Mechanism of Chalcone Synthase

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Polyketide synthases (PKS) assemble structurally diverse natural products using a common mechanistic strategy that relies on a cysteine residue to anchor the polypeptide during a series of decarboxylative condensation reactions that build the final reaction product. Crystallographic and functional studies of chalcone synthase (CHS), a plant-specific PKS, indicate that a cysteine-histidine pair (Cys164-His303) forms part of the catalytic machinery. Thiol-specific inactivation and the pH dependence of the malonyl-CoA decarboxylation reaction were used to evaluate the potential interaction between these two residues. Inactivation of CHS by iodoacetamide and iodoacetic acid targets Cys164 in a pH-dependent manner (pKa5 = 5.50). The acidic pKa4 of Cys164 suggests that an ionic interaction with His303 stabilizes the thiolate anion. Consistent with this assertion, substitution of a glutamine for His303 maintains catalytic activity but shifts the pKa of the thiol to 6.61. Although the H303A mutant was catalytically inactive, the pH-dependent incorporation of [14C]idoacetamide into this mutant exhibits a pKa5 = 7.62. Subsequent analysis of the pH dependence of the malonyl-CoA decarboxylation reaction catalyzed by wild-type CHS and the H303Q and C164A mutants also supports the presence of an ion pair at the CHS active site. Structural and sequence conservation of a cysteine-histidine pair in the active sites of other PKS implies that a thiolate-imidazolium ion pair plays a central role in polyketide biosynthesis.

Polyketide synthases (PKS)1 from bacteria, fungi, and plants produce an array of natural products (1–4). Many polyketides possess pharmacological properties and are used as antibiotics, immunosuppressants, anti-cancer agents, and anti-fungal agents (5–6). Despite the structural complexity of these compounds, a common chemical strategy underlies the biosynthetic mechanisms of different PKS. The initial reaction step involves loading a starter molecule onto an active site cysteine through an acyltransferase activity. Following formation of the primed acyl-enzyme complex, a decarboxylative condensation reaction extends the reaction intermediate. The elongation step can be repeated until an appropriate chain length is reached and the reaction product released. This process is analogous to the reactions catalyzed by fatty acid synthases (FAS) (7). Recent structural and kinetic studies of chalcone synthase (CHS), a plant-specific PKS, have elucidated the basis of polyketide formation in plants and provide a model for understanding the reaction mechanism of other PKS.

Unlike the modular PKS, such as 6-deoxyerythronolide B synthase, which are large protein assemblies with distinct active sites that catalyze each elongation step (1–2, 4, 6), the plant-specific PKS function as homodimeric iterative PKS (monomer, molecular mass ~ 42 kDa) that perform consecutive elongation reactions at two independent active sites (3, 8). CHS uses p-coumaryl-CoA as a starter molecule and three malonyl-CoA extender molecules to form a tetraketide intermediate that is cyclized into 4,2,4,6-tetrahydroxycalcone (chalcone) (Fig. 1a) (9). This activity is central to the biosynthesis of anti-microbial isoflavonoid phytoalexins, anthocyanin floral pigments, and flavonoid inducers of Rhizobium nodulation genes (10, 11). Also, flavonoids are of interest as pharmacological agents (12–14) and are constituents in plant-rich diets associated with a reduced risk of cardiovascular disease and some forms of cancer (15, 16).

The three-dimensional structure of alfalfa CHS2 provides a view of the active site that catalyzes chalcone formation (Fig. 1b) (17). Four residues (Cys164, His303, Asn336, and Phe215) form the catalytic center of CHS and are strictly conserved in other CHS-like enzymes, including 2-pyrene synthase (18), stilbene synthase (19), bibenzyl synthase (20), acridone synthase (21), and the rppA CHS-like protein (22). Based upon structural and functional studies of CHS (17, 23, 24), the proposed reaction mechanism involves Cys164 acting as the nucleophilic thiolate in the loading reaction and as the covalent thioester-anchor for the acyl-enzyme chain during the elongation reactions (Fig. 1c). In addition, His303 and Asn336 catalyze the decarboxylation of malonyl-CoA in the elongation reaction and stabilize the transition state during the condensation phases of polyketide formation. Phe215 may orient substrates and reaction intermediates at the active site.

In the crystal structure of CHS (17), the Sy of Cys164 forms a hydrogen bond with the Ne of His303, which is 3.5 Å distant (Fig. 1b). Previous mutagenesis studies suggest that His303 does not act as a general base by abstracting a proton from Cys164 to form the reactive thiolate required for chalcone formation (24). Rather, at physiological pH, these two residues may form a stable imidazolium-thiolate ion pair. In other enzymes, notably the cysteine proteases (25–28), the thiolate anion of the catalytic cysteine is stabilized by an imidazolium
Cysteine-Histidine Dyad in Chalcone Synthase

**Fig. 1.** CHS reaction and active site structure. *a*, overall CHS reaction; *b*, CHS active site. The catalytic cysteine and conserved histidine are shown along with the terminal end of a CoA molecule and adjacent residues that have had their catalytic roles investigated previously. The hydrogen bond between Cys164 and His303 is indicated by the dotted line. This view is oriented looking down the pantothenyl arm of the CoA. *c*, proposed reaction mechanism for CHS. Loading, decarboxylation, and elongation steps are shown. *R* is the coumaroyl moiety in the first reaction cycle, coumaroyl-acetyl group in the second cycle, and a coumaroyl-diacyetyl group in the third cycle. The proposed cysteine-histidine ionic interaction in the loading is indicated.

ion on an adjacent histidine. The structural proximity of Cys164 and His303 in the CHS active site raises the potential for a similar mechanistic feature in this PKS.

This paper describes the use of thiol-specific inactivators to evaluate the reactivity of Cys164, to determine the pKₐ of the active site cysteine, and to establish the role of His303 in maintaining the reactivity of Cys164. In addition, the pH dependence of the malonyl-CoA decarboxylation reactions catalyzed by wild-type CHS and the H303Q and C164A mutants was examined. These studies demonstrate that Cys164 is a reactive thiolate anion with an acidic pKₐ that is modulated by interaction with His303. Combined with previous crystallographic and kinetic studies, this work provides insight into the mechanism of CHS and suggests that a thiolate-imidazolium ion pair plays a significant role in both polyketide and fatty acid biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—[2-¹⁴C]Malonyl-CoA (45.8 mCi/mmol) was purchased from PerkinElmer Life Sciences. [2-¹⁴C]Iodoacetic acid (53.0 mCi/mmol) and [1-¹⁴C]Iodoacetamide (59.0 mCi/mmol) were from Amersham Pharmacia Biotech. G-25 Sephadex Quick-spin columns were bought from Roche Molecular Biochemicals. Iodoacetamide, iodoacetic acid, and malonyl-CoA were obtained from Sigma. p-Coumaroyl-CoA was synthesized using the method of Stoeckigt and Zenk (29) with reagents purchased from Aldrich or Sigma. Electrospray mass spectroscopy to confirm the identity of the synthesis product was performed by the Mass Spectrometry Facility of the Scripps Research Institute.

**Preparation of Wild-type and Mutant CHSs**—The CHS C164A, H303Q, and H303A mutants were generated previously (24). Wild-type and mutant CHS proteins were expressed and purified to homogeneity and adjacent residues that have had their catalytic roles investigated previously.

**CHS Assay**—The standard assay for determining CHS activity was conducted in 100-μl systems containing 100 mM potassium phosphate buffer (pH 7.0), 50 μM [2-¹⁴C]malonyl-CoA (50,000 cpm), and 15 μM coumaroyl-CoA at 25 °C (24). Reactions were initiated by the addition of enzyme and were quenched by ethyl acetate extraction. Extracts were evaporated to dryness and re-dissolved in methanol. Scintillation counting to detect radioactivity in the methanol sample was performed in Ecolume scintillation fluid.

**Inactivation Studies**—Wild-type CHS (10 μg) was incubated in 30-μl volumes using a triple buffer system (30, 31) (50 mM AMPSO, 50 mM sodium phosphate, and 50 mM sodium pyrophosphate, pH 7.0) in the presence of 0–50 μM iodoacetamide or iodoacetic acid at 25 °C. Similar experiments with the H303Q mutant (10 μg) were performed in the presence of 0–500 μM iodoacetamide or 0–1000 μM iodoacetic acid under the same reaction conditions. All reactions were initiated by the addition of inactivator. Aliquots (2 μl) were withdrawn from the incubation mixture and diluted into the standard assay system, and the amount of enzyme activity remaining determined. All inactivation experiments were monitored relative to a control sample without inactivator, which is set to 100% activity at each time point. This control accounts for the loss of enzyme activity under experimental conditions. Inactivation data were plotted as log (% initial enzyme activity) versus time. Semi-log plots were fitted to the first-order equation, −dE/dt = k(t), where it is assumed that the disappearance of enzyme activity over time is related to the concentration of either iodoacetamide or iodoacetic acid, (I), multiplied by k, a rate constant. This allowed determination of the half-life for inactivation (t½) at each inactivator concentration. Kitz-Wilson analysis of the data was used to generate the limiting constant for inactivation (kₐmax) and Kᵢ by plotting t½ versus 1/[inactivator], (mM).

**Determination of Active Site Labeling**—Wild-type CHS (10 μg), H303Q mutant (10 μg), or C164A mutant (25 μg) were incubated with either 5 μM [2-¹⁴C]iodoacetamide or 10 μM [1-¹⁴C]iodoacetic acid at 25 °C in the triple buffer system (pH 7.0) as described above. Two aliquots were removed at each time point. One sample was assayed for CHS activity. The second sample was diluted 50-fold with triple buffer and loaded onto a G-25 Sephadex Quick-spin column. The radiolabeled
RESULTS

Kinetics of CHS Inactivation by Iodoacetamide and Iodoacetic Acid—Affinity-labeling agents and enzyme inactivators provide useful probes of active site chemistry in enzymes. Thiolspecific compounds, such as iodoacetamide and iodoacetic acid, capitalize on the ability of a sulphydryl group to be sufficiently nucleophilic to rapidly react in solution. Early work on CHS from cell suspension cultures qualitatively demonstrated that iodoacetamide and iodoacetic acid inactivated the enzyme (9, 34). Using homogenous recombinant protein, the inactivation of CHS by iodoacetamide and iodoacetic acid was re-examined.

CHS is inactivated by both compounds in a pseudo-first-order kinetic manner (Fig. 2, a and c). Inactivation of CHS by iodoacetamide and iodoacetic acid displayed second-order rate constants ($k_2$) of 1390 and 635 M$^{-1}$ s$^{-1}$, respectively, as determined by Kitz-Wilson analysis (Fig. 2, b and d).

Identification of Inactivator Attachment Site—The CHS active site contains one cysteine residue (Cys$^{164}$) (17, 24). To confirm that Cys$^{164}$ is specifically targeted by iodoacetamide and iodoacetic acid, purified wild-type CHS and the C164A mutant were incubated with [14C]iodoacetamide and [14C]iodoacetic acid. Incorporation of [14C]iodoacetamide into wild-type CHS coincided with a loss of activity for chalcone formation (Fig. 3). Similar results were obtained when wild-type CHS was incubated with radioactive iodoacetic acid (not shown). To ensure that modification of the active site thiol was covalent, wild-type CHS and inactivated wild-type CHS were dialyzed against reaction buffer and re-assayed for CHS activity. After dialysis, enzyme treated with iodoacetamide or iodoacetic acid lacked activity, whereas untreated wild-type enzyme retained catalytic activity. Stoichiometries of 0.80 mol [14C]iodoacetamide/mol of CHS monomer and 0.68 mol [14C]iodoacetic acid/mol of CHS monomer were determined from the amount of radiolabeled inactivator incorporated into the enzyme. Because CHS is a homodimeric protein with two active
sites that are catalytically independent (7), the stoichiometries suggest that both inactivators target the active site cysteine of each monomer. Incubation of the C164A mutant with [14C]iodoacetamide (Fig. 3) or [14C]iodoacetic acid (not shown) under identical reaction conditions as wild-type CHS showed that negligible amounts of radioactivity were incorporated into the mutant protein. Therefore, Cys164 is the specific residue targeted by both iodoacetamide and iodoacetic acid that results in loss of CHS activity.

The pKₐ of the Catalytic Cysteine—In the acyltransferase reaction that loads the starter molecule onto CHS, a thiol anion is required as the nucleophile. However, physiological pH is 1 unit below the accepted pKₐ value range of 8.0–8.5 for a cysteine sulfhydryl (35). Therefore, the pKₐ of the catalytic cysteine in CHS must shift below 7.0 to serve as an effective nucleophile. To determine the pKₐ of Cys164, the pH dependence of inactivation was evaluated using iodoacetamide. The second-order rate constants (k₂) for CHS inactivation by iodoacetamide were determined over a pH range from 4.62 to 8.27. Wild-type CHS inactivated with a pKₐ of 5.50 ± 0.10 and exhibited a 1-log-unit change in the value of k₂ over a single pH unit (Fig. 4), consistent with the titration of a single ionizable group (36).

Effect of the Conserved Histidine on Inactivation Kinetics—Inactivation of the CHS H303Q mutant by iodoacetamide and iodoacetic acid followed pseudo-first-order kinetics (Fig. 5) or [14C]iodoacetic acid did not show a second-order rate constant kᵢ that is required for malonyl-CoA decarboxylation. Therefore, Cys164 is mutated to aprotic residues; this suggests that the pH dependence of CHS inactivation by iodoacetamide was monitored (squares) to determine the pKₐ of the catalytic cysteine. The pH dependence of inactivation was evaluated using iodoacetamide. The second-order rate constants (k₂) for CHS inactivation by iodoacetamide were determined over a pH range from 5.04 to 8.50 (Fig. 7). Because of the multiple catalytic steps involved in chalcone formation, each requiring different protonation states of CHS or the substrates and intermediates, the malonyl-CoA decarboxylation reaction was examined to simplify interpretation of these experiments. The pH dependence of kᵢ for wild-type CHS displays a convex-shaped profile that levels off at both high and low pH with a pKₐ of 6.94 ± 0.09. In both the H303Q and C164A mutants, this break point is eliminated. The kᵢ profile for wild-type CHS reveals that the enzyme-substrate complex required for malonyl-CoA decarboxylation undergoes a change in protonation state, which does not occur when either His303 or Cys164 is mutated to aprotic residues; this suggests that the rate-limiting step in the decarboxylation reaction is altered in each mutant. The log(kᵢ/kᵢ₉₀₀₀) versus pH profiles for malonyl-CoA decarboxylation catalyzed by wild-type CHS, the H303Q mutant, and the C164A mutant each exhibited a similar break point in the basic region with extrapolated pKₐ values of 9.23 ± 0.73, 9.46 ± 1.99, and 9.32 ± 1.68, respectively. The presence of this break point in the kᵢ/kᵢ₉₀₀₀ profiles, and not the kᵢ profile,
of each protein suggests that a basic residue is involved in substrate binding. This inflection point may represent any of the three arginine or lysine residues on the surface of CHS that interact with the phosphate moieties of CoA substrates (17, 24).

**DISCUSSION**

Iodoacetamide and iodoacetic acid inactivate CHS through specific modification of Cys 164 at the active site. This result agrees with mutagenesis experiments of CHS in which substitution of the cysteine with a serine or alanine results in a complete loss of chalcone formation (23–24). Importantly, the p\(_{Ka}\) of Cys164 indicates that a thiolate anion is present at the CHS active site at physiological pH to serve as the nucleophile in the loading reaction and as the attachment site of the polyketide during the elongation reactions. The acidic p\(_{Ka}\) of Cys164 also explains why the sulfhydryl group of this residue is oxidized to sulfinic acid (Cys-SO\(_2\)H) in several crystal structures of CHS (17, 24). Similar oxidation of the active site thiol in the cysteine proteases also occurs (37, 38). In these enzymes, the reactive thiolate forms an ion pair with the imidazolium ion of an adjacent histidine residue (27). Although the p\(_{Ka}\) value determined for Cys164 in CHS is not as low as the 3.3–4.0 p\(_{Ka}\) of the thiolate present at the active site of the cysteine proteases (25–27), the observed p\(_{Ka}\) of the CHS active site thiol is significantly shifted from the accepted p\(_{Ka}\) value of 8.0–8.5 for a cysteine sulphydryl moiety (35). A reduction in p\(_{Ka}\) of this magnitude requires stabilization by the local environment. The proximity of His303 to Cys164 in the CHS structure suggests that the histidine, as an imidazolium cation, stabilizes the thiolate anion (Fig. 8).

Consistent with the presence of an ion pair at the CHS active site, the reactivity and p\(_{Ka}\) of Cys164 shifted when mutations of His303 were made. Substitution of a glutamine for His 303 also maintains the thiolate of Cys 164, but the resulting nucleophile is less reactive than in wild-type CHS. In the three-dimensional structure of the H303Q mutant (24), substitution of a glutamine for His303 is isosteric with the amide nitrogen of Gln303 hydrogen bonding the sulfhydryl of Cys164. Hydrogen bonding and a partial positive charge on the amide nitrogen arising from resonance stabilization promote formation of the thiolate at Cys164, albeit less efficiently and with a corresponding shift in p\(_{Ka}\) (Fig. 8).

No stabilizing effect would be expected from the side chain of Ala303 in the H303A mutant (Fig. 8). As described previously (24), the lack of malonyl-CoA decarboxylation and chalcone formation activities in this mutant underscores the mechanis-
tic importance of the histidine. Although the p\(K_a\) for iodoacetamide labeling of the H303A mutant is shifted to 7.62, the observed p\(K_a\) of still 0.4–0.9 pH units below that of a free cysteine. Asn\(^{336}\) is another polar residue near Cys\(^{164}\) (4.1 Å), but substitution of the asparagine with an alanine does not alter the inactivation kinetics of the N336A mutant or the p\(K_a\) of Cys\(^{164}\) in this mutant.\(^2\) Since no other direct interactions occur with Cys\(^{164}\), the local environment may alter the p\(K_a\) of this residue. Cys\(^{164}\) is at the N terminus of \(\alpha\)-helix 9 in the CHS structure and the helix-dipole may further reduce the thiol’s p\(K_a\) in the absence of other interactions (40–42).

If Cys\(^{164}\) and His\(^{303}\) form a stable ion pair, then the ionization states of both residues should be thermodynamically linked and a second inflection point observed in the pH profiles for inactivation. For example, in papain the p\(K_a\) of the active site histidine shifts from 4.3 to 8.5 when the active site thiol is deprotonated (25–26). Since the structure of wild-type CHS indicates that interaction between Cys\(^{164}\) and His\(^{303}\) occurs (17, 24), it is likely that the absent inflection point is beyond the pH range studied. Therefore, the potential p\(K_a\) of His\(^{303}\) in the free enzyme must be greater than 9.0, which is also consistent with the activity of the H303Q mutant, in which the glutamine’s amide p\(K_a\) is approximately 17.0 (39). Under physiological conditions, a stable thiolate-imidazolium ion pair in the CHS active site would maintain the nucleophilic thiolate required for the loading reaction.

The pH dependence of the malonyl-CoA decarboxylation reaction also supports the importance of a charged interaction at the CHS active site. In wild-type enzyme, the presence of an imidazolium ion would enhance formation of an enolate anion in the decarboxylation reaction (43). Since the \(k_{cat}/K_M\) profile corresponds to the protonation state of the enzyme-substrate complex, the assignment of the observed p\(K_a\) is problematic. Most likely, the observed break point represents enolization of malonyl-CoA during the decarboxylation reaction. Mutation of either His\(^{303}\) or Cys\(^{164}\) to an aprotic side chain eliminates the observed inflection point, suggesting that the rate-determining step in the decarboxylation reaction is altered. Although the H303Q and C164A mutants retain hydrogen bond donors at the active site as a glutamine and a histidine, respectively, the ionic pair at the active site is disrupted. Loss of the imidazolium ion in these mutants would slow substrate enolization and eliminate the observed pH dependence on the decarboxylation reaction.

Since the active site residues of CHS are conserved among CHS-like PKS, including 2-pyrene synthase (18), stilbene synthase (19), bibenzyl synthase (20), acridone synthase (21), and the rppA CHS-like protein (22), these enzymes likely retain a thiolate-imidazolium ion pair at their active sites. In addition, homology of the CHS active site residues with those of the FAS and other PKS (44), such as 6-deoxoerythronolide B synthase and actinorhodin synthase, suggests that a similar ion pair may be a defining feature in these enzymes.

The functional studies of FAS II and III demonstrate that the role of the catalytic cysteine in these enzymes is identical to that of Cys\(^{164}\) in CHS (45, 46). Also, the rapid inactivation of FAS by iodoacetamide implies that the active site thiol is highly reactive (47–48). Although studies on FAS III demonstrate the importance of the active site histidine in the overall reaction mechanism (42, 46), the effect of this residue on the p\(K_a\) of the catalytic cysteine in FAS has not been evaluated. Currently, no detailed structural information is available on the modular or heterodimeric iterative PKS, but mechanistic analysis of actinorhodin synthase suggests that a cysteine-histidine dyad is an essential catalytic component (49). In the reaction mechanism of actinorhodin synthase, a cysteine (Cys\(^{169}\)) serves as the attachment point for the polyketide chain (49–51), and a histidine (His\(^{346}\)) may activate the thiol in the loading and elongation reactions.

Stabilization of negatively charged thiolates by imidazolium ions at the active sites of PKS eliminates the need for formal proton transfers such as those governed by general acid-base catalysis. Intuitively, the catalytic advantage of this mechanism may derive from limiting bond making and bond breaking steps to thioester formation and breakdown without additional proton transfers to and from the thiolate and imidazolium ions and their respective acids and bases.

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\(^2\) J. M. Jez and J. P. Noel, unpublished observations.
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