Concerted and Stepwise Dehydration Mechanisms Observed in Wild-Type and Mutated *Escherichia coli* dTDP-Glucose 4,6-Dehydratase†

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ABSTRACT: The conversion of dTDP-glucose into dTDP-4-keto-6-deoxyglucose by *Escherichia coli* dTDP-glucose 4,6-dehydratase (4,6-dehydratase) takes place in the active site in three steps: dehydrogenation to dTDP-4-ketoglucose, dehydration to dTDP-4-ketoglucose-5,6-ene, and rereduction of C6 to the methyl group. The 4,6-dehydratase makes use of tightly bound NAD⁺ as the coenzyme for transiently oxidizing the substrate, activating it for the dehydration step. Dehydration may occur by either of two mechanisms, enolization of the dTDP-4-ketoglucose intermediate, followed by elimination (as proposed for β-eliminations by Gerlt, J. A., and Gassman, P. G. (1992) *J. Am. Chem. Soc.* 114, 5928–5934), or a concerted 5,6-elimination of water from the intermediate. To assign one of these two mechanisms, a simultaneous kinetic characterization of glucosyl C5(1H/2H) solvent hydrogen and C6(18OH/18O) solvent oxygen exchange was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The reaction of the wild-type enzyme is shown to proceed through a concerted dehydration mechanism. Interestingly, mutation of Asp135, the acid catalyst, to Asn or Ala alters the mechanism, allowing enolization to occur to varying extents. While aspartic acid 135 is the acid catalyst for dehydration in the wild-type enzyme, the differential enolization capabilities of D135N and D135A dehydratases suggest an additional role for this residue. We postulate that the switch from a concerted to stepwise dehydration mechanism observed in the aspartic acid variants is due to the loss of control over the glucosyl C5–C6 bond rotation in the active site.

dTDP-glucose 4,6-dehydratase catalyzes the conversion of dTDP-glucose into dTDP-4-keto-6-deoxyglucose. The homodimeric *Escherichia coli* 4,6-dehydratase (RfFg) contains one irreversibly bound NAD⁺ per subunit and catalyzes a net redox neutral reaction through a mechanism that includes four chemically distinct species (Scheme 1). The first step, 4-dehydrogenation of substrate, is a critical activating step for β-elimination of water between C5 and C6. In the absence of activation by the adjacent ketone group, deprotonation at C5 (pKₐ > 40) would be impossible under any conceivable conditions in an enzymatic active site. While the 4-keto functionality acidifies the C5 proton, bringing its pKₐ into the range of 18–19 (1), it also creates additional possibilities for the catalytic mechanism of dehydration. In particular, water elimination might proceed more quickly by enolization to an enol (or enolate) intermediate, as depicted in Scheme 2, than by concerted elimination. In Schemes 1 and 2 NAD⁺, NADH, and all dTDP sugars remain bound to the active site throughout the course of a single turnover.

### Scheme 1

Concerted Water Elimination

In enzyme-catalyzed α-β elimination reactions where activation by the carbonyl functionality may not be sufficient, enol formation with concerted α-proton abstraction and stabilization of the enolate has been proposed to explain the observed rate enhancements (2). Certainly such a mechanism presents additional opportunities for enzymatic catalysis, such as protonation of the enolate oxygen, or electrostatic catalysis of the enolate formation. Mechanisms for the 4,6-dehydratase that include an enol/enolate have appeared consistently in the literature, the earliest by Gabriel and Lindquist (3) and the most recent by Allard and co-workers (4).

A recent study has shown that the 4,6-dehydratase active site contains a base, Glu136, and an acid, Asp135, that

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catalyze dehydration (5). While the elimination of water has been shown to proceed with syn stereochemistry with respect to the C5(H) and C6(OH) (6), no information has been published that would help to distinguish between the concerted and stepwise mechanisms for dehydration outlined in Schemes 1 and 2. It is important for the precise understanding of the function of 4,6-dehydratase that the mechanistic relevance of enolization be determined.

During the 4,6-dehydratase-catalyzed conversion of substrate to product, there is complete exchange of the C5(H) with solvent hydrogen (3, 7). To accomplish this, Glu136 must exchange rapidly with solvent prior to the reprotonation of C5, which occurs in the reduction of the dTDP-4-keto-glucose-5,6-ene by NADH (Scheme 1). This C5(H/D) exchange has also been observed in the absence of net catalysis. In our recent pre-steady-state analysis of wt 4,6-dehydratase (8), using dTDP-glucose-d7 as the substrate, we observed the transient formation of C5-exchanged substrate (dTDP-glucose-d6). It became apparent that C5(H/D) and C6-(16O/18O) substrate exchange reactions could be used as tools to detect the involvement of an enolate intermediate in the chemistry of water elimination. If water elimination occurs in concert with C5(H) abstraction, then the rates of deuterium exchange at C5 and [18O]water exchange at C6 should be identical. However, if an enol/enate intermediate forms to any significant extent prior to water elimination, one would expect the rate of solvent hydrogen exchange from C5 to exceed the [18O]water exchange from C6. In this study we present the kinetic analysis of C5 solvent hydrogen (D/16O/18O) substrate exchange reactions could be used as kinetic tools to detect the involvement of an enolate intermediate in the chemistry of water elimination. If water elimination occurs in concert with C5(H) abstraction, then the rates of deuterium exchange at C5 and [18O]water exchange at C6 should be identical. However, if an enol/enate intermediate forms to any significant extent prior to water elimination, one would expect the rate of solvent hydrogen exchange from C5 to exceed the [18O]water exchange from C6. In this study we present the kinetic analysis of C5 solvent hydrogen (D/H) and C6-(16O/18O) substrate exchange reactions catalyzed by wt 4,6-dehydratase and two Asp135 variants (D135N and D135A dehydratases). In addition to being the acid catalyst for dehydration, active site residue Asp135 has been found to be an important controlling determinant for enolization.

### Stepwise Elimination

[18O]Water. A 1 mL sample of [18O]water (~80% enriched and containing HCl) was generously provided by W. W. Cleland. The HCl was removed first by adding dry Tris base until the solution was weakly alkaline and then by performing a two-bulb distillation. To determine the ratio of 16O to 18O, approximately 0.5 mg of PCs was quenched in 40 µL of the 18O-enriched water. The resulting HCl was neutralized with an excess of solid imidazole, and the 31P NMR spectrum was obtained in 500 µL of D2O. The relative abundance of 16O and 18O in phosphate was determined by integrating the five peaks of the phosphate signal [centered at 2.1 ppm; 0.021 ppm separation (10); the peaks, from downfield to upfield, correspond to 0–4 atoms of 18O in phosphate] with the program Peakfit (SPSS Inc.). The percentage enrichment of 18O was found to be 78%.

Exchange Reactions. Three 100 µL reaction mixtures were assembled, each containing 1 mM dTDP-glucose-d7, 1 mM DTT, 1 mg/mL BSA (fraction V), 10 mM ammonium acetate, [18O]water, and 1.8, 24.7, or 14.7 µM wt, D135A, or D135N 4,6-dehydratase, respectively. All of the reagents (except for the [18O]water) were carefully adjusted to pH 7.5 prior to mixing. Each tube contained minimal amounts of components dissolved in natural abundance H2O that contribute to the ultimate isotopic dilution of 18O. The final percentage of [18O]water in the wt, D135A, and D135N reaction mixtures was 72% in each case. Reactions were initiated at 18 °C by addition of enzyme. For analysis, MALDI-TOF MS samples were prepared by quenching 10 µL aliquots of each reaction, at various times, in 100 µL of 70 °C ethanol (8). Precipitated protein was removed by centrifugation, and the ethanolic supernatant was concentrated to dryness in a speed-vac concentrator (Savant) at room temperature. The dried residue was dissolved in 6 µL of H2O and subjected to MALDI-TOF mass spectrometric analysis.

Data Processing. Mass spectral peak areas were obtained using the program Peakfit (SPSS). The substrate dTDP-glucose-d7 (monoisotopic mass 571.2, detected in the negative ion mode [M – H] at 570.2) displayed an isotopic envelope consisting of four peaks with 2.3% of the area at m/z = 569.2, 72.1% at m/z = 570.2, 17.8% at m/z = 571.2, and 7.8% at m/z = 572.2 (errors are ±0.1%). The 569.2 peak was present because the glucose-d7 used in the synthesis of dTDP-glucose-d7 contained only 98% deuterium enrichment; the 571.2 and 572.2 peaks were due to the natural abundance of 13C throughout the molecule. Identical isotopic envelopes are assumed for the C5(D) and C6(13O) exchanged dTDP-glucose species. The relative amounts of each of the four possible exchanged substrate species (dTDP-[6-16O]glucose-d7, dTDP-[6,18O]glucose-d7, dTDP-[6-18O]glu-
cose-d₆, and dTDP-[6-¹⁸O]glucose-d₆) were obtained from the solution to the system of four algebraic expressions:

1. area of 569.2 peak = 72.1% (dTDP-[6-¹⁶O]glucose-d₆) + 2.3% (dTDP-[6-¹⁸O]glucose-d₆)
2. area of 570.2 peak = 17.8% (dTDP-[6-¹⁶O]glucose-d₆) + 72.1% (dTDP-[6-¹⁸O]glucose-d₆)
3. area of 571.2 peak = 7.8% (dTDP-[6-¹⁶O]glucose-d₆) + 17.8% (dTDP-[6-¹⁸O]glucose-d₆)
4. area of 572.2 peak = 7.8% (dTDP-[6-¹⁶O]glucose-d₆) + 72.1% (dTDP-[6-¹⁸O]glucose-d₆)

As little or no signal was detectable at m/z = 568.2, 573.2, 574.2, and 575.2 expressions were not derived for these masses. The concentration of each species in a given sample was then calculated using the total areas for the isotopic envelope of product (major peak at m/z = 551) and deconvoluted substrate species normalized to the starting concentration of 1 mM. Assuming an insignificant ¹⁸O isotope effect for the C₆(OH) exchange reaction, it is possible to correct for partial solvent ¹⁸O enrichment. To make this correction, the [dTDP-[6-¹⁸O]glucose-d₆] was divided by the fraction of ¹⁸O in each reaction (0.72 in each case as measured above), and the increase was subtracted from the experimentally observed [dTDP-[6-¹⁶O]glucose-d₆].

RESULTS

The rates of glucosyl C₅(D) solvent hydrogen and C₆-(OH) solvent oxygen exchange into the substrate were simultaneously determined by mass spectrometric characterization during the multiple turnover conversion of dTDP-glucose-d₆ to product by wt, D135N, and D135A 4,6-dehydratase, in ¹⁸O-enriched water. Representative mass spectra, showing the complete set of substrate peaks for wt and D135A reactions, are presented in Figure 1. It is obvious from these raw data that different exchange properties are manifested in the reactions of wt and D135A dehydratases. The relative abundance of substrate and product molecules
mechanisms. Biological reactions with carbocationic intermediates are not observed, except in cases of allylic stabilization of the carbocation, as in polyterpene cyclases, and glycosyl transfer reactions through oxocarbenium ion intermediates.

The major qualitative difference among the three progress curves in Figure 2 is the rate of formation and accumulation of substrate with the C5 solvent hydrogen exchanged that has not also acquired $^{18}$O, that is, dTDP-$[6-^{16}$O]$\text{glucose-d}_6$. The reaction of wt dehydratase shows no accumulation of dTDP-$[6-^{16}$O]$\text{glucose-d}_6$, only the transient accumulation of doubly exchanged substrate (dTDP-$[6-^{16}$O]$\text{glucose-d}_6$). In contrast, the reaction of D135A dehydratase shows a rapid burst of C5 solvent hydrogen exchanged substrate (dTDP-$[6-^{16}$O]$\text{glucose-d}_6$) preceding the slower transient formation of doubly exchanged substrate (dTDP-$[6-^{18}$O]$\text{glucose-d}_6$). Unlike the wt enzyme, the D135N dehydratase allows some singly exchanged substrate to accumulate but does not show the same rapid burst of proton exchange catalyzed by D135A.

To enable quantitative interpretation of our exchange results, all three sets of progress curves were fitted to a comprehensive minimal kinetic model shown in Figure 3. In this model dTDP-$\text{glucose-d}_7$ may be depleted through one or both of two irreversible steps. The first ($k_1$) leads directly to C5 solvent hydrogen exchanged substrate (dTDP-$[6-^{16}$O]$\text{glucose-d}_6$) and requires dehydrogenation to the 4-ketone, 5-enolization, enzyme-base solvent hydrogen exchange, and re-formation of substrate. The second ($k_2$) leads to a common intermediate, I, which must be the dTDP-4-ketoglucose-5,6-ene as it lacks both the C5(H) and the C6(OH) but is capable of reacting with water in the active site in reverting to the substrate. Because the reaction was carried out in $^{16}$O$\text{water}$, re-formation of substrate from I can only generate dTDP-$[6-^{18}$O]$\text{glucose-d}_6$. This process was modeled as a reversible step ($k_s$, $k_o$). The reincorporation of $^{16}$O into I, yielding the other two substrate forms (dTDP-$[6-^{16}$O]$\text{glucose-d}_7$ and dTDP-$[6-^{16}$O]$\text{glucose-d}_6$), is disallowed because of isotopic dilution of $^{16}$O. These steps were modeled as irreversible ($k_3$, $k_s$). The same rate constant ($k_3$) was used for the forward reaction of both C5 protonated substrates, as experimental error will be of the same or larger magnitude as the $^{18}$O isotope effect. Transformation of I into product was modeled with an irreversible step ($k_5$).

At first glance, analysis of the reaction might appear to be subject to simplification by steady-state approximations. Because substrate is in excess of enzyme (1 mM dTDP-$[6-^{16}$O]$\text{glucose-d}_7$; wt, D135A, and D135N are 1.8 – 24.7 $\mu$M) and the isotopically exchanged forms of substrate (dTDP-$[6-^{16}$O]$\text{glucose-d}_6$ and dTDP-$[6-^{18}$O]$\text{glucose-d}_6$) are diluted into a large pool of dTDP-$[6-^{16}$O]$\text{glucose-d}_7$, the reaction can only be accurately described using a series of differential equations. As the reaction progresses, the product and substrate will compete for the enzyme. For these reasons substrate and product binding steps ($k_{on}$, $k_{oa}$) were included in the differential kinetic model. Although $K_D$ for the substrate has not been determined, its value can be estimated from other constants. The $K_M$ is 5 $\mu$M (9). The substrate analogue dTDP-xylene binds wt 4,6-dehydratase with a $K_D$ of 7 $\mu$M, and the product binds with a $K_D$ of 25 $\mu$M (5). For the purposes of modeling the exchange reactions the value of $K_D$ for the substrate was assumed to be 10 $\mu$M. Equilibrium binding was also assumed with $k_{on}$ being fixed at
1000000 \text{ M}^{-1} \text{ s}^{-1} \text{ (approximately } k_{\text{cat}}/K_{M} = 820000 \text{ M}^{-1} \text{ s}^{-1}; 9) \text{ and } k_{\text{off}} \text{ at } 10 \text{ s}^{-1} \text{ (consistent with } K_{D} \sim 10 \mu \text{M}). \text{ Binding isotope effects were assumed to be insignificant for the present purposes, and identical } k_{\text{on}} \text{ and } k_{\text{off}} \text{ values were used for all three substrate species. Values for } k_{\text{on}} \text{ and } k_{\text{off}} \text{ were } 1000000 \text{ M}^{-1} \text{ s}^{-1} \text{ and } 10 \text{ s}^{-1} \text{ for wt and } 100000 \text{ M}^{-1} \text{ s}^{-1} \text{ and } 1 \text{ s}^{-1} \text{ for D135N and D135A. The fitting program, Dynafit (11), did not converge with slower } k_{\text{on}} \text{ and } k_{\text{off}} \text{ for wt or faster } k_{\text{on}} \text{ and } k_{\text{off}} \text{ for the reaction of D135N. The data for D135A were fitted with either set of } k_{\text{on}} \text{ and } k_{\text{off}} \text{ values with negligible effects on the fitted parameters. The progress curve fits are shown as solid and dashed lines in Figure 4, and the kinetic parameters derived from these fits are listed in Table 1.}

The failure of dTDP-glucose-\text{d}_{6}-C\text{6}\text{16}O \text{ to accumulate in the wt progress curves makes it impossible to derive a value for } k_{1}, \text{ indicating that } k_{1} \ll k_{3}. \text{ The large differences in rate constants between wt and the Asp135 variants are not apparent in Figure 2 because of differences in enzyme concentration and time domains. In each case the major contributor to overall rate limitation in product formation is } k_{7}. \text{ The published decrements in } k_{\text{cat}} \text{ for D135N and D135A compared with wt } k_{\text{cat}} \text{ (124- and 223-fold, respectively) are consistent with the 50- and 107-fold decrements observed for } k_{7}, \text{ requiring only an additional 2-fold contribution from other steps. All of the other constants in the Asp135 variant reactions have suffered major decrements, the largest being for } k_{3} \text{ in the reaction of D135A, which is nearly 5000-fold below the wt value. The value of } k_{3} \text{ for D135N dehydratase is only 400-fold below the wt value.}

![Figure 3: Kinetic mechanism for the action of 4,6-dehydratase. This is the minimal kinetic mechanism used to fit all three data sets shown in Figure 2. Steps indicated by } k_{1}, k_{3}, \text{ and } k_{5} \text{ are irreversible because of infinite dilution of exchanged DH\text{16}O \text{ and H\text{16}O into solvent H\text{18}O. Listed } m/z \text{ values are for the monoanionic (M-H) nucleotide sugar species as detected during negative ion mode MALDI-TOF mass spectrometric analysis. Intermediate I is included in this model to provide appropriate connectivity between detectable nucleotide sugar species. From previous studies (8), I will predominantly be dTDP-4-ketoglucose-5,6-ene, but since enzyme concentrations are very low compared to dTDP-glucose, I is undetectable.}

![Figure 4: Effects of C5-C6 bond rotomeric configuration on chemistry. Newman projections through the C5-C6 bond show the importance of rotomeric conformation to elimination chemistry. In panel A, the configuration is optimal for the concerted syn elimination. In panel B, elimination cannot occur as a result of insufficient orbital overlap; deprotonation of C5 results in delocalization of the nascent carbanion into the adjacent ketone functionality (enolization). Both water elimination and enolization are initiated by C5H/D abstraction. To judge the extent of the effects of the mutation of Asp135 on dehydration, it is valuable to compare the sum of the rate constants for enolization } (k_{1}) \text{ and concerted elimination } (k_{3}) \text{ to the wt } k_{1}. \text{ Quite remarkably, } k_{1} + k_{3} \text{ (listed in Table 1) values are very similar for the D135A and D135N dehydratases and are 370-fold lower than } k_{3}.
The ratio of \( k_3 \)  because of the 100% incorporation of deuterium at C5 in the product generated in D\(_2\)O (3, 7). While one might be concerned that the eliminated water molecule could be retained in the wt active site, the absence of accumulating dTDP-[6-\(^{18}\)O]glucose-\(d_6\) in the wt profile shows that water is readily released from the active site.

The essential facts about the kinetics of the elimination reaction are as follows: (a) The solvent exchanges of C5-\(H\) for H and C6(\(OH\)) for \(^{18}\)OH in the substrate occur at the same rate. No difference can be detected. The simplest interpretation of this fact is that the dehydration proceeds in a concerted step, as shown in Scheme 1. (b) A more complex interpretation would be that the elimination is stepwise as in Scheme 2, where the rate constant for elimination of C6-\(OH\) from the enol(ate) is larger that the rate constant for enol(ate) formation. Then, no enol(ate) would accumulate, as previously reported (8), and the solvent exchange of C5-(\(D\)) would occur before reverse addition of water to the dTDP-4-ketoglucose-5,6-ene. According to this interpretation, a slowing of the elimination step in Scheme 2 would allow the buildup of dTDP-4-ketoglucose, and its enolization and solvent exchange of C5(\(D\)) would then be observed. One way to slow the second step is to abolish the elimination through the substitution of dTDP-D-xylose for dTDP-glucose.

In earlier studies (5, 9) we examined the reaction of dTDP-D-xylose and solvent exchange of C5(\(H\)), dTDP-D-xylose at the active site rapidly undergoes the dehydrogenation by NAD\(^+\) \((k = 1 s^{-1})\) to 73% dTDP-4-ketoxylose and NADH at equilibrium. Thus, the C5(\(H\)) is activated for enolization, but the pseudointermediate cannot undergo either the overall reaction or the elimination of water. Despite the fact that the pseudointermediate builds to 73% in the active site, wild-type 4,6-dehydratase catalyzes the C5(\(H,D\)) exchange with a rate constant of only 0.001 s\(^{-1}\) and for D135A 0.002 s\(^{-1}\). These exchange rate constants are many orders of magnitude lower than \( k_{cat} \) for the overall reaction (5 s\(^{-1}\)) and many orders of magnitude slower than the hydration and reduction of dTDP-4-ketoglucose-5,6-ene \((k = 3 s^{-1})\) to dTDP-glucose (8). The essential absence of solvent exchange cannot be attributed to a forward commitment in this case. The simplest interpretation of all the results is that abstraction of the C5(\(H,D\)) from dTDP-4-ketoglucose is mechanistically connected to the elimination of C6(\(OH\)).

It is difficult to envision a scenario where net C5(\(H\)) exchange from the enzyme active site occurs rapidly from the 4,6-dehydratase/dTDP-4-ketoglucose-5,6-ene complex but several orders of magnitude more slowly from the 4,6-dehydratase/dTDP-4-ketoglucose-5-enol(ate) complex. Under this scenario the reaction would appear to be concerted, with equal C5(\(H\)) and C6(\(OH\)) exchange rates, but could proceed by either stepwise or concerted mechanisms. In this scenario Glu136-COOH exchanges rapidly with solvent in the intermediate with dTDP-4-ketoglucose-5,6-ene but not in the intermediate with dTDP-4-ketoglucose-5-enol(ate). While one might imagine that the two enzyme intermediate complexes could have radically different exchange properties, this extreme case seems unlikely once the significantly slower reactions of the Asp135 variants are examined. The D135N reaction proceeds with nearly identical C5(\(H\)) and C6(\(OH\)) exchange rates, meaning that the slow exchange from the enol(ate) complex is significantly (another 2 orders of magnitude) slower than was needed to make a stepwise wt reaction appear concerted, yet D135A allows rapid solvent exchange.

Table 1: Kinetic Parameters Derived from Steady-State Exchange Progress Curves

<table>
<thead>
<tr>
<th>Reaction</th>
<th>wt rate (s(^{-1}))</th>
<th>D135A rate (s(^{-1}))</th>
<th>D135N rate (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_1 )</td>
<td>0.0003 ((±0.0006))</td>
<td>0.0004 ((±0.0002))</td>
<td>0.0005 ((±0.0008))</td>
</tr>
<tr>
<td>( k_2 )</td>
<td>1.5 ((±0.7))</td>
<td>0.0022 ((±0.0003))</td>
<td>0.0004 ((±0.0009))</td>
</tr>
<tr>
<td>( k_3 )</td>
<td>0.13 ((±0.01))</td>
<td>0.0008 ((±0.0003))</td>
<td>0.0001 ((±0.0003))</td>
</tr>
<tr>
<td>( k_4 )</td>
<td>0.0014 ((±0.0004))</td>
<td>0.0003 ((±0.0005))</td>
<td>0.0004 ((±0.0009))</td>
</tr>
<tr>
<td>( k_5 )</td>
<td>0.0007 ((±0.0008))</td>
<td>0.0054 ((±0.0009))</td>
<td>0.0000 ((±0.0009))</td>
</tr>
<tr>
<td>( k_6 )</td>
<td>0.0 ((±0.0))</td>
<td>0.09 ((±0.2))</td>
<td>0.07 ((±0.02))</td>
</tr>
</tbody>
</table>

* Rate constants were obtained using the kinetic mechanism shown in Figure 3 and the fitting program Dynafit (11). The rate constant \( k_1 \) in wt consistently approached a meaninglessly small number with a large error as the other rate constants converged. This step could be removed from the kinetic mechanism used to fit the wt data without affecting the values of the other constants. This expression corresponds to the k in Scheme 2, where the rate constant for elimination of C6-\(OH\) is readily released from the active site. Clearly our mechanistic interpretations of concurrent exchange of C5-(\(D\)) for H and C6(\(OH\)) for \(^{18}\)OH in the substrate occur at the same rate. No difference can be detected. The simplest interpretation of this fact is that the dehydration proceeds in a concerted step, as shown in Scheme 1. (b) A more complex interpretation would be that the elimination is stepwise as in Scheme 2, where the rate constant for elimination of C6-\(OH\) from the enol(ate) is larger than the rate constant for enol(ate) formation. Then, no enol(ate) would accumulate, as previously reported (8), and the solvent exchange of C5-(\(D\)) would occur before reverse addition of water to the dTDP-4-ketoglucose-5,6-ene. According to this interpretation, a slowing of the elimination step in Scheme 2 would allow the buildup of dTDP-4-ketoglucose, and its enolization and solvent exchange of C5-(\(D\)) would then be observed. One way to slow the second step is to abolish the elimination through the substitution of dTDP-D-xylose for dTDP-glucose. In earlier studies (5, 9) we examined the reaction of dTDP-D-xylose and solvent exchange of C5(\(H\)), dTDP-D-xylose at the active site rapidly undergoes the dehydrogenation by NAD\(^+\) \((k = 1 s^{-1})\) to 73% dTDP-4-ketoxylose and NADH at equilibrium. Thus, the C5(\(H\)) is activated for enolization, but the pseudointermediate cannot undergo either the overall reaction or the elimination of water. Despite the fact that the pseudointermediate builds to 73% in the active site, wild-type 4,6-dehydratase catalyzes the C5(\(H,D\)) exchange with a rate constant of only 0.001 s\(^{-1}\) and for D135A 0.002 s\(^{-1}\). These exchange rate constants are many orders of magnitude lower than \( k_{cat} \) for the overall reaction (5 s\(^{-1}\)) and many orders of magnitude slower than the hydration and reduction of dTDP-4-ketoglucose-5,6-ene \((k = 3 s^{-1})\) to dTDP-glucose (8). The essential absence of solvent exchange cannot be attributed to a forward commitment in this case. The simplest interpretation of all the results is that abstraction of the C5(\(H,D\)) from dTDP-4-ketoglucose is mechanistically connected to the elimination of C6(\(OH\)).
hydrogen exchange from the same complex. This scenario is more complex and less reasonable than the concerted mechanism, given the large differences in 4,6-dehydratase/dTDP-4-ketoglucose-5,6-ene vs 4,6-dehydratase/dTDP-4-ketoglucose-5-enol(ate) exchange rates needed to explain the wt and D135N results and the large increase in the 4,6-dehydratase/dTDP-4-ketoglucose-5-enol(ate) exchange rate caused by the D135A variant.

Available facts do not support a stepwise mechanism but are consistent with a concerted mechanism. Deletion of the acid catalyst in D135N and especially D135A dramatically lowers the rate and, as is typical in such studies with enzymes, allows a different, in this case, stepwise, mechanism to take over.

Both Asp135 variants show significantly lower values of all five rate constants, \( k_1, k_2, k_3, k_a, \) and \( k_r \), relative to wt. The circular dichroism spectra of wt, D135N, and D135A are indistinguishable, so significant changes in secondary structure are not responsible for the lower values (9). One would expect \( k_1, k_2, k_3, \) and \( k_a \) to be affected by changes in catalysis of dehydration and thus be susceptible to variation of Asp135 and/or Glu136. It is perhaps less obvious why variation of Asp135 would affect \( k_r \), as this constant solely governs the rate for the irreversible reduction of the intermediate dTDP-4-keto-glucose-5,6-ene (I) to the product dTDP-4-keto-6-deoxyglucose. The \(~100\)-fold lower value of \( k_r \) indicates that mutation of Asp135 is somehow harming the catalysis of rereduction. One possibility is that the Asp135 variants are altering the ability of Glu136 to function as the acid catalyst. With the alteration of Asp135, acid catalysis of water elimination may be accomplished by another active site amino acid residue, potentially Glu136. This scenario would leave Glu136 in the wrong protonation state (deprotonated) for the reduction step. Conceivably, both base and acid catalysis by Glu136 could occur by the mechanism shown in Scheme 3. Given the observed rapidity of solvent hydrogen exchange from Glu136, prototropic rate limitation for the following step is unlikely (5). Alternatively, one might expect significant changes in the acid/base properties of Glu136 to result from the removal of the adjacent Asp135 carboxylic acid functionality. These two residues probably function together in the active site to promote net catalysis efficiently. The Asp135 variants may also have other effects on the reduction step that we cannot yet describe in detail.

Differences between the kinetics and means of substrate depletion by wt, D135N, and D135A suggest multiple functions for Asp135. While both D135N and D135A show similar rates of substrate depletion \( (k_r + k_3) \), D135A, D135N, and wt 4,6-dehydratases show large differences in the partitioning between enolization and concerted dehydration \( [k/(k_1 + k_3)] \). The lower rates of substrate depletion for the two Asp135 variants (370-fold slower than that for wt) may be largely due to the removal of the acid catalyst for dehydration. It is possible that the lower rates are caused by the partial incapacitation of Glu136, which will become a weaker base once the adjacent Asp135 acid functionality is removed.

By functioning in concert, Asp135 and Glu136 catalyze water elimination in a manner that does not necessitate formation of a high energy intermediate. Removal of either the acid or base would force the reaction through a higher energy transition state and would slow the rate as the \( \Delta\Delta G^{\text{act}} \) is made larger. Enolization leads to the higher energy carbanionic intermediate with delocalization of the nascent negative charge at C5 into the 4-keto functionality. In the action of the wt 4,6-dehydratase, where concerted acid/base dehydration catalysis is maintained, enolization cannot contribute further to transition state stabilization and occurs too slowly to be observed in the presence of the more energetically favorable concerted reaction.

The large observed difference in partitioning between enolization and concerted elimination by D135N and D135A forces one to consider how small structural differences between these variants might guide the reaction through one or the other pathway. One way that the different partitioning may be explained is by invoking an increase in rotational freedom about the C5–C6 bond resulting from alterations in hydrogen-bonding potential as Asp135 is changed to asparagine and alanine. The stereochemical course of the dehydration reaction has been shown to be syn, as depicted in a Newman projection in Figure 4A (6). It is reasonable to propose that Asp135, as the acid catalyst for water elimination, also donates a hydrogen bond to the C6(OH). This hydrogen-bonding interaction may be important in preventing the C6(OH) from assuming rotational conformations that are suboptimal for elimination, as would be the case as the C6-(OH) approaches a position orthogonal to the C5(H) (Figure 4B). Loss of control over rotational freedom would likely favor enolization, as productive orbital overlap for elimination would be less frequent at best and disfavored at worst. As depicted in Figure 5, wt and D135N are capable of asserting this rotational control through hydrogen-bonding interactions, while D135A is not. It is also possible that nonproductive C5–C6 bond rotational conformers are excluded by steric means, although given Asp135’s role in acid catalysis, hydrogen bonding is probably also involved.

Other 4,6-dehydratases, like GDP-mannose 4,6-dehydratase and CDP-glucose 4,6-dehydratase, might contain analogous active site amino acid configurations to promote concerted acid/base catalysis of dehydration and to control C5–C6 bond rotation. The crystal structure of GDP-mannose 4,6-dehydratase (PDB 1DB3) shows a glutamate residue, analogous to Glu136, that has been identified as the base that deprotonates C5, but a serine residue is in place of Asp135 (12). It is possible that some other residue may provide acid catalysis, although it is hard to speculate what that residue might be given that the structure does not contain GDP-mannose. Amino acid sequence alignments show that conserved lysine and aspartate residues from the CDP-glucose 4,6-dehydratase correspond to the Glu136 and Asp135 from dTDP-glucose 4,6-dehydratase, although no
Gerlt and Gassman described concerted acid/base catalysis of enolization as a means of explaining rapid catalytic turnover of $\beta$-elimination reactions despite large $pK_a$ gaps between the substrate $\alpha$-carbon acids and the active site bases (2). Kinetic isotope effect studies of enoyl-CoA hydratase (crotonase) indicate that it catalyzes a concerted hydration at the $\beta$-position of an $\alpha$-$\beta$ unsaturated thioester (14, 15). Enoyl-CoA hydratase has other similarities to 4,6-dehydratase in that it employs two carboxylic acid amino acid active site residues, Glu144 and Glu164 (analogous to Glu136 and Asp135, respectively, in 4,6-dehydratase), for acid/base catalysis of a concerted syn hydration (16). Gerlt and Gassman concede that concerted acid/base catalysis of $\beta$-elimination reactions would resolve similar $pK_a$ discrepancies in dehydration reactions without necessitating the formation of an enol intermediate (2). Concerted acid/base catalysis of either elimination or enolization may be employed in enzyme-catalyzed reactions to decrease energy barriers in cases where large $pK_a$ differences exist between active site residues and substrates.

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REFERENCES


FIGURE 5: Enolization and concerted water elimination in wt, D135N, and D135A. Three panels postulate mechanistic explanations for the observed exchange results of wt, D135N, and D135A. The partitioning between enolization and elimination is indicated in each panel. Dotted arrows represent a less frequent electronic motion. In the wt reaction a hydrogen bond locks the C6(OH) into position for a concerted acid/base-catalyzed syn elimination. The D135N variant can still provide a hydrogen-bonding interaction with the C6(OH), but it cannot function as an acid catalyst. As a result the elimination is slowed, but enolization only occurs during 7% of substrate depletion. The D135A variant is incapable of both hydrogen bonding and acid catalytic functions, and thus the reaction is slowed, and enolization occurs 90% of the time.

Structural data are available to confirm these assignments (13).