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Enzyme Catalytic Promiscuity: The Nonheme Fe^{2+} Center of β-Diketone-Cleaving Dioxygenase Dke1 Promotes Hydrolysis of Activated Esters

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Natural enzymes are often described as highly efficient and finely tuned catalysts for specific chemical transformations of biological relevance. Some of these enzymes, however, are promiscuous in a sense that they catalyze secondary reactions, chemically distinct from the reaction promoted in the conversion of the physiological substrate.[1] Catalytic promiscuity has drawn mechanistic attention as it necessitates that different reaction coordinates be accommodated by a single enzyme active site.[1a] Furthermore, it presents an often untapped source of potentially useful enzymatic functionality.[2] Active-site redesign, by using minimal structural modifications of the original catalytic center, has been exploited successfully with some enzymes to revive latent alternative reactivity present in the native form.[1–3]

β-Diketone-cleaving enzyme Dke1 is a nonheme Fe^{2+}-dependent dioxygenase from *Acinetobacter johnsonii* that converts 2,4-pentanedione and O_2 into methyglyoxal and acetate (Scheme 1).[4] The metal cofactor of Dke1 is coordinated by the triad His62, His64, and His104 (Figure 1) and is absolutely required for oxygenase activity.[5] Other metal ions, like Zn^{2+}, Ni^{2+} or Cu^{2+}, that compete with Fe^{2+} for binding to the 3-His site are completely inactive in the enzymatic reaction with O_2.[5] We show here that the catalytic center of Dke1 in its native Fe^{2+} form catalyses the hydrolysis of 4-nitrophenylesters of short-chain alkanoic acids, which is an accidental type[2a] of catalytic promiscuity. A non-native Zn^{2+} form of the enzyme was likewise active as esterase—about ten-times more so than the Fe^{2+} enzyme. Substitution of His104 by Glu, which destroys the oxygenase activity,[5] was fully compatible with the

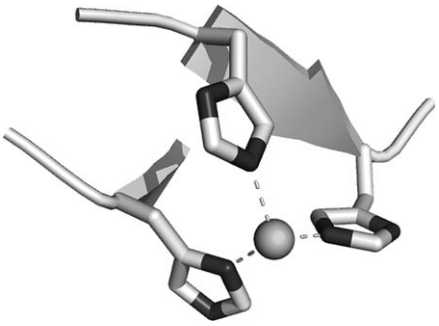
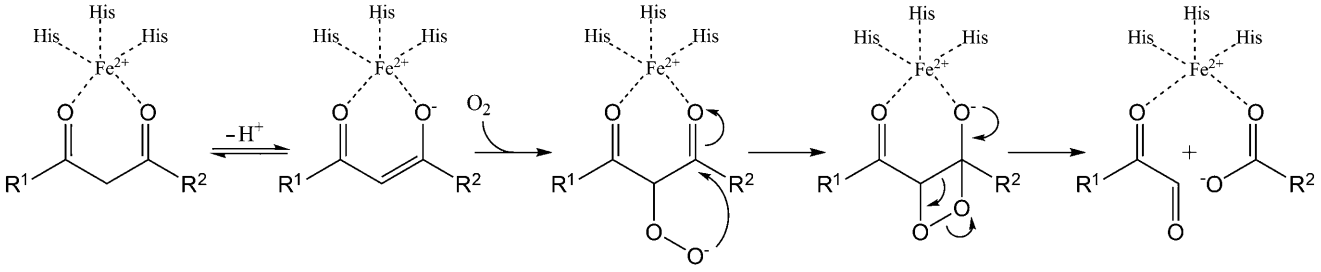


Figure 1. Metal cofactor coordination by the 3-His center of Dke1. The picture was derived from the crystal structure of a Zn^{2+} form of Dke1 (PDB ID: 3BAL) that is inactive as dioxygenase[4,5] but promotes ester hydrolysis (this work).

function of a Fe^{2+}/Zn^{2+} esterase. The type of catalytic promiscuity (oxygenase→esterase) seen for Dke1 is novel, and with the exception of methionine aminopeptidase,[6] for which a role for Fe^{2+} as cofactor has been proposed, hydrolytic activity of a catalytic Fe^{2+} site appears to have limited precedence in reported enzymes (see later).

When a purified preparation of Dke1 (5 μM Fe^{2+} sites) was incubated in the presence of 4-nitrophenylpropionate (pNPP; 3 mM in 20 mM Tris-Cl buffer, pH 7.5; 25 °C) formation of 4-nitrophenol occurred at a spectrophotometric rate (405 nm) that was 40-fold faster than the rate of the uncatalyzed hydrolysis of the ester substrate under otherwise identical conditions lacking the enzyme. Apo-Dke1, from which Fe^{2+} had been removed by exhaustive dialysis in the presence of the metal chelator FereneS, was completely inactive towards pNPP. Dke1 preparations showing a variable Fe^{2+} content between 0.03 and 0.70 (mol metal per mol enzyme subunit of 16 kDa) displayed specific activities that increased linearly with increasing Fe^{2+} occupancy of the active site (Figure S1 in the Supporting



Scheme 1. Proposed catalytic mechanism of O_2-dependent C—C bond cleavage in 2,4-pentanedione (R^1=R^2=CH_3) and related β-diketones by Dke1. A β-keto-enolate coordinated to the nonheme Fe^{2+} center is thought to be the reactive form of the substrate. The type of cleavage shown takes place in the case that R^2 is more electron-withdrawing than R^1.[4b]

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Information). Fe^{2+} in solution (33–167 μM) did not promote hydrolysis of NPP. The nonheme Fe^{2+} center of Dke1 is therefore essential for the esterase activity of the enzyme.

Variants of Dke1 in which the native Fe^{2+} had been substituted by another divalent metal were either totally inactive toward pNPP (Cu^{2+}, Mn^{2+}, Ni^{2+}) or showed activity (Zn^{2+}) comparable to that seen for the Fe^{2+} enzyme. However, when applied in solution, none of the metals used for replacement of Fe^{2+} caused rate acceleration in hydrolysis of pNPP. Table 1 summarizes the kinetic parameters of Fe^{2+} and Zn^{2+} forms of these site-directed substitutions[5] could explain the absence of Fe^{2+} esterase activity in the four Dke1 variants, it is interesting that the corresponding Zn^{2+} enzymes were also inactive despite the fact that, according to literature,[5] binding of Zn^{2+} has been hardly affected in the His62 and His64 mutants as compared to wild-type Dke1. The presence of esterase activity in a His104→Glu enzyme (H104E) that was nonreactive as β-diketone-cleaving dioxygenase is therefore striking. Both Fe^{2+} and Zn^{2+} forms of H104E were active, as shown in Table 1. However, binding of Fe^{2+} appeared to have been strongly impaired as a result of the substitution His104 by Glu, arguably explaining the relatively low k_{cat} for the Fe^{2+} as compared to the Zn^{2+} dependent catalytic reaction with this mutant.

Table 1. Kinetic parameters for hydrolysis of ester substrates by Fe^{2+} and Zn^{2+} forms of wild-type Dke1 and the metal-site variant H104E at pH 7.5.

Parameter		Wild-type enzyme		H104E	
		Fe^{2+}	Zn^{2+}	Fe^{2+}	Zn^{2+}
k_{cat} [s^{-1}]	pNPP	3.0×10^{-2}	2.7×10^{-2}	0.3×10^{-2}	4.5×10^{-2}
	pNPA	0.5×10^{-2}	1.3×10^{-2}	0.7×10^{-2}	3.1×10^{-2}
	pNPB	0.7×10^{-2}	0.6×10^{-2}	0.1×10^{-2}	0.7×10^{-2}
K_M [μM]	pNPP	4.2×10^{2}	61	4.3×10^{2}	9.2×10^{2}
	pNPA	1.0×10^{3}	2.6×10^{2}	1.8×10^{3}	2.1×10^{3}
	pNPB	2.5×10^{2}	17	94	1.7×10^{2}
k_{cat}/K_M [$M^{-1}s^{-1}$]	pNPP	71	4.5×10^{2}	7.4	48
	pNPA	5.1	49	3.6	14
	pNPB	29	3.6×10^{2}	12	41
k_{uncat} [s^{-1}]	pNPP		3.6×10^{-4}		
	pNPA		1.0×10^{-3}		
	pNPB		n.d.[c]		
$k_{cat}/K_M k_{uncat}$ [M^{-1}][b]	pNPP	2.0×10^{5}	1.2×10^{6}	2.0×10^{4}	1.3×10^{5}
	pNPA	5.0×10^{3}	4.8×10^{4}	3.5×10^{3}	1.4×10^{4}
	pNPB	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]

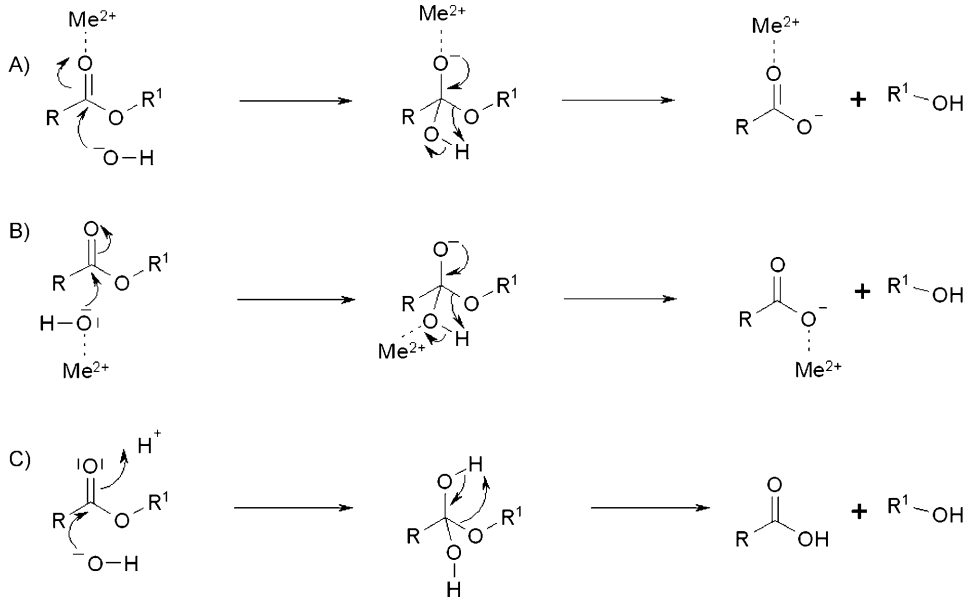
[a] Relative s.d. on k_{cat} and K_M was ≤15 and ≤20%, respectively; [b] k_{uncat} was determined at a constant substrate concentration of 4.2 mM; [c] not determined: uncatalyzed rate was too low to be determined precisely. pNPP: 4-nitrophenylpropionate; pNPA: 4-nitrophenylacetate; pNPB: 4-nitrophenlybutyrate.

There are two principle mechanisms by which Fe^{2+}/Zn^{2+} centers of the native 3-His form of Dke1 or the mutant 2-His–1-Glu form thereof might provide catalytic assistance to 4-nitrophenylester hydrolysis (Scheme 2). Uncatalyzed hydrolysis is thought to occur through simultaneous attack of hydroxide and hydronium to form a *gem*-diol intermediate, the elimination of which yields the products (Scheme 2C).[8] We measured the pH dependence of the conversion of pNPP by the different Fe^{2+}/Zn^{2+} enzyme preparations, considering that pH-rate profiles might be mechanistically revealing. Results are shown as double-log plots in Figure 2. The apparent k_{cat} for wild-type enzyme (Fe^{2+}, Zn^{2+}) was level at low pH (≤7.0–7.5) and increased as the pH was raised in the range ~7.5–11 (Figure 2A). Interestingly, the pH dependence of k_{cat} for H104E (Zn^{2+}) was level at high pH (≥9.5) and decreased below an apparent p*K* of ~9 (Figure 2B). Unlike the wild-type enzyme (Fe^{2+}, Zn^{2+}) the activity of H104E (Zn^{2+}) did not reach a constant level at pH values down to 6.0. Hydrolysis of pNPP by H104E (Fe^{2+}) was pH dependent in the range 6.5–11.0. These pH dependencies generally support a role for base catalysis in the enzymatic reactions, and arguably involve participation from a metal–hydroxide species (Scheme 2B). However, the pH effects on k_{cat} for wild-type enzyme and H104E were clearly not uniform. It is, therefore, conceivable for Dke1 (and would be consistent with studies of other metalloenzymes like carbonic anhydrase and small molecule models thereof[9]) that changes in the primary coordination sphere can result in a slightly altered catalytic action of the active-site metal. However, a more detailed examination of a potential ligand field effect in Fe^{2+}/Zn^{2+} Dke1 was considered to be beyond the scope of this work.

Dke1 for hydrolysis of 4-nitrophenylesters of alkanoic acids differing in chain length from two to four carbon atoms. The Zn^{2+} enzyme was the more efficient catalyst (in k_{cat}/K_M terms) of the two Dke1 preparations. Turnover of substrate (k_{cat}) was only about two (pNPP and 4-nitrophenylacetate; pNPA) or three orders of magnitude (4-nitrophenylbutyrate; pNPB) slower than O_2-dependent conversion of 2,4-pentanedione, the presumed natural reaction of the Fe^{2+} enzyme.[4a] The observation that irrespective of the metal cofactor used Dke1 could be saturated with substrate concentrations in the micromolar range suggests a mechanism in which the applied 4-nitrophenlyesters bind to the Fe^{2+}/Zn^{2+} active site of the enzyme where catalytic assistance to their hydrolysis is provided. The rate acceleration contributed by the two nonheme metal centers over the respective uncatalyzed reaction (k_{uncat}) at pH 7.5 was in the range 15–120-fold (Table 1). A catalytic proficiency ($k_{cat}/K_M\ k_{uncat}$)[7] of between 2.0×10^5 and 1.2×10^6 M^{-1} was calculated from the data in Table 1.

Mutants of Dke1 in which the native 3-His center of nonheme Fe^{2+} had been changed at position His62 (to Glu) and at position His64 (to Glu, Asp, or Asn) were nonreactive as Fe^{2+}- and Zn^{2+}-dependent esterases. While disruption of the catalytic Fe^{2+} site that was previously shown to occur as a result of

Despite notable efforts toward design of artificial metalloenzymes from nonenzymatic protein scaffolds,[10] there is current-



Scheme 2. Possible mechanisms for catalytic participation of the Dke1 Fe^{2+}/Zn^{2+} center in hydrolysis of ester substrates. A) Lewis acid stabilization; B) activation of water for attack on carbon; C) uncatalyzed hydrolysis.

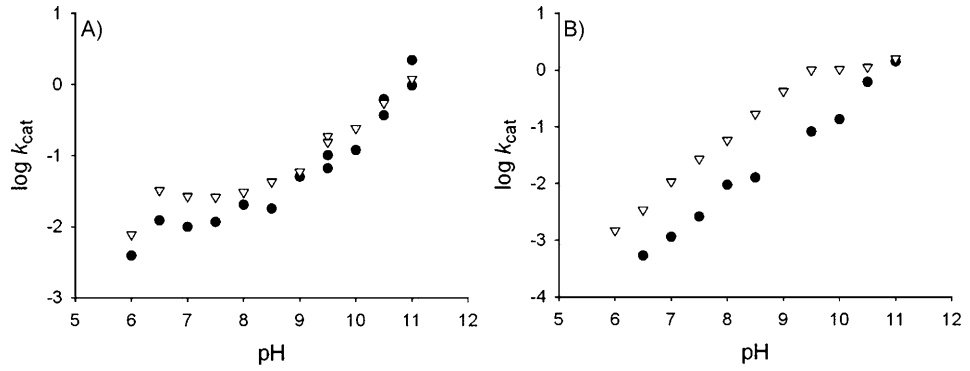


Figure 2. The pH profiles of k_{cat} (s^{-1}) for hydrolysis of pNPP by: A) wild-type Dke1, and B) H104E; ●: Fe^{2+} enzymes; ▽: Zn^{2+} enzymes. Note that all initial rates used in determination of k_{cat} were carefully corrected for the corresponding rates of uncatalyzed hydrolysis.

diol dioxygenases, both of which catalyze the oxidative cleavage of catechol, are interesting examples in which hydrolysis of oxidized reaction intermediates (intradiol: anhydride; extradiol: lactone) promoted by metal-bound hydroxide constitutes the final step of the proposed enzymatic mechanism.[12] The extradiol dioxygenase MhpB from *Escherichia coli* was shown to provide Fe^{2+}-dependent catalysis to the hydrolysis of a seven-membered lactone analogue that was chemically synthesized to mimic the putative reaction intermediate.[12c] Di-zinc aminopeptidase provides an interesting case of reverse (hydrolase→oxidase) catalytic promiscuity for which substitution of the native Zn^{2+} by Cu^{2+} has yielded a proficient catechol oxidase dependent on H_2O_2.[13] Also relevant, incorporation of vanadate into hydrolase phytase has generated a novel peroxidase.[14] Generally, metal exchange was in several enzymes accompanied by reactivity change.[3c–e]

Dke1 belongs to the cupin protein superfamily, a large and widely spread group of β-barrel proteins in which a mononuclear metal center is both a conserved feature of the structure and a rich source of functional diversity.[15] The metal binding residues of Dke1 (Figure 1) are contributed from two highly conserved core motifs that provide the signature for the superfamily. Metal exchange, as shown for Fe^{2+} and Zn^{2+} forms of Dke1, could be one way of introducing new reactivity patterns in existing enzymes. Cupins have evolved diverse enzymatic functions spanning primary E.C. classes 1, 3,[16] 4, and 5. Design of biocatalysts with novel or improved functionalities could focus on interconversion of homologous enzymes or the exploitation of accidental catalytic promiscuity, like the one observed in Dke1.

ly limited evidence for catalytic promiscuity in natural enzymes displaying activity dependent on a metal cofactor. Carbonic anhydrase, which like Dke1 utilizes a 3-His motif for coordination of its native Zn^{2+} cofactor, shows Zn^{2+}/Co^{2+} dependent esterase activity.[11] The catalytic efficiency of carbonic anhydrase for hydrolysis of pNPP ($516\ M^{-1}s^{-1}$; pH 8.5) can be compared with that of wild-type Dke1 in the Zn^{2+} form.[11e,f] Rational design and directed-evolution strategies have been successfully applied to enhance the activity and the substrate scope of carbonic anhydrase as an ester hydrolase,[11d] and show that these secondary activities of metalloenzymes present a potential opportunity for biocatalytic synthesis. Although recent work on methionine aminopeptidase reveals that Fe^{2+} sites in proteins are capable of promoting a hydrolysis reaction,[7] the *promiscuous function* of an oxygenase Fe^{2+} catalytic center as an esterase active site has not been described before. However, Fe^{3+}-dependent intradiol dioxygenases and Fe^{2+}-dependent extra-

Experimental Section

Experimental details (chemicals and reagents; site-directed mutagenesis, enzyme production and purification; metal exchange studies; enzymatic assays; steady-state kinetic analysis) are provided in the Supporting Information.

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