EPR-kinetic isotope effect study of the mechanism of radical-mediated dehydrogenation of an alcohol by the radical SAM enzyme DesII

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The radical *S*-adenosyl-L-methionine enzyme DesII from *Streptomyces venezuelae* is able to oxidize the C3 hydroxyl group of TDP-D-quinovose to the corresponding ketone via an α -hydroxyalkyl radical intermediate. It is unknown whether electron transfer from the radical intermediate precedes or follows its deprotonation, and answering this question would offer considerable insight into the mechanism by which the small but important class of radical-mediated alcohol dehydrogenases operate. This question can be addressed by measuring steady-state kinetic isotope effects (KIEs); however, their interpretation is obfuscated by the degree to which the steps of interest limit catalysis. To circumvent this problem, we measured the solvent deuterium KIE on the saturating steady-state concentration of the radical intermediate using electron paramagnetic resonance spectroscopy. The resulting value, 0.22 ± 0.03 , when combined with the solvent deuterium KIE on the maximum rate of turnover (*V*) of 1.8 ± 0.2 , yielded a KIE of 8 ± 2 on the net rate constant specifically associated with the α -hydroxyalkyl radical intermediate. This result implies that electron transfer from the radical intermediate does not precede deprotonation. Further analysis of these isotope effects, along with the pH dependence of the steady-state kinetic parameters, likewise suggests that DesII must be in the correct protonation state for initial generation of the α -hydroxyalkyl radical. In addition to providing unique mechanistic insights, this work introduces a unique approach to investigating enzymatic reactions using KIEs.

enzymology | desosamine | catalytic commitment | solvent isotope effect | AdoMet

The enzyme-catalyzed dehydrogenation of an alcohol to a carbonyl is one of the most common and fundamental reactions of both primary metabolism and secondary metabolism. In the majority of cases studied, these reactions proceed via hydride transfer to an organic cofactor, such as the nicotinamide moiety of NAD(P)⁺ or the isoalloxazine group of FAD among others (1, 2). In contrast, much less is understood regarding radical-mediated dehydrogenation of an alcohol, because only a handful of enzyme examples have been described. Of the radical alcohol dehydrogenases, galactose oxidase is the most extensively studied, and it is known to use a Cu^{II} ion coordinated to a protein-derived 3'-(*S*-cysteinyl)tyrosyl radical to catalyze dehydrogenation of the C6 hydroxyl group of galactose (3, 4). In contrast, BtrN and the anaerobic sulfatase maturing enzymes (anSMEs) are radical *S*-adenosyl-L-methionine (SAM) enzymes (5–7) responsible for the dehydrogenation of the C3 hydroxyl group of 2-deoxy-scyllo-inosamine (8, 9) and the hydroxyl/sulfhydryl moieties of serine/cysteine residues (10–12), respectively. In addition to the primary [4Fe-4S]¹⁺ cluster, which reduces SAM (1) to methionine (2) and the 5'-deoxyadenosyl radical (17; see Fig. 4) to initiate radical catalysis, BtrN (13, 14) and anSMEs (15–18) possess one or more auxiliary [4Fe-4S]_{aux} clusters, which have been proposed to coordinate the hydroxyl group to be oxidized in the substrate.

The enzyme DesII from *Streptomyces venezuelae* is also a radical SAM enzyme and catalyzes the deamination of TDP-4-amino-4,6-

dideoxy-D-glucose (4) to TDP-4,6-dideoxy-3-keto-D-glucose (5) in the biosynthetic pathway of TDP-D-desosamine (19, 20) (Fig. 1A). DesII has also been shown to accept TDP-D-quinovose (6) as a substrate; however, rather than being dehydrated, the C3 hydroxyl group is oxidized to yield TDP-6-deoxy-3-keto-D-glucose (7) (19) (Fig. 1A). This dehydrogenation is characterized by a 1:1 stoichiometry of SAM reduction vs. TDP-D-quinovose oxidation, and the reduced [4Fe-4S]¹⁺ cluster is regenerated during each catalytic cycle in the absence of an additional source of reducing equivalents beyond the substrate (6) itself (21). Therefore, the dehydrogenation of TDP-D-quinovose by DesII is controlled well despite not being the biosynthetic function of this enzyme.

It has been hypothesized that coordination of the alcohol to either the Cu^{II} ion in galactose oxidase or the [4Fe-4S]_{aux}²⁺ cluster in the radical SAM enzymes plays a key role both in generating the substrate radical and in its controlled oxidation to the carbonyl product (4, 13). The metal ion or cluster may act as a Lewis acid to facilitate deprotonation of the hydroxyl group, making it a stronger reductant, and thereby promote the initial hydrogen atom abstraction from the substrate. Furthermore, direct coordination of the nascent substrate radical to the Cu^{II} center or [4Fe-4S]_{aux}²⁺ cluster would permit rapid inner-sphere electron transfer to complete the dehydrogenation reaction. However, the results of iron-sulfur titration experiments (19), as well as the presence of only four cysteine residues (22) in the primary sequence of DesII, indicate that this enzyme possesses only a single [4Fe-4S] cluster. Thus, it is unlikely that the C3 hydroxyl of TDP-D-quinovose is coordinated to an active site metal ion. Furthermore, electron paramagnetic resonance (EPR) characterization of the DesII-catalyzed dehydrogenation demonstrated formation of an α -hydroxyalkyl radical intermediate (8) rather than the corresponding ketyl radical (9) (23) (Fig. 1B).

These observations have led to a hypothesis regarding oxidation of the initial substrate radical 8 as shown in Fig. 1B (Upper). Deprotonation of the α -hydroxyalkyl radical could be facilitated by the ~5-unit decrease in the pK_a of α -hydroxyalkyl radicals vs. the corresponding alcohols (24). The resulting short-lived ketyl radical would then represent a significantly stronger reductant, given the reduction potential of -1.5 V for a ketone vs. -0.9 V for the corresponding conjugate acid (24–26). Thus, the deprotonation of 8 to 9 could facilitate outer-sphere electron transfer from the radical intermediate to reduce the oxidized [4Fe-4S]_{aux}²⁺ cluster,

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Fig. 1. (A) Deamination and dehydrogenation reactions catalyzed by DesII. (B) Hypotheses for oxidation of the α -hydroxyalkyl intermediate during dehydrogenation of 6.

which has a midpoint potential of around -1 V in aprotic solvents (27–29). Nevertheless, a much more positive reduction potential (-0.5 V) has been measured for the $[4\text{Fe-4S}]^{2+}$ cluster within the active site of the radical SAM enzyme lysine-2,3-aminomutase (30). Therefore, one cannot necessarily rule out the possibility that electron transfer occurs before deprotonation to generate the protonated ketone (10) as an intermediate (Fig. 1B, Lower).

In principle, these hypotheses could be tested by measuring the solvent deuterium kinetic isotope effect (KIE) on the net rate constant governing oxidation of the α -hydroxyalkyl radical (8 $\rightarrow$ 7). Although this KIE will be reflected in the isotope effects on the steady-state kinetic parameters V and V/K , its expression may be diminished depending on the degree to which this process limits turnover (31–33). Although adjustments in pH can help to make a pH-dependent step more rate-limiting (34), this too depends on the kinetic mechanism and can be further complicated by solvent deuterium equilibrium isotope effects (EIEs). Herein, we use EPR to measure the solvent deuterium isotope effect on the steady-state concentration of the radical intermediate. This allows us to take advantage of Cleland's theory of net rate constants (35) to isolate the KIE on the net rate constant specifically associated with the radical intermediate. This approach is reminiscent of recent studies regarding the steady-state concentrations of stable intermediates associated with thiamine-dependent enzymes (36–39). Our results suggest that DesII must be in the correct protonation state to form the initial α -hydroxyalkyl radical and that it is subsequently deprotonated either before or concerted with its oxidation (Fig. 1B, Upper).

Results and Discussion

Steady-State Characterization of DesII. Inspection of the initial rate curves at pH 8.0 indicated substrate inhibition by TDP-D-quinovose, with inhibition being most pronounced as SAM becomes saturating (*SI Kinetic Analysis*). Inhibition by SAM was not observed in the concentration range investigated. Such a pattern is consistent with sequential uncompetitive substrate inhibition in which TDP-D-quinovose (6) binds after SAM (40). Inhibition arises from binding of TDP-D-quinovose to form a dead-end complex with the enzyme and the reduction products of SAM (i.e., 2 and 3) following dissociation of the oxidation product (7). Fitting of the initial rates, given this model of substrate inhibition (*SI Kinetic Analysis*), yielded a 95% confidence interval for the reciprocal substrate inhibition constant $1/K_{Di}^{Sub}$ (at pH 8.0), which does not contain 0, implying an improvement in the fit vs. no substrate inhibition. Michaelis constants of 27 ± 6 μM and 200 ± 50 μM were obtained for SAM and TDP-D-quinovose, respectively. A second model of substrate inhibition was also considered in which TDP-D-quinovose binds the free enzyme to form a dead-end complex; however, this model did not demonstrate a significant improvement in the fit.

A similar result has also been reported for the radical SAM dehydrogenase BtrN (8); however, BtrN demonstrates a significantly smaller inhibition constant of $K_{Di}^{Sub} \approx 0.2$ mM compared with 2 mM for DesII. Dissociation constants for substrate inhibition are apparent and depend on both the intrinsic equilibrium associated with substrate binding to form the dead-end complex as well as the relative rates of product dissociation (40). Therefore, the weaker substrate inhibition seen with DesII may be a combination of more rapid dissociation of the SAM reduction products compared with BtrN as well as weaker substrate binding, because 6 is not the natural substrate of DesII.

Dependence of Steady-State Kinetic Parameters on pH. Initial rates determined at different pH values with saturating concentrations of SAM were fit using the following structural relation for substrate inhibition:

$$v = \frac{V_s}{K_M^{Sub} + s + s^2/K_{Di}^{Sub}}, \quad [1]$$

where s denotes the initial concentration of (6). Estimates of V , $(V/K)_{Sub}$, and corresponding SEs were obtained at each pH from these fits. As shown in Fig. 2, $(V/K)_{Sub}$ decreases at high pH with an apparent pK_a of 10 ± 0.1 . This implies an ionizable group on either the free substrate (6) or the binary complex of enzyme and SAM that must be protonated before forming the ternary Michaelis complex, because V does not show a similar decrease. The N3 nitrogen of TDP is expected to have a pK_a of ~ 10 (41). Therefore, it is proposed that the N3 nitrogen in the thymine ring of the TDP-sugar must be in a neutral state to form a productive ternary Michaelis complex or substrate inhibition complex,* because substrate inhibition was also noted to be weaker under more alkaline conditions.

Both V and $(V/K)_{Sub}$ decrease as the pH is lowered. The apparent pK_a s associated with the acid limb of $(V/K)_{Sub}$ and V are 7.8 ± 0.2 and 8.9 ± 0.1 , respectively. The pK_a values for both the first and second deprotonation events of the diphosphate moiety of the TDP-sugar are expected to be no greater than 7. Therefore, these dependences are likely the result of an ionizable group in the DesII-SAM binary complex that is also able to undergo proton exchange with solvent following substrate (6) binding. The minimal kinetic model shown in Fig. 3A is thus proposed, consistent with the observed pH dependence of the acid limbs of both the $(V/K)_{Sub}$ - and V -pH titrations.

*Closer inspection of the basic limb for the pH titration of $(V/K)_{Sub}$ suggests a wavelike pattern consistent with weak substrate binding when deprotonated; however, the error associated with the $(V/K)_{Sub}$ measurements precludes a more quantitative examination of this hypothesis.

Fig. 2. Dependence of DesII steady-state parameters V (●) and $(V/K)_{\text{Sub}}$ (○) on pH. The ordinate denotes the base-10 logarithm of the steady-state parameter ($P = V$ or $(V/K)_{\text{Sub}}$) normalized vs. the minimum value (P_{min}) observed. The results for V are shifted one log unit along the ordinate for presentation purposes. Error bars denote ± 1 SE of the observed parameter at the given pH following propagation through the logarithm. Fitted lines and the apparent pK_a values were estimated by weighted nonlinear regression as described in *SI Kinetic Analysis*.

Solvent Deuterium KIEs.[†] Solvent deuterium KIEs were measured by direct comparison (42) at pH 8.0 and pH 10.0. Kinetic parameters at pH 8.0 were obtained from fits to Eq. 1, where s is the concentration of **6**, whereas Eq. 2 was used at pH 10.0:

$$v = \frac{V_s}{K_M^{\text{Sub}} + s} \quad [2]$$

because substrate inhibition was negligible at this pH. At pH 8.0 and pH 10.0, the $^{D2O}(V/K)_{\text{Sub}}$ effects were 1.5 ± 0.4 and 1.1 ± 0.1 , respectively, which are not significantly different from unity within error. In contrast the ^{D2O}V isotope effect at pH 8.0 was 2.7 ± 0.4 and decreased to 1.8 ± 0.2 at pH 10.0.

The ^{D2O}V isotope effect at the pH optimum of 10.0 can be considered the isotope effect on the pH-independent value of V , and will therefore be denoted $^{D2O}V_0$. Only the net rate constant k_9 (Fig. 3) is considered able to exhibit a large KIE, because it describes the net transformation **8**→**7**, which includes a solvent-exchangeable proton transfer event. The magnitude of $^{D2O}V_0$ therefore indicates that either only a relatively small solvent deuterium isotope effect exists on k_9 or that it does not contribute significantly to V_0 (further discussion is provided in *SI Kinetic Analysis*). The difference in the ^{D2O}V isotope effects measured at pH 8.0 vs. pH 10.0 is best explained by a mismatch between the isotope effects on the apparent acidity constant for V , denoted $^{D2O}K_{\text{app}}^V$, and the acidity constant for the Hepes buffer used at a constant buffer ratio corresponding to pH 8.0 (43). The latter is equal to the ratio $[\text{H}_3\text{O}^+]/[\text{D}_3\text{O}^+]$ in the H_2O vs. D_2O reactions, and it is denoted H/D for simplicity. The ratio $^{D2O}K_{\text{app}}^V/(H/D)$ can be computed from the values measured for K_{app}^V , ^{D2O}V , and $^{D2O}V_0$, and it is equal to 1.6 ± 0.5 (*SI Kinetic Analysis*). This suggests a discrepancy of ~ 0.2 units between the isotope effects on pK_{app}^V and the pK_a of Hepes.

[†]The typical convention for isotope effect notation is followed (40), such that the solvent deuterium isotope effect on some parameter x is denoted ^{D2O}x and is equal to the ratio of the parameter determined in H_2O divided by that determined in D_2O , (i.e., $^{D2O}x = x_{\text{H}_2\text{O}}/x_{\text{D}_2\text{O}}$).

Interpretation of the solvent deuterium isotope effects on $(V/K)_{\text{Sub}}$ is complicated by the observed sensitivity to both increasing and decreasing pH. Nevertheless, neither value of $^{D2O}(V/K)_{\text{Sub}}$ measured at either pH 8.0 or pH 10.0 was significantly different from unity. This may again be explained by either a small solvent deuterium isotope effect on k_9 or a large forward commitment (42, 44) on the proton transfer step. In the latter case, the commitment may originate from “sticky” substrate binding (40, 42) or an irreversible step, such as formation of the substrate radical, preceding any solvent-exchangeable proton transfer (e.g., $k_6, k_8 \rightarrow 0$) (Fig. 3).

Solvent Deuterium Isotope Effect on ϵ_{rad}^V . The solvent deuterium isotope effect on the steady-state concentration of the α -hydroxyalkyl radical under saturating substrate concentrations, denoted $^{D2O}\epsilon_{\text{rad}}^V$, was measured using EPR (X-band, 100 K). Reactions were run in H_2O and D_2O at buffer (Hepes) ratios corresponding to pH 8.0, because no radical signal was observed at the optimum pH of 10.0. Experiments were performed using two different batches of reconstituted DesII with reactions freeze-quenched in EPR tubes at either 15 s or 20 s. Signal intensities were determined based on double-integration of the EPR spectra using Bruker WIN-EPR V2.22 Rev. 12 software and were normalized for the instrument gain used in each acquisition. The gain-normalized EPR signal intensities in H_2O were divided by those in D_2O to provide the isotope effects. As described in *Supporting Information*, these ratios were next corrected for any differences in the concentration of active enzyme in the H_2O vs. D_2O preparations, and then for the subsaturating ($>80\%$) concentration of substrate used in the experiments.

Fig. 3. Hypothetical kinetic mechanisms A–D discussed in the text to address the observed decrease in V and V/K as the pH is lowered. Intermediates E and EH denote the binary complexes between DesII and SAM in the unprotonated and protonated states, respectively. TDP-D-quinovose, in which the C3 hydroxyl is protonated, is represented by SH (with concentration s). The conversions $EHS \rightleftharpoons EHS^*H$ and $ESH \rightleftharpoons ES^*H$ denote collectively the reductive homolysis of SAM and subsequent generation of the substrate radical by the protonated and unprotonated enzyme forms, respectively. The α -hydroxyalkyl radical observed by EPR (23) thus corresponds to both EHS^*H and ES^*H . The net rate constant k_9 describes both the electron transfer and deprotonation events of the substrate radical (**8**→**7**). Complete release of products and binding of SAM, which is saturating, are described by k_{11} .

Whether the reactions were quenched at 15 s vs. 20 s, quench times had no significant effect on the observed isotope effect. The isotope effect was also not significantly dependent on whether the experiment was conducted with 0.8 mM vs. 1.0 mM initial substrate. Furthermore, the isotope effects measured with the first batch vs. the second batch of reconstituted DesII were the same within error. Finally, there was no evidence of premature release of the substrate radical from the enzyme active site. All these controls are detailed in *SI EPR Measurements*. Thus, the final pooled result of 10 independent measurements in both H₂O and D₂O yielded an isotope effect of $^{D_2O}\epsilon_{rad}^V = 0.22 \pm 0.03$, implying a greater steady-state concentration of the α -hydroxyalkyl radical intermediate in D₂O compared with H₂O.

The observed value of $^{D_2O}\epsilon_{rad}^V$ provides additional insight into the kinetic mechanism of DesII. Given the mechanism shown in Fig. 3A, the majority of the forward reaction flux under saturating (V) conditions will be along the lower, deprotonated pathway at pH 10.0, whereas at pH 8.0, the flux will primarily be through the upper, protonated path (kinetic equations are provided in *SI Kinetic Analysis*). The concentration of the α -hydroxyalkyl substrate radical observed by EPR depends on the steady-state accumulation of both *EHS*[•]*H* and *ES*[•]*H* (Fig. 3), which will be a function of the kinetic partitioning between these EPR-active species and the remaining EPR-silent intermediates. In the case that protonation of the enzyme were to have no effect on radical generation (i.e., $k_7 = k_5$ and $k_8 = k_6$), one would expect that accumulation of the substrate radical species would be the same in both H₂O and D₂O as the pH is decreased (i.e., $^{D_2O}\epsilon_{rad}^V \rightarrow 1$). This is because the mechanism in Fig. 3A predicts that the majority of active enzyme will pile up in the *EHS**H* and *EHS*[•]*H* forms as the pH is reduced (further details are provided in *SI Kinetic Analysis*). Partitioning between *EHS**H* and *EHS*[•]*H* should be the same whether in H₂O or D₂O, because no proton transfer is expected during either reductive homolysis of SAM or H-atom abstraction from the substrate, described collectively by k_7 (and k_8). This expectation of the mechanism shown in Fig. 3A is therefore in discordance with the observed value for $^{D_2O}\epsilon_{rad}^V$.

One possible explanation for this discrepancy is that on freezing of the EPR samples, reactions in H₂O had consumed significantly more substrate than those in D₂O on account of the solvent deuterium isotope effect. This would lead to a significantly lower degree of enzyme saturation with substrate in H₂O than D₂O and, consequently, a value of $^{D_2O}\epsilon_{rad}^V$ less than unity. This possibility seems unlikely because no significant decrease in signal intensity was observed at later quench times in the first set of EPR experiments. Furthermore, during the second experimental trial, reactions prepared under conditions analogous to those for freeze-quenching were also quenched at 15 s using EDTA. HPLC analysis of these reactions indicated less than 5% turnover in either H₂O or D₂O, and the turnover rates inferred from these controls were consistent with those from corresponding activity assays. Therefore, no significant deviation from the initial conditions was observed in either the H₂O or D₂O reactions at the time of freeze-quenching.

A second explanation for the difference between the observed and expected values of $^{D_2O}\epsilon_{rad}^V$ based the mechanism shown in Fig. 3A is that this model or its accompanying assumptions are incorrect. Because dehydrogenation of TDP-D-quinovose is not the biosynthetic function of DesII, it stands to reason that there may not be an enzymatic residue responsible for deprotonation of the α -hydroxyalkyl radical. Instead, hydroxide itself could fulfill this role in a specific base mechanism, such as that shown in Fig. 3D. However, this mechanism again predicts $^{D_2O}\epsilon_{rad}^V \rightarrow 1$, with decreasing pH due to pile up in the *ESH* and *ES*[•]*H* complexes, which partition based on the isotopically (D₂O vs. H₂O) independent rate constants k_5 and k_6 . For similar reasons, the same result is also obtained in the case of the mechanism shown in Fig. 3A if the protonation and deprotonation events were also not at

thermodynamic equilibrium. Therefore, the kinetic mechanisms shown in Fig. 3A and D are disfavored.

The kinetic model shown in Fig. 3A presupposes that only oxidation of the α -hydroxyalkyl radical (**8**→**7**) is pH-sensitive, because it is this process that explicitly involves a proton transfer event. There is no reason, however, to rule out a priori the possibility that the enzyme must be in the appropriate protonation state to generate the substrate radical (**6**→**8**) efficiently. In terms of the mechanism shown in Fig. 3A, this hypothesis implies that the transformation *EHS**H* → *EHS*[•]*H* is disfavored compared with the transformation *ESH* → *ES*[•]*H*, such that k_5 is significantly greater than k_7 . In the limiting case that k_7 and k_8 become vanishingly small, the mechanism shown in Fig. 3A reduces to that shown in Fig. 3B. The mechanism shown in Fig. 3B again predicts enzyme pile-up in the *EHS**H* and *EHS*[•]*H* complexes as the pH is decreased and substrate becomes saturating. However, in contrast to the situation in Fig. 3A, where a direct connection exists between these intermediates, partitioning between *EHS**H* and *EHS*[•]*H* in Fig. 3B depends on that between the minor species *ESH* and *ES*[•]*H*. This, in turn, will be sensitive to whether the steady state is established in H₂O or D₂O, because it depends not only on k_5 and k_6 but also on k_9 , which may exhibit a significant solvent deuterium KIE (details are provided in *SI Kinetic Analysis*).

It should be pointed out that a nonunit value of $^{D_2O}\epsilon_{rad}^V$ may also be observed in a mechanism where only the radical generation step is pH-dependent (i.e., *EHS*[•]*H* does not form, as shown in the Fig. 3C). Here, the concentration of *ES*[•]*H* is dependent on both k_9 as well as the acid-base equilibrium between *ESH* and *EHS**H*, which may also demonstrate a solvent deuterium EIE (*SI Kinetic Analysis*). Therefore, the minimal kinetic mechanisms described by Fig. 3B and C could adequately account for the pH dependence of the

Fig. 4. Mechanistic proposal for the DesII-catalyzed dehydrogenation reaction. It should be emphasized that other pH-sensitive interactions between the substrate and enzyme are expected in addition to that involving the hypothesized general base.

dehydrogenation reaction, as well as the nonunit value observed for $^{D2O}\epsilon_{rad}^V$.

The proposed pH dependence of the radical generation step ($ESH \rightleftharpoons ES^{\bullet}H$) is consistent with current hypotheses regarding radical catalysis of alcohol dehydrogenation (4, 13). In these cases (galactose oxidase, BtrN, and anSMEs), formation of a Lewis adduct between the hydroxyl to be oxidized and a Lewis acid (e.g., an active site metal ion) has been suggested. Although the observed DesII substrate radical does indeed appear to be protonated (23) and there is no reason to expect coordination of the C3 hydroxyl group to an active site metal ion (19), it nevertheless remains a possibility that the C3 hydroxyl group of **6** engages as a donor in a hydrogen-bonding interaction before hydrogen atom abstraction (e.g., **12** in Fig. 4). If this interaction and other pH-dependent interactions are necessary for polarizing the C3 hydroxyl or the proper positioning of the substrate in the active site, a reduction in activity would be expected on disruption at suboptimal pH.

Isotope Effect on the Net Rate Constant k_9 . The key difference between the mechanisms shown in Fig. 3B and C is that the former predicts a pH dependence of the step involving oxidation of the α -hydroxyalkyl radical, whereas the latter does not. Because the proton on the α -hydroxyalkyl radical (**8**) must be removed in any case, pH independence would imply that electron transfer precedes proton transfer (Fig. 1B, Lower). The resulting protonated ketone (**10**) would have a pK_a less than 0, and thus would be readily deprotonated at any of the pH values assayed. These hypotheses can be interrogated using Cleland's theory of net rate constants (35) to determine the solvent deuterium KIE on k_9 within the framework of the two mechanisms.

The solvent deuterium KIE on the net rate constant k_9 is related to the maximum steady-state velocity at suboptimal pH according to the expressions

$$^{D2O}k_9 = \frac{^{D2O}V_0}{^{D2O}\epsilon_{rad}^V} \frac{^{D2O}K_{app}^V}{^{D2O}K_2} \quad [3]$$

for the mechanism shown in Fig. 3B and

$$^{D2O}k_9 = \frac{^{D2O}V_0}{^{D2O}\epsilon_{rad}^V} \frac{^{D2O}K_{app}^V}{(H/D)} \quad [4]$$

for the mechanism shown in Fig. 3C, where H/D again corresponds to the $[H_3O^+]/[D_3O^+]$ ratio at the pH 8.0 Hepes buffer ratio (derivations are provided in *SI Kinetic Analysis*). The isotope effect on k_9 for the mechanism shown in Fig. 3C can therefore be obtained from Eq. 4 using the measured values for $^{D2O}K_{app}^V/(H/D)$ (1.6 ± 0.5), $^{D2O}V_0$ (1.8 ± 0.2), and $^{D2O}\epsilon_{rad}^V$ (0.22 ± 0.03) to give the result $^{D2O}k_9 = 13 \pm 4$. This isotope effect is at the upper limit of the semiclassical expectation for a primary deuterium KIE (45). It is therefore incompatible with the predicted large internal forward commitment due to irreversible electron transfer preceding deprotonation of the resulting protonated ketone. In other words, proton transfer should contribute little to k_9 , which would largely reflect electron transfer, and $^{D2O}k_9$ should approximate unity.

Given the mechanism shown in Fig. 3B, Eq. 3 cannot be solved without some knowledge of $^{D2O}K_2$. As discussed in *SI Kinetic Analysis* (Eq. S74), the acidity constants K_2 and K_{app}^V are not necessarily equivalent; however, their corresponding isotope effects, $^{D2O}K_2$ and $^{D2O}K_{app}^V$, will become equal as either product dissociation becomes very fast (i.e., k_{11} becomes large) and/or oxidation of the substrate radical outpaces its formation (i.e., $k_9 > k_5, k_6$). Under these circumstances, Eq. 3 can be solved for $^{D2O}k_9$ to yield a value of 8 ± 2 , which again approximates the semiclassical value for a primary deuterium KIE. This result is fully compatible with the mechanism shown in Fig. 3B and implies that deprotonation of the α -hydroxyalkyl

radical takes place either before or concerted with electron transfer back to the $[4Fe-4S]^{2+}$ cluster.

Mechanistic Implications. Based on the kinetic analysis described above, we propose the mechanism described in Fig. 4 for the dehydrogenation reaction. In this mechanism, DesII possesses an active site base that can exist in either a protonated or unprotonated state in the free enzyme (**11**, or binary complex with SAM). On binding of SAM (**1**) and substrate (**6**), a hydrogen bond is formed between this active site residue and the C3 hydroxyl group of TDP-D-quinovose in the ternary Michaelis complex (**12**). Disruption of this as well as any other pH-sensitive interactions in the protonated form of the enzyme may then lead to a weaker Michaelis complex and may help to explain the smaller value observed for $pK_{app}^{V/K}$ vs. pK_{app}^V .

The combined process of reductive homolysis of SAM (**12**→**13**) and hydrogen atom abstraction from C3 by the resulting 5'-deoxyadenosyl radical (**13**→**14**) leads to the initial α -hydroxyalkyl radical (the conversion **12**→**14** may also be concerted). Efficient generation of the substrate radical (**12**→**14**) is expected to require the enzyme to be in the appropriate ionization state due to the nonunit value of $^{D2O}\epsilon_{rad}^V$ at suboptimal pH.

Following formation of the substrate radical **14**, it can subsequently partition forward to form the oxidized product (**16**), partition backward to regenerate the Michaelis complex (**12**), or undergo protonation to form a dead-end complex also observable by EPR. The large forward commitment on $^{D2O}(V/K)_{Sub}$ is consistent with slow partitioning of **14** back to the Michaelis complex (**12**) vs. oxidation of **14** to **16**, but it may also be explained by sticky substrate binding. The KIE of ~ 8 on the net rate constant governing forward partitioning of **14** to **16** implies that the active site base deprotonates the α -hydroxyalkyl radical either before or in concert with electron transfer back to the oxidized $[4Fe-4S]^{2+}$ cluster. In the former event that this occurs in a stepwise fashion, the short-lived ketyl radical of intermediate complex **15** is expected. Dissociation of the final product complex then completes the catalytic cycle.

Conclusions

If the isotope effect on the steady-state concentration of an enzyme intermediate can be measured during catalytic turnover, the isotope effect on the associated net rate constant can also be determined. A net rate constant can, in some sense, be thought of as governing the "rate of decay" of an intermediate under steady-state conditions (33, 35). Therefore, knowledge of isotope effects on intermediate concentrations can be used to parse steady-state isotope effects into more readily interpretable components. This approach was used here to demonstrate that oxidation of the α -hydroxyalkyl radical intermediate during the DesII-catalyzed dehydrogenation of TDP-D-quinovose is limited by a solvent exchangeable proton transfer reaction. The method should prove useful in the study of other complex reactions for which relative changes in the concentration of catalytic intermediates can be measured.

Materials and Methods

All reactions were prepared and run in a Coy anaerobic glove box under an atmosphere of $>98\%$ N_2 and $\sim 1.5\%$ H_2 with <1 ppm O_2 . Details regarding DesII expression, purification, and reconstitution have been previously reported (19, 21). Initial rates were determined discontinuously using relative HPLC peak integrations. EPR analysis of freeze-quenched reactions was performed with a Bruker EMX plus spectrophotometer at the Electron Paramagnetic Resonance Spectroscopy Facility in the Department of Chemistry and Biochemistry, University of Texas at Austin. All spectra were obtained at 100 K by field-sweep and the X-band (9.4 GHz) microwave frequency. Details regarding all experimental procedures, as well as representative EPR spectra and instrument parameters, can be found in *SI Materials and Methods* and *SI EPR Measurements*, respectively.

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