turnvover Transient Kinetics. Rapid-mixing stopped-flow experiments were performed under conditions in which the substrate concentration limited the reaction to less than a single turnover of the total active site concentration present. The kinetic transients were recorded with the aim of characterizing microscopic steps leading to and from the Dke1−β-keto−enolate complex (Scheme 1). Figure 4 shows representative single absorbance traces for the reaction of Dke1 with four selected β-diketones in the presence of an air-saturated initial c_{O_2}. Equations 3 and 4 were used to fit the appropriate part of the time course from each stopped-flow experiment, as described in the Experimental Section. Table 1 summarizes pseudo-first-order rate constants of formation (k_1) and disappearance (k_2) of the MLCT band at λ_3 and compares them to the corresponding steady-state kinetic parameters. Note that all results are from measurements at a single dioxygen concentration (260 μM), which is not saturating in the steady state. However, as already shown in Figure 1, the dependence of k_2 on c_{O_2} for the conversion of DFPD was exactly the same as that of k_{cat}^{app} for the same reaction. The results in Table 1 reveal that k_1 and k_2 were strongly influenced by the substrate structure, whereby electron-withdrawing substituents slowed the formation and even more dramatically the decay of the chromophoric complex. k_2 and k_{cat}^{app} are congruent, and consequently their correlations with ϵ_{HOMO} are almost superimposable, which implies that the intrinsic nucleophilic reactivity of the substrate governs k_2 in analogy to k_{cat}^{app}. By contrast, log k_1 cannot be so clearly correlated against the chosen reactivity scale, indicating that factors other than the electronic ones also influence binding. The analysis of kinetic transients recorded at wavelengths diagnostic of selected free and Dke1-bound substrates, namely TFPD, DFPB, and TFPB, also showed that within the limits of experimental error enzymatic cleavage of the β-keto−enolate and loss of the MLCT band occurred at the same pre-steady-state rate (data not shown).

The plot of k_2 against c_{O_2} in Figure 1 thus contains additional information of mechanistic relevance. k_2 is a pseudo-first-order rate constant, and for the simple reaction, E−S + O_2 ↔ E−P, where E−S is the Dke1−PD complex and E−P is the Dke1−product complex, it is described by the relationship $k_2 = k_{+2}[O_2] + k_{-2}$ where k_{+2} and k_{-2} are rate constants in the forward and

(12) Eng, S. J.; Motekaitis, R. J.; Martell, A. E. *Inorg. Chim. Acta* **1998**, *278*, 170−177.

(13) (a) Ryle, M. J.; Padmakumar, R.; Hausinger, R. P. *Biochemistry* **1999**, *38*, 15278−15286. (b) Hegg, E. L.; Whiting, A. K.; Saari, R. E.; McCracken, J.; Hausinger, R. P.; Que, L., Jr. *Biochemistry* **1999**, *38*, 16714−16726.

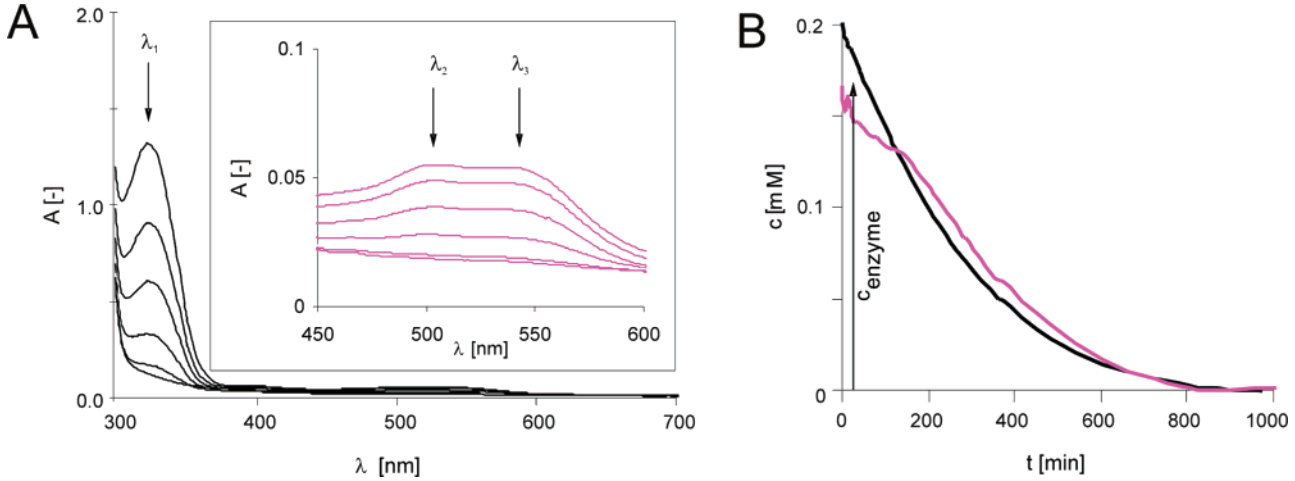


Figure 3. Spectra of a Dke1−TFPB complex and time-resolved analysis of band decay at $\lambda_1 - \lambda_3$ during enzymatic substrate conversion in the presence of 260 μM dioxygen at 25 °C. The complex was formed by mixing Dke1 (175 μM active sites) and TFPB (200 μM), each dissolved in an air-saturated Tris/HCl buffer (20 mM, pH 7.5). (A) Wavelength scans performed at incubation times of 0, 150, 300, 500, 750, and 1000 min show depletion of the TFPB-enolate band (λ_1) and the bands at λ_2 and λ_3 that are lacking in the free enzyme but appear in the Dke1−TFPB complex (enlarged inset). (B) Superimposition of the time courses of band decay at λ_1 (black) and $\lambda_{2,3}$ (pink).

Table 2. Characterization of β-Diketone Binding to Dke1 Using UV−Visible and Fluorescence Spectroscopy

S	$\epsilon_1(\lambda_1)^a$ $mM^{-1}\,cm^{-1}$ (nm)	$\epsilon_2(\lambda_2)^a$ $mM^{-1}\,cm^{-1}$ (nm)	$\epsilon_3(\lambda_3)^a$ $mM^{-1}\,cm^{-1}$ (nm)	$K_d{}^b$ μM
PD	2.2 (280)	0.27 (355)	0.25 (420)	0.75
DFPD	10 (290)	0.22 (380)	0.28 (450)	5.8
TFPD	14 (290)	0.28 (382)	0.27 (450)	6.7
DFPB	13 (324)	0.40 (510)	0.37 (530)	4.1
TFPB	16 (322)	0.34 (505)	0.35 (540)	2.2
2′HAP	3.3 (322)	0.25 (472)	0.25 (500)	22

[a] The wavelengths of maximum absorption (λ_1) for the free *cis*-β-keto−enolate form of the substrate (S) and the anaerobic Dke1-ligand complex (λ_2, λ_3) are given together with the corresponding molar extinction coefficients (ϵ). Note that the molarity of Fe^{2+}-containing active sites is the basis for the calculation of ϵ. [b] The K_d values are from fits of eq 2 to data from fluorescence titrations carried out at 4 °C and have a standard deviation of ±25%. Data obtained at 25 °C yield similar K_d values with, however, greater standard deviation.

reverse direction of the reaction with O_2, respectively. The finding that this plot passes through the origin means that $k_{-2} \approx 0$, or in other words, MLCT band decay in the presence of O_2 is not detectably reversible. Therefore, this implies that the O_2-dependent conversion of Fe^{2+}-bound diketonate is the first irreversible step of the catalytic cycle of Dke1 and is rate-determining overall.

Addition of 200 μM TFPB ($\cong 20 \times K_d$) to air-saturated solutions of 300 μM Dke1 did not cause O_2 depletion at a rate appreciably faster than that expected from k_{cat}^{app} for the conversion of TFPB. This result implies that formation of the Dke1−TFPB complex does not promote O_2 binding to the active site, which should lead to an observable, stepwise decrease of c_{O_2} at high enzyme concentrations, nor does it induce significant O_2 reduction prior to the rate-limiting step, which should be mirrored by an increased dioxygen consumption rate under the described single-turnover conditions. On the other hand, MLCT band decay was detected only in the presence of O_2. These results together with the fact that the enzyme is not saturable with dioxygen in the steady state (Figure 1) strongly indicate that it is the chemical reaction of the binary complex with O_2 that is rate-determining.

The Redox Potential of the Cofactor. Why is it that Dke1-bound substrates are so efficiently primed for reaction with O_2, whereas individually the β-dicarbonyl compounds and Dke1 are essentially inert? There is evidence from the studies of heme proteins[14] that the redox potentials of their respective iron centers (E_0) may be altered in response to substrate binding. The log $k_{cat}^{app} - \epsilon_{HOMO}$ correlation for Dke1 implies the possibility that E_0 of the Fe^{2+} cofactor is lowered upon formation of the binary complex, thereby enhancing the stability of Fe^{3+} and thus favoring one-electron reduction of dioxygen by Fe^{2+} in the rate-determining step (Scheme 1). We plotted literature values of E_0 for mononuclear iron complexes of the form $Fe^{3+}L_3$, where L is the enolate of PD or a substituted derivative thereof,[15] against the corresponding ϵ_{HOMO} values of L. A good linear correlation was obtained ($r^2 > 0.90$; Figure 5) in which the midpoint potential decreased with increasing ϵ_{HOMO} from +0.07 V for $Fe(TFPB)_3$ to 0.68 V for $Fe(PD)_3$. By way of comparison, the standard redox potential of the unligated Fe^{2+}/Fe^{3+} couple is +0.77 V, suggesting that substrate-derived donor ligands to the Fe^{2+} cofactor of Dke1 could indeed make one-electron reduction of O_2 more favorable.

2′HAP can be seen as a *cis*-β-keto−enol structure with the enol double bond fixed in the aromatic ring. It is totally unreactive toward oxygenative carbon−carbon bond cleavage by Dke1 and a potentially useful probe through which the steps involved in dioxygen reduction by the enzyme might be dissected kinetically. Its ϵ_{HOMO} is similar to that of PD, and its E_0 can be compared to that of PD and 2-acetylcyclohexanone (ACH), as shown in Figure 5. Except for a partial delocalization in the aromatic ring, the HOMO of 2′HAP is localized at the same positions as that of ACH and β-diketonate structures in general, that is, at the central carbon atom and the nearby oxygen atoms (see the Supporting Information). Formation of a Dke1−2′HAP complex gives MLCT bands at 472 and 500 nm (Table

(14) (a) Denisov, I. G.; Makris, T. M.; Sligar, S. G.; Schlichting, I. *Chem. Rev.* **2005**, *105*, 2253−2277. (b) Ost, T. W.; Clark, J.; Mowat, C. G.; Miles, C. S.; Walkinshaw, M. D.; Reid, G. A.; Chapman, S. K.; Daff, S. *J. Am. Chem. Soc.* **2003**, *125*, 15010−15020. (c) Miles, C. S.; Ost, T. W.; Noble, M. A.; Munro, A. W.; Chapman, S. K. *Biochim. Biophys. Acta* **2000**, *1543*, 383−407. (d) Daff, S. N.; Chapman, S. K.; Turner, K. L.; Holt, R. A.; Govindaraj, S.; Poulos, T. L.; Munro, A. W. *Biochemistry* **1997**, *36*, 13816−13823. (e) Sligar, S. G. *Biochemistry* **1976**, *15*, 5399−5406.
(15) Endo, K.; Furukawa, M.; Yamatera, H.; Sano, H. *Bull. Chem. Soc. Jpn.* **1980**, *53*, 407−410.

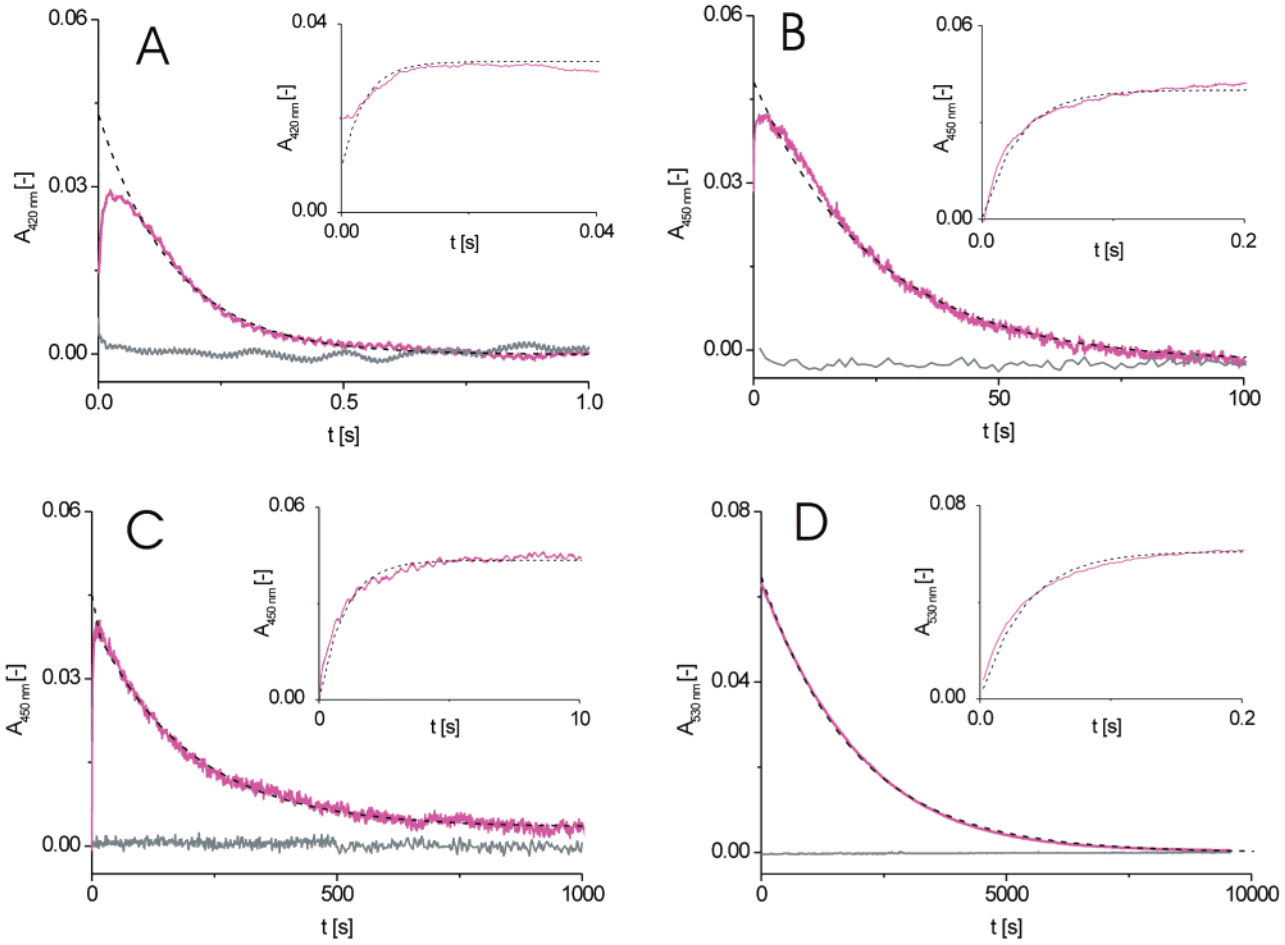


Figure 4. Representative absorbance traces at λ_3 in single-turnover stopped-flow reactions of Dke1 (220 μM active sites) and PD (A), DFPD (B), TFPD (C), or DFPB (D), each at a concentration of 200 μM. c_{O_2} was 260 μM and constant, and all experiments were performed in 20 mM Tris/HCl buffer, pH 7.5, at 25 °C. The insets of panels A−D show the part of the time course enlarged in which binary complex formation occurs. Solid pink lines are the experimental absorbance traces, and the broken lines are nonlinear fits to eq 4 or 3 (inset). Gray lines show the control containing substrate in air-saturated buffer but lacking Dke1.

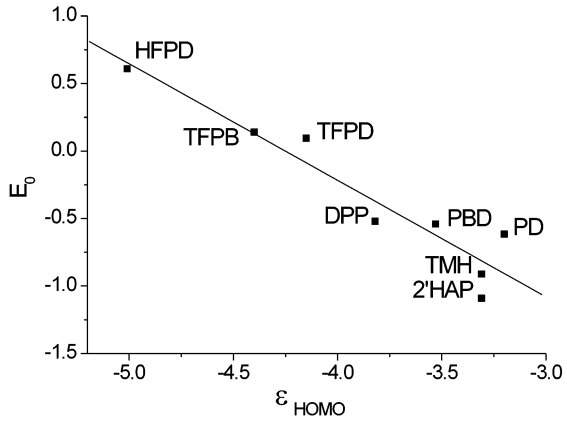


Figure 5. Correlation of E_0 values of Fe^{3+}−β-diketonate complexes with the ϵ_{HOMO} values of the corresponding free ligands. The midpoint potentials are from the literature[15] except the E_0 value of 2′HAP, which was determined experimentally (see the Supporting Information). ϵ_{HOMO} values are from Table 1. Newly introduced abbreviations are: HFPD, 1,1,1,5,5,5-hexafluoropentanedione; TMH, 2,2,6,6-tetramethyl-3,5-heptanedione; DPP, 1,3-diphenylpropanedione.

2), suggesting that enzyme-bound 2′HAP and ACH share considerable electronic similarities. Therefore, if one-electron reduction of O_2 by substrate-bound Fe^{2+} was the rate-limiting step of the enzymatic reaction, one would expect that formation of the Dke1−2′HAP complex might trigger Fe^{2+} into Fe^{3+} conversion concomitant with the evolution of superoxide but uncoupled from C−O bond formation, which in the case of 2′HAP is greatly disfavored because it would destroy ring aromaticity. Accelerated autoxidation of the 2′HAP-bound Dke1, compared with free enzyme, would result, leading to rapid inactivation. Increased autoxidation of NHMEs has previously been causally related to a ligand-induced oxidation of Fe^{2+}.[7] The observed O_2 consumption rate of 8×10^{-6} s^{-1} for the Dke1−2′HAP complex (0.2 mM), however, corresponds to the basal autoxidation rate of unliganded Dke1 in the presence of O_2 (7.5×10^{-6} s^{-1}), which is low but clearly significant. The finding essentially rules out that E_0 of the Fe^{2+} cofactor is markedly influenced upon binding of 2′HAP.[16]

Discussion

The Rate-Limiting Step of the Dke1 Catalytic Cycle. Results of steady-state kinetic analysis for the reactions of Dke1 with dioxygen in the presence of a β-diketone substrate and in

(16) The rate of dioxygen consumption and concomitant c_{O_2}-dependent release of metal cofactor from the enzyme into solution as Fe^{3+} constitutes the major mechanism of enzyme inactivation in Dke1 (Straganz, G. D.; Nidetzky, B., Graz University of Technology, Graz, Austria. Unpublished results, 2005). The causal relationship of steps involved in Dke1 autoxidation is that one-electron reduction of dioxygen by the Fe^{2+} cofactor precedes the dissociation of Fe^{3+}, which is released into solution because in its oxidized form the metal has no detectable affinity for the active site. Details of the Dke1 inactivation pathway will be reported elsewhere.

the absence thereof (referred to here as autoxidation) are consistent with a ternary complex enzymatic mechanism that is formally random. The apparent K_m values for the substrate are independent of c_{O_2}, which indicates that binding of substrate does not enhance the affinity of Dke1 for dioxygen and vice versa. Considering catalytic efficiencies for substrate that are ~50 times the corresponding k_{cat}^{app}/K_m values for dioxygen, a kinetic pathway in which substrate binds before dioxygen is clearly preferred under the experimental conditions used in this study.

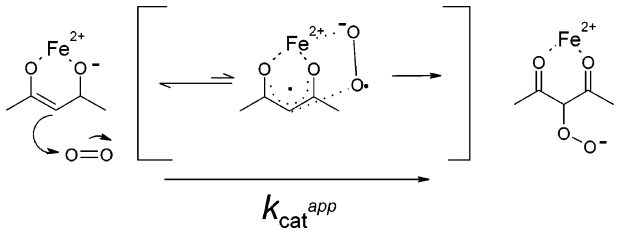
The evidence from steady-state and transient kinetic studies of the Dke1-catalyzed conversion of β-diketone substrates differing more than 10^5-fold in reactivity toward dioxygen in the presence of the enzyme suggests a reaction coordinate in which the chemical transformation of the enzyme-bound *cis*-β-keto−enolate into an alkyl peroxidate intermediate is the rate-limiting step. Linear free-energy relationship analysis has revealed the pronounced increase of k_{cat}^{app} (or the mechanistically synonymous transient rate constant k_2) in response to a decrease of ϵ_{HOMO}, which implies that the observable catalytic rate is controlled by the nucleophilic participation of the bound substrate in an irreversible chemical reaction with dioxygen. There is limited precedent among NHMEs regarding the use of structure−reactivity correlations of kinetic substituent effects to address mechanistic questions. Electron-withdrawing substituents were shown to slow the reactions catalyzed by the Fe^{3+}-dependent enzymes protocatechuate dioxygenase[17] and catechol-1,2-dioxygenase,[18] but because of kinetic complexity it remained difficult to consolidate the results into a chemical mechanism of the enzymatic reduction of dioxygen. The existence of a spectroscopically accessible intermediate on the reaction profile of Dke1 provided us with the powerful opportunity to examine internal steps of the catalytic cycle that involve dioxygen chemistry. These critical steps are, with the exception of α-ketoacid-dependent dioxygenases[11,13,19] and a recent example of a homoprotocatechuate-2,3-dioxygenase that utilizes a colored model substrate,[20] spectroscopically inaccessible for mechanistic study in NHMEs. This article describes for the first time a comprehensive quantitative structure−activity relationship analysis of the substituent effect on the kinetically unmasked chemical step of the reaction catalyzed by a non-heme Fe^{2+}-dependent dioxygenase. The results can be parsed unambiguously into substrate-derived catalytic factors used by Dke1 to achieve efficient reduction of dioxygen coupled with C−O bond formation.[21]

The Proposed Mechanism of Dioxygen Reduction. The apparent turnover number of the Dke1-catalyzed conversion of β-diketones is linearly dependent on the dioxygen concentration, implying that, under the conditions used and within the limits of detection by the analytical methods, O_2 does not combine with the substrate-bound enzyme to accumulate a ternary complex in a precatalytic equilibrium at the steady state. Transient kinetic analysis for the reaction of Dke1 with DFPD leads to the same conclusion. A two-step mechanism in which dioxygen binding to the Dke1−substrate complex precedes the slow reaction would lead to a hyperbolic dependence of k_2 on c_{O_2}. The observed linear correlation is thus consistent with binding and reaction taking place in one kinetic step. A very low binding affinity of O_2 to ligand-bound Dke1 is also emphasized by results of titration experiments showing that saturation of Dke1 with a slowly reacting substrate (TFPB) or an inactive analogue (2′HAP) does not cause significant depletion of dioxygen (260 μM) from the bulk solution. At the high concentration of enzyme employed (0.3 mM) in the experiments, a fractional saturation of the Dke1−ligand complex with O_2 of less than 0.03 would have been detectable. Summarizing, enzymatic reaction is thus suggested to take place from an encounter complex with dioxygen at the rate determined by k_{cat}^{app}.[22]

The conversion of Dke1-bound β-diketone into the proposed alkylperoxidate intermediate (Scheme 1) requires the net transfer of two electrons from the substrate to dioxygen. Considering that a one-step reduction of triplet dioxygen by the singlet substrate giving the singlet diketone−peroxide is formally spin-forbidden, we asked the question of the relative timing of electron-transfer steps during the Dke1-catalyzed reduction of dioxygen and the possible role of the Fe^{2+} cofactor in triggering the reaction. Assuming that autoxidation of the Fe^{2+} cofactor serves as a surrogate of one-electron transfer from Fe^{2+} to dioxygen during the normal catalytic cycle of Dke1 as it has been invoked previously,[7] our results do not support a mechanism in which the reduction of dioxygen to a superoxide via the Fe^{2+}−substrate complex is rate-determining. If that was the case, the E_0 of the metal cofactor in the Dke1−2′HAP complex, which according to studies of homologous metal−β-diketonate complexes in solution should have a low lying value in comparison with the Fe^{2+}/Fe^{3+} couple in the free enzyme, would be expected to stimulate strongly (that is, by several orders of magnitude as seen for k_{cat}^{app}) the rate of enzyme autoxidation. Considering a nonfavorable overall equilibrium for one-electron reduction of dioxygen by Fe^{2+},[6b] we emphasize that autoxidation of unliganded Dke1 took place at a finite rate, implying that an incremental change of this rate in response to ligand binding would not have escaped detection by the analytical methods under the experimental conditions used. Note that, while it is clear that a potential partial electron transfer from iron to dioxygen does not determine the catalytic rate, our results do not exclude the possibility that it has some role in activating the O_2 cosubstrate for reaction. However, putative formation of the reactive $Fe^{3+}O_2^{\bullet-}$ complex would then have to occur in a precatalytic equilibrium that probably lies far on the side of $Fe^{2+}O_2$.[23] The enzymatic reaction would then take place from the very small portion of activated complex, in our opinion leading to an extremely inefficient enzyme.

(17) Walsh, T. A.; Ballou, D. P. *J. Biol. Chem.* **1983**, *258*, 14413−14421.
(18) (a) Ridder, L.; Briganti, F.; Boersma, M. G.; Boeren, S.; Vis, E. H.; Scozzafava, A.; Veeger, C.; Rietjens, I. M. *Eur. J. Biochem.* **1998**, *257*, 92−100. (b) Walsh, T. A.; Ballou, D. P.; Mayer, R.; Que, L., Jr. *J. Biol. Chem.* **1983**, *258*, 14422−14427.
(19) Grzyska, P. K.; Ryle, M. J.; Monterosso, G. R.; Liu, J.; Ballou, D. P.; Hausinger, R. P. *Biochemistry* **2005**, *44*, 3845−3855.
(20) Groce, S. L.; Miller-Rodeberg, M. A.; Lipscomb, J. D. *Biochemistry* **2004**, *43*, 15141−15153.
(21) The clear electronic disposition of the Dke1−substrate complex for reaction eliminates the possibility that active-site conformational factors, which have been invoked with α-ketoglutarate-dependent non-heme Fe^{2+} enzymes,[4a] primarily drive the process of O_2 reduction by Dke1.
(22) Obviously one could define a second-order rate constant k_{cat}' (μM^{-1} s^{-1}) which incorporates the dependence of the apparent k_{cat} on c_{O_2}. However, the results for three different β-diketones in Figure 1 show clearly that the dependence of k_{cat}' on intrinsic nucleophilic reactivity of the substrate would be exactly the same as it appears in a correlation that uses apparent k_{cat} values. The conclusion is also valid for the transient rate constants.
(23) For the metallocenter of various NHMEs the binding of Fe^{2+} to O_2, which is accompanied by some charge transfer to O_2, has been estimated to be endergonic by ~10 kcal/mol.[4b,6]

Scheme 2. Suggested Mechanism of Concerted Dioxygen Reduction and C−O Bond Formation by Dke1[a]



[a] The structure in brackets depicts a hypothetical transition state in which the first electron has been transferred to O_2.

We would like to therefore suggest an alternative mechanism that involves the direct attack of dioxygen by the Fe^{2+}-bound substrate in the rate-controlling step, as outlined in Scheme 2. Coordination of the substrate to Fe^{2+} such that an orbital mixing of its HOMO with the HOMO of the metal can take place, similar to what has been observed in 2,3-dihydroxybiphenyl-1,2-dioxygenase,[4b] is proposed to prime the substrate for the reaction with dioxygen in a now spin-allowed process. We envision a cataytic reaction profile in which one-electron transfer from the substrate to dioxygen is thermodynamically unfavorable and requires coupling to a second, fast step of C−O bond formation that pulls the reaction toward the virtually irreversible peroxidate formation. The chemical steps of the Dke1-catalyzed reaction therefore take place in a concerted manner. The immediate implications of this are that dioxygen reduction and C−O bond formation must occur in one single catalytic step and electron transfer and recombination of the intermediates cannot be kinetically resolved.[24] The transition state (TS) of the reaction is thus expected to resemble, structurally and charge-wise, the product of the electron transfer from the substrate to dioxygen, as illustrated in Scheme 2. The subsequent recombination to the alkylperoxidate intermediate is kinetically and thermodynamically advantageous and thus expected not to contribute to TS energy. The lack of measurable activity with 2′HAP which cannot form the peroxidate is thus understood in the context of a thermodynamically unfavorable equilibrium for reduction of dioxygen by the activated substrate that in the case of 2′HAP cannot be overcome by subsequent C−O bond formation.

The Role of the Cofactor in the Reactions Catalyzed by Dke1 and Related NHMEs. We think that the Fe^{2+} cofactor in Dke1 contributes to catalysis in several ways. First and perhaps most importantly, it brings about coordination of the substrate and may activate it for attack on dioxygen, as suggested earlier in this article. Second, it may provide electrostatic stabilization to the TS of dioxygen reduction. One possibility is that reduction is facilitated by coordination of a transient superoxide species to Fe^{2+}, as it enables the formation of the $[Fe^{2+}O_2^{\bullet-} \leftrightarrow Fe^{3+}O_2^{2-}]$ resonance structure, which formally represents the thermodynamically more favorable two-electron reduction of dioxygen.[6] However, the direct interaction of O_2 with iron is debatable. The emerging concept of substrate activation as one catalytic factor in the reactions of Fe^{2+}-dependent NHMEs is supported by recent reports on quercetin dioxygenases. In the crystal structure of an anaerobic substrate-bound complex of Cu^{2+}-containing quercetin dioxygenase the ligand adopts a distorted conformation which was thought to reflect some radical character of the bound substrate, induced by charge transfer from the substrate to Cu^{2+}.[9] The proposed mechanism is that the thus primed substrate attacks molecular oxygen directly, or dioxygen is reduced by reoxidation of Cu^{+} and both substrate and superoxide radical recombine. Related studies have revealed quercetin dioxygenases that can utilize Fe^{2+} or Cu^{2+} as a cofactor,[25] and remarkably, they do so with comparable enzymatic activities. A detailed structural and mechanistic characterization of Fe^{2+}-dependent quercetin dioxygenase is presently not available. However, an interesting result from electron spin resonance studies is that the Fe^{2+} cofactor is shielded from interactions with the O_2 surrogate nitric oxide by the enzyme-bound quercetin.[26] It suggests the intriguing possibility that carbon−carbon bond-cleaving dioxygenase reactions may proceed without any direct interaction of dioxygen and Fe^{2+} in cases when substrate activation is involved.

Acknowledgment. We thank Dr. Mario Klimacek for assistance with stopped-flow measurements and Dr. Claudia Muresanu for redox potential measurements.

Supporting Information Available: Plots for determination of the apparent ϵ_1 for the β-diketone substrates used in this study, results of fluorescence titration, determination of E_0 of 2′HAP, and a graphical representation of the HOMOs of ACH and 2′HAP. This material is available free of charge via the Internet at http://pubs.acs.org.

JA042313Q

(24) (a) The results point out a major difference between Dke1 and catechol-cleaving dioxygenases,[24b] which otherwise share the requirement for substrate-derived electrons to drive the catalytic reaction. Choice of an appropriate substrate made it possible to uncouple superoxide production from subsequent substrate transformations catalyzed by the latter enzymes. Considering Scheme 2, similar approaches would seem to be elusive for Dke1. (b) Mayer, R.; Widom, J.; Que, L., Jr. *Biochem. Biophys. Res. Commun.* **1980**, *92*, 285−291.

(25) (a) Bowater, L.; Fairhurst, S. A.; Just, V. J.; Bornemann, S. *FEBS Lett.* **2004**, *557*, 45−48. (b) Barney, B. M.; Schaab, M. R.; LoBrutto, R.; Francisco, W. A. *Protein Expression Purif.* **2004**, *35*, 131−141.

(26) Gopal, B.; Madan, L. L.; Betz, S. F.; Kossiakoff, A. A. *Biochemistry* **2005**, *44*, 193−201.