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Electronic Substituent Effects on the Cleavage Specificity of a Non-Heme Fe^{2+}-Dependent β-Diketone Dioxygenase and Their Mechanistic Implications

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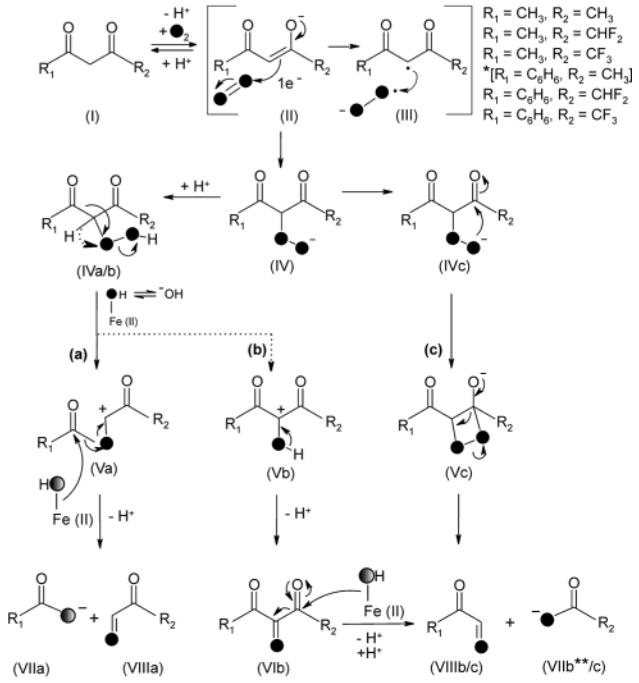
The enzymatic fission of C−C bonds by the incorporation of one atom of molecular oxygen into each site of bond cleavage is a key step of specialized pathways through which microorganisms assimilate xenobiotic and environmentally toxic compounds. It is an intriguing type of reaction that has no immediate counterpart in organic chemistry and is thus of a fundamental interest. In nature, it is generally performed by C−C bond-cleaving dioxygenases that require a non-heme metal cofactor for activity (NHMCDs). Among the NHMCDs, intradiol and extradiol-cleaving catechol dioxygenases have been studied in great detail.[1] By contrast, mechanistic studies of the cleavage of aliphatic C−C bonds are sparse,[2] limiting the current view of the chemistry employed by NHMCDs to the conversion of aromatic substrates.

Acinetobacter johnsonii acetylacetone dioxygenase (Dke1)[3] is a representative Fe(II)-dependent NHMCD that acts on aliphatic substrates. It catalyzes oxidative degradation of 2,4-pentanedione (Scheme 1) and related β-dicarbonyl compounds capable of undergoing enolization to a *cis*-β-keto−enol structure. Here, we report a quantitative structure−activity relationship analysis of electronic substituent effects on the cleavage specificity of Dke1, which is a novel approach to explore the mechanisms of NHMCDs. The results, together with evidence from ^{18}O labeling experiments, support a nucleophilic mechanism of C−C bond cleavage involving a dioxetane intermediate (see Scheme 1c). These findings are relevant in consideration of the mechanistic proposals for extradiol- and intradiol-cleaving catechol dioxygenases that invoke Criegee rearrangement (Scheme 1a).[1,4]

The Dke1-catalyzed conversion of acetylacetone (2,4-pentanedione) was carried out under an $^{18}O_2$ atmosphere, and the incorporation of ^{18}O label into the products was monitored by solid-phase microextraction GC−MS (see the Supporting Information for experimental setup and detailed results). We determined 97 ± 2% and 70 ± 5% incorporation of one ^{18}O into acetate and methylglyoxal, respectively, and found no double labeling of either product. Incomplete ^{18}O incorporation of methylglyoxal reflects the kinetic competition between the enzymatic reaction and the loss of label to water solvent due to aldehyde group hydratation (see Supporting Information). The observed pattern of ^{18}O labeling is fully consistent with a dioxygenase reaction in which one atom from molecular oxygen is incorporated into each site of C−C bond cleavage. Therefore, the plausible mechanism of Dke1 involves a C-3 peroxide (IV),[5] and this central intermediate may undergo conversion to products via three different routes, as outlined in Scheme 1: Criegee rearrangement (a), hydrogen migration (b), and formation and subsequent cleavage of a dioxetane intermediate (c).

Scheme 1 shows that the completeness of incorporation of ^{18}O into acetate is of a mechanistic relevance. Partial exchange of label with solvent is not consistent with a dioxetane intermediate but may accompany Criegee rearrangement or hydrogen migration.[6]

Scheme 1. Three Routes of C−C Bond Cleavage by Dke1[a]



[a] Paths a and b show rearrangement of a common peroxide intermediate (IV) via acyl group migration (solid arrow) and hydrogen migration (dashed arrow), respectively, followed by attack of Fe^{2+}-bound OH^- (which may partially equilibrate with solvent) to yield products. Path c shows nucleophilic attack of the peroxidate to generate a dioxetane that decomposes to the products. Mechanisms a−c will differ in substituent effects on the cleavage pattern. *For 1-phenyl-1,3-butanedione, cleavage is expected adjacent to the benzoyl moiety in all three routes. **Labeling in (VIIb) may be partial.

To confirm the results of ^{18}O incorporation, we carried out a reverse labeling experiment in $H_2{}^{18}O$ as solvent and using unlabeled O_2. No ^{18}O was incorporated into acetate from the C−C bond cleavage (see Supporting Information for detailed results). This is in line with all three mechanistic routes in Scheme 1.

To differentiate between the intermediacy of a dioxetane or a rearrangement product, we investigated the cleavage pattern for the Dke1-catalyzed degradation of a homologous series of asymmetric β-carbonyl substrates in which the electronic properties of R_1 and R_2 (Scheme 1) were different and systematically varied. Considering that the conversion of (I) is quasi-irreversible, the observed product pattern immediately mirrors the ratio of cleavage velocities at the two candidate C−C bonds and is not attenuated by substituent effects on the thermodynamic equilibrium constant of the reaction. The broad substrate tolerance of Dke1 allowed us to analyze electronic substituent effects on cleavage specificity without adding a strong steric bias. Introduction of electron-withdrawing fluorine

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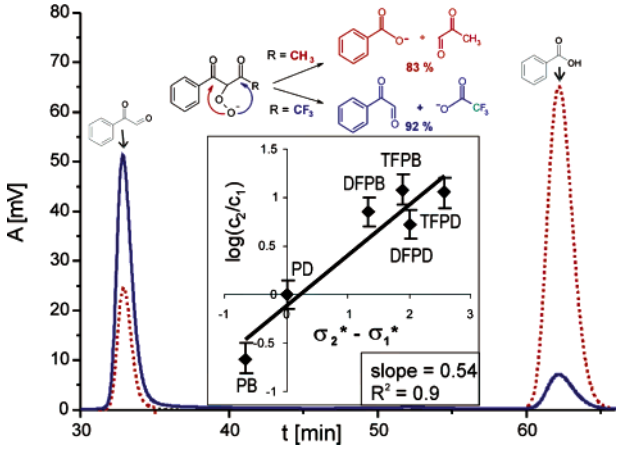

Figure 1. HPLC chromatogram of the products of Dke1-catalyzed conversion of 4,4,4-trifluoro-1-phenyl-1,3-butanedione (blue solid line) and 1-phenyl-1,3-butanedione (red dashed line). Inset: Correlation of logarithmic cleavage ratios of methylgyloxal (c_2) and acetate (c_1) or, in the case of benzoylic substrates (R_1 = C_6H_6), phenylglyoxal (c_2) and benzoate (c_1), which are formed by cleavage adjacent to R_2 and R_1, respectively, and the corresponding $\Delta\sigma^*$ values.[7] Substrates investigated were: 1-phenyl-1,3-butanedione (PB), 4,4-difluoro-1-phenyl-1,3-butanedione (DFPB), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TFPB), 2,4-pentanedione (PD), 1,1-difluoro-2,4-pentanedione (DFPD), and 1,1,1-trifluoro-2,4-pentanedione (TFPD).

atoms into R_2 should strongly enforce the susceptibility of the adjacent carbonyl group for nucleophilic attack by the distal oxygen of the C-3 peroxide anion, thus favoring bond cleavage next to the substituted acetyl group in the case of a dioxetane intermediate. In the Criegee mechanism, by contrast, those moieties that can best stabilize a positive charge migrate preferentially, implying a low migratory aptitude for a fluorinated acetyl group compared to the nonfluorinated counterpart. The selected acetylacetone derivatives were incubated in a concentration range of 0.2−3.0 mM with 20 μM purified Dke1 at 25 °C until all substrate was converted (2−4 h). The cleavage ratio was obtained from the product concentrations measured by HPLC (Figure 1).

We observe a marked preference for enzymatic cleavage of the bond adjacent to the most electron-deficient carbonyl carbon. The electronic substituent effect is strong, as emphasized by the full reversal of the product ratio during enzymatic conversion of benzoylic substrates upon changing R_2 from methyl (0.2) to trifluoromethyl (12). The pronounced correlation between C−C bond cleavage and the TAFT factor (σ^*)[7] of the adjacent substituents strongly supports a mechanism of C−C bond fission via nucleophilic attack at carbonyl carbon. A Criegee mechanism of Dke1 governed by electronic migratory aptitudes is not consistent with the observed correlation in Figure 1. A primary stereoelectronic effect that has been shown to control Criegee rearrangement in sterically rigid systems[8] will not attenuate the clearcut mechanistic distinction for Dke1, because conformational restrictions are clearly lacking in the β-dicarbonyl substrates used here. With the widely held assumption that the group that migrates does so from a position antiperiplanar to the oxygen−oxygen bond of the peroxide (IVa), the stereoelectronic analysis of the 1,1-difluoroacetylacetone-peroxide does not reveal conflicts in dipole orientation for a conformer in which the electronically favored $CH_3C{=}O$ group is properly aligned for migration. Finally, we rule out that the cleavage pattern reflects a substituent effect on the enol−enol equilibrium of β-dicarbonyl compounds. Compared to acetylacetone, this equilibrium changes by a factor of maximally 1.5 in response to the electronic properties of the substituents investigated.[9]

What, however, cannot be discounted on the basis of the available evidence, is a rearrangement that involves migration of a hydrogen and subsequent cleavage of the resulting tricarbonyl intermediate in the active site (Figure 1b). By analogy, rearrangement of C-3 substituted substrates such as 3-methyl- (MPD) and 3-phenyl-pentane-2,4-dione (PPD) might take place via migration of a methyl and phenyl group, respectively, in which case the expected formation of the methyl- and phenyl-ester of acetate would be diagnostic. Alternatively, if exchange of an iron-bound alkoxide with bulk water occurred (see Scheme 1b in which OR^- would replace OH^-), methanol and phenol would be expected as products. Within limits of detection (≥0.1 mol % of PPD consumed), the Dke1-catalyzed conversion of PPD did not yield an ester or alcohol product, essentially eliminating the possibility that migration of a phenyl cation occurs. Therefore, this supports the notion that cleavage of C−C bonds by Dke1 does not occur via migration of the C-3 substituent. Consequently, we propose that C−C bond cleavage in Dke1 proceeds via nucleophilic attack of the carbonyl group by the peroxidate moiety leading to a dioxetane intermediate, which then undergoes cleavage to acetate and methylglyoxal (Scheme 1c).[10]

Supporting Information Available: Experimental setup and results of the ^{18}O isotope incorporation experiments, detailed results of the substrate cleavage pattern, a pictorial representation of the calculated HOMO for acetylacetonate, and a stereoelectronic analysis of a Criegee intermediate for the reaction with DFPD. This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) Me^{2+}-dependent NHMCDs are thought to activate molecular oxygen through electron transfer from the deprotonated, metal bound substrate.[1,2] We perform Hartee-Fock calculations of the electronic state of monoanionic acetylacetone and find that the HOMO is centered at C-3 and oxygen. Nucleophilic attack by C-3 of the substrate would directly lead to the peroxide intermediate. Single electron transfer from the substrate via the metal yields a conjugated radical that is located both on C-3 and the two oxygen atoms. The resulting activated oxygen species will subsequently add to C-3 (see (II) and (III), Scheme 1). Therefore, irrespective of the exact mechanism of oxygen activation in Dke1, the same central peroxide intermediate will occur and is the point of departure for our mechanistic considerations.

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(10) Considering that conversion of a dioxetane ring is a highly exothermic reaction, we looked at the occurrence of luminescence during the Dke1-catalyzed cleavage of pentanedione at 25 °C and pH 7.5. We did not observe any, even at high enzyme concentrations of 200 μM. However, we emphasize that the nature of the rate-limiting step in the reaction of Dke1 is not known and the fraction of enzyme-bound dioxetane intermediate may be too small to allow detection of luminescence.

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