Roles of Substrate Distortion and Intramolecular Hydrogen Bonding in Enzymatic Catalysis by Scytalone Dehydratase

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ABSTRACT: Alternative substrates and site-directed mutations of active-site residues are used to probe factors controlling the catalytic efficacy of scytalone dehydratase. In the E1cb-like, *syn*-elimination reactions catalyzed, efficient catalysis requires distortion of the substrate ring system to facilitate proton abstraction from its C2 methylene and elimination of its C3 hydroxyl group. Theoretical calculations indicate that such distortions are more readily achieved in the substrate 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one (DDBO) than in the physiological substrates vermelone and scytalone by ∼2 kcal/mol. A survey of 12 active-site amino acid residues reveals 4 site-directed mutants (H110N, N131A, F53A, and F53L) have higher relative values of k_{cat} and k_{cat}/K_m for DDBO over scytalone and for DDBO over vermelone than the wild-type enzyme, thus suggesting substrate-distortion roles for the native residues in catalysis. A structural link for this function is in the modeled enzyme−substrate complex where F53 and H110 are positioned above and below the substrate's C3 hydroxyl group, respectively, for pushing and pulling the leaving group into the axial orientation of a *pseudo*-boat conformation; N131 hydrogen-bonds to the C8 hydroxyl group at the opposite end of the substrate, serving as a pivot for the actions of F53 and H110. Deshydroxyvermelone lacks the phenolic hydroxyl group and the intramolecular hydrogen bond of vermelone. The relative values of k_{cat} (95) and k_{cat}/K_m (1800) for vermelone over deshydroxyvermelone for the wild-type enzyme indicate the importance of the hydroxyl group for substrate recognition and catalysis. Off the enzyme, the much slower rates for the solvolytic dehydration of deshydroxyvermelone and vermelone are similar, thus specifying the importance of the hydroxyl group of vermelone for enzyme catalysis.

Involvement of substrate substituents in promoting substrate reactivity on enzymes [substrate-assisted catalysis (*1*−*5*)] and enzyme residues in perturbing substrate geometry [substrate distortion (*6*−*10*)] are among recognized factors that can enhance rates of enzyme catalysis. Here these influences are investigated with respect to the catalytic mechanism of scytalone dehydratase. Scytalone dehydratase (SD, EC 4.2.1.94),[1] the single dehydratase operating within the fungal melanin biosynthesis pathway of *Magnaporthe grisea*, is an efficient catalyst of the dehydration of its physiological substrates, scytalone and vermelone (Figure 1) (*11*−*21*). SD (*22*−*31*) and 3HNR (1,3,8-trihydroxynaphthalene reductase) (*32*−*39*), one of two naphthol reductases in the pathway (*40*, *41*), are the biochemical targets of fungicides in development and practice for protecting rice plants from blast. *M. grisea* is the fungal agent of blast disease in rice (*42*−*45*) and the source of fungal melanin biosynthesis enzymes under recent study (*11*−*14*, *16*−*21*, *26*−*31*, *35*−*41*). Biochemical and structural studies are in accord with assigning the binding of potent inhibitors of SD (*11*−*13*, *16*−*18*, *21*, *26*−*31*) and 3HNR (1,3,8-trihydroxynaphthalene reductase) (*35*−*39*) as overlapping the naphthol substrate (product) binding sites. Adding to their inherent value in inhibitor design, X-ray structures of the enzyme−inhibitor complexes are useful in modeling substrates in the respective active sites (*11*, *14*, *16*−*18*, *35*, *38*, *41*). Owing, in part, to its having a 3−5-fold larger binding pocket resulting in many more protein contacts with inhibitors (*18*, *38*), the potent inhibitors of SD function ($K_i \sim 10$ pM) have 1000-fold greater affinity than the potent 3HNR inhibitors ($K_i \sim 10$ nM). SD is the target of more recently developed fungicides that have lower application rates for disease control than the older fungicides that target 3HNR.

E1cb (elimination first-order conjugate base) and E1cb-like mechanisms (*46*−*48*) are proposed for the solvolytic and SD-catalyzed dehydrations of scytalone (*11*, *14*−*16*, *18*, *49*, *50*). When a *pseudo*-boat conformation of scytalone is docked in the active site of SD as shown in Figure 2, the substrate orientation is ideal for the *syn*-elimination of the

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[1] Abbreviations: SD, scytalone dehydratase; DDBO, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one; DHV, deshydroxyvermelone; 3HN, 1,3,8-trihydroxynaphthalene; 3HNR, 1,3,8-trihydroxynaphthalene reductase; E1cb, elimination first-order conjugate base; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital.

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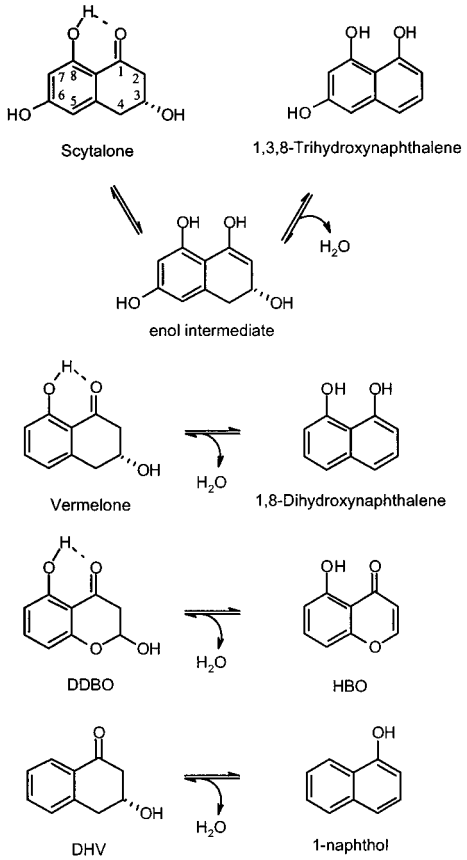

FIGURE 1: Dehydration reactions catalyzed by scytalone dehydratase. Scytalone and vermelone are physiological substrates for the enzyme. DDBO is a synthetic, nonphysiological substrate (*11*, *19*); due to the rapid opening and closing of its heterocyclic ring, DDBO has no lasting stereochemistry, and it is fully dehydrated by scytalone dehydratase. DHV (deshydroxyvermelone) is a synthetic, nonphysiological substrate prepared from 1,3-dihydroxynaphthalene for this work.

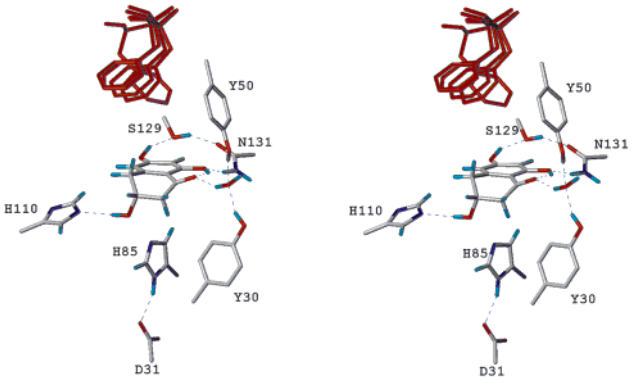

FIGURE 2: Model of scytalone in the active site of scytalone dehydratase. In the stereoview, residues important for catalysis are shown. The reactive conformation of scytalone was docked into the active site of the SD crystal structure (PDB entry 3STD) as described (*14*). F53 is shown in red for the X-ray structures with PDB entry codes 3STD, 4STD, 5STD, 6STD, and 7STD. After overlaying the backbone atoms of the N-terminal α-helix onto those of 3STD as described (*28*), F53 residues from the other structures were transferred to the model. F53 belongs to a flexible loop and is the most mobile active-site residue among structures. Hydrogen bonds are indicated by dashed lines.

elements of water with the participation of two tyrosine residues, two histidine residues, an asparagine, a serine, and a water molecule. Enolization occurs with the hydroxyl groups of tyrosines 30 and 50 assisting the acidification of an active-site water molecule that protonates the carbonyl oxygen of scytalone in concert with the abstraction of the *pro-R* hydrogen (*14*) from the C2 position by the imidazole group of H85 (*11*). The basicity of H85 is increased by pairing its imidazole with the carboxylate of D31 (*11*). Kinetic isotope effects determined from single-turnover experiments (H/D ∼ 6) indicate that abstraction of the *pro-R* hydrogen from scytalone is rate-limiting (*14*). Subsequent hydroxide elimination from C3 is assisted by proton donation from the (now protonated) imidazolium group of H85 and by hydrogen-bonding between the substrate's C3 hydroxyl group and the imidazole group of H110. Tautomerization to generate 1,3,8-trihydroxynaphthalene completes the catalytic cycle.

In addition to its two physiological substrates, the synthetic substrate DDBO is efficiently dehydrated by SD; indeed, it is a better substrate for SD than scytalone or vermelone (*11*, *16*, *19*−*21*). In contrast to scytalone and vermelone, DDBO contains a ring oxygen atom that makes the adjacent carbon atom anomeric (Figure 1). In contrast to scytalone, vermelone and DDBO lack a second phenolic hydroxyl group. Previously, it was shown that the preference for DDBO over vermelone was greater for two mutant enzymes (N131A and H110N) than for the wild-type enzyme, suggesting that the anomeric carbon atom in DDBO assists the catalytically impaired enzymes in the elimination reaction (*11*). In this work, we propose a mechanism for the DDBO effect that involves residues H110 and N131 and extends to residue F53 in deforming the C−OH group of substrates to its reactive state for elimination. Another nonphysiological substrate, as determined by this work, is deshydroxyvermelone (DHV) (Figure 1). In comparison to vermelone, a hydrogen atom replaces the C8 hydroxyl group, and an intramolecular hydrogen bond is lost. The 45-fold larger K_m for vermelone caused by the N131A mutation suggests that the hydrogen bond between the C8 hydroxyl group of vermelone and the carboxamide of N131 is important for substrate recognition (*11*). The C8 hydroxyl group of vermelone could also contribute to catalysis by promoting enolization through its intramolecular hydrogen bond with the C1 carbonyl oxygen. Recently, Kirby and co-workers have demonstrated that one properly positioned intramolecular hydrogen-bonding can stabilize the transition state by as much as 14 kcal/mol (*51*). Therefore, we also examine the solvolytic and enzyme-catalyzed rates of DHV dehydration for comparison with vermelone, DDBO, and scytalone.

EXPERIMENTAL PROCEDURES

Materials and General Methods. DDBO (*19*), scytalone (*11*), and vermelone (*11*) were prepared as described. UV/VIS spectrophotometric analyses were performed on a HP 8452A diode array spectrophotometer (Hewlett-Packard). Homogeneous wild-type SD (*52*) and mutants (*11*, *16*) were prepared as before. 3HNR was purified to homogeneity as described (*53*). Unless indicated differently, other reagents were obtained from Sigma-Aldrich. Data were fitted to the equations through linear or nonlinear regression analysis using Grafit 4 (Erithacus Software).

Preparation of Deshydroxyvermelone (DHV). DHV was prepared using a modification of a published procedure (*54*),

Table 1: Steady-State Kinetic Parameters for Scytalone Dehydratase and Mutants with Substrate Scytalone at pH 7.0 and 25 °C[a]

enzyme	k_{cat}(scytalone) (s^{-1})	k_{cat}/K_m(scytalone) ($\mu M^{-1}\ s^{-1}$)	K_m(scytalone) (μM)	k_{cat}(DDBO)/ k_{cat}(scytalone)	[k_{cat}/K_m](DDBO)/ [k_{cat}/K_m](scytalone)
wild-type[b]	73 ± 8	2.2 ± 0.3	33 ± 3	5.5 ± 0.6	12 ± 2
F53A[c]	5.0 ± 0.6	0.011 ± 0.0005	450 ± 76	22 ± 3	100 ± 6
F53L[c]	12 ± 0.5	0.086 ± 0.003	140 ± 10	10 ± 0.9	310 ± 90
F158L	40 ± 3	0.37 ± 0.03	110 ± 17	7.0 ± 0.7	12 ± 1
F162L[c]	8.0 ± 0.4	0.043 ± 0.002	190 ± 16	5.8 ± 0.5	17 ± 2
M69A	85 ± 26	0.14 ± 0.01	620 ± 230	1.8 ± 0.6	25 ± 2
M69L	78 ± 2	1.5 ± 0.05	53 ± 3	6.9 ± 0.4	15 ± 2
V75A	9.9 ± 0.4	0.054 ± 0.003	180 ± 2	2.1 ± 0.1	20 ± 2

[a] Standard errors from fitting to eq 1 are indicated with the values. Values for k_{cat}(DDBO) and [k_{cat}/K_m](DDBO) are from ref *16*. [b] Means and standard deviations from 7 determinations. [c] Corrected for active-site content as described (*16*).

the main change being that homogeneous 3HNR was employed. The 75 mL reaction contained 100 mM sodium phosphate, pH 7.0, 300 mg of glucose 6-phosphate, 130 mg of 1,3-dihydroxynaphthalene, 500 mg of NADPH, 44 mg of 3HNR, 30 mg of dithiothreitol, and 100 units of glucose-6-phosphate dehydrogenase. The reaction was initiated with 3HNR and incubated 1.5 h under N_2 at 22 °C. The reaction mixture was extracted 4 times with ethyl acetate. The organic layers were combined and dried with sodium sulfate. Ethyl acetate was removed, and the residue was chromatographed on silica gel with ethyl ether as the eluant to afford 50 mg of DHV. The ^{1}H NMR spectrum corresponded to that reported previously (*54*) with the presence of a minor unidentified component. Because of the impurity, the concentration of DHV was determined after dehydration using SD (see *Dehydration Reactions*) and comparison to the UV−Vis spectrum of authentic 1-naphthol in 100 mM sodium phosphate, pH 7.0, at 25 °C. The product of DHV dehydration has an identical UV−Vis spectrum to 1-naphthol above 240 nm.

Dehydration Reactions. Steady-state kinetic parameters for the substrates DDBO, scytalone, and vermelone were determined for the wild-type SD and the mutants at pH 7.0 and 25 °C as described (*11*). In a similar fashion, reactions (1 mL, 30 s) with varied concentrations of DHV contained 100 mM sodium phosphate, pH 7.0 at 25 °C. After initiation of reactions with enzyme, progress was monitored continuously at 290 nm using a delta extinction coefficient (product − substrate) of 2.87 $mM^{-1}\ cm^{-1}$. Initial rate data were fitted to eq 1, where v is the observed velocity, k_{cat} is the maximum velocity, A is the DHV concentration, and K_m is the Michaelis constant. Competitive inhibition of the enzyme-catalyzed dehydration of scytalone was determined under similar conditions with varied concentrations of scytalone and DHV. Initial rates of scytalone dehydration were monitored at 352 nm and fitted to eq 2, where v is the observed velocity, k_{cat} is the maximum velocity, A is the scytalone concentration, I is the DHV concentration, K_m is the Michaelis constant for scytalone, and K_{is} is the inhibition constant for DHV. Values of k_{cat} for DDBO versus temperature were determined between 11 and 30 °C for the wild-type SD in 1 mL reactions (10 s) containing 1.0 mM DDBO [K_m(DDBO) = 15 μM] in 100 mM sodium phosphate, pH 7.0.

$$v = \frac{k_{cat}A}{K_m + A} \quad (1)$$

$$v = \frac{k_{cat}A}{A + K_m(1 + I/K_{is})} \quad (2)$$

Solvolytic rates of dehydration were determined at pH 7.0 (100 mM sodium phosphate) and at temperatures ranging from 25 to 58 °C. Reactions (2 mL) containing 2 mM substrate in sterile plastic tubes were incubated in thermostated water baths. Aliquots (0.1 mL) were withdrawn periodically (up to 60 days) and stored at −80 °C until analysis. Concentrations of scytalone and DDBO remaining in the time-dependent samples were determined spectrophotometrically by end-point analysis using SD as described (*11*). Concentrations of vermelone and DHV remaining in the samples were determined in a similar fashion using delta extinction coefficients (product − substrate) of 5050 $M^{-1}\ cm^{-1}$ at 332 nm for vermelone and 11 100 $M^{-1}\ cm^{-1}$ at 252 nm for DHV. Solvolytic dehydration rates were determined by fitting the remaining substrate concentration versus incubation time to a simple exponential with a zero end point. Thermodynamic parameters were extracted from the rate data using known relationships (*55*).

Theoretical Procedures. All calculations were carried out using the Gaussian 98 program (*56*). Geometrical optimizations were performed with the standard 6-31G(d) basis set by using a hybrid density functional theory method (B3LYP) (*57*, *58*). It has been shown recently that B3LYP is a reasonable method for treating the intramolecular hydrogen-bonding interaction such as exists in vermelone (*59*).

RESULTS

Enzyme-Catalyzed Dehydrations. Steady-state kinetic parameters for the SD-catalyzed dehydrations of scytalone are listed in Table 1 for the wild-type and the mutant SD enzymes prepared for this work. The k_{cat} parameter of the wild-type SD is limited by events after the dehydration reaction by a factor of 6 when either scytalone (k_{cat} = 73 s^{-1}) or vermelone (k_{cat} = 73 s^{-1}) is the substrate and very little when DDBO (k_{cat} = 400 s^{-1}) is the substrate as the three substrates are dehydrated by wild-type SD in the pre-steady-state at about 400 s^{-1} (*11*). We have postulated that aromatization after the dehydration of scytalone or vermelone is rate-limiting to k_{cat} (*11*); aromatization does not occur in the case of DDBO. The k_{cat} values of mutant enzymes severely compromised in catalysis (F53A, F53L, F162L, and V75A) should reflect the true rates of dehydration for both substrates since, in these cases, dehydration is presumably slower than aromatization. As for the wild-type SD, the k_{cat} values for the F158L, M69A, and M69L mutants with the substrates scytalone and vermelone likely underestimate their catalytic power for dehydrating scytalone. Also listed in Table 1 are the relative values of k_{cat} and k_{cat}/K_m for the substrates DDBO and scytalone. The relative values (DDBO/

Table 2: Steady-State Kinetic Parameters for Scytalone Dehydratase and Mutants with Substrate Vermelone at pH 7.0 and 25 °C[a]

enzyme	k_{cat}(vermelone) (s^{-1})	k_{cat}/K_m(vermelone) ($\mu M^{-1} s^{-1}$)	K_m(vermelone) (μM)	k_{cat}(DDBO)/ k_{cat}(vermelone)	[k_{cat}/K_m](DDBO)/ [k_{cat}/K_m](vermelone)
wild-type[b]	73 ± 4	1.9 ± 0.2	38 ± 6	5.5 ± 0.3 (5.1 ± 0.3)[c]	14.2 ± 1.8 (13.2 ± 2.3)[c]
F53A[d]	4.9 ± 0.2	0.021 ± 0.0006	240 ± 20	22.4 ± 1.2 (26.5 ± 2.3)[c]	52.4 ± 2.4 (53 ± 3)[c]
F53L[d]	13 ± 0.7	0.15 ± 0.01	91 ± 11	9.2 ± 0.9 (10.8 ± 0.8)[c]	180 ± 55 (160 ± 28)[c]

[a] Standard errors from fitting to eq 1 are indicated with the values. Values for k_{cat}(DDBO) and [k_{cat}/K_m](DDBO) are from ref *16*. [b] Means and standard deviations from 7 determinations. [c] Values from a second determination. [d] Corrected for active-site content as described (*16*).

scytalone) for the two parameters are similar to the wild-type SD for the mutants F158L, F163L, M69A, M69L, and V75A. The F53A and F53L mutants show considerably larger ratios, suggesting that their mutations impose a preference for the anomeric center of DDBO. A preference for DDBO over scytalone was also recorded for the H110N and N131A mutants (*11*).

To make comparisons between more similar substrates, the relative substrate specificities for the two mutant enzymes (F53A and F53L) were determined for the substrates DDBO and vermelone, which differ in chemical structure by having a ring oxygen and a ring methylene. Steady-state kinetic parameters for the SD-catalyzed dehydration of vermelone and the relative values of k_{cat} and k_{cat}/K_m for the substrates DDBO and vermelone are listed in Table 2 for the wild-type and the F53 mutants. The individual enzymes have similar values for the kinetic parameters with scytalone and vermelone as substrates. Correspondingly, similar high ratios of the relative values of k_{cat} and k_{cat}/K_m for the F53 mutants with the DDBO/scytalone pair are found for the DDBO/vermelone pair. Similar values for the kinetic parameters of scytalone and vermelone held by SD mutants are not always found; e.g., S129A is 820-fold more specific for vermelone than scytalone according to the ratio of k_{cat}/K_m (*11*).

Steady-state kinetic parameters determined at pH 7.0 and 25 °C for the SD-catalyzed dehydration of DHV are 0.77 ± 0.03 s^{-1}, 1.03 ± 0.06 $mM^{-1} s^{-1}$, and 0.75 ± 0.07 mM for k_{cat}, k_{cat}/K_m, and K_m, respectively. In comparison to vermelone, the relative values (vermelone/DHV) for k_{cat} and k_{cat}/K_m are 95 and 1800, respectively. DHV is a competitive inhibitor with respect to scytalone in the enzyme-catalyzed dehydration reaction with a determined K_{is} of 0.97 ± 0.10 mM. This value is similar to the K_m value and shows that a potent inhibitor of the enzyme was not copurified in the DHV preparation.

The temperature dependence of k_{cat} for DDBO with the wild-type SD was used to calculate the thermodynamic parameters in units of kcal/mol: E_a, 13.7 ± 0.1; ΔH, 13.1 ± 0.1; $-T\Delta S$, 0.8 ± 0.1; and ΔG, 13.9 ± 0.1.

Solvolytic Dehydrations. At 25 °C and pH 7.0, the first-order rates of solvolytic dehydration of DHV and vermelone are (8.07 ± 0.007) × 10^{-7} s^{-1} and (1.85 ± 0.06) × 10^{-6} s^{-1}, respectively. For comparison, the corresponding rates for scytalone and DDBO were previously determined as (1.17 ± 0.007) × 10^{-7} s^{-1} and (5.23 ± 0.09) × 10^{-6} s^{-1} (*11*). A temperature study of the dehydration rates in the same medium yields the Arrhenius plots of Figure 3. The dehydration rates at 25 °C and the slopes of the lines of Figure 3 were used for calculating the thermodynamic parameters listed in Table 3. In comparison to the SD-catalyzed dehydration, the solvolytic dehydration rate of DDBO is slower by a factor of 7.7 × 10^7, which is reflected mainly in the difference between the values of the enthalpy terms.

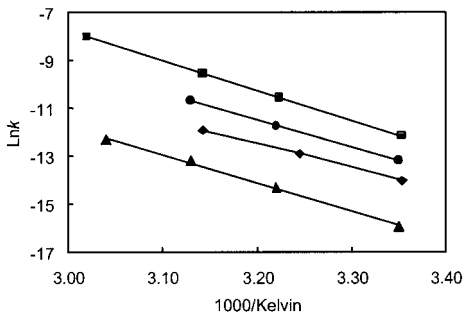

FIGURE 3: Arrhenius plots for the solvolytic dehydration rates of SD substrates. Natural logarithm of the rates (in s^{-1} units) are plotted versus the inverse of the temperature in degrees kelvin times 1000. (▲) Scytalone. (●) Vermelone. (■) DDBO. (◆) DHV.

Table 3: Thermodynamic Parameters (kcal/mol) at 25 °C for the Solvolytic Rates of Dehydration of SD Substrates at pH 7.0

parameter	scytalone	vermelone	DDBO	DHV
ΔG	26.8 ± 0.7	25.2 ± 0.2	24.6 ± 0.1	25.7 ± 0.3
E_a	23.2 ± 0.7	22.6 ± 0.2	24.8 ± 0.1	20.3 ± 0.3
ΔH	22.6 ± 0.7	22.0 ± 0.2	24.2 ± 0.1	19.7 ± 0.3
$-T\Delta S$	4.2 ± 0.7	3.2 ± 0.2	0.4 ± 0.1	6.0 ± 0.3

Theoretical Calculations. A theoretical method at the B3LYP/6-31G(d) level was used to estimate the energy required for distorting vermelone and DDBO to their corresponding eclipsed conformations with the H−C2−C3−O(−H) torsion angle being 0°. In principle, the C3 hydroxyl could be in three possible orientations. We therefore considered all three possibilities in the theoretical study. Geometry of each conformer was fully optimized with the *pseudo*-boat conformation wherein the H(*pro-R*)−C2−C3−O(−H) torsion angle is fixed at 0°. Additional energetic evaluations were also carried out at the B3LYP/6-311+G-(2d,p) level of theory; the calculated energy differences only changed slightly. The energy difference reported here compares the most stable equatorial conformation and the most stable eclipsed boat conformation. In the cases of vermelone and scytalone, the calculated energy differences between the boat conformation and the envelope conformation with an equatorial C3−OH are 6.0 and 6.1 kcal/mol, respectively. The corresponding energy difference for DDBO is 3.9 kcal/mol. Thus, DDBO is easier to deform to the higher energy eclipsed boat conformation than vermelone and scytalone by 2.1 and 2.2 kcal/mol, respectively.

DISCUSSION

The thermodynamic parameters determined for dehydration of scytalone, vermelone, DDBO, and DHV in aqueous solution in the absence of enzyme serve as reference points for the enzyme-catalyzed reactions. An E1cb mechanism has been demonstrated for scytalone (*15*), and by inference applies to the solvolytic dehydrations of vermelone and DHV.

Vermelone serves as the central molecule for making comparisons with the other substrates because it has single substitution changes with respect to DDBO, DHV, and scytalone. As reflected in the Gibbs free energy values (ΔG), solvolytic dehydration of DDBO is faster than that of scytalone, vermelone, or DHV. In an E1cb mechanism, breaking the C−OH bond is rate-limiting, and the ring oxygen of DDBO could promote breaking of the bond by lowering the activation energy (E_a) of the transition state. However, the activation energy for the aqueous dehydration of DDBO is higher than that of the other SD substrates, indicating that an anomeric effect is not operative in the rate enhancement through an E1cb mechanism. Rapid heterocyclic ring opening in DDBO to the rapidly enolizable β-keto-aldehyde precludes use of D_2O exchange to probe directly the E1cb mechanism for this substrate. Replacement of the methylene group of vermelone with the oxygen atom of DDBO causes unfavorable interactions between the lone-pair electrons of the ring oxygen atom and the developing partial negative charge during enolization, thus destabilizing the enolate intermediate of the E1cb mechanism. As enolization of DDBO is considered to be slower than that of vermelone and its hydroxide elimination is considered to be faster, the dehydration of DDBO occurs through either E1 or E2 mechanisms. DDBO dehydrates faster than vermelone because of the smaller entropic term, and the true mechanism of dehydration must accommodate this fact. The small entropic term favors the E2 mechanism because there is little charge accumulation in the transition state that would enforce a loss of freedom of motion by solvent water molecules as occurs in the E1 mechanism (or an E1cb mechanism).

The small difference in activation energies between scytalone and vermelone is likely due to the fact that a significant fraction of scytalone exists in anionic form under the pH 7.0 solvolysis conditions [the pK_a for the C6 hydroxyl group of scytalone is 7.3 (*11*)], which disfavors the enolization step of the E1cb mechanism. In comparing the activation energies for DHV and vermelone, the intramolecular hydrogen bond seems counterproductive to the dehydration in aqueous solution; in vermelone, there is a competing nonproductive deprotonation of the C8 phenol that would disfavor subsequent deprotonation α to the carbonyl. The larger entropic term for DHV than that for vermelone may be attributed to the need for DHV to recruit an additional water molecule to satisfy the loss of the C8 hydroxyl group of vermelone.

Stereochemistry is anti for the aqueous dehydration reactions rather than syn as occurs in the active site of the enzyme (*14*, *15*). Also, the SD-catalyzed dehydration rates (k_{cat}) are faster than the solvolytic dehydration rates by factors of 7.7×10^7, 9.5×10^5, 6.2×10^8, and 3.9×10^7 for DDBO, DHV, scytalone, and vermelone, respectively. However, some of the points raised for the aqueous dehydration of the substrates pertain to the enzyme-catalyzed reactions. First, SD does not bind the anionic form of scytalone, as reflected in the pK_a value of 7.9 for k_{cat}/K_m and the pK_a value of 7.3 for the C6 hydroxyl group of scytalone (*11*). Thus, SD avoids binding a less reactive (anionic) form of scytalone. Second, the thermodynamic parameters for the aqueous dehydration of DDBO argue against the involvement of an anomeric effect in the rate enhancement in an E1cb mechanism, and the same arguments suggest that one is not operative in the SD-catalyzed dehydration of DDBO. Third, the syn stereochemistry of the enzyme-catalyzed reaction is best explained by a conformational flip of the substrate from the envelope conformation of the ground state (the preferred conformation in the solvolytic dehydration) (*15*) to a higher energy boat conformation.

The syn stereochemistry of the SD-catalyzed dehydration reactions requires a good orbital overlap between the HOMO (highest occupied molecular orbital) of the breaking C−H (or the enolate in a stepwise reaction) and the LUMO (lowest unoccupied molecular orbital) of the C3−O(−H) in the transition state. The H−C2−C3−O(−H) dihedral angle determines how well the orbitals overlap, and the maximum overlap is reached at a 0° dihedral angle. The efficacy of enzymatic catalysis, to some extent, is determined by how efficiently an enzyme active site can facilitate such geometrical distortion. According to our theoretical calculations, DDBO is much more readily distorted into the eclipsed conformation than either scytalone or vermelone. The easier deformation of DDBO into its reactive conformation than either scytalone or vermelone is an appealing explanation for DDBO being the better substrate for SD.

Because of the inherent limitation imposed by the physiological substrates in adopting conformations favorable for dehydration, one might suspect that SD has adapted to mediate the formation of reactive conformations of scytalone and vermelone as part of its catalytic power. The ability to promote the formation of such reactive conformations [or near-attack conformers (NACs) as coined by Bruice] by a pre-organized active site has been suggested to be a critical factor in enzyme catalysis in general (*60*−*62*). To probe for SD residues that may function to organize the physiological substrates into their reactive conformations (NAC), we compare kinetic parameters for DDBO and the physiological substrates for the wild-type and site-directed mutants of SD. This study, together with a previous study (*11*), has examined 18 site-directed mutants of 12 active-site residues for their relative substrate specificities for scytalone and DDBO. Among these, 8 mutants (5 residues) have significantly larger relative specificities (DDBO/scytalone) than the wild-type. One of these residues is S129, whose hydroxyl group is important for recognizing scytalone. However, the hydroxyl group is unimportant for recognizing DDBO, vermelone, or inhibitors and unimportant for organizing the active site through its hydrogen bond to the side chain amide of N131. Furthermore, it does not fit our criterion for a substrate organizer because its relative specificity (DDBO/vermelone) is similar to the wild-type SD. Only 4 mutants (H110N, N131A, F53A, and F53L) of 3 active-site residues have significantly higher relative substrate specificities (DDBO/vermelone) than the wild-type SD.

According to our model of SD complexed with scytalone (Figure 2), H110 and F53 are positioned above and below the substrate's C3 position. H110 is hydrogen-bonded to the substrate's C3 hydroxyl group and may be considered to function in selecting the axial conformation of the leaving group. F53, the most mobile active-site residue as determined from comparisons of X-ray structures of several SD inhibitor complexes (Figure 2) (*18*, *28*), may be considered to function by pushing down the C3 position of the substrate so that it attains the productive geometry. In order for the influences of H110 and F53 to have an effect on the orientation of the

C3 group, there must be a pivot to direct their leverage. This role is supplied by N131, which is hydrogen-bonded to the C8 hydroxyl group at the other end of the substrate. The only other candidates for this anchoring role are S129, Y30, Y50, and H85. S129 can be ruled out as having such a role since the S129A mutant has wild-type kinetic parameters with vermelone. Replacing tyrosines 30 and 50, which are indirectly hydrogen-bonded to the substrate's carbonyl oxygen atom through a water molecule, with phenylalanines affords similar affinities for substrates and carbonyl-containing inhibitors to the wild-type SD (*11*, *16*). The Y30F and Y50F mutants have similar relative values (DDBO/vermelone) for k_{cat}/K_m as the wild-type SD, and the role of the tyrosines in catalysis is strictly to acidify the water molecule for promoting the enolization reaction. H85, whose role is to serve as a general base and a general acid in the catalytic cycle, is so severely compromised in catalysis upon mutation to asparagine that the mutant's kinetic parameters for scytalone could not be measured (*11*). However, an efficient mechanism for catalysis would enroll H85 to assist in forming a NAC by positioning the C2 *pro-R* hydrogen atom.

To probe the role of the substrate's intramolecular hydrogen bond in the enzymatic reaction, we compare the k_{cat} and k_{cat}/K_m values for vermelone and deshydroxyvermelone. The relative values of k_{cat} and k_{cat}/K_m for vermelone over deshydroxyvermelone are 95 and 1800, respectively, for the wild-type enzyme, underscoring the importance of the hydrogen-bonding interaction in the enzymatic reaction. This is in stark contrast to the 2-fold difference in rates observed for the two solvolytic dehydrations. Assuming that the ratio of K_m values reflects the contribution of the intermolecular hydrogen bond between the N131 side chain NH_2 group and the vermelone C8 hydroxyl group, the ratio of k_{cat} values would reflect the contribution of the intramolecular hydrogen bond between vermelone's C8 hydroxyl group and the carbonyl oxygen at the C1 position in stabilizing the negative charge development during enolization. Proton donation from N131 to the C8 hydroxyl group allows for more facile donation of a proton from the hydroxyl group to the C1 carbonyl oxygen in the transition state. The ratio of 95 for k_{cat} values corresponds to 2.7 kcal/mol in transition-state stabilization by the intramolecular hydrogen bond while the ratio of K_m values corresponds to 1.7 kcal/mol, reflecting the intermolecular hydrogen bond between N131 and vermelone's C8 hydroxyl group. Alternatively, although in our model of the SD−DHV complex there is not enough room for doing so, a possibility exists for a surrogate water molecule in the active site of the enzyme to assume the role of the C8 hydroxyl group of vermelone. Water molecule(s) is (are) anticipated to replace the C8 hydroxyl group interactions of vermelone in the aqueous dehydration of DHV in the absence of enzyme, accounting for the large entropic penalty. In the scenario on the enzyme, the different kinetic parameters between DHV and vermelone would then reflect the more effective contribution of vermelone's C8 hydroxyl group relative to such a water molecule.

REFERENCES

1. Carter, P., and Wells, J. A. (1987) *Science 237*, 394−399.
2. Wang, Q., and Withers, S. G. (1995) *J. Am. Chem. Soc. 117*, 10137−10138.
3. Tews, I., Terwisscha van Scheltinga, A. C., Perrakis, A., Wilson, K. S., and Dijkstra, B. W. (1997) *J. Am. Chem. Soc. 119*, 7954−7959.
4. Ryan, M., Liu, T., Dahlquist, F. W., and Griffith, O. H. (2001) *Biochemistry 40*, 9743−9750.
5. Kosloff, M., and Selinger, Z. (2001) *Trends Biochem. Sci. 26*, 161−166.
6. Sykes, B. D., and Dolphin, D. (1971) *Nature 233*, 421−422.
7. Belasco, J. G., and Knowles, J. R. (1980) *Biochemistry 19*, 472−477.
8. Sulzenbacher, G., Driguez, H., Henrissat, B., Schuelein, M., and Davies, G. J. (1996) *Biochemistry 35*, 15280−15287.
9. Hokenson, M. J., Cope, G. A., Lewis, E. R., Oberg, K. A., and Fink, A. L. (2000) *Biochemistry 39*, 6538−6545.
10. Fort, S., Coutinho, P. M., Schulein, M., Nardin, R., Cottaz, S., and Driguez, H. (2001) *Tetrahedron Lett. 42*, 3443−3446.
11. Basarab, G. S., Steffens, J. J., Wawrzak, Z., Schwartz, R. S., Lundqvist, T., and Jordan, D. B. (1999) *Biochemistry 38*, 6012−6024.
12. Jordan, D. B., Basarab, G. S., Steffens, J. J., Lundqvist, T., Pfrogner, B. R., Schwartz, R. S., and Wawrzak, Z. (1999) *Pestic. Sci. 55*, 277−280.
13. Steffens, J. J., Basarab, G. S., Schwartz, R. S., Lundqvist, T., Wawrzak, Z., and Jordan, D. B. (1999) in *Modern Fungicides and Antifungal Compounds II* (Lyr, H., Russell, P. E., Dehne, H.-W., and Sisler, H. D., Eds.) pp 121−130, Intercept Ltd., Andover, Hampshire, U.K.
14. Jordan, D. B., Zheng, Y.-J., Locket, B. A., and Basarab, G. S. (2000) *Biochemistry 39*, 2276−2282.
15. Basarab, G. S., Jordan, D. B., and Zheng, Y.-J. (2000) *Org. Lett. 2*, 1541−1544.
16. Jordan, D. B., Basarab, G. S., Steffens, J. J., Schwartz, R. S., and Doughty, J. G. (2000) *Biochemistry 39*, 8593−8602.
17. Lundqvist, T., Rice, J., Hodge, C. N., Basarab, G. S., Pierce, J., and Lindqvist, Y. (1994) *Structure 2*, 937−944.
18. Wawrzak, Z., Sandalova, T., Steffens, J. J., Basarab, G. S., Lundqvist, T., Lindqvist, Y., and Jordan, D. B. (1999) *Proteins: Struct., Funct., Genet. 35*, 425−439.
19. Thompson, J. E., Basarab, G. S., Pierce, J., Hodge, C. N., and Jordan, D. B. (1998) *Anal. Biochem. 256*, 1−6.
20. Thompson, J. E., and Jordan, D. B. (1998) *Anal. Biochem. 256*, 7−13.
21. Basarab, G. S., and Jordan, D. B. (1999) *Biochem. Biophys. Res. Commun. 263*, 617−620.
22. Kurahashi, Y., Sakawa, S., Kinbara, T., Tanaka, K., and Kagabu, S. (1997) *Nippon Noyaku Gakkaishi 22*, 108−112.
23. Tsuji, G., Takeda, T., Furusawa, I., Horino, O., and Kubo, Y. (1997) *Pestic. Biochem. Physiol. 57*, 211−219.
24. *Agrow* (1997) PJB Publications Ltd., Richmond, U.K., Vol. 287, pp 21−22.
25. Sieverding, E., Hirooka, T., Nishiguchi, T., Yamamoto, Y., Spadafora, V. J., and Hasui, H. (1998) in *Proceedings of the 1998 Brighton Conference—Pests and Diseases*, Vol. 2, pp 359−366, British Crop Protection Council, Brighton, England.
26. Chen, J. M., Xu, S. L., Wawrzak, Z., Basarab, G. S., and Jordan, D. B. (1998) *Biochemistry 37*, 17735−17744.
27. Jordan, D. B., Lessen, T., Wawrzak, Z., Bisaha, J. J., Gehret, T. C., Hansen, S. L., Schwartz, R. S., and Basarab, G. S. (1999) *Bioorg. Med. Chem. Lett. 9*, 1607−1612.
28. Basarab, G. S., Jordan, D. B., Gehret, T. C., Schwartz, R. S., and Wawrzak, Z. (1999) *Bioorg. Med. Chem. Lett. 9*, 1613−1618.
29. Jennings, L. D., Wawrzak, Z., Amorose, D., Schwartz, R. S., and Jordan, D. B. (1999) *Bioorg. Med. Chem. Lett. 9*, 2509−2514.
30. Jordan, D. B., and Basarab, G. S. (2000) *Bioorg. Med. Chem. Lett. 10*, 23−26.
31. Jennings, L. D., Rayner, D. R., Jordan, D. B., Okonya, J., Basarab, G. S., Amorose, D. K., Anacelerio, B. M., Gehret, T. C., Lee, J. K., Schwartz, R. S., and Whitmore, K. A. (2000) *Bioorg. Med. Chem. 8*, 897−907.
32. Ishida, M., and Nambu, K. (1975) *Noyaku Kagaku 3*, 10−26.
33. Tokousbalides, M. C., and Sisler, H. D. (1978) *Pestic. Biochem. Physiol. 8*, 26−32.

34. Woloshuk, C. P., Wolkow, P. M., and Sisler, H. D. (1981) *Pestic. Sci. 12*, 86−90.
35. Andersson, A., Jordan, D. B., Schneider, G., and Lindqvist, Y. (1996) *Structure 4*, 1161−1170.
36. Thompson, J. E., Basarab, G. S., Andersson, A., Lindqvist, Y., and Jordan, D. B. (1997) *Biochemistry 36*, 1852−1860.
37. Liao, D.-I., Basarab, G. S., Gatenby, A. A., and Jordan, D. B. (2000) *Bioorg. Med. Chem. Lett. 10*, 491−494.
38. Liao, D.-I., Basarab, G. S., Gatenby, A. A., Valent, B., and Jordan, D. B. (2001) *Structure 9*, 19−27.
39. Jordan, D. B., Basarab, G. S., Liao, D.-I., Johnson, W., Winzenberg, K., and Winkler, D. A. (2001) *J. Mol. Graphics Model1. 9*, 434−447.
40. Thompson, J. E., Fahnestock, S., Liao, D.-I., Valent, B., and Jordan, D. B. (2000) *J. Biol. Chem. 275*, 34867−34872.
41. Liao, D.-I., Thompson, J. E., Fahnestock, S., Valent, B., and Jordan, D. B. (2001) *Biochemistry 40*, 8696−8704.
42. Ou, S. H. (1985) in *Rice Diseases*, 2nd ed., pp 109−201, C. A. B. International, Slough, U.K.
43. Bell, A. A., and Wheeler, M. H. (1986) *Annu. Rev. Phytopathol. 24*, 411−451.
44. Chumley, F. G., and Valent, B. (1990) *Mol. Plant-Microbe Interact. 3*, 135−143.
45. Howard, R. J., and Valent, B. (1996) *Annu. Rev. Microbiol. 50*, 491−512.
46. Gerlt, J. A., Kozarich, J. W., Kenyon, G. L., and Gassman, P. G. (1991) *J. Am. Chem. Soc. 113*, 9667−9669.
47. Gerlt, J. A., and Gassman, P. G. (1992) *J. Am. Chem. Soc. 114*, 5928−5934.
48. Gerlt, J. A., and Gassman, P. G. (1993) *Biochemistry 32*, 11943−11952.
49. Anderson, V. E. (1998) in *Comprehensive Biological Catalysts*, Vol. 2, pp 115−133, Academic Press, New York.
50. Zheng, Y.-J., and Bruice, T. C. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 4158−4163.
51. Hartwell, E., Hodgson, D. R. W., and Kirby, A. J. (2000) *J. Am. Chem. Soc. 122*, 9326−9327.
52. Lundqivst, T., Weber, P. C., Hodge, C. N., Braswell, E. H., Rice, J., and Pierce, J. (1993) *J. Mol. Biol. 232*, 999−1002.
53. Andersson, A., Jordan, D., Schneider, G., Valent, B., and Lindqvist, Y. (1996) *Proteins: Struct., Funct., Genet. 24*, 525−527.
54. Simpson, T. J., and Weerasooriya, M. K. B. (2000) *J. Chem. Soc., Perkin Trans. 1*, 2771−2775.
55. Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219−310.
56. *Gaussian 98* (1998) Revision A.5, Gaussian, Inc., Pittsburgh, PA.
57. Becke, A. D. (1993) *J. Chem. Phys. 98*, 5648−5652.
58. Lee, C., Yang, W., and Parr, R. G. (1988) *Phys. Rev. B 37*, 785−789.
59. Schiott, B., Iversen, B. B., Madsen, G. K. H., and Bruice, T. C. (1998) *J. Am. Chem. Soc. 120*, 12117−12124.
60. Bruice, T. C., and Benkovic, S. J. (2000) *Biochemistry 39*, 6267−6274.
61. Almarsson, O., and Bruice, T. C. (1993) *J. Am. Chem. Soc. 115*, 2125−2138.
62. Luo, J., Kahn, K., and Bruice, T. C. (1999) *Bioorg. Chem. 27*, 289−296.

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