Mechanistic Roles of Thr134, Tyr160, and Lys 164 in the Reaction Catalyzed by dTDP-Glucose 4,6-Dehydratase†

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ABSTRACT: *Escherichia coli* dTDP-glucose 4,6-dehydratase and UDP-galactose 4-epimerase are members of the short-chain dehydrogenase/reductase SDR family. A highly conserved triad consisting of Ser/Thr, Tyr, and Lys is present in the active sites of these enzymes as well in other SDR proteins. Ser124, Tyr149, and Lys153 in the active site of UDP-galactose 4-epimerase are located in similar positions as the corresponding Thr134, Tyr160, and Lys164, in the active site of dTDP-glucose 4,6-dehydratase. The role of these residues in the first hydride transfer step of the dTDP-glucose 4,6-dehydratase mechanism has been studied by mutagenesis and steady-state kinetic analysis. In all mutants except T134S, the k_{cat} values are more than 2 orders of magnitude lower than of wild-type enzyme. The substrate analogue, dTDP-xylose, was used to investigate the effects of the mutations on rate of the first hydride transfer step. The first step becomes significantly rate limiting upon mutation of Tyr160 to Phe and only partly rate limiting in the reaction catalyzed by K164M and T134A dehydratases. The pH dependence of k_{cat}, the steady-state NADH level, and the fraction of NADH formed with saturating dTDP-xylose show shifts in the pK_a assigned to Tyr160 to more basic values by mutation of Lys164 and Thr134. The pK_a of Tyr160, as determined by the pH dependence of NADH formation by dTDP-xylose, is 6.41. Lys164 and Thr134 are believed to play important roles in the stabilization of the anion of Tyr160 in a fashion similar to the roles of the corresponding residues in UDP-galactose 4-epimerase, which facilitate the ionization of Tyr149 in that enzyme [Liu, Y., et al. (1997) *Biochemistry 35*, 10675−10684]. Tyr160 is presumably the base for the first hydride transfer step, while Thr134 may relay a proton from the sugar to Tyr160.

The short-chain dehydrogenase/reductase (SDR) family consists of members in all kingdoms that catalyze a wide range of reactions, including alcohol and steroid dehydrogenation, carbonyl reduction, and ketoacyl reduction (*1*). Even though there is very little sequence identity (15−30%) among the enzymes of the SDR family, the three-dimensional structures of the N-terminal 180-odd residues are conserved (*1*−*3*). The cofactor NAD(H) or NADP(H) common to all SDR enzymes is bound to a Rossmann-fold (*4*) located in the N-terminal region which includes the highly conserved motif, GlyXXXGlyXGly (*1*). The most conserved amino acid is a Tyr residue found in the other conserved primary sequence motif, TyrXXXLys, which has been shown by chemical modification and site-directed mutagenesis to be critical for catalytic activity of the SDR enzymes (*5*−*8*). These motifs and the tertiary structure of the N-terminal region are also common to both dTDP-glucose 4,6-dehydratase (dehydratase)[1] and UDP-galactose 4-epimerase (epimerase)[1] which are members of a sub-family included in the SDR family (*1*, *2*, *9*).

Nucleotide-linked-6-deoxyhexoses are important precursors for the biosynthesis of antibiotics such as streptomycin and of the saccharides of the outer membrane lipopolysaccharides (LPS) (*10*). In the *Escherichia coli* chromosome the genes of the *rff* cluster encode enzymes involved in the biosynthesis of the enterobacterial common antigen, among which is dehydratase encoded by the *rffG* gene (*11*). Dehydratase catalyzes the tranformation of dTDP-glucose into dTDP-4-keto-6-deoxyglucose (*12*, *13*). Its primary amino acid sequence is 25% identical to that of *E. coli* UDP-galactose-4-epimerase (*14*). The three-dimensional structures of the two enzymes are highly conserved (*9*, *15*), including the active sites, with one NAD^+ irreversibly bound per subunit (*14*, *16*). In the reaction mechanisms of dehydratase (*14*, *17*) and epimerase (Scheme 1) the first intermediate is a nucleotide-linked-4-keto-pyranose, which is formed by concomitant oxidation of C-4 of the pyranose moiety and reduction of NAD^+ to NADH. Following this step the two mechanisms diverge. In the epimerase mechanism the hydride is transferred from NADH to the opposite face of the 4-keto group to give the product, following rotation about the bond between the anomeric oxygen and the β-phosphorus

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[1] Abbreviations: SDR, short-chain dehydrogenase/reductase; dehydratase, dTDP-glucose 4,6-dehydratase; epimerase, UDP-galactose 4-epimerase; PGM, phosphoglucomutase; NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); dTDP-glucose, thymidine 5′-diphospho-α-D-glucose; wt, wild-type; dTTP, thymidine 5′-triphosphate; TNM, tetranitromethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; OD_{600}, optical density at 600 nm.

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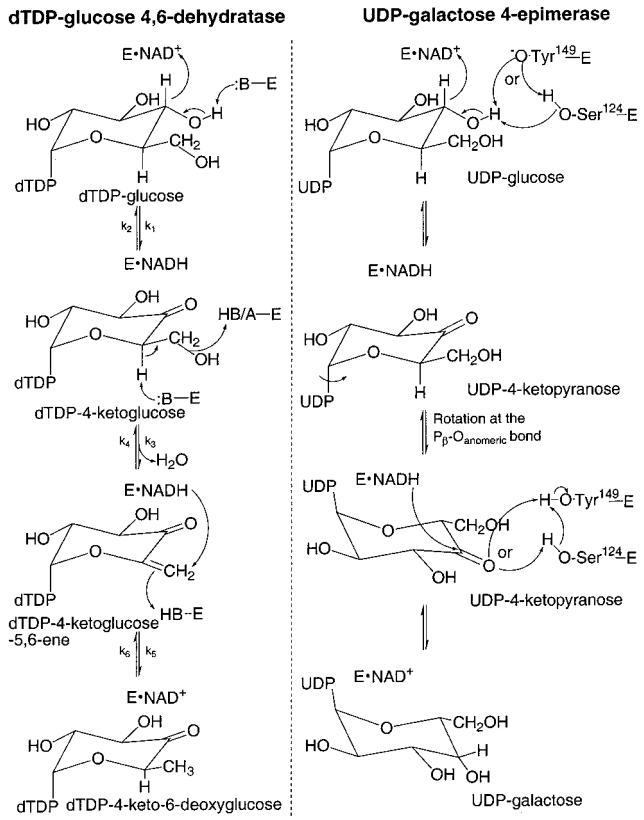
Scheme 1



of the nucleotide. The dehydratase mechanism is chemically more complex. The 4-keto intermediate undergoes dehydration between C-5 and C-6 yielding dTDP-4-ketoglucose-5,6-ene (*14*). The product dTDP-4-keto-6-deoxyglucose is then formed by hydride transfer from NADH to C-6, with oxidation of NADH to NAD^+.

In some SDR proteins such as mouse lung carbonyl reductase (*18*), *drosophila* alcohol dehydrogenase (*19*), 3β,-17β-hydroxysteroid dehydrogenase (*20*), GDP-mannose 4,6-dehydratase (*21*), and sepiapterin reductase (*22*) a conserved triad formed by Ser/Thr, Tyr, and Lys has been shown by mutagenesis studies to be important for activity. This catalytic triad is present in the active sites of both dehydratase and epimerase (*9*, *15*). The mechanistic roles of Tyr149, Lys153, and Ser124 of epimerase have been thoroughly investigated by kinetic and crystallographic studies (*23*−*25*). Tyr149 is the base catalyst that either directly or through a proton relay mediated by Ser124 abstracts the proton of the 4-OH group of the pyranose moiety of the substrate (*24*). Lys153 enhances the chemical reactivity of the coenzyme NAD^+ (*23*). In light of the similarities between the epimerase and dehydratase we have investigated by mutagenesis and kinetic studies the roles of the triad Thr134, Tyr160, and Lys164 in the catalytic mechanism of the dehydratase.

EXPERIMENTAL PROCEDURES

Materials and Methods. All deoxyoligonucleotides were from Life Technologies and further purified by urea-denaturing acrylamide gel electrophoresis. Carrier free [^{32}P]-orthophosphate (10 mCi/mL) in 1 N HCl was from American Radiolabeled Chemical Inc. Glycogen phosphorylase and phosphoglucomutase were from Boehringer Manheim. All buffers, dTTP, UDP-glucose pyrophosphorylase, and glycogen were from Sigma. All solvents, dTMP-morpholidate, α-D-glucose-1-phosphate, and α-D-xylose-1-phosphate were from Aldrich. The wt dehydratase was a generous gift from Adrian D. Hegeman. dTDP-glucose and dTDP-xylose were synthesized and purified using the Moffatt procedure (*27*) from dTMP-morpholidate and α-D-glucose-1-phosphate and α-D-xylose-1-phosphate, respectively. DNA sequencing was performed by the University of Wisconsin Biotechnology Center Nucleic Acid and Protein Facility.

Construction of Expression Plasmids for Variants of 4,6-Dehydratase. The pTZ18UgalSDo355CBPF plasmid with chitin binding protein/intein/ dehydratase fusion expression construct was a generous gift from Adrian D. Hegeman. Mutations were introduced in pTZ18UgalSDo355CBPF using a Stratagene QuickChange kit. The oligonucleotide primers used for site-directed mutagenesis are listed as Supporting Information. The variant plasmids were transformed into *Epicurian coli* XL1-Blue supercompetent cells (Stratagene) and purified using a QIAprep Miniprep kit (QIAGEN). The mutations introduced were verified by sequencing the entire ORF for each variant. The overexpression and purification of the variants are described as Supporting Information.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectroscopy was performed on an AVIV 62A DS spectrometer with a temperature control unit. All the variant samples were exchanged into 50 mM potassium phosphate, pH 7.5, using G50 spin columns. UV CD spectra (300 to 185 nm) of the mutants (0.2−0.4 A_{280}) were acquired at 25 °C.

Synthesis of [β-^{32}P]dTDP-Glucose. [β-^{32}P]dTDP-Glucose was synthesized from glycogen, [^{32}P]orthophosphate and dTTP using PGM, glycogen phosphorylase, UDP-glucose pyrophosphorylase, and inorganic pyrophosphatase and purified as described by Hegeman *et al.* (*26*).

Determination of the NAD^+/NADH Content in the Variants. The amount of NAD^+ bound in each variant was determined using a modified procedure described by Klingenberg (*28*). The fluorescence spectra were obtained with a Perkin-Elmer MPF-3 spectrofluorimeter with the excitation wavelength at 340 nm and the emission wavelength at 460 nm. Both slits were 10 nm. To 20 μL of enzyme solution (0.8 mM) was added 100 μL of 1 N $HClO_4$, and the mixture was vortexed and centrifuged. The supernatant was collected and to the precipitated enzyme were added 100 μL of 100 mM glycine, pH 8.7. The solution was vortexed, and after centrifugation the supernatants were combined and titrated to neutrality with 5 N KOH. The solution was centrifuged and the supernatant collected. To the potassium perchlorate precipitate 100 μL of 100 mM glycine, pH 8.7, were added, and the solution was centrifuged again. The supernatants were combined and 220 μL of the supernatant was diluted in 770 μL of 0.1 M glycine, pH 8.7, with 5 μL of absolute ethanol, and the fluorescence emission at 460 nm was measured (F_1). To this solution 5 units of alcohol dehydrogenase were added and the emission at 460 nm again recorded (F_2). Finally 5 μL of a NAD^+ solution of known concentration was added and the fluorescence at 460 nm measured again (F_3). The concentration of NAD^+ from the enzyme was determined from the following equation:

$$[NAD^+]_{sample} = [(F_2 - F_1)/(F_3 - F_2)] \times [NAD^+]_{standard}$$

The amount of NADH bound in each variant was determined after denaturation of 20 μL of the enzyme solution in 100 μL of 6 M guanidine hydrochloride, filtration using a microcon 10 (Amicon), and dilution to 3 mL with 100 mM glycine, pH 8.7. The fluorescence of the solution was measured with excitation at 340 nm and detection at 460 nm with a Perkin-Elmer MPF-3 spectrofluorimeter. A calibration curve was measured using standard solutions of NADH.

Rates of Reduction of Dehydratase with Dimethylamine Borane. To 1 mg/mL of enzyme in 1 mM EDTA, 1 mM DTT, 100 mM Tris, pH 7.5, dimethylamine borane was added to a final concentration of 200 mM. The reduction of NAD^+ was followed at 25 °C by monitoring the formation of the peak at 355 nm.

Determination of Steady-State Rate Constants. Reactions were run at 37 °C in 100 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA with substrate concentrations varying from 1 to 150 μM. [β-^{32}P]-dTDP-Glucose was added to give a solution of 5000 cpm/μL. Aliquots of the reaction were spotted on a silica gel TLC plate (Whatman LK6DF silica gel 60 Å). After drying the plates, the substrate ($R_f = 0.3$) and the product ($R_f = 0.4$) were separated using the solvent system methanol/acetic acid/triethylamine/ethanol (30:5:2:70). Detection and quantitation was accomplished by phosphoimagery (Molecular Dynamics). Assay reactions were run in triplicate. The kinetic constants were obtained by fitting the data to $v = VA/(K + A)$ using the program HYPER (*29*).

Log k_{cat} vs pH Profiles for Dehydratase and Variants. Dehydratase activity can be measured by monitoring the formation of dTDP-4-keto-6-deoxyglucose in the stopped point assay described by Okazaki et al. (*30*). Assay reactions were run in triplicate at 18 °C in 1 mM DTT, 1 mM EDTA and with the 0.08 M piperazine, 0.1 M HEPES buffer system described by Hegeman et al. (*26*). The detection limit of the product in this assay allowed determination only of k_{cat}. The substrate concentrations used were 0.8 and 1.2 mM. The pH profiles were fitted to $\log k_{cat} = \log[c/(1 + [H^+]/K_1 + K_2/[H^+])]$ using the program BELL (*29*).

pH Dependence of the Fraction of NADH with Saturating dTDP-Glucose or dTDP-Xylose. The fraction of NADH formed during steady-state turnover was measured in a pH range from 6.0 to 10.0 at 16 °C for a solution of 0.025 mM in 1 mM DTT, 1 mM EDTA, and in the 0.08 M piperazine, 0.1 M HEPES buffer system. The dTDP-glucose concentrations varied from 2 to 5 mM. dTDP-xylose experiments were done by adding dTDP-xylose to the reaction mixture until no further increase in absorbance at 355 nm was detected. Enzyme-bound NADH was detected on a Cary 300 Scan UV−vis spectrophotometer by its absorbance at 355 nm and quantified by the extinction coefficient of 6 mM^{-1} cm^{-1} (*14*). The fraction of NADH formed in the reaction with dTDP-glucose was determined as described by Hegeman et al. (*26*). The fraction of NADH formed in the reaction with saturating dTDP-xylose at equilibrium was determined from the difference in absorbance before the addition of dTDP-xylose and the maximum absorbance measured after addition of dTDP-xylose and correction for the dilution factor.

The pH profiles of the dTDP-xylose reaction were fitted to $\log k_{cat} = \log[c/(1 + [H^+]/K_1)]$ using the program HABELL (*29*). The rate of approach to equilibrium with saturating dTDP-xylose was measured at 16 °C in 1 mM DTT, 1 mM EDTA, 100 mM Tris, pH 7.5, with 0.025 mM enzyme. The dTDP-xylose concentrations used were 12, 8, 6, and 4 mM. The rate of formation of NADH was measured at 355 nm using an OLIS Inc. RSM-1000 stopped flow spectrophotometer in the reaction catalyzed by T134A dehydratase or with a Cary 300 Scan UV−vis spectrophotometer in the reaction catalyzed by the K164M dehydratase.

RESULTS

Coenzyme Content and CD Spectra of the Mutated Dehydratases. The purified dehydratase variants contained coenzyme in the reduced form, as shown by the absorbance peak centered at 355 nm in their absorption spectra. The amount of bound NADH present in the variants ranged from 8 to 98%. In the Y160A variant, almost all the coenzyme (98%) was tightly bound NADH. Other variants with a high percentage of bound NADH were K164M, T134V, and Y160F with, respectively, 62, 56, and 35% of reduced coenzyme. The bound NADH was oxidized to NAD^+ by adding dTDP-4-keto-6-deoxyglucose. When all the coenzyme was in the oxidized form, the dTDP-sugars were removed from the enzyme using a G50 spin column equilibrated with the appropriate buffer and 1 mM DTT. The enzymes thus treated were used in all experiments unless indicated otherwise. The total content of reduced and oxidized coenzyme present was measured on the purified variants, which had not been treated with product. One mole of coenzyme is present per mol of subunit in all cases except the Lys164 variants. K164M and K164A dehydratases contained 0.79 and 0.31 of coenzyme per monomer, respectively. When exogenous NAD^+ was added to these variants, neither an increase in bound dinucleotide content nor an increase in activity were observed. Proper folding of the secondary structural elements of the variants was established by circular dichroism spectroscopy. The CD spectra of the mutants (not shown) are essentially identical to the wt CD spectrum (*26*). Therefore the observed decreased activity of the variants is not due to major structural changes in the enzyme.

Steady-State Kinetics. The steady-state kinetic constants measured at 37 °C and at pH 7.5 for each variant are summarized in Table 1. Threonine 134 is not a conserved residue in the enzymes of the SDR family (*1*) and is often substituted by a serine residue, as in the epimerase (*24*). The T134S dehydratase has similar steady-state kinetic constants to the wild-type, showing that this conservative mutation does not affect catalysis. The kinetic parameters for the Lys164 variants have been corrected for the amount of cofactor bound. Mutation of Lys164 to Ala or Met causes a decrease in k_{cat} by factors of 34 or 96, respectively. These are the only mutations studied that slightly affect the value of K_m, which increased by factors of 15 and ~9-fold for K164A and K164M dehydratases, respectively. All other variants display changes in k_{cat} of ~200-fold and in k_{cat}/K_m of 200−800-fold.

The pH dependencies of k_{cat} for the wt and for T134A, Y160F, and K164M dehydratases were measured at 18 °C using the spectrophotometric dehydratase assay and are shown in Figure 1. The profiles are similar and are all bell-

Table 1: Steady State Kinetic Parameters for dTDP-Glucose 4,6-Dehydratase Variants and Wild-Type Enzyme Measured at 37 °C and pH 7.5

dehydratase	K_m (mM)	fold increase	k_{cat}/K_m (mM^{-1} s^{-1})	fold decrease	k_{cat} (s^{-1})	fold decrease
wt[a]	0.0060 ± 0.0007		820 ± 60		4.9 ± 0.2	
T134A	0.007 ± 0.001	1.2	2.9 ± 0.4	283	0.021 ± 0.002	233
T134V	0.020 ± 0.002	3.3	1.04 ± 0.07	788	0.0207 ± 0.0005	237
T134S	0.022 ± 0.003	3.7	109 ± 8	7.5	2.4 ± 0.2	2
Y160A	0.017 ± 0.001	2.8	1.20 ± 0.09	683	0.0198 ± 0.0005	247
Y160F	0.0073 ± 0.0007	1.2	3.5 ± 0.3	234	0.0258 ± 0.0007	190
K164A[b]	0.09 ± 0.01	15	1.58 ± 0.1	820	0.14 ± 0.01	34
K164M[b]	0.052 ± 0.009	8.7	1.0 ± 0.1	837	0.051 ± 0.004	96

[a] Ref *26*. [b] Kinetic rate constants corrected for the amount of bound coenzyme present in the variant.

Table 2: Thermodynamic and Kinetic pK_a Values Obtained from the HABELL or BELL Fits in Figures 1, 2, and 3

dehydratase	pK_a from ss NADH with dTDP-glucose, Figure 2	pK_a from log k_{cat} vs pH, Figure 1	pK_a from fraction of NADH with saturating dTDP-xylose, Figure 3
wt[a]	6.60 ± 0.06	6.87 ± 0.08	6.41 ± 0.07
T134S	6.65 ± 0.06	6.51 ± 0.09	
T134A	7.4 ± 0.2	6.9 ± 0.1	7.40 ± 0.07
K164M	7.7 ± 0.1	7.84 ± 0.08	8.2 ± 0.1
K164A	8.0 ± 0.2		
Y160F	6.6 ± 0.1		

[a] Ref *26*.

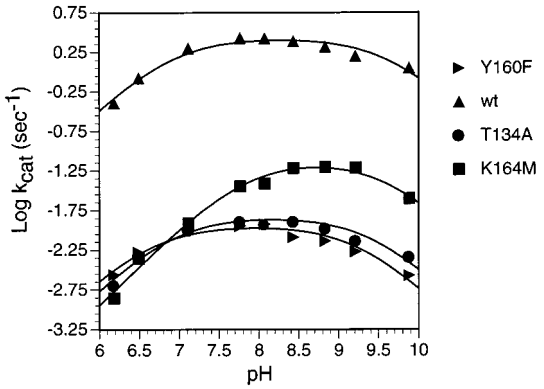


FIGURE 1: Log k_{cat} vs pH profiles for the reactions catalyzed by wild-type, T134A, Y160F, and K164M dehydratases at 18 °C. The pK_a values from the fitted curves are in Table 2.

shaped. The pK_a values on the acidic side are 6.87 ± 0.08, 6.6 ±0.1, 6.9 ± 0.1, and 7.84 ± 0.08 for wt, Y160F, T134A, and K164M dehydratases, respectively. On the basic side, the pK_a values are 9.6 ± 0.1, 9.3 ± 0.1, 9.4 ± 0.1, and 9.6 ± 0.1 for wt, Y160F, T134A, and K164M dehydratases, respectively (Table 2). The pH-independent constants for each pH profile are in Table 3.

pH Dependence of the Fraction of NADH with Saturating dTDP-Glucose. The pH dependence of the fraction of NADH observed in the reactions catalyzed by wt dehydratase (*26*) and T134S, T134A, K164A, and K164M dehydratases is shown in Figure 2. The fraction of bound NADH is an indication of the formation of the dTDP-4-ketoglucose and dTDP-4-ketoglucose-5,6-ene in the steady state. The highest percentage of NADH was observed at basic pHs and was identical for the wt (*26*) and T134S dehydratase (44%). T134A and K164M dehydratases displayed lower steady-state levels of NADH than wt, with 8.6 and 10.0% (Table 3), respectively, while the steady-state level of bound NADH for K164A dehydratase was 20%. Bound NADH was not

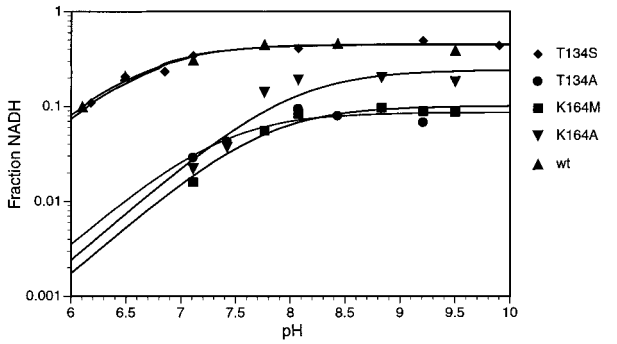


FIGURE 2: Fraction of steady-state NADH with saturating dTDP-glucose measured at 16 °C over a range of pH. The pK_a values determined from the fitted curves are in Table 2.

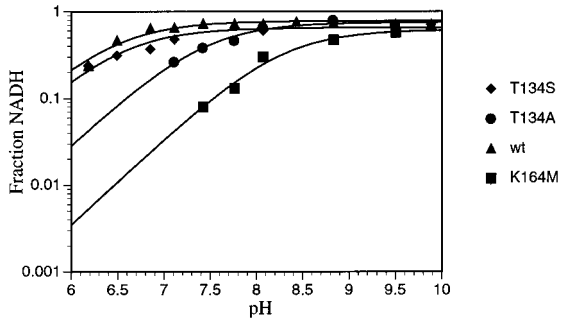


FIGURE 3: Dependence on pH of the equilbrium fraction of NADH measured at 16 °C with saturating dTDP-xylose for the wild-type and variant enzymes. The pK_a values from the fitted curves are in Table 2.

observed in the steady state of the reaction catalyzed by Y160F dehydratase at 16 °C in the pH range tested for the other variants, with the limit of detection being 0.5%. The pH profiles measured (Figure 2) show pK_a values of 6.60 ± 0.06, 6.65 ± 0.06, 7.4 ± 0.2, 7.7 ± 0.1, and 8.0 ± 0.2 for wt (*26*), T134S, T134A, K164M, and K164A dehydratases, respectively (Table 2).

pH Dependence of the Fraction of NADH with Saturating dTDP-Xylose. In the reaction catalyzed by dehydratase with dTDP-xylose, dTDP-4-ketoxylose is formed with concomitant reduction of NAD^+ to NADH as shown in Scheme 2 (*26*). When saturating dTDP-xylose is used, the equilibrium is shifted in the direction of the reduced coenzyme. The maximum amount of NADH was measured under saturating conditions at 16 °C and at different pHs (Figure 3). The pH profiles showed pK_a values of 6.41 ± 0.07, 6.51 ± 0.09, 7.40 ± 0.07, and 8.2 ± 0.1 for wt (*26*), T134S, T134A, and K164M dehydratases, respectively (Table 2). The plateau shown in Figure 3 corresponds to 70−80% of NADH formed for wt (*26*), T134S, and T1324A dehydratases. At equilib-

Table 3: Net Rate Constants k_1' and k_5' for the Reactions of Wild-Type and Vaiants Measured at 16 °C with Saturating dTDP-Glucose

dehydratase	$k_1'k_5'/(k_1' + k_5')^a$ (s^{-1})	$k_1'/(k_1' + k_5')^b$	k_1' (s^{-1})	fold decrease	k_5' (s^{-1})	fold decrease
wt[c]	2.7 ± 0.2	0.44	4.81		6.16	
T134A	0.015 ± 0.002	0.086	0.016	295	0.17	36
K164M	0.08 ± 0.01	0.10	0.086	56	0.77	8
Y160F	0.011 ± 0.001	not observed				

[a] pH independent k_{cat} value. [b] Steady-state level of NADH at plateau. [c] Ref *26*.

Table 4: Rate Constants for the Reaction of Wild-Type and Variants Measured at 16 °C with Saturating dTDP-Xylose

dehydratase	$(k_3^{xyl} + k_4^{xyl})^a$ (s^{-1})	$k_3^{xyl}/(k_3^{xyl} + k_4^{xyl})^b$	k_3^{xyl} (s^{-1})	fold reduction	k_4^{xyl} (s^{-1})	fold reduction
wt[c]	1.4	0.73 (0.73)	1.0		0.4	
T134A	0.0028	0.46 (0.79)	0.0013	770	0.0015	267
K164M	0.000 26	0.13 (0.57)	3.4×10^{-5}	29 400	2.26×10^{-4}	1770

[a] Rate of approach to equilibrium at pH 7.5. [b] Fraction of NADH at equilibrium at pH 7.5. Fractions of NADH at equilibrium on the high pH plateau are in parentheses. [c] Ref *26*.

rium a lower level of bound NADH (57%) is observed for the reaction catalyzed by the K164M dehydratase.

The level of NADH measured under saturating conditions at pH 7.5 and at 16 °C is shown in Table 4 and corresponds to $k_3^{xyl}/(k_3^{xyl} + k_4^{xyl})$, assuming that all enzyme is in the bound nucleotide-sugar form. The rates of approach to equilibrium were measured at pH 7.5 and at 16 °C for some of the variants and are listed in Table 4. This rate corresponds to the sum of k_3^{xyl} and k_4^{xyl}. The calculated values of k_3^{xyl} and k_4^{xyl} are in Table 4. Bound NADH was not observed in the reaction of Y160F dehydratase under saturating conditions at 16 °C and at pH 8.07 and 9.5. These reactions were also run at 25 and 37 °C at pH 8.07 and 9.5 and allowed to proceed for 24 h. In no case was NADH observed.

DISCUSSION

The Tyr149-X-X-X-Lys153 located in the epimerase active site is characteristic of all members of the SDR family. Some SDR proteins contain a Ser/Thr residue corresponding to Ser124 in the epimerase. The structure of the active site of the epimerase/NADH abortive complex with UDP-glucose (*15*) shows Lys153 hydrogen bonded to the 2′ and 3′ hydroxyl groups of the ribosyl ring of NADH, Tyr149 near the nicotinamide ring and Lys153 and Ser124 hydrogen bonded to the 4-hydroxyl group of the glucose moiety (*15*). When the partially refined three-dimensional structure of dehydratase with NAD^+ bound (*9*) is aligned with the structure of the abortive complex of epimerase (*15*), the similarity between the two active sites becomes striking (Figure 4). When comparing these two active sites caution should be taken considering that different enzyme forms are overlaid; the epimerase is in the abortive complex state with substrate bound while the dehydratase is in the oxidized state without substrate. The catalytic triad in the epimerase active site is located in a similar position as the corresponding catalytic triad, Thr134, Tyr160, and Lys164, in the dehydratase active site (Figure 4).

The replacement of Thr134 with a serine has little effect on the activity of the dehydratase. Similarly, the Ser124Thr mutant of epimerase retains all the wild-type properties (*2*). Therefore the first position in the catalytic triad can be occupied by either a serine or a threonine.

The epimerase mechanism is highly compromised when Tyr149 or Ser124 are mutated to phenylalanine or alanine, causing a drop in k_{cat} of 10 000 and 3000, respectively (*24*). The double mutant Y149F/S124A of epimerase displays very low activity, 10^{-7} times that of wild-type epimerase (*32*). These residues are clearly involved in general acid/base catalysis of hydride transfer. Conservative and null mutations have been introduced in dehydratase in order to investigate the role of the triad in the first step of the dehydratase that corresponds to the epimerase reaction. Mutations of either Tyr160 or Thr134 cause a decrease in k_{cat} of only 200-fold. The modest drop in steady-state kinetic parameters for Y160F, Y160A, T134A and T134V dehydratases should be considered in the context of the chemically more complicated mechanism of dehydratase, in contrast to the mechanism of epimerase. Kinetic isotope effects (*26*) and pre-steady-state (*14*) experiments on the wt dehydratase indicate that the chemistry is at least partly rate limiting and that the reduction of the glucose-5,6-ene intermediate and the ketonization of dTDP-glucose occur at similar rates, with the reduction of the C5−C6 bond slightly slower. Therefore the loss in activity which arises from mutation of a residue involved in general acid/base catalysis in the first step is partly disguised by the effects of rate limitation at other steps in the reaction of wt dehydratase. It is likely, however, that in the Tyr160 mutant some other residue can act as the general base.

The assignment and measurement of the pK_a value of Tyr149 in epimerase was accomplished by observing the dependence of the intensity of the charge-transfer band on pH in wt epimerase and by the absence of the charge-transfer band in Y149F epimerase (*24*). In epimerase the charge-transfer band arises from interaction of the nicotinamide ring of NAD^+ and the phenolate of Tyr149 (*24*). When this charge-transfer interaction is interrupted by mutating Tyr149 to Phe, the chemical reactivity of NAD^+ toward reducing agents such as sodium cyanoborohydride increases. These peculiar characteristics of epimerase are not present in the dehydratase. The pseudofirst-order rate constants for reduction of NAD^+ by dimethylamineborane for wt and the Y160 dehydratase are very similar, 4.2×10^{-4} and 2.3×10^{-4} s^{-1}, respectively. Similar rates of reduction were measured for the other variants.

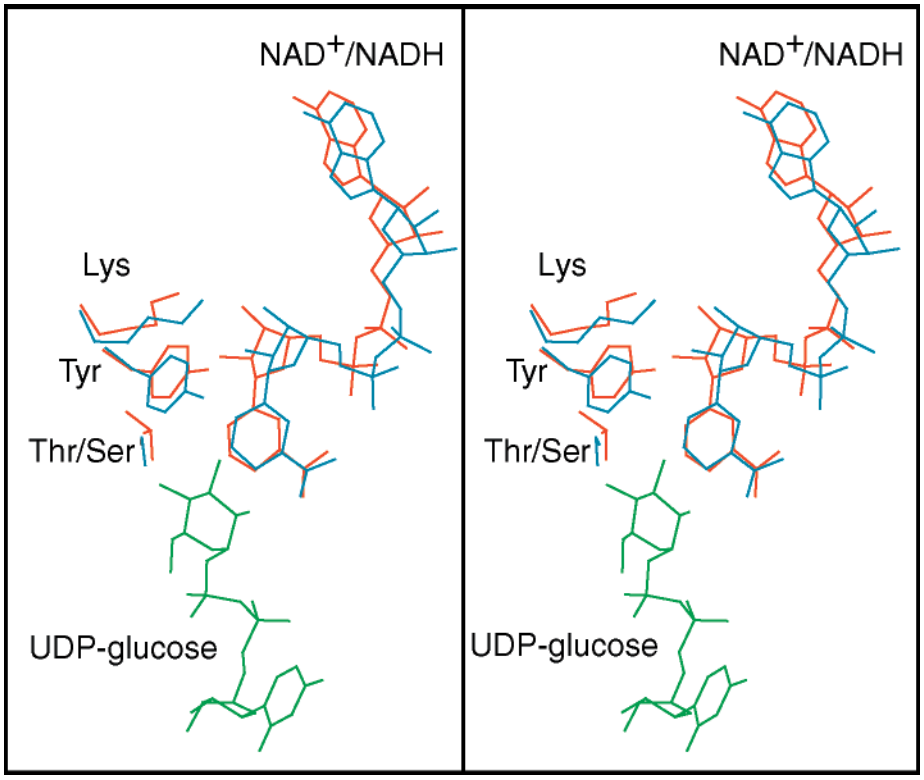
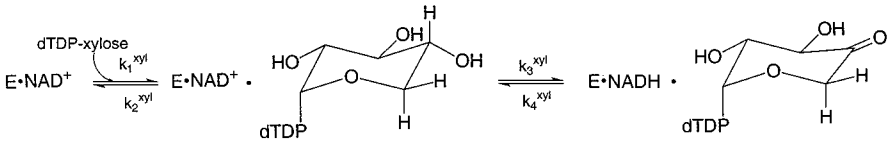


FIGURE 4: An overlay of the active sites of dTDP-glucose 4,6-dehydratase in red (PDB filename 1BKX) and UDP-galactose 4-epimerase in blue (PDB filename 1XEL). UDP-glucose (in green) is in the epimerase active site. The alignment was performed using MolView (*34*).

Scheme 2



Upon binding of a uridine nucleotide, the chemical reactivity of NAD^+ in epimerase is enhanced by charge repulsion between the positive Lys153 and the nicotinamide ring of NAD^+ (*23*, *33*). The coenzyme in dehydratase is not activated by nucleotide binding (*26*) and Lys164 has no effect on its chemical reactivity. A shared property between the epimerase and dehydratase is the role of the Lys residue in binding the coenzyme as shown by their crystal structures. Alanine and methionine variants of Lys164 in the dehydratase and of Lys153 in the epimerase (*23*) do not contain a full complement of coenzyme and display a slight increase in the K_m value for the substrate.

The absence of a 6-hydroxyl methyl group in the xylose moiety of dTDP-xylose allows the reaction of dehydratase to proceed only through the first oxidation step, as shown in Scheme 2. The dTDP-4-ketoxylose/NADH form of dehydratase is in equilibrium with the dTDP-xylose/NAD^+ form when saturating levels of dTDP-xylose are used. This equilibrium depends on k_3^{xyl} and k_4^{xyl} only, since all the enzyme is in the bound nucleotide-sugar state. When dTDP-xylose is oxidized to dTDP-4-ketoxylose by NAD^+, a proton is generated and the equilibrium for the formation of dTDP-4-ketoxylose is affected by the state of protonation of dehydratase. As shown in Figure 2, the equilibrium is shifted toward dTDP-4-ketoxylose/NADH when the pH changes from 6.0 to more basic values for wild-type and for T134S, T134A, and K164M dehydratases. This indicates that the state of protonation of an ionizable residue in the active site affects the equilibrium. Interestingly, in the reaction catalyzed by the Y160F dehydratase, NADH formation was not observed. Y160F is the only variant among those tested here and in previous work (*26*) which appears to shift the equilibrium reaction drastically toward dTDP-xylose/NAD^+, even at high pH values. Therefore Tyr160 is a key residue in the equilibrium reaction and its state of protonation affects the fraction of NADH formed (Scheme 3). The net charge of the NAD^+/dTDP-xylose/Tyr160 complex when Tyr160 is deprotonated is zero and it stays zero in the NADH/dTDP-4-ketoxylose/Tyr160 complex. When this balance of charge is disrupted by protonation of Tyr or by removal of the hydroxyl group of Tyr160 by mutation, the equilibrium becomes very unfavorable for formation of dTDP-4-ketoxylose, since there is no acceptor for the proton produced in the oxidation. The pH dependence of the fraction of NADH with dTDP-xylose thus reflects changes in the protonation state of Tyr160 (Scheme 3).

The pK_a value of Tyr160 was determined as 6.41 by fitting the pH dependence of the fraction of NADH with dTDP-xylose in the reaction catalyzed by wild-type dehydratase. A similar unusually low pK_a value of 6.1 for a tyrosine residue has been assigned for Tyr149, the corresponding residue in epimerase. In epimerase, the low pK_a value arises from stabilization of the phenolate form of Tyr149 by the positive charges of Lys153 and of the nicotinamide ring and

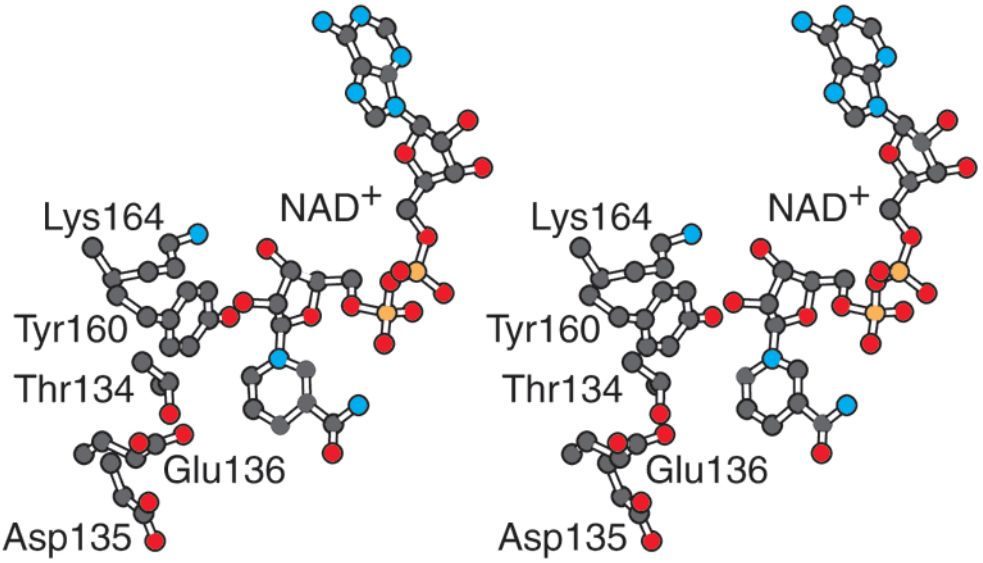
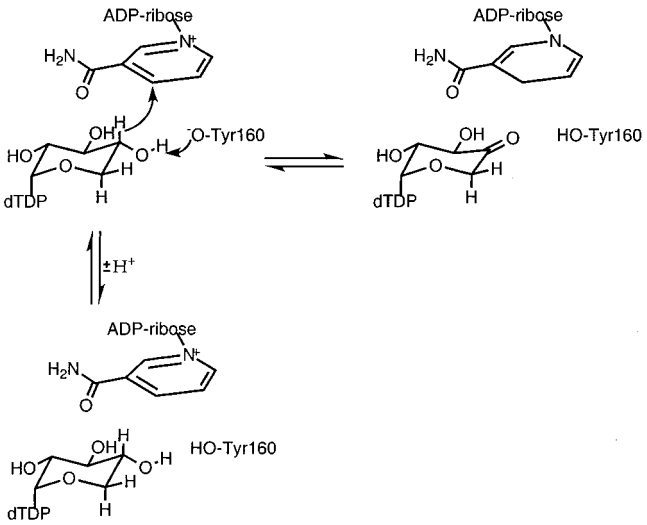


FIGURE 5: Stereoview of the active site structure of dTDP-glucose 4,6-dehydratase (PDB filename 1BKX) with bound NAD^+ using MolView (*34*).

Scheme 3



to a smaller extent by Ser124 (*24*). Mutation of Ser124 causes a change in the pK_a value of Tyr149 of 0.6 pH units in the epimerase (*24*). In the dehydratase the pK_a values determined after mutating either Thr134 to Ala or Lys164 to Met are shifted to the basic side (Figure 1). Lys164 is the major factor in the stabilization of the anion of Tyr160 as shown by the shift in pK_a value of 1.8 pH units upon mutation. The effect of the K164M mutation in the stabilization of Tyr160 anion is evident by the decrease of 29400-fold in k_3^{xyl} and of 1770-fold in k_4^{xyl} at pH 7.5 (Table 4).

The steady-state level of NADH observed in the reaction catalyzed by dehydratase with saturating dTDP-glucose corresponds to the amount of enzyme with dTDP-4-keto-glucose-5,6-ene/NADH bound, since dTDP-4-ketoglucose does not accumulate (*26*). The fraction of NADH observed is equal to the reciprocal of the net rate constant that follows dTDP-4-ketoglucose-5,6-ene/NADH ($1/k_5'$) divided by the sum of the reciprocals of the net rate constants which contribute to the overall rate limitation ($1/k_1' + 1/k_5'$) affect the steady-state NADH level observed. The net rate constant k_3' was not included in the analysis because from previous experiments it has been shown that dTDP-4-ketoglucose does not accumulate and that the dehydration step does not contribute to the overall rate limitation (*26*). The net rate constants for the initial hydride transfer and for the final reduction steps have been determined for wild-type dehydratase and for the variants (Table 3), using the steady-state NADH level at high pH [equal to $k_1'/(k_1' + k_5')$] and the pH independent k_{cat} values [equal to $k_1'k_5'/(k_1' + k_5')$] (Scheme 1).

In Figure 1, the steady-state NADH levels at different pH values are shown for the reaction catalyzed by wt and variants of dehydratase. The reactions catalyzed by K164A, K164M, and T134A dehydratases show a drop in steady-state NADH level at low pH. Considering the role of the corresponding residues in the epimerase mechanism, the steady-state NADH levels observed for these mutants are consistent with a decrease in the net rate of initial dehydrogenation at C-4, which becomes ~10 times slower than the net rate of the reduction of dTDP-4-ketoglucose-5,6-ene (Table 3). As is the case with dTDP-xylose, the first hydride transfer step becomes very unfavorable upon mutation of Tyr160. This unfavorable equilibrium for hydride transfer leads to a very low steady-state level of NADH and reduced k_{cat}, since the reduction of dTDP-4-ketoglucose-5,6-ene is not greatly slowed by the Tyr160 mutation.

The steady-state NADH levels in wild-type and the variants depend on changes in the protonation state of the active site (Figure 2). Similar pK_a values (Table 2) to those measured in the dTDP-xylose reaction were determined by fitting the curves of Figure 2 and by fitting log k_{cat} vs pH profiles (Figure 1). However, the pK_a values determined in this case are kinetic values. We here observed the same shift toward more basic value for the pK_a measured in the reaction catalyzed by T134A and K164M dehydratases that was observed in the reaction with dTDP-xylose and in the reaction of the corresponding mutants in epimerase (*24*). The assignment of the pK_a values observed in the log k_{cat} vs pH profiles and in the steady-state level of NADH profiles is complicated by the complexity of the reaction observed. However, the decrease observed in k_3^{xyl} and the decrease in steady-state NADH level with substrate in the reaction of T134A and K164M dehydratases shows that in these reactions the first

hydride transfer step becomes at least partly rate limiting. It is likely that the pK_a values determined for these mutants represents the pK_a values of Tyr160 which acts as the base catalyst in the dehydrogenation of C-4, especially considering the similarity of the pK_a values to those determined for the dTDP-xylose reaction. Since the first hydride step is slightly faster than formation of the product in the reaction of wild-type enzyme (*26*) and for the T134S-dehydratase, the assignment of the determined pK_a values is potentially jeopardized. However, the similarity with the pK_a values determined in the dTDP-xylose experiments tends to indicate that these values can be assigned to Tyr160.

The log k_{cat} vs pH profile of the Y160F dehydratase shows a pK_a value in the acidic range shifted ~0.3 pH units toward more acidic values. In the epimerase reaction, it is believed, based on pH profile studies, that in the absence of the Tyr149 the hydride transfer is catalyzed by Ser124 (*32*). In the active site of epimerase lacking the phenolate oxygen of Tyr149, the anion of Ser124 is stabilized by the positive charge of Lys153, causing a drop in pK_a from 13.4 to 9.4 (*32*). In the active site of the crystal structure of the abortive complex of epimerase, the only group other than Tyr149 near the hydroxyl group of the glucosyl C-4 of UDP-glucose is Ser124 (*15*). In the active site of dehydratase, other functional residues than Thr134 are located near the hydroxyl group of the glucosyl C-4 of dTDP-glucose, specifically Glu136 and Asp135 (Figure 5) (*26*). It is therefore possible that, in the absence of Tyr160, either Glu136 or Asp 135 catalyzes the first hydride transfer step by acting as the base catalyst and that the pK_a determined in the pH profile of the reaction catalyzed by Y160F variant could correspond to that of either Glu136 or Asp135. Alternatively, the proton generated by the hydride transfer could be released into solution while the protonation states of these two acidic groups may affect the conformation of the protein and thus the rate of hydride transfer.

CONCLUSIONS

We believe that Tyr160 acts as the base catalyst in the first hydride transfer and has a pK_a value of 6.41. When the interactions between the positive charge of Lys164 and Tyr160 is disrupted by mutation of the lysine residue, a shift in kinetic pK_a of 1.8 pH units is observed. The shifts in pK_a values observed upon mutation of Thr134 and Lys164 are similar to the shifts in the pK_a of Tyr149 in the epimerase observed upon mutation (*24*). The Lys164 and Thr134 residues are likely to play a role in the first step of the dehydratase reaction by depressing the pK_a of Tyr160 from 10.2 to 6.41. In addition, Thr134 may act as a proton relay, as appears to occur in the epimerase reaction.

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SUPPORTING INFORMATION AVAILABLE

Table of primers used for mutagenesis of dTDP-glucose 4,6-dehyratase. This material is available free of charge via the Internet at http://pubs.acs.org.

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