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pH-Rate Profiles Establish that Polyketide Synthase Dehydratase Domains Utilize a Single-Base Mechanism

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Abstract

FosDH1 from module 1 of the fostriecin polyketide synthase (PKS) catalyzes the dehydration of a 3-hydroxybutyryl-SACP to the (*E*)-3-butenoyl-SACP. The steady-state kinetic parameters, k_{cat} and k_{cat}/K_m , were determined over the pH range 3.0 to 9.2 for the FosDH1-catalyzed dehydration of the *N*-acetylsteamine thioester, 3-hydroxybutyryl-SNAC (**3**), to (*E*)-3-butenoyl-SNAC (**4**). The pH rate profiles for both $\log(k_{\text{cat}})$ and $\log(k_{\text{cat}}/K_m)$ each corresponded to a single pH-dependent ionization to give an active site general base, with a calculated $\text{p}K_a$ 6.1 ± 0.2 for k_{cat} and $\text{p}K_a$ 5.7 ± 0.1 for k_{cat}/K_m . These results are inconsistent with the commonly suggested “two-base” (base-acid) mechanism for the dehydratases of PKS and fatty acid biosynthesis and support a simple one-base mechanism in which the universally conserved active site His residue acts as the base to deprotonate C-2 of the substrate, then redonates the proton to the C-3 hydroxyl group to promote C–O bond cleavage and elimination of water. The carboxylate of the paired Asp or Glu residue is thought to bind and orients the hydroxyl group of the substrate in the stereoelectronically favored conformation.

Graphical Abstract

PKS dehydratases utilize a one-base mechanism

One or more dehydratase (DH) domains are present in all of the dozens of biochemically characterized modular polyketide synthases (PKSs), as well as in the nearly 15,000 predicted modular PKSs that have been revealed by bacterial genome mining.^{1–4} Each such DH domain is responsible for the dehydration of a specific 3-hydroxyacyl-acyl carrier protein (ACP) or 2-alkyl-3-hydroxyacyl-ACP intermediate to give the corresponding enoyl-ACP or 2-alkylenoyl-ACP. These unsaturated intermediates are in turn either reduced by a paired enoyl reductase domain to the corresponding saturated acyl-ACP or, more commonly,

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Conflicts of interest

There are no conflicts to declare.

Electronic Supplementary Information (ESI) available: LC-MS data, steady-state kinetic plots, Table of pH-dependent kinetic parameters.

passed directly to the proximal downstream PKS module for a further round of polyketide chain elongation and modification.

Polyketide synthase DH domains exhibit significant levels of mutual amino acid sequence similarity and protein structural homology to the DH domains of type I fatty acid synthases (FASs), as well as to the closely related discrete DH enzymes, FabA and FabZ, of type II bacterial fatty acid biosynthesis. All known PKS and FAS dehydratase protein structures display characteristic $\beta + \alpha$ double hotdog folds, in which each active site harbours a universally conserved dyad of His and Asp or Glu residues, typically separated by 4.1 Å, that are positioned above a largely hydrophobic substrate binding pocket.^{5–12} Site-directed mutagenesis experiments have confirmed that these paired His and Asp or Glu residues are each essential to the catalytic activity of DH enzymes.¹¹ DH domains from several modular PKSs,^{6, 13–16} as well as FabA and FabZ proteins^{17, 18} and the analogous DH domain of the multidomain yeast FAS,¹⁹ have all been shown to catalyse stereospecific *syn* dehydration of their respective 2-methyl-3-hydroxyacyl-ACP or 3-hydroxyacyl-ACP substrates.

In early speculation on the mechanism of the β -hydroxydecanoyl dehydratase-isomerase (FabA) and the closely related dehydratase (FabZ) of *E. coli*, Schwab^{18, 20, 21} extended the original ideas of Rose and Hanson on the relationship between enzyme stereospecificity and mechanism²² to point out that the demonstrated overall *syn* (suprafacial) stereochemistry of the dehydratase-catalyzed reaction favors a one-base mechanism (while not excluding an alternative “two-base” (more correctly, base-acid) process), whereas the alternative *anti* (antarafacial) steric course would be compatible only with a “two-base” (base-acid) mechanism (barring rotation of the reaction intermediate in the dehydratase active site) (Scheme 1). Nonetheless, there have been no reported experiments directed at distinguishing between these canonical one-base and base-acid mechanisms. Indeed, since 1996, following the structural characterization of FabA and FabZ proteins from a variety of microbial sources, a base-acid dehydration mechanism has come to be commonly invoked,^{8–13} inspired largely by the observed spatial relationship between the substrate and the His – Asp/Glu dyads, in which the universally conserved active site His residue is proposed to serve as the general base to remove the H-2 proton of the 3-hydroxyacyl-ACP substrate, with the paired Asp or Glu side chain carboxyl thought to act as the general acid to protonate the 3-hydroxyl oxygen and thus promote the requisite cleavage of the C–O bond (Scheme 1b). As superficially appealing as such proposals might seem, however, the base-acid mechanism has an intrinsic flaw, since it would require that the pK_a of the normally acidic Asp carboxylate (solution $pK_a \sim 4$) exceed that of the His imidazolium/imidazole pair (solution $pK_a \sim 6$). Although the observed pK_a of an active site amino acid side chain can differ by 2–3 pH units from those of the corresponding free amino acids (and in one case an active site Asp has been reported to have a $pK_a > 9$),²³ to date there has been no direct experimental evidence to support the requisite perturbations of the pK_a of the active site Asp/Glu or His residues of any PKS or FAS dehydratase. Although some investigators have pointed out this apparent conundrum,^{8, 24} to the best of our knowledge there have been no published studies of the pH dependence of any FAS dehydratase, and only a single determination of the pH rate profile for the k_{cat}/K_m of a PKS DH domain.²⁵ We now report that the pH-rate behavior of both k_{cat} and k_{cat}/K_m of a prototypical PKS DH domain corresponds to a single ionization to an active basic form, consistent only with a single-base dehydration mechanism, and

therefore inconsistent with an alternative base-acid mechanism requiring two pH-dependent ionizations.

Results

We have recently established that FosDH1, from module 1 of the fostriecin polyketide synthase, catalyzes the reversible stereospecific dehydration of (3*R*)-3-hydroxybutyryl-SFosACP1 (**1**) to (*E*)-2-butenoyl-SFosACP1 (**2**) (Scheme 2). FosDH1 is a prototypical dehydratase, as typified by the presence of the universally conserved active site His-Asp dyad, housed within characteristic DHXXXGXXXXP and DXXXXH motifs that are common to PKS and FAS DH domains and proteins.

In order to determine the pH dependence of the FosDH1-catalyzed dehydration reaction, we chose, as surrogates for the natural -S-FosACP1 substrate and product, the corresponding *N*-acetylcysteamine analogues, (3*R*)-3-hydroxybutyryl-SNAC (**3**) and (*E*)-2-butenoyl-SNAC (**4**), respectively. The commonly used SNAC thioesters lack ionizable substituents, unlike the natural -SFosACP1 thioester or the -SCoA analog, thereby avoiding any confounding pH-dependent effects on substrate binding to the FosDH1 active site or perturbation of established DH-ACP charge-charge surface interactions.^{9, 26} The FosDH1-catalyzed dehydration of **3** to **4**, as well as the reverse hydration reaction, were conveniently monitored by high performance liquid chromatography – mass spectrometry (LC-MS) analysis of ethyl acetate extracts of periodically withdrawn samples of each incubation mixture (Figures S1-S3). The identity of the individual substrate and product peaks was confirmed by MS-MS analysis. At pH 7.2, FosDH1 exhibited k_{cat} for **3** of $24 \pm 2 \text{ min}^{-1}$ and K_{m} $65 \pm 7 \text{ mM}$, corresponding to a $k_{\text{cat}}/K_{\text{m}}$ $370 \text{ M}^{-1}\text{min}^{-1}$. The reverse reaction, the FosDH1-catalyzed hydration of 2-butenoyl-SNAC (**4**) to 3-hydroxybutyryl-SNAC (**3**), had k_{cat} $12 \pm 2 \text{ min}^{-1}$ and K_{m} $23 \pm 7 \text{ mM}$ ($k_{\text{cat}}/K_{\text{m}}$ $520 \text{ M}^{-1}\text{min}^{-1}$).

To determine the pH dependence of the FosDH1-catalyzed dehydration of (3*R*)-3-hydroxybutyryl-SNAC (**3**), the steady state kinetic parameters were determined in a series of incubations carried out from pH 3.0 to 9.2 (Figure S4, Table S1). FosDH1 had negligible dehydratase activity below pH 5.0, with increasing activity at pH 6.0 that reached a maximum from pH 7.0 – 9.2 (Figures 1, S5, S6). The observed pH dependence of k_{cat} was fit to a single acid-base ionization^{27, 28} with a calculated $\text{p}K_{\text{a}}$ 6.1 ± 0.1 , while the pH dependence of $k_{\text{cat}}/K_{\text{m}}$ showed to a single ionization, $\text{p}K_{\text{a}}$ 5.7 ± 0.1 .

Discussion

The observation of a single ionization in the pH–rate profiles for both $\log(k_{\text{cat}})$ and $\log(k_{\text{cat}}/K_{\text{m}})$ is inconsistent with a base-acid (“two-base”) mechanism for the FosDH1-catalyzed dehydration of (3*R*)-3-hydroxybutyryl-SNAC (**3**), which would have given rise to a bell-shaped pH rate profile. The observed pH dependence fully supports a single-base mechanism, with the observed $\text{p}K_{\text{a}}$ 6.1 for k_{cat} , which reflects the properties of the enzyme under saturating conditions, consistent with the action of the basic active site His residue. The fact that the observed dehydratase activity shows no decrease up to pH 9.2 is incompatible with any mechanism in which the active site Asp serves as a general acid,

thereby excluding the commonly invoked “two-base” (base-acid) mechanism for the DH-catalyzed dehydration.

A recently published study of PicDH2, the DH domain from module 2 of the picromycin PKS, included determination of the pH dependence of the $\log (V_{\max}/K_m)$ of PicDH2, which exhibited a half-bell-shaped profile.²⁵ The observed pK_a 7.0 ± 0.1 was assigned to the deprotonation of the active site His residue. In spite of the evidence for only a single ionization over the pH range 6.6 – 9.0, the authors invoked the commonly proposed “two-base” DH mechanism, suggesting that the active site Asp residue would serve as the proton source. In any case, the pH dependence of k_{cat}/K_m reflects only the properties of the free enzyme at the limit of zero substrate concentration. By contrast, the pH dependence of k_{cat} reflects the catalytically relevant pK_a values of the enzyme with bound substrate.

In light of the compelling evidence for a single-base DH mechanism, what then might be the role of the carboxylate of the essential active site Asp residue? We suggest that rather than Asp acting as a general acid, the primary role of the negatively charged Asp side chain is to bind and orient the substrate by an essential H-bond to the hydroxyl group of the 3-hydroxyacyl-SACP substrate (Scheme 4a). In the reverse hydration reaction, the carboxylate of this Asp side chain would similarly H-bond to the nucleophilic water. Strong experimental analogy supporting this mechanistic model for DH domains can be found in the results of extensive protein structural, site-directed mutagenesis, pH rate, kinetic, and spectroscopic studies of the analogous enoyl-SCoA hydratase (ECH) reaction of the fatty acid oxidation pathway.^{29–31} Although enoyl-SCoA hydratase has a protein structure and primary amino acid sequence that are entirely distinct from those of the FAS and PKS DH domains, the underlying biochemical reaction mechanism for the Michael addition of water to an 2-enoyl-SCoA substrate bears striking similarities to the reverse of the DH-catalyzed dehydration reaction (Scheme 4b). In place of the conserved His – Asp/Glu dyad of the dehydratases, the active site of enoyl-CoA hydratase harbours a pair of catalytically essential Glu residues that play an analogous catalytic role. Thus the E164 carboxylate has been shown to serve as the single active site base for the deprotonation of the nucleophilic water and reprotonation at C2 of the resulting enolate, while the carboxylate of E144 binds and orients this active site water. Consistent with this mechanism, it has been established that both protons of the nucleophilic water are incorporated into the 3-hydroxybutyryl-SCoA product.³⁰

Conclusions

The pH dependence of both k_{cat} and k_{cat}/K_m for the dehydration of 2-hydroxybutyryl-SNAC (**3**) to 2-butenoyl-SNAC (**4**), catalysed by FosDH1, a prototypical PKS dehydratase, each exhibited a single ionization from inactive to active form with a pK_a ~6. Combined with the *syn* stereochemistry established for many DH-catalyzed eliminations, these results are incompatible with the commonly invoked two-base (base-acid) dehydratase mechanism, and are instead fully consistent with a one-base mechanism in which the active site His residue serves as the base that first removes the H-2 proton of the substrate, then transfers this proton directly to the hydroxyl group of the intermediate enolate, thereby facilitating cleavage of the C–O bond with net elimination of water. The negatively charged carboxylate

of the active site Asp is unable to serve as a general acid, but instead binds and orients the hydroxyl group of the 3-hydroxyacyl thioester substrate in the preferred stereo-electronic conformation for elimination of water.

Experimental section

Materials and methods

Isopropylthio- β -D-galactopyranoside (IPTG) and ampicillin were purchased from Thermo Scientific. *E. coli* BL21(DE3) was purchased from New England BioLabs. All other chemical reagents were purchased from Sigma-Aldrich and utilized without further purification. Amicon Ultra Centrifugal Filter Units (Amicon Ultra-15 and Amicon Ultra-4, 30,000 MWCO) were purchased from Millipore. Pre-charged 5 mL HisTrapTM FF columns were purchased from GE Healthcare Life Sciences. (3*R*)-3-hydroxybutyryl-SNAC (**3**) was synthesized from (3*R*)-hydroxybutyric acid and SNAC, as previous described.³² (*E*)-2-Butenoyl-SNAC (**4**) was synthesized from (*E*)-2-butenoyl chloride and *N*-acetylcysteamine, as previous described.^{32, 33} General methods were as previously described.³⁴ Growth media and conditions used for *E. coli* strains and standard methods for handling *E. coli* in vivo and in vitro were those described previously,³⁴ unless otherwise noted. All proteins were handled at 4 °C unless otherwise stated. Protein concentrations were determined according to the method of Bradford,³⁵ using a Tecan infinite M200 Microplate Reader with bovine serum albumin as the standard. Protein purity and size was estimated using SDS-PAGE, visualized using Coomassie Blue stain, and analyzed with a Bio-Rad ChemiDoc MP System. A Thermo LXQ LC-mass spectrometer equipped with Surveyor HPLC system and a Phenomenex Jupiter C16 column (150 mm $\times$ 2 mm, 5.0 μ m) was utilized for analysis of SNAC esters.

Protein Expression and Purification.

Recombinant FosDH1 was expressed and purified by procedures similar to those previously described.²⁴ Competent *E. coli* BL21(DE3) cells were transformed with the pET28a vector containing FosDH1 with codons optimized for expression in *E. coli* and the resultant recombinants were cultured under standard conditions. A single recombinant clone was inoculated into 10 ml LB medium containing 50 μ g/ml kanamycin and grown at 37 °C overnight. The overnight culture was transferred into 500 ml of Super Broth with 50 μ g/ml kanamycin in a 2.5-L flask and grown until an OD₆₀₀ of 0.4–0.8. The cultures were cooled to 18 °C and 0.2 mM IPTG was added to induce protein expression. The cell culture was continuously grown for an additional 40–48 h at 18 °C and cells were then harvested by centrifugation at 4,200 *g* for 20 min. The resulting cell pellet was dissolved in 35 ml lysis buffer (1 M NaCl, 50 mM sodium phosphate, 75 mM imidazole, 1 mg/ml of lysozyme, pH 7.8) and stored at –80 °C. Frozen cells in lysis buffer were thawed at room temperature, followed by sonication. The cell supernatant and pellet were separated by centrifugation at 23,000 *g* for 30 min. The supernatant was filtered using a 0.8 μ m filter and loaded onto a pre-charged 5-ml HisTrapTM FF column (GE Healthcare). The column was washed with 25 mL lysis buffer and then 25 mL washing buffer (50 mM sodium phosphate, 1 M NaCl, 75 mM imidazole, pH 7.6). Proteins were eluted by elution buffer (150 mM NaCl, 50 mM phosphate, 150 mM imidazole, pH 7.5). The eluted fractions were collected, concentrated

with an Amicon filter (MWCO 30,000), and the buffer was exchanged with exchange buffer (50 mM sodium phosphate, 10% glycerol, 100 mM NaCl, pH 7.2), concentrated, and stored at -80°C until use. Protein purity was assessed as $>90\%$ by 12% acrylamide SDS-PAGE, and the N-terminal His-tagged proteins were utilized without further modification.

Determination of initial velocity conditions.

A typical assay consisted of FosDH1 (1.25, 2.5 or 5.0 μM) and 10 mM substrate **3** or **4** in a total volume of 700 μL of 50 mM phosphate buffer (2.5 mM Tris(2-carboxyethyl)phosphine (TCEP) (pH 7.2). At 5, 10 and 15 min time points, 100 μL of reaction mixture was withdrawn and added to 100 μL of 0.5 M HCl to quench the reaction, then extracted with ethyl acetate ($3 \times 500 \mu\text{L}$). After evaporation of the solvent, the concentrated organic extract was dissolved in 200 μL methanol and analyzed by HPLC-MS. HPLC was carried out at a flow rate of 0.2 ml/min at room temperature. Eluent A was 0.1% formic acid in water, and eluent B was 100% acetonitrile. HPLC conditions used were 5%–95% buffer B for 10 min, 95% buffer B for another 1 min, 100%–5% buffer B for 3 min and then 5% buffer B for 4 min. Each reaction was performed in duplicate. Progress curves at varying enzyme concentrations were generated and the initial velocity at each enzyme concentration was obtained (Figures S1 and S2). The amount of enzymatic product formed at each time point was calculated using equation 1:

$$A_p = A_s * P_p / (P_s + P_p) \quad (1)$$

Where A_p : amount of product; A_s : initial amount of substrate; P_p : relative MS intensity of product in MS spectra of retention time between 1 min and 10 min; P_s : relative MS intensity for substrate in MS spectra of retention time between 1 min and 10 min.

Kinetic analysis of FosDH1-catalyzed dehydration and hydration reactions by LC-MS/MS.

Enzymatic reactions were carried out in a total volume of 250 μL under initial velocity conditions containing FosDH1 (2.5 μM), reaction buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.2) and substrates **3** or **4** at variable concentrations (0.5, 1, 4, 10, 20, 60 mM). The incubation mixture also contained $\sim 1\%$ each of glycerol from the protein solution and DMSO from the stock solutions of **3** or **4**. After incubation at room temperature for 15 min, 100 μL reaction mixture was transferred into a new Eppendorf tube, acidified with 100 μL of 1 M HCl, and extracted with ethyl acetate ($3 \times 500 \mu\text{L}$). The concentrated organic extract was dissolved in 200 μL methanol and analyzed by HPLC-MS. Parallel control incubations for each concentration of substrate were performed without the addition of enzyme. Each reaction and analysis were performed in duplicate (Figure S3). The steady-state kinetic parameters were determined by fitting the observed rate and substrate concentration data to the Michaelis-Menten equation by non-linear least squares regression using GraphPad Prism 7.0d. Reported standard deviations in the steady-state kinetic parameters represent the calculated statistical errors in the non-linear, least squares regression analysis.

pH dependence profile of FosDH1.

The effect of pH on the steady-state kinetic parameters for FosDH1-catalyzed dehydration of **3** was determined using a series of 50 mM buffers of increasing pH: citric acid-sodium citrate (pH 3.0, 4.0, 5.0 and 6.0), HEPES (pH 7.0 and 8.0), and sodium carbonate (pH 9.2). At each pH value (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.2), the incubations were conducted with 3-hydroxybutyryl-SNAC (**3**, 0.5, 1, 4, 10, 20, 60 mM) under the conditions described above. The k_{cat} and k_{cat}/K_m values at each pH were obtained by fitting the observed initial rate and substrate concentration data to the Michaelis-Menten equation by non-linear least squares regression using GraphPad Prism 7.0d. The $\text{p}K_a$ value of the ionizable group was obtained by fitting the observed values of k_{cat} and k_{cat}/K_m at each pH 5.0 – 9.2 to eq. 2 and 3, respectively, corresponding to the observed half-bell shape with a single acidic break (Figures S4-S6, Table S1):

$$\log(k_{\text{cat}}) = \log C / (1 + [\text{H}^+] / K_a) \quad (2)$$

$$\log(k_{\text{cat}} / K_m) = \log C / (1 + [\text{H}^+] / K_a) \quad (3)$$

where, C is the pH-independent plateau value of k_{cat} or k_{cat}/K_m , $[\text{H}^+]$ is the hydrogen ion concentration, and K_a is the dissociation constant of the acid.^{27, 28} Two independent replicates of each pH rate series were carried out. The data for each replicate were also analysed over the entire experimental pH range 3.0 – 9.2 using an alternative formulation of the Logistical equations for the pH behaviour of k_{cat} or k_{cat}/K_m , as described in the Electronic Supporting Information, with each plot displaying a single $\text{p}K_a$ (Figure S6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
pH dependence of FosDH1 (Replicate 1). A. $\log(k_{cat})$ vs pH. B. $\log(k_{cat}/K_m)$ vs. pH.

Scheme 1.

Dehydratase mechanistic models. a. One-base (B:) mechanism. b. Base-acid (B: - AH) mechanism.

Scheme 2.

FosDH1-catalyzed dehydration of (3*R*)-**1** to (E)-**2** by the fostriecin PKS.

Scheme 3.

Reversible FosDH1-catalyzed dehydration of (3*R*)-3 to (E)-4.

Scheme 4.

One-Base dehydration and hydration mechanisms. a) Dehydration by PKS and FAS DH domains. b) Enoyl-CoA hydratase.