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Kinetic and CD/MCD Spectroscopic Studies of the Atypical, 3-His Ligated, Non-heme Fe²⁺ Center in Diketone Dioxygenase: the Role of Hydrophilic Outer Shell Residues in Catalysis

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Abstract

Diketone cleaving enzyme (Dke1) is a dioxygenase with an atypical, 3 histidine ligated, mononuclear non-heme Fe²⁺ center. To assess the role in enzyme catalysis of the hydrophilic residues in the active site pocket, residues Glu98, Arg80, Tyr70 and Thr107 were subjected to mutational analysis. Steady state and pre-steady state kinetics indicated a role for Glu98 in promoting both substrate binding and O₂ reduction. Additionally, the Glu98-substitution eliminated the pH dependence of substrate binding ($k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ -pH profile) present in wild type Dke1 ($\text{p}K_{\text{a}}$ 6.3±0.4 and 8.4±0.4). MCD spectroscopy revealed that the Glu98→Gln mutation leads to the conversion of the six-coordinate (6C) resting Fe²⁺ center present in the wild type enzyme at pH 7.0 to a mixture of 5C and 6C sites. The 6C geometry was restored with a pH-shift to 9.5 which also resulted in ligand field (LF) energy splittings identical to that found for wild type (WT) Dke1 at pH 9.5. In WT Dke1 these LF transitions are shifted up in energy by ~300 cm⁻¹ at pH 9.5 relative to pH 7.0. These data, combined with CD pH titrations which reveal a $\text{p}K_{\text{a}}$ of ~8.2 for resting WT Dke1 and the Glu98→Gln variant, indicate the deprotonation of a metal ligated water. Together, the kinetic and spectroscopic data reveal a stabilizing effect of Glu98 on the 6C geometry of the metal center, priming it for substrate ligation. Arg80 and Tyr70 are shown to promote O₂ reduction, while Thr107 stabilizes the Fe(II) cofactor.

Mononuclear non-heme Fe²⁺ centers are important for the catalysis of a variety of highly specific O₂-dependent reactions(1-3). A common rationale is often employed in these enzymes: a facial triad of two histidines and one glutamate/aspartate together with 2-3 water molecules ligate the resting Fe²⁺ cofactor. In the presence of all reaction components, a catalytically competent 5-coordinate (5C) metal center is formed, which allows O₂ binding and reduction, coupled with substrate oxidation (3). The facial triad is considered to play various roles including (i) creating a high affinity Fe²⁺ binding site, (ii) providing a geometric scaffold that allows O₂ coordination to the Fe²⁺ center upon cosubstrate and/or substrate binding to the enzyme, and (iii) maintaining the appropriate redox-potential of the metal center.

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Supporting Information Available: QSAR analysis of Dke1-Fe²⁺-diketonate complex decay and the electronic substrate structure for wild-type and variant Dke1; k_{cat} - pH and $k_{\text{cat}}/K_{\text{M}}$ - pH profiles for Dke1 and variants; Stopped flow binding curves of PD to Dke1 wild-type and variants; CD-Spectra of Dke1 wild type and the Glu98Gln variant at varying pH; Overlay of the MCD spectra of WT and E98Q Dke1 at pH 9.5. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The facial triad is embedded in the protein environment, which typically affords selective binding and appropriate positioning of the substrate and, if required, co-substrate. The active site pocket may also provide proteinogenic residues that promote reaction steps via acid-base catalysis or H-bonding interactions. In the reaction mechanism of extradiol catechol ring-cleaving dioxygenases, results from detailed pre-steady state and steady state kinetic, mutational and computational studies have demonstrated roles for specific hydrophilic active site residues in substrate binding, O₂ reduction and C-C bond cleavage (4-7). Additionally, the H-bonding network in the active site will contribute to the de/stabilization of outer and inner shell water which can participate in catalysis. Metal-bound waters, furthermore, directly determine the metal center coordination number and, consequently, cofactor stability and the catalytic competence of the metal center. While a 6C metal center is believed to protect Fe²⁺ from uncoupled oxidation by O₂ when only cofactor is present, formation of a 5C center upon ligation of all (co)substrates is required for efficient catalysis. The facial triad itself has been shown to contribute directly to the H-bonding network of non-heme iron enzymes. In the α -ketoglutarate dependent dioxygenases, the carboxylate ligand of the facial triad plays a role in stabilizing water coordination through a H-bonding interaction between the non-coordinating oxygen of the carboxylate and the coordinated water (8).

In recent years, a small number of O₂ dependent non-heme iron enzymes have been found that show distinct metal binding motifs (9). The rationale for these deviations and the resulting consequences are under active investigation. In the case of the α -ketoglutarate- and O₂-dependent halogenases SyrB2 (10) and CytC3 (11), the natural substitution of the metal ligating carboxylate of the facial triad with alanine leaves space for a halide ion, which is the substrate in the halogenation reaction. This mechanistically driven deviation from the facial triad motif comes at the cost of losing a high affinity Fe²⁺ binding site in the resting enzyme.

In the C-C bond cleaving dioxygenase Dke1, which displays a high affinity 3 His Fe²⁺ binding site (Figure 1, PDB: 3bal (12)), a clear rationale for this deviation in metal binding motif, which it shares only with cysteine dioxygenase, is still missing (Footnote 2). We have studied the role of the first coordination sphere of Dke1, which constitutes the primary enzyme of the acetylacetone degradation pathway in *Acinetobacter johnsonii* (13,14), in metal binding and catalysis. Spectroscopic investigations, employing a combination of near-infrared circular dichroism (CD), magnetic circular dichroism (MCD), and variable-temperature, variable-field (VTVH) MCD spectroscopies have revealed that Dke1 follows the general mechanistic strategy of O₂ activation by the facial triad ligated Fe²⁺ center,, showing a 6C resting site and partial conversion to a 5C species upon substrate ligation (15). Mutation of the first coordination sphere to a facial triad leads to the destruction of the high-affinity metal binding site (16) preventing study of a restructured facial triad.

Here we report a detailed investigation of the role of the outer shell residues in the enzyme's structural stability and reactivity. Previous steady state and single turnover enzyme kinetics together with quantitative structure/activity relationship (QSAR) studies have allowed dissection of particular steps of the chemical reaction and have implied a chemical mechanism (17,18). The O₂ reduction is the rate limiting step of the enzymatic reaction under physiological conditions, and O₂ reduction and C-O bond formation are concerted. In this study, these methods are combined with mutational and CD and MCD spectroscopic methods to gain detailed insight into the interplay of second shell residues and the structure and function of the 3-His metal center. Results reveal marked effects of particular outer shell residues on cofactor stability, active site geometry, substrate binding and O₂ reduction rates. The roles of the respective amino acid residues in specific reaction steps are discussed.

Footnote 2Sequence alignments with cysteine dioxygenase suggest a 3-His metal binding motif also for cysteamine- and 3-mercaptopropionate dioxygenase. Direct evidence from protein structures however is not available.

2. Materials and Methods

2.1 Materials

2,4-Pentanedione (PD), 1,1,1-trifluoro-PD (TFPD), 4,4-difluoro-1-phenyl-1,3-butanedione (DFPB), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TFPB), phenyl-1,3-butanedione (PB) and other chemicals were obtained from Sigma Aldrich (St. Louis, MO, U.S.A.) except 1,1-difluoro-2,4-pentanedione (DFPD) which was from Matrix Scientific (Columbia, SC, U.S.A.). All substrates were purchased at highest available quality (>97% pure) if not otherwise stated. *Pfu* DNA polymerase was from Promega (Madison WI, U.S.A.). The other enzymes for molecular biology were purchased from MBI Fermentas (St. Leon-Rot, Germany). The expression vector pKYB1 was from New England Biolabs (Beverly, MA, U.S.A.).

2.2 Methods

2.2.1. Site-directed mutagenesis and enzyme preparation—Site-directed mutations were introduced via a standard two stage PCR protocol (19). Two separate primer extension reactions were performed using the respective forward and reverse oligonucleotide primer (Table 1) and the plasmid vector pKYB1- *dke1 Strep* (20) as a template. The latter contains the full-length *dke1* gene fused to an oligonucleotide encoding a 10-amino acid C-terminal affinity tag (21). Note that we have demonstrated, previously, that heterologous expression and the affinity tag do not alter the biochemical and catalytic properties of Dke1 (20), which we originally isolated from *A. johnsonii* (13,14). For the introduction of multiple mutations the respective mutation bearing analogous vector was used. The PCR protocol used 150 ng of template in a total volume of 25 μ L. Four cycles of separate amplification with 3U of *Pfu* DNA polymerase (Promega, Madison WI, U.S.A.) were followed by 18 cycles of amplification of the combined reaction mixtures using an annealing temperature of 62 °C. The final extension phase was 15 min at 72 °C. After digestion of the template DNA with *DpnI*, the amplified plasmid vectors were transformed into electro-competent cells of *E. coli* BL21(DE3) (Stratagene, La Jolla, CA, U.S.A.) according to standard procedures. Plasmid DNA from positive clones was isolated via a miniprep kit (Wizard Plus SV Minipreps, Promega) and subjected to dideoxy sequencing of the entire *dke1* gene, to verify that the clone bore the desired mutation and that no errors had been introduced into the sequence during amplification via PCR.

Production of recombinant Dke1 wild-type and variants Dke1 was done according to the procedures described previously (20). Purification was carried out according to reported protocols, using affinity chromatography with a 1-mL *Strep*-Tactin column (IBA, Goettingen, Germany) and subjected to buffer exchange by three passages of desalting via NAP-25™ columns (GE Healthcare, Chalfont St. Giles, Great Britain). using 20 mM Tris/HCl buffer, pH 7.5. Protein solutions were concentrated (≥ 10 mg mL⁻¹, > 95 % purity) with Vivaspin centrifugation concentrator tubes (Vivascience, Hannover, Germany) and stored at -20°C.

2.2.2. Fe²⁺ content and protein concentration—The content of protein-bound Fe²⁺ in purified enzyme preparations was determined spectrophotometrically, whereby the formation of a colored solution complex between Fe²⁺ and ferene S ($\epsilon_{592\text{ nm}} = 35.5\text{ mM}^{-1}\text{ cm}^{-1}$) (22,23) was monitored in an over-night measurement. All assays were performed at 25°C in 20 mM Tris/HCl buffer (pH 7.5), containing ferene S in excess (20 mM) and 2 mM-ascorbic acid as a reductant, to maintain Fe²⁺ in its reduced form over time. Blanks in the absence of protein were measured and subtracted. Protein concentrations were determined by measuring the absorbance at 280 nm of the appropriately diluted purified enzyme preparation in Tris buffer (20 mM, pH 7.5) and applying a theoretically calculated absorption coefficient from the respective sequence based on the method of Edelhoch (24) and the parameters of Pace (25) using the program ProtParam (<http://www.expasy.ch>). Note that this method, which uses native

protein, gave the same results as the standard procedure, which uses denaturing conditions (6M Guanidine-HCl buffer, 20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 6.5) within an experimental error of 5%. The method was furthermore validated using the BCA™ Method (Pierce, Rockford, IL, U.S.A.). Apparent 1st order iron detachment rates were determined using the Program Microcal Origin™.

2.2.3 Steady state kinetics—The standard assay of Dke1 activity was carried out at 25° C using 0.2 mM PD dissolved in air-saturated 20mM Tris/HCl buffer, pH 7.5. The rate of substrate depletion was measured spectrophotometrically at 280 nm and 25° C with a DU 800 UV–Vis Spectrophotometer (Beckmann Coulter, Inc., Fullerton, CA, U.S.A.).

Steady-state kinetic studies with PD and TFPD at different pH values were performed in 100 mM MES (pH 5.0 -6.5) and Tris (pH 6.5 -10.5) buffer, whereby the absorbance trace at the wavelength of maximum substrate absorbance was monitored (18) (Footnote 3). The pH dependence of extinction coefficients had been determined previously (18) and reaction rates were calculated accordingly. Specific activities and apparent $k_{\text{cat}}^{\text{app}}$ values were generally related to the concentration of Fe^{2+} containing enzyme active sites, which were calculated from the iron content in the gel filtrated protein preparation as described (vide supra).

$K_{\text{M}}^{\text{app}}$ and $k_{\text{cat}}^{\text{app}}$ values were obtained in two ways: (i) A series of classical initial-rate measurements at varied substrate concentrations and a non-linear fit of the Henri–Michaelis–Menten equation for a single-substrate enzymatic reaction to the data was performed. (ii) To validate initial rate data, the integrated form of the Michaelis Menten equation to expanded reaction time courses was applied, whereby 50 μM of substrate were used and rates were corrected for the decrease in O_2 concentration. It was shown that both approaches of kinetic analysis yielded consistent estimates for the apparent catalytic center activity ($k_{\text{cat}}^{\text{app}}$) and Michaelis–Menten constant ($K_{\text{M}}^{\text{app}}$) at 260 μM O_2 , whereby the integrated approach allows for a better assessment of rates at low substrate concentrations.

2.2.4. Substrate binding kinetics—Formation of the enzyme- Fe^{2+} –substrate complex was measured with a stopped-flow UV/Vis-spectrophotometer (SF61DX2, Hi-Tech, UK) housed in argon-flushed glove box (Belle Technology, UK), monitoring the prototypical low energy transition of the substrate ligated metal center at the wavelength of its maximum absorbance, λ_{max} (18). Stock solutions of substrate (300 μM) and enzyme (300 μM Fe^{2+} , 600 μM enzyme subunits) were used, which had been made anaerobic by repeated flushing with nitrogen. Note that the substrate concentration was fully saturating at the steady-state ($K_{\text{M}}^{\text{app}} < 10 \mu\text{M}$). The reaction was carried out at 25 °C in an air-saturated 20mM Tris/HCl buffer, pH 7.5.

Binding kinetics showed biphasic behavior and were best fit via the following formula describing double exponential decay:

$$A = a_0 - a_1 e^{-(k_1 t)} - a_2 e^{-(k_2 t)}$$

Whereby A is the absorbance, a_0 is the total amplitude, a_1 and a_2 are the amplitudes of the respective binding-steps that proceed at first order rate constants of k_1 and k_2 , respectively. All measurements were carried out in triplicate and resulting rate constants were averaged.

Footnote 3 Given that Dke1 activity is insensitive to a increase of ionic strength, as tested at salt concentrations up to 1 M NaCl, the change of ionic strength at various pH values was neglected.

2.2.5. CD and MCD spectroscopy—Apo-enzyme was prepared by dialyzing Dke1 against 20 mM EDTA in Tris/HCl buffer 20 mM, pH 7.5 at 4°C for 2 days using a Slide-A-Lyzer™ cassette (Pierce, Rockford, IL, U. S. A.), with repeated buffer exchange and another 2 days against the Tris buffer without EDTA. The resulting apo-enzyme was then buffer exchanged into 100 mM Tris/HCl in D₂O at the appropriate pD, using a 4-mL Amicon Ultra-4 filter with a 10 kDa cutoff membrane (Millipore), and the protein was then brought to a monomer concentration of ~1-2 mM. Reagents were made anaerobic by purging with argon gas on a Schlenk line. Dke1 was made anaerobic by alternating cycles of evacuation and argon purge on the Schlenk line at 0°C. All samples were prepared under an inert atmosphere inside a N₂-purged ‘wet box’ to maintain an O₂ free environment. Ferrous ammonia sulfate was added in microliter quantities to a final concentration of 90% of the monomer concentration. The samples were then transferred into an anaerobic cuvette and incubated for ~1 h in the glove box, prior to measurement. Saturating amounts of deuterated sucrose were added anaerobically to protein preparations as a glassing agent for MCD measurements to a final concentration of ~1mM active sites. CD measurements were taken without and with sucrose to ensure that the Fe²⁺ site was unaffected by the glassing agent. (Note that sucrose, rather than glycerol, was used as a glassing agent as the latter affected the NIR CD spectrum of the Fe²⁺ active site.)

Near-IR (600-2000 nm) CD and MCD data were recorded on a Jasco J-200D spectropolarimeter with a liquid N₂-cooled InSb detector and equipped with an Oxford Instruments SM4000-7 tesla (T) superconducting magnet. CD measurements were made at 5°C in an anaerobic cuvette. CD spectra were corrected for buffer and protein baselines by subtraction, and MCD spectra were corrected for the natural CD and zero-field baseline effects by averaging the positive and negative field data at a given temperature.

3. Results

3.1 Kinetic parameters and pH profile of WT-Dke1

The steady state kinetic parameters of WT Dke1 at pH 7.5 towards a range of dicarbonyl substrates have been investigated previously (17,18,25), (Footnote 4). In order to gain information on the ionizable groups involved in catalysis, $k_{\text{cat}}^{\text{app}}$ and $k_{\text{M}}^{\text{app}}$ values were determined using PD for WT Dke1 at pH values between 5 and 10. Fig. 2A shows these values as a function of pH.

The log ($k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$) versus pH, showed a clear bell shape with inflections in the acidic and basic pH regions with a slope of +1 and -1. Data were fit to equation 2:

$$Y = \text{LOG} (C / (1 + (K_1 / [\text{H}^+] / K_2)))$$

where Y is log ($k_{\text{cat}}^{\text{app}}$) or log ($k_{\text{cat}}^{\text{app}} / K_{\text{M}}^{\text{app}}$), C is the pH-independent value of Y, and K_1 and K_2 are the macroscopic dissociation constants for the ionizable groups being titrated. $[\text{H}^+]$ is the proton concentration. From this fit, $\text{p}K_{\text{a}}$ values of 6.3 ± 0.4 and 8.4 ± 0.4 were determined. Given that $k_{\text{cat}}^{\text{app}}$ mirrors the O₂ reduction rate, which is the first step of the chemical reaction (18), this indicates that some pre-catalytic step is influenced by either enzyme or substrate ionization and is therefore pH dependent. It should be noted that the substrate, PD, has a $\text{p}K_{\text{a}}$ of 8.9 and is considered to bind as a monoanion to the metal center (18,26). As a result, a change in its protonation state may well affect substrate affinity and substrate binding rates.

Footnote 4^{It} should be noted that Dke1 could not be saturated with O₂ up to $c_{\text{O}_2} = 1.2$ mM, preventing the determination of kinetic constants under saturating conditions.

The $k_{\text{cat}}^{\text{app}}$ values are constant over the pH range investigated, with only a slight decrease of the $k_{\text{cat}}^{\text{app}}$ observed above pH 10 and below pH 5.5, where protein instability began to interfere with activity measurements. Due to this protein instability, it was not possible to determine whether the slight curvature in the extremes of the plots reflects a protonation equilibrium. Data were fit by equation 2 (vide supra) and the resulting curve eliminated the possibility of a pK_{a} in the range of pH 4.2 – 10.0 (Fig. 2A).

To test if the protonation state of the substrate is affecting the enzyme kinetics, $k_{\text{cat}}/K_{\text{M}}^{\text{app}}$ -pH profiles for Dke1 were determined using TPD, the trifluorinated substrate analogue of PD, which has a pK_{a} of 6.6 (18,26), more than 2 log units lower than that of PD. The resulting $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ pH profile showed an inflection in the basic region with a pK_{a} value of 8.1 ± 0.2 , (Table 2 and supporting info, Figure S2B), the same as that for PD within experimental error. Therefore, the basic pK_{a} found in the $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ pH profile was clearly not due to substrate deprotonation. For pH values < 6.0 no reliable data could be obtained due to enzyme inactivation and slow reaction rates and a low extinction coefficient with TPD.

3.2 Kinetic parameters and pH profiles of second sphere variants of Dke1

3.2.1 Kinetic parameters—In order to gain insight into the role of H-bonding residues in dicarbonyl cleavage, hydrophilic amino acid residues with a potential role in catalysis were identified and subjected to mutagenesis. The structure of Dke1 reveals a mononuclear metallocenter, ligated by three histidines, His62, His64 and His 104, in an active site pocket predominantly lined with hydrophobic residues and accessible via a hydrophobic tunnel (PDB code: 3bal). Only four hydrophilic amino acid residues are present in the substrate binding pocket. A hydrophilic gate formed by Arg80, Glu98 and Tyr70 connects the hydrophobic cavity with the exterior (Figure 1). The glutamate residue, Glu98, is a second shell residue, positioned adjacent to the metal-ligating His104, with its proximal carboxylate O in H-bonding distance of the ϵ^2 N-H group of the histidine (2.82 Å). Furthermore, Glu98 forms a salt bridge with Arg80. This structure H-bonds to a water molecule, which is further H-bonded to Tyr70, forming the hydrophilic gate. The fourth hydrophilic residue found in the active site is Thr107, which is situated in the outer coordination shell of Fe^{2+} . All four residues were subjected to mutagenesis, and the resulting protein variants were characterized to elucidate their role in catalysis.

Variants were constructed, expressed and purified where Tyr70, Arg80, Glu98 and Thr107 were, individually and in combination, substituted with alanine. Additionally, the ‘structurally conservative’ mutants Tyr70→Phe and Glu98→Gln were produced. Iron contents were determined for all variants. Notably, for Thr107→Ala, the iron content was <5 % in all preparations, and reconstitution of this enzyme variant with Fe^{2+} could not be achieved, indicating a disruption of the metal binding site. All other variants showed iron contents of >50 % after purification, indicative of an intact metal binding site and allowing further characterization.

The steady state kinetic properties of all variants and typical Fe^{2+} content are given in Table 2 and summarized below. The substitution of Glu98 with alanine showed a remarkable, ~100-fold reduction in $k_{\text{cat}}^{\text{app}}$ (0.074 s^{-1}). The analogous substitution with glutamine resulted in a similarly diminished reaction rate ($k_{\text{cat}}^{\text{app}} = 0.050 \text{ s}^{-1}$). A comparable decrease of $k_{\text{cat}}^{\text{app}}$ by two orders of magnitude was also found for the Arg80→Ala variant ($k_{\text{cat}} = 0.126 \text{ s}^{-1}$). The substitution of Tyr70 with alanine resulted in a 60 fold decrease in $k_{\text{cat}}^{\text{app}}$ while the structurally conservative mutation to phenylalanine lead to a minor, 3 fold decrease of the catalytic rate. No major changes were found for the $K_{\text{M}}^{\text{app}}$ values of the variants investigated. Thus, mutations perturbing the ‘hydrophilic gate’, formed by Glu98, Arg80 and Tyr70, led to a reduction of $k_{\text{cat}}^{\text{app}}$ by two orders of magnitude. Dke1 variants bearing double- and triple mutations were constructed in order to characterize the interplay of the particular mutations. Simultaneous

substitution of Glu98 and Arg80 with alanine resulted in $k_{\text{cat}}^{\text{app}}$ values comparable to the respective single mutations. The additional exchange of Tyr70, thus completely abolishing the hydrophilic gate, had an additional diminishing effect on $k_{\text{cat}}^{\text{app}}$.

In a previous study on wild type Dke1 it was determined that the reduction of O_2 , which coincided with the enzyme-substrate complex decay rate ($k_{\text{decay}}^{\text{app}} = k [\text{O}_2]$), constituted the rate limiting step of catalysis ($k_{\text{cat}}^{\text{app}}$). This rate was characterized by a strong dependence of $k_{\text{cat}}^{\text{app}}$ on the potential of the substrate to donate electrons which is mirrored by the energy level of its highest occupied molecular orbital (ϵ_{HOMO}) (18). Analogous correlations of $k_{\text{decay}}^{\text{app}}$ with the ϵ_{HOMO} of the substrate, obtained for the above variants, show that the nature of the rate-limiting step has not changed upon mutation (see supporting information, Figure S1).

3.2.2 pH profiles—In order to investigate the apparent role of ionizable groups in the pre-catalytic steps of reactivity in Dke1, pH profiles of the steady-state kinetic parameters for the variants Glu→98Ala, Arg80→Ala and Tyr70→Phe were determined. Importantly, the $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ -pH profiles of the Glu→98Ala and Glu→98Gln variants of Dke1 showed an apparent elimination of both the acidic and basic pK_{a} effects (Figure 2B and supporting info Figure S2A), (Footnote 5). Alternatively, for the variants Arg80→Ala and Tyr70→Phe, the basic pK_{a} values were conserved, demonstrating that these residues are not associated with the basic $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ -pH curve inflection (supporting information, Figure S2C and S2D). As in WT Dke1, the $k_{\text{cat}}^{\text{app}}$ rates were not pH dependent in all cases, over the entire pH range investigated. Results from $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ -pH profiles are summarized in Table 2. The $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ -pH profiles and $k_{\text{cat}}^{\text{app}}$ -pH profiles for all variants are shown in the supporting information.

3.3 Stopped flow kinetic analysis of substrate binding

In order to further define the pH-dependence of the precatalytic steps and, in particular, the apparent elimination of the pH dependence of the $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ -pH profiles for the Glu98 variants, the substrate net-binding rates of wild type Dke1 and variants were determined by stopped flow kinetic measurements. Substrate ligation to the metal center was monitored by measuring the formation of an absorption band which evolves upon ligation of a diketonate to the Fe^{2+} cofactor and has been suggested previously to result from a MLCT transition (18).

Substrate binding was measured using PD and DPD as ligands. The latter substrate binds more slowly than PD, and allows for the initial, fast binding phase to be monitored (18). Variation of ligand concentration (0.15–5 mM) did not lead to a change in the binding rate under the conditions used (inset, Figure 3A), which implies that ligation of the diketonate to the metal cofactor was measured under saturating conditions, and substrate ligation is not the first kinetic step of substrate binding. This is in line with a scenario where the rates observed mirror the net rate of exchange of water and substrate ligands at the Fe^{2+} center and not prebinding steps.

Binding rates of DPD to the variants Glu98→Gln, Glu98→Ala, Tyr70→Phe and Arg80→Ala were measured at pH 7.5 (Figure 3A, Table 3). Generally, curves were best fit with a double exponential decay, indicating a biphasic binding event. In the E98Q and E98A variants the binding velocity is decreased by two (k_1) and three (k_1) orders of magnitude, respectively. The substitutions of Tyr70→Ala and Arg80→Ala only caused a moderate, ten-fold, reduction in binding rates. Binding curves with PD as a ligand showed the same general trend as found for DPD (see supporting information, Figure S3A), where, with the exception of the Glu98

Footnote 5Protein instability precludes measurements of the $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ above pH 10. The curvature in the $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ pH profile at high pH cannot be fit with equation 2, ruling out a $\text{pK}_{\text{a}} < 10$.

variants, the initial binding phase was too fast to be assessed. Results are summarized in Table 3.

In order to further investigate the elimination of the basic pK_a effect on the k_{cat}^{app}/K_M^{app} -pH profile upon Glu98 substitution, binding kinetics for WT Dke1 and the Glu98→Gln variant were determined at varying pH. Measurements were performed at pH's of 7.5 and 9.5, which are approximately one log unit below and one unit above the pK_a of 8.4, determined from the k_{cat}^{app}/K_M^{app} -pH profile of WT Dke1 (vide supra). The resulting curves obtained for DPD ligation are shown in Figure 3B and the associated rate constants are given in Table 3. The low and high pH forms of the Glu98→Gln variant clearly show different binding characteristics, with the low pH form showing a biphasic binding behavior with a pronounced slower phase compared to the apparently monophasic high pH form.

In WT Dke1, the analogous shift resulted in a ~30 (k_I) and ~80-fold (k_{II}) decrease in binding rates. PD binding curves, again, showed the same trend as found for DPD (see supporting information, Figure S3B), however, fast substrate binding rates and a probable change of substrate protonation state in the pH range investigated influence the substrate binding rates and complicate the data analysis. The sensitivity and robustness of substrate binding rates of WT Dke1 and the Glu98 variants, respectively, to a pH shift from 7.5 to 9.5 mirror their k_{cat}^{app}/K_M^{app} -pH profile characteristics.

Taken together, the results from stopped flow kinetics show that Glu98 promotes substrate binding at $pH < pK_a$ 8.4. Given that a pK_a of 8.4 is extraordinarily high to be associated with glutamic acid ionization and taking into account the fact that there is no other ionizable group in the active site, one explanation for this effect is a possible role of metal bound water in the basic pK_a process. (Note that Fe^{2+} -bound water usually shows a pK_a of ~9, depending on the environment.) A combination of near-infrared (NIR) CD and MCD was employed to directly probe the coordination at the Fe^{2+} site as a function of pH in WT Dke1 and its E98Q variant.

3.4 NIR-CD and MCD spectroscopy of the metal center in wild type Dke1 and its Glu98Gln variant

CD and MCD are spectroscopic techniques that provide information about the coordination environment and ligand field at a metal cofactor site (27,28). In this way, these techniques can report changes at the active site complementary to the kinetic results. Wild type (WT) Dke1 and the E98Q variant were studied with CD and MCD spectroscopy to explore the pH dependence observed in the kinetic measurements. (pD's are used here as the data must be obtained in D_2O .) A detailed description of the effects of the 3His ligand set on the Fe^{2+} center in Dke1 relative to the two His-, one carboxylate facial triad is forthcoming (15) and will not be covered here.

WT Dke1—CD spectra of WT Dke1 were taken between pD 7.0 and 9.5. The CD spectrum at pD 7.0 shows two transitions, one at 8,000 (positive feature) and one at 10,500 cm^{-1} (negative feature). Upon increasing pD, the spectrum shifts to one dominant positive peak at ~ 11,000 cm^{-1} (Figure 4A and supporting information Figure S4A). A plot of $\Delta\epsilon$ versus pD for the transition at 11,300 cm^{-1} fits to a titration curve with a pK_a of 8.2 ± 0.2 (Figure 4A, inset).

To investigate whether the signals observed in CD were due to a change in the ligand field at the iron center or to a conformational change in the protein, low temperature MCD spectra were taken of the pD 7.0 and 9.5 samples. At pD 7.0, two positive transitions are observed centered at ~ 10,100 cm^{-1} and split by ~ 1900 cm^{-1} , which is characteristic of a 6 coordinate site. (From extensive studies on structurally defined Fe^{2+} model complexes, 6 coordinate Fe^{2+} exhibits 2 transitions in the ~10,000 cm^{-1} region, split by ~2,000 cm^{-1} , 5 coordinate shows 1 transition at ~10,000 cm^{-1} and one at ~5,000 cm^{-1} and 4 coordinate only shows transitions

in the 5,000 – 7,000 cm^{-1} region.) (27,28) Upon increase of the pD to 9.5, a very similar two transition pattern is observed; however, the transitions are shifted up in energy by $\sim 300 \text{ cm}^{-1}$ compared to pD 7.0 (Figure 4B).

Dke1 Glu98→Gln variant—To further explore the apparent effect of Glu98 on the pK_a of substrate binding, CD and MCD experiments were done in parallel to those described above for the wild type enzyme. The CD spectra for the E98Q variant show one main peak at $\sim 11,000 \text{ cm}^{-1}$ that shifts up in energy (to $\sim 11,300 \text{ cm}^{-1}$) and up in intensity with increasing pD (Figure 5A, and supporting information, Figure S4B). The inset of Figure 5A shows a plot of this intensity versus pD for E98Q Dke1. These data can be fit to a pD equilibrium with a pK_a of 8.2 ± 0.2 . The low temperature MCD spectrum of the E98Q variant at pD 7.0 shows a significant spectral change compared to wild type. A low energy feature at $\sim 5,000 \text{ cm}^{-1}$ is now observed, characteristic of the presence of a 5C Fe^{2+} species. The more intense asymmetric peak centered at $\sim 9,700 \text{ cm}^{-1}$ suggests two overlapping contributions and since a 5C component can only contribute two transitions in the NIR spectral region, the third peak indicates the additional presence of a 6C Fe^{2+} component. Upon increase of the pD to 9.5 in the E98Q variant, the spectrum changes to two transitions centered at $\sim 10,400 \text{ cm}^{-1}$. The spectrum indicates that only a 6C Fe^{2+} species is now present. Additionally, these transitions occur at the same energies as those observed for the WT enzyme at pD of 9.5 (supporting information Figure S5).

3.5 Auto-oxidation studies of the E98Q variant

In order to determine whether the change of metal center geometry to a partial five-coordinate species had an impact on the auto-oxidation rate of the Glu98Gln variant, the O_2 consumption of the resting Glu98Gln variant was monitored at 25°C . Notably, iron detachment rates ($\sim 0.0003 \text{ s}^{-1}$), estimated by the fereneS method (23), are 2-3 orders of magnitude faster than apparent auto-oxidation rates determined for WT Dke1 at air saturation (18). Therefore free iron will be present in equilibrium throughout the enzymic auto-oxidation ($K_{\text{d-Fe}} = 5 \text{ }\mu\text{M}$ for WT Dke1 (16)), and consequently, the specific rate of auto-oxidation ($k_{\text{autoox}}^{\text{app}}$) will be complex, representing the oxidation of both bound and unbound iron. For the E98Q variant a biphasic trace for O_2 consumption was observed, with a fast decay of $\sim 15 \text{ }\mu\text{M O}_2$ followed by a slow rate of O_2 consumption (Figure S6, supporting information). The first, fast, phase of O_2 consumption shows the typical oxidation rate of unbound Fe^{2+} ($k_{\text{Fe}} = 0.016 \text{ mM}^{-1} \text{ s}^{-1}$, Figure S7, supporting information) which is generally expected to be present in Fe(II) containing enzyme preparations in equilibrium. The second phase reflects the auto-oxidation of the E98Q variant and has a specific rate of autooxidation ($k_{\text{autoox}}^{\text{app}}$) of $1.2 \times 10^{-6} \text{ s}^{-1}$ related to Fe^{2+} . This value compares well to the slightly higher rate of autooxidation in Dke1 WT ($k_{\text{autoox}}^{\text{app}} = 7.5 \times 10^{-6} \text{ s}^{-1}$) (18), showing that the apparent auto-oxidation rate in the Glu98Gln variant is not elevated compared to wild type Dke1.

Discussion

The hydrophilic residues in the substrate binding pocket of Dke1 are crucial for enzyme function in terms of (i) cofactor stability, (ii) stabilization of the six coordinate geometry at the metal center and (iii) promotion of the transition state for O_2 reduction.

The effect of 2nd shell residues on cofactor stability in Dke1

Thr107, stabilizes Fe^{2+} binding and mutation leads to loss of cofactor affinity and function. In the crystal structure of Dke1 (Figure 1), Thr107 is in H-bonding distance of putative metal-bound waters (note that first shell water molecules are missing in the crystal structure), suggesting a stabilization of the water ligands. Destabilization of the metal center by an order of magnitude following mutation of a 2nd shell threonine has been reported previously for the 3-His ligated metallocenter of Zn^{2+} -dependent carbonic anhydrase. In this case, the crystal

structure of the variant showed subtle geometric changes in the water shell (29,30). In Dke1, for which a K_d of 5 μM for Fe^{2+} has been determined previously (16), a decrease in affinity by an order of magnitude will lead to elimination of the high affinity metal binding site.

Alternatively, Glu98, which is also located in the 2nd shell of the metal center and interacts with the ϵ^2 nitrogen of His 104 by a 2.86 Å H-bond, shows no stabilizing effect on the metal center. The Glu98 variants of Dke1 actually lead to a 4-fold decrease in Fe^{2+} detachment rates from 0.002 s^{-1} to 0.0005 s^{-1} . Second sphere carboxylate residues H-bonded to the non-coordinated nitrogen of a metal-ligated histidine have been reported to increase metal-cofactor stability when the carboxylate acts as a strong H-acceptor (31,32). In Dke1, the H-bonding interaction of Glu98 with Arg80 (Figure 1) lowers the pK_a of Glu98 and likely prevents a similar role for Glu98 here.

Stabilization of the 6C Fe^{2+} center

A detailed kinetic analysis of wild type Dke1 revealed new insight into the precatalytic steps of diketonate cleavage. Taking into account the proposed mechanism of Dke1 (18), where the diketonate coordinates to the Fe^{2+} cofactor and the substrate ligated metal center reduces O_2 via an encounter complex, precatalytic steps mirror the event of substrate binding. The $k_{\text{cat}}^{\text{app}}/K_M^{\text{app}}$ pH-profile shows a bell shape with inflections in the acidic (pK_a 6.3) and basic (pK_a 8.4) ranges, corresponding to a pH dependence of binding rates, as $k_{\text{cat}}^{\text{app}}$ values are insensitive to pH in the range investigated (Figure 2). Protein instability prevented a detailed characterization of the low pH effect. The high pK_a was characterized further by stopped flow measurements and CD and MCD spectroscopy.

Transient substrate binding kinetics of WT enzyme generally showed a > 30 fold decrease of the rate of substrate ligation to Fe^{2+} at pH 9.5 compared to pH 7.5, which parallels the pH effect seen in the $k_{\text{cat}}^{\text{app}}/K_M^{\text{app}}$ profile. Note that substrate binding rates determined were independent of DPD concentration in the range of 0.15 mM to 5 mM showing that substrate ligation to the metal center is not the first kinetic step of substrate binding. It is therefore reasonable to view the velocities determined as net rates of the ligand binding to the metal center and independent of access to the site. A reduction in the rate of binding at high pH suggests a stronger ligand set at the Fe^{2+} center. This is consistent with deprotonation of one of the water ligands at the Fe^{2+} center. The CD pH titration, which directly probes the Fe^{2+} center, revealed a pK_a of 8.2 for WT Dke1. The shift of the ligand field in the MCD data to higher energy (Figure 4B) indicates a change at the Fe^{2+} center to a stronger ligand. These data support a coordinated water-hydroxide equilibrium in WT Dke1. Hydroxide will be displaced more slowly than water by the substrate ligand, leading to decreased binding rates (Footnote 6). This is mirrored by an increased K_M^{app} value in the enzyme kinetics of Dke1 and, consequently, the inflection of the $k_{\text{cat}}^{\text{app}}/K_M^{\text{app}}$ -pH profile. A water-hydroxide equilibrium at this pH range is not commonly found for mononuclear non-heme Fe^{2+} sites and represents a significant difference between the 3His ligation to Fe^{2+} in Dke1 and the facial triad Fe^{2+} ligation seen for many other ferrous enzymes. This depressed pK_a of bound water likely reflects the reduced donation from the third histidine ligand compared to the carboxylate found in the standard facial triad enzymes.

Investigation of the Glu98→Gln variant by CD spectroscopy showed a pH-dependent signal change with a pK_a at pD ~8.2, analogous to the wild type enzyme. In the MCD data of E98Q Dke1 at pD 7.0, a mixture of 5C and 6C species is present. The presence of this 5C Fe^{2+} species indicates that there is a reduced affinity for H_2O in the absence of the carboxylate. Previous

Footnote 6 Note that the MCD spectra show a complete conversion to the PD-ligated metal center at pD 7.0 (14) In the kinetics, extinction coefficients of the enzyme-substrate complex are not significantly changed at high pH or with DPD as a ligand. This indicates that despite a shift in net binding rates, at equilibrium, substrate ligation to the metal center is > 90%.

work has suggested that a carboxylate hydrogen bond can stabilize water binding to an Fe^{2+} site. (8) The MCD data for the E98Q variant at high pH show a completely 6C site. Hydroxide binds more tightly than water to the Fe^{2+} center, and increasing the pH results in deprotonation of the water ligand giving an hydroxide that stays bound due to its increased affinity for Fe^{2+} . Combined, these data strongly support the model that the pK_a reflects deprotonation of a metal-bound water ligand. Therefore, Glu98 plays a role, directly or indirectly, in the stability of the bound water.

In the $k_{\text{cat}}^{\text{app}}/K_M^{\text{app}}$ -pH profile of E98Q, both of the pK_a effects have been eliminated. The acidic pK_a may mirror slowed binding due to the protonation of Glu98, however protein instability prevented further exploration of this effect. Pre-steady state kinetic substrate binding studies of the Glu98→Gln and Glu98→Ala variants showed a marked, > 100 fold, decrease in net binding rates. Contrary to the wild type enzyme, substrate ligation rates at pH 7.5 and pH 9.5 were in the same time regime (Figure 3B) with k_1 values of $0.30 \pm 0.02 \text{ s}^{-1}$ and $0.28 \pm 0.03 \text{ s}^{-1}$, respectively, consistent with the pH profile results that substrate binding velocities show no marked pH dependence between pH 7.5 and 9.5. The MCD data on E98Q show that the water-hydroxide equilibrium observed in WT is still present in this variant. Thus, the elimination of the pH effect on the E98Q variant is not due to the direct elimination of a water-hydroxide equilibrium at the Fe^{2+} active site but a more indirect effect of a protein structural change due to the mutation in the vicinity of the site.

Effect of the hydrophilic gate

Glu98, Arg80 and Tyr70 form a hydrophilic gate that allows small hydrophilic molecules like water and the metal ion cofactor, to access the hydrophobic active site. Mutation of each residue to the smaller alanine destroys this gate. O_2 reduction rates in these variants are diminished by 2 orders of magnitude, confirming their crucial role in catalysis. The $k_{\text{cat}}^{\text{app}}$ rates are insensitive to pH in the range investigated, showing that acid-base catalysis is not involved in the rate limiting step of O_2 reduction. One role of the gate may be in providing an H-bond that stabilizes the developing negative charge on O_2 in the transition state of the rate determining step of O_2 reduction, a scenario invoked for the early steps of the mechanism in Mn^{2+} dependent catechol dioxygenase (33). Loss of this H-bond can cause a 100 to 1000-fold reduction in the rate (10-20 kJ/mol). In the crystal structure of Dke1, Glu98, Arg80 and Tyr70 are oriented such that they could position one water molecule to provide this H-bond. Alternatively, destruction of the gate will allow more water molecules to enter the active site and this may interfere with O_2 reduction – Further studies will be required to characterize the impact of active site hydrophilicity on catalysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Dke1	Diketone Cleaving Enzyme
6C, 5C	six-coordinate, five-coordinate

LF	ligand field
CD	circular dichroism
(VTVH) MCD	variable-temperature, variable-field magnetic circular dichroism
QSAR	quantitative structure/activity relationship
PD	2,4-Pentanedione (PD)
TFPD	1,1,1-trifluoro-PD
DFPB	4,4-difluoro-1-phenyl-1,3-butanedione
TFPB	4,4,4-trifluoro-1-phenyl-1,3-butanedione
PB	phenyl-1,3-butanedione
DFPD	1,1-difluoro-2,4-pentanedione
WT	wild type

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Figure 1. Metallocenter and H-bonding network in Dke1, the primary enzyme of the acetylacetone degradation pathway in *A. johnsonii* (PDB: 3bal, (12)). First sphere histidines and hydrophilic active site residues are labeled accordingly. Note the structure is for the Zn substituted form and no H₂O was resolved. Carbons, oxygens, nitrogens and sulfurs are depicted in grey, red, blue and yellow, respectively. H-bonds are in blue.

Figure 2. k_{cat}^{app}/K_M^{app} - and k_{cat}^{app} profiles for A - wild type Dke1 and B - the Glu98→Gln variant, obtained at 25°C in air saturated buffer.

Figure 3. Stopped flow traces of the Dke1-metallocenter with 1,1-difluor-2,4-pentanedione (DPD). Traces were averaged from triplicate measurements. Panel A shows substrate binding rates for Dke1 wild type (violet) and the outer sphere variants Tyr70Phe (blue), Arg80Ala (teal), Glu98Ala (dark grey), Glu98Gln (black). The inset shows substrate ligation to the Fe²⁺ center at varying DPD concentrations. Panel B shows the pH dependence of substrate binding to Dke1 wild-type and the E98Q variant: traces show wild type Dke1 at pH 7.5 (violet) and at pH 9.5 (red), while the respective traces obtained for the Glu98→Gln variant are in black (pH 7.5) and blue (pH 9.5). Analogous rates for binding of 2-4 pentanedione are presented in the supplementary information section.

Figure 4. CD and LT MCD data for WT Dke1. A – CD for pD 7.0 (purple) and 9.5 (green). Inset gives intensity at 11,300 cm⁻¹ as a function of pD. B – LT MCD at 5K and 7T for pD 7.0 (purple, $\Delta\epsilon \times 1.5$) and 9.5 (green).

Figure 5. CD and LT MCD data for E98Q Dke1. A – CD for pD 7.0 (red, $\Delta\epsilon \times 4$) and 9.5 (teal). Inset gives intensity at 11,300 cm^{-1} as a function of pD. B – LT MCD at 5K and 7T for pD 7.0 (red) and 9.5 (teal).

Table 1

Primers and template-DNA used in this study to generate Dke1 variants. Mutations introduced are underlined in the primer sequence.

Target-variant	Template: ^a	Primer (5' → 3')
T107A	WT ^b	fw: 5'-CATTGCATGGTAAAGCTTTCTTTCC rev: 5'-CGACAGGAAAGAAAGCTTTACCATC
E98Q	WT	<div><div>fw: 5'-CCAGCTACGGTTTTGCGTCTTCAGGTGCA</div><div>rev: 5'-CAATGCACCTGAAGACGCAAAACCGTAGCT</div></div>
E98A	WT	fw: 5'-CCAAGCTACGGTTTTGCGTCTTCAGGTGCA rev: 5'-CAATGCACCTGAAGACGCAAAACCGTAGCT
R80A	WT	fw: 5'-GAAAAATGGAAGTGGGCTGGTGGCGA rev: 5'-CTTGCTCGCCACCAGCCACTTCCAT
E98A_R80A	E98A	fw: 5'-GAAAAATGGAAGTGGGCTGGTGGCGA rev: 5'-CTTGCTCGCCACCAGCCACTTCCAT
Y70F	WT	fw: 5'-CATGCTGGCCCCGGGGGAATTTTCCTGAC rev: 5'-CCCTTAGTCAGGAAAAATTCCCCGGGGGC
E98A_R80A_Y70F	E98A_R80A	fw: 5'-CATGCTGGCCCCGGGGGAATTTTCCTGAC rev: 5'-CCCTTAGTCAGGAAAAATTCCCCGGGGGC
Y70A	WT	<div><div>fw: 5'-CATGCTGGCCCCGGGGGAATTTTCCTGAC</div><div>rev: 5'-CCCTTAGTCAGGAAAAATTCCCCGGGGGC</div></div>
E98A_R80A_Y70A	E98A_R80A	fw: 5'-CATGCTGGCCCCGGGGGAATTTTCCTGAC rev: 5'-CCCTTAGTCAGGAAAAATTCCCCGGGGGC

^aThe template was the pKYB1- *dke1*_Strep vector, bearing the respective, outlined mutation.

^bWild type plasmid.

Table 2

Steady state kinetic properties of Dke1 WT and variants. Kinetic constants were determined in air saturated buffer, at pH 7.5 and 25°C. the typical iron content of an enzyme preparation (Fe^{2+}) is given and $\text{p}K_{\text{a}2}$ values obtained for the ‘basic’ inflection in the $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ - pH correlation of WT and each variant is given. The $k_{\text{cat}}^{\text{app}}$ values showed are relative standard deviation of < 20 %. The experimental error of all Fe^{2+} contents was < 10 % and corresponded to their total iron determined by ICP-MS (error < 1%)

Variant	$k_{\text{cat}}^{\text{app}}$ [s^{-1}]	$K_{\text{M}}^{\text{app}}$ [mM s^{-1}]	Fe^{2+} [%]	$\text{p}K_{\text{a}2}$
WT	6.6	9.1±1.5 (21)	70	8.4±0.4 (8.1±0.2) ^a
E98A	0.074	4.1±2	60	- / > 10
E98Q	0.050	2.9±2	71	- / > 10
R80A	0.126	37±15	50	9.0 ± 0.5
Y70F	1.27	8.5±3	64	9.1 ± 0.5
Y70A	0.13	35±15	51	n. d.
T107A	0.1	n.d. ^b	5	n. d.
E98A_R80A	0.098	35±15	47	n. d.
E98A_R80A_Y70F	0.033	n.d. ^b	38	n. d.
E98A_R80A_Y70A°	0.007	n.d. ^b	68	n. d.

^b No variation of catalytic rates at substrate concentrations of 400μM and 600 μM were obtained, therefore the resulting velocities were considered to mirror enzyme saturation and, therefore, $k_{\text{cat}}^{\text{app}}$.

^a The $\text{p}K_{\text{a}}$ for the substrate TPD is given in brackets.

Table 3

Kinetic 1st order rate constants of substrate ligation for Dke1 wild type and variants from stopped flow kinetic studies. Data were obtained at 25°C and at air saturation. The experimental error of all rate constants is >20 %.

Dke1 variant	Substrate	pH	$k_f \text{ s}^{-1}$	$k_r \text{ s}^{-1}$
WT	PD	7.5	> 2000	180
WT	DPD	7.5	55	10
E98Q	PD	7.5	1.3	0.15
E98Q	DPD	7.5	0.28	0.02
E98A	DPD	7.5	0.23	0.008
R80A	PD	7.5	56	-
R80A	DPD	7.5	13.5	0.75
Y70F	PD	7.5	7.15	-
Y70F	DPD	7.5	1.9	0.30
WT	PD	9.5	330	25
WT	DPD	9.5	1.6	0.13
E98Q	PD	9.5	1.7	0.12
E98Q	DPD	9.5	0.3	-