Acid−Base Catalysis by UDP-Galactose 4-Epimerase: Correlations of Kinetically Measured Acid Dissociation Constants with Thermodynamic Values for Tyrosine 149[†]

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ABSTRACT: The steady-state kinetic parameters for epimerization of UDP-galactose by UDP-galactose 4-epimerase from *Escherichia coli* (GalE), Y149F-GalE, and S124A-GalE have been measured as a function of pH. The deuterium kinetic isotope effects for epimerization of UDP-galactose-C-d_7 by these enzymes have also been measured. The results show that the activity of wild-type GalE is pH-independent in the pH range of 5.5−9.3, and there is no significant deuterium kinetic isotope effect in the reaction of UDP-galactose-C-d_7. It is concluded that the rate-limiting step for epimerization by wild-type GalE is not hydride transfer and must be either a diffusional process or a conformational change. Epimerization of UDP-galactose-C-d_7 by Y149F-GalE proceeds with a pH-dependent deuterium kinetic isotope effect on k_{cat} of 2.2 ± 0.4 at pH 6.2 and 1.1 ± 0.5 at pH 8.3. Moreover, the plot of log k_{cat}/K_m breaks downward on the acid side with a fitted value of 7.1 for the pK_a. It is concluded that the break in the pH−rate profile arises from a change in the rate-limiting step from hydride transfer at low pH to a conformational change at high pH. Epimerization of UDP-galactose-C-d_7 by S124A-GalE proceeds with a pH-independent deuterium kinetic isotope effect on k_{cat} of 2.0 ± 0.2 between pH 6 and 9. Both plots of log k_{cat} and log k_{cat}/K_m display pH dependence. The plot of log k_{cat} versus pH breaks downward with a pK_a of 6.35 ± 0.10. The plot of log k_{cat}/K_m versus pH is bell-shaped, with fitted pK_a values of 6.76 ± 0.09 and 9.32 ± 0.21. It is concluded that hydride transfer is rate-limiting, and the pK_a of 6.7 for free S124A-GalE is assigned to Tyr 149, which displays the same value of pK_a when measured spectrophotometrically in this variant. Acid−base catalysis by Y149F-GalE is attributed to Ser 124, which is postulated to rescue catalysis of proton transfer in the absence of Tyr 149. The kinetic pK_a of 7.1 for free Y149F-GalE is lower than that expected for Ser 124, as proven by the pH-dependent kinetic isotope effect. Epimerization by the doubly mutated Y149F/S124A-GalE proceeds at a k_{cat} that is lower by a factor of 10^7 than that of wild-type GalE. This low rate is attributed to the synergistic actions of Tyr 149 and Ser 124 in wild-type GalE and to the absence of any internal catalysis of hydride transfer in the doubly mutated enzyme.

The interconversion of UDP-galactose[1] and UDP-glucose according to eq 1 is an essential step in the metabolism of carbohydrates in all cells and is catalyzed by UDP-galactose 4-epimerase, GalE, in *Escherichia coli* (*1*).

(1)

Purified, homogeneous GalE is a homodimer with one molecule of tightly bound NAD^+ in each subunit (*2*, *3*). NAD^+ functions as the essential coenzyme for promoting epimerization at hexopyranosyl C4 of substrates by a mechanism in which reversible dehydrogenation at C4 according to eq 2 is the essential chemical process (*4*).

$$\text{E}-\text{NAD}^+ + \text{UDP-galactose} \rightleftharpoons \text{E}-\text{NADH}-\text{UDP-4-ketohexopyranose} \rightleftharpoons \text{E}-\text{NAD}^+ + \text{UDP-glucose} \quad (2)$$

Nonstereospecific hydride abstraction from C4 of the hexopyranosyl ring by NAD^+ at the active site produces UDP-4-ketohexopyranoside and NADH as transient intermediates, which are not free to dissociate from the enzyme in the normal course of catalysis. Nonstereospecific reduction of the 4-ketone by NADH completes the epimerization process. Nonstereospecific hydride transfer is postulated to be facilitated by rotation of the ketoheoxpyranosyl intermediate about the P_β−O bond connecting the nucleotide moiety with the anomeric oxygen atom of the keto sugar (*4*−*6*).

GalE is a member of a superfamily of $NAD(P)^+$-dependent enzymes that incorporate a lysyl residue, a tyrosyl residue,

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[1] Abbreviations: GalE, UDP-galactose 4-epimerase from *E. coli*; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; UDP-glucose, uridine-5′-diphosphate glucose; UDP-galactose, uridine-5′-diphosphate galactose; UMP, uridine 5′-monophosphate; UDP, uridine 5′-diphosphate; EDTA, ethylenediaminetetraacetate.

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and usually a seryl or threonyl residue in the active site (*8*−*10*). In GalE, these are Lys 153, Tyr 149, and Ser 124. Lys 153 is hydrogen bonded to the 2′-OH and 3′-OH groups of the nicotinamide ribosyl moiety of NAD^+, and Tyr 149 and Ser 124 interact with the hexopyranosyl rings of the substrates (*7*, *11*).

The proton abstraction from the hexopyranosyl C4(OH) concomitant with hydride transfer from C4 to NAD^+ is believed to be driven by Tyr 149 acting as the base catalyst (*7*). The proximity of Tyr 149 to hexopyranosyl C4(OH) of the substrate, together with its measured pK_a of 6.08, constitutes the available evidence in support of its postulated function in general acid−base catalysis. The pK_a of Tyr 149 has not heretofore been kinetically correlated with catalysis of hydride transfer, mainly because the tritium kinetic isotope effect in reactions of nucleotide [4-^{3}H]glucose is so small in magnitude as to imply that hydride transfer is not the rate-limiting step (*12*, *13*). Mutation of Tyr 149 or Ser 124 generates variant forms of epimerase that display very low activities. We report here results of kinetic studies designed to determine whether hydride transfer limits the rates of catalysis by mutated GalEs, and if so whether correlations of pH dependence with the thermodynamically measured values of pK_a for Tyr 149 implicate this residue in acid−base catalysis.

EXPERIMENTAL PROCEDURES

Materials. NAD^+, UDP-glucose dehydrogenase, UDP-galactose, UDP-glucose, and bicine were purchased from Sigma. HEPES (enzyme grade) and NaOH were purchased from Fisher Scientific. Piperazine and KCl were obtained from Aldrich and Mallinckrodt, respectively. Sheer-Lock microtubes were from Research Products International Corp., and D-glucose-C-d_7 was from Cambridge Isotope Laboratories and Isotec Inc.

UDP-galactose-C-d_7 was synthesized enzymatically from glucose-C-d_7. UDP-glucose-C-d_7 was first synthesized by reaction of glucose-d_7 with ATP and UTP in the presence of Mg^{2+}, hexokinase, phosphoglucomutase, glucose 1,6-diphosphate, and UDP-glucose pyrophosphorylase, as described previously (*14*). UDP-glucose-C-d_7 was then transformed into the equilibrium mixture of UDP-glucose-C-d_7 and UDP-galactose-C-d_7 by the action of GalE, which was removed by gel filtration chromatography. The mixture of UDP-glucose-C-d_7 and UDP-galactose-C-d_7 was subjected to the action of UDP-glucose dehydrogenase and excess NAD^+ to convert UDP-glucose-C-d_7 into UDP-glucuronate-d_5, which was separated from UDP-galactose-C-d_7 by anion exchange chromatography over a column of QAE-Sephadex eluted with triethylamine bicarbonate. After rotary evaporation in vacuo of triethylamine bicarbonate, enzymatic assays of UDP-galactose-C-d_7 with UDP-glucose dehydrogenase and NAD^+ revealed the presence of significant contamination by UDP-glucose-C-d_7. Therefore, the UDP-galactose-d_7 was again treated with UDP-glucose dehydrogenase and excess NAD^+, and the UDP-galactose-C-d_7 was again purified by chromatography. After the second round of enzymatic oxidation of UDP-glucose-C-d_7 and chromatography, assays of UDP-galactose-C-d_7 with UDP-glucose dehydrogenase showed it to be free of detectable UDP-glucose-C-d_7. Spectrophotometric assays of UDP-galactose-C-d_7 at 260 nm were in agreement with enzymatic assays based on the coupled actions of GalE and UDP-glucose dehydrogenase, showing that the compound was UDP-galactose-C-d_7 free of UDP-glucose and other nucleotides.

Enzyme Preparations. Wild-type GalE was expressed in *E. coli*, and specifically mutated epimerases Y149F-GalE and S124A-GalE were prepared as described previously (*7*, *15*). The enzymes were purified free of NADH-containing abortive complexes as previously described (*16*). The GalE concentrations in solutions were determined spectrophotometrically by dividing the absorbance at 280 nm by 1.81 mL mg^{-1} cm^{-1} (*3*). GalE was routinely assayed during purification by coupling the conversion of UDP-galactose to UDP-glucose with NADH production by UDP-glucose dehydrogenase, according to the method of Wilson and Hogness (*1*, *3*).

pH−Rate Profiles. Adaptation of the standard assay procedure to the construction of pH−rate profiles proved to be impractical due to stability and activity problems with UDP-glucose dehydrogenase. Therefore, an end-point assay was adopted in which the initial, timed reactions of GalE with UDP-galactose at a given pH were conducted at 27 °C within small tubes for intervals ranging from 0 to 10 min and then stopped by heating the mixtures to 100 °C. Reaction mixtures were buffered in the pH range of 5.5−9.7 with 0.025 M Hepes/piperazine, and the ionic strength (I) was adjusted with KCl to 0.1. Aliquots of the stopped reaction mixtures were than analyzed at pH 9.0 (0.1 M sodium bicinate buffer) by use of UDP-glucose dehydrogenase and NAD^+ for the UDP-glucose formed in the initial timed reactions. The amount of NADH produced in the second stage was measured fluorimetrically in a Perkin-Elmer MPF-3 spectrofluorimeter, with excitation at 340 nm and detection at 460 nm. Standard solutions of NADH were used with each fluorimetric assay for calibration purposes. Initial rates were measured in triplicate and plotted versus substrate concentrations, and KalleidaGraph was used to evaluate k_{cat} and K_m at each pH. The profiles of log k_{cat} and log k_{cat}/K_m versus pH were plotted and the data computer fitted to eq 3 or 4, using the HABELL or BELL program, respectively, of Cleland (*17*)

$$\log y = \log\left(\frac{c}{1 + [H^+]/K_a}\right) \tag{3}$$

$$\log y = \log\left(\frac{c}{1 + [H^+]/K_{a1} + K_{a2}/[H^+]}\right) \tag{4}$$

In fitting the data, y values were the experimental pH-dependent values of k_{cat} or k_{cat}/K_m, c was the pH-independent value arising from the fitting procedure, and K_a values were the fitted values of acid dissociation constants.

Deuterium Kinetic Isotope Effects. The deuterium kinetic isotope effects on k_{cat} and k_{cat}/K_m for the reactions of GalE, Y149F-GalE, and S124A-GalE were measured at various pHs. Rates were measured by the end-point assay. The substrates used for the measurements of kinetic isotope effects were UDP-galactose and UDP-galactose-C-d_7 that had been synthesized from D-glucose or D-glucose-C-d_7 by the same procedure.

Table 1: Deuterium Kinetic Isotope Effects in the Epimerization of UDP-Galactose-C-d_7[a]

epimerase	pH	$^{D}k_{cat}$[b]		pH	$^{D}k_{cat}$[b]
wild type	8.3	1.3 ± 0.2	S124A-GalE	9.0	1.9 ± 0.1
Y149F-GalE	8.3	1.1 ± 0.5	S124A-GalE	7.8	2.1 ± 0.2
Y149F-GalE	6.2	2.2 ± 0.4	S124A-GalE	6.3	2.1 ± 0.2

[a] The only C−H bond cleavage in epimerization occurs at galactosyl C4(H), and it is on this cleavage that a deuterium kinetic isotope effect could be observable. UDP-galactose-C-d_7 was used as the substrate to detect this isotope effect because it could be synthesized from commercially available glucose-C-d_7, and any secondary isotope effects from deuterium bonded to other carbon atoms should not be detectable by the methods that were used. [b] Deuterium kinetic isotope effects on k_{cat} measured at 27 °C.

RESULTS

Deuterium Kinetic Isotope Effects. Epimerization by GalE displays very small tritium kinetic isotope effects (k_H/k_T = 1.7−3.5) in the reactions of UDP-[4-^{3}H]glucose and dTDP-[4-^{3}H]glucose (*12*, *13*). The low values of tritium isotope effects indicate that hydride transfer may not be rate-limiting for the wild-type enzyme, with tritium transfer becoming partially rate limiting. According to this interpretation, the intrinsic tritium isotope effects are probably masked by rate limitation at another step. This matter has been addressed by the measurement of deuterium kinetic isotope effects in the epimerization of UDP-galactose-C-d_7 by wild-type GalE and mutated forms of this enzyme, with the results presented in Table 1. Wild-type GalE does not display a significant deuterium kinetic isotope effect at pH 8.3. This is in agreement with the tritium isotope effects and suggests that a process other than hydride transfer is rate-limiting. Inasmuch as hydride transfer is expected to be subject to acid−base catalysis, a further indication that hydride transfer is not rate-limiting is the absence of any pH dependence in the action of wild-type GalE, as shown in the next section.

In contrast to wild-type GalE, mutations of Tyr 149 or Ser 124 lead to species of epimerase that display significant deuterium kinetic isotope effects. Y149F-GalE displays pH dependence in the expression of isotope effects in the epimerization of UDP-galactose-C-d_7. At pH 8.3, there is no significant isotope effect, but at pH 6.3, the isotope effect is 2.2. The theory of pH dependence in kinetic isotope effects suggests that hydride transfer is rate-limiting at pH 6.3 but not at pH 8.3 for this species of epimerase (*18*, *19*). On this basis, epimerization should be pH-dependent for Y149F-GalE, showing a downward break on the acid side and a plateau at higher pHs, and this is verified below. Epimerization of UDP-galactose-C-d_7 by S124A-GalE proceeds with a deuterium kinetic isotope effect (k_H/k_D) of ∼2 throughout the pH range of 6.3−9.0. These data suggest that in the case of S124A-GalE hydride transfer is rate-limiting throughout this pH range.

pH−Rate Profiles for GalE and Y149F-GalE. The plots of log k_{cat} and log k_{cat}/K_m versus pH for epimerization of UDP-galactose by GalE and Y149F-GalE are shown in Figure 1. The action of GalE is almost independent of pH between pH 5.5 and 9.7. The rates show a tendency to drift down at lower pHs, but no break in the pH−rate profiles can be assigned. In view of the absence of a deuterium kinetic isotope effect, and small values of tritium kinetic isotope effects (*12*, *13*), hydride transfer is unlikely to be rate-limiting

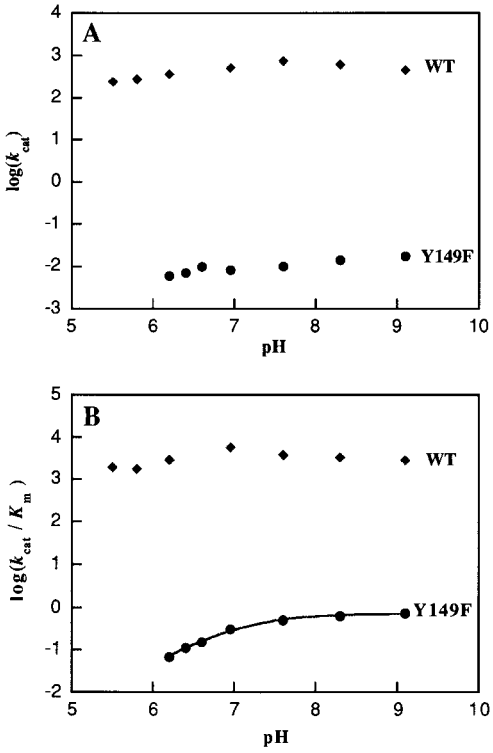


FIGURE 1: pH−rate profiles for epimerization of UDP-galactose by wild-type GalE and Y149F-GalE. Values of k_{cat} and K_m were determined in the pH range of 5.5−9.3 as described in Experimental Procedures. Panel A depicts plots of log k_{cat} vs pH for epimerization by wild-type GalE (upper) and Y149F-GalE (lower). Panel B depicts plots of log k_{cat}/K_m vs pH. The only plot that shows significant pH dependence is that of log k_{cat}/K_m for Y149F-GalE. The solid line arises from fitting the data to eq 3, resulting in a pK_a value of 7.13 ± 0.04.

in the action of wild-type GalE. The rate data could not be extended below pH 5.5 because of the instability of GalE at lower pHs. The pH−rate profile is in accord with rate limitation by pH-independent processes rather than hydride transfer, which is expected to require general base catalysis.

In the case of Y149F-GalE, the rates are 4 orders of magnitude lower than that of wild-type GalE. However, the pH−log k_{cat} profile does not break between pH 6 and 9, although the rates drift downward at lower pHs. The plot of log k_{cat}/K_m versus pH turns down on the acidic side of the profile, and when fitted to eq 3 (half-bell) gives a pK_a value of 7.13 ± 0.04. Evidently, the free Y149F-GalE undergoes a mechanistically significant ionization. The relationship of the kinetically measured pK_a of 7.13 to ionization at the active site is taken up in the Discussion.

pH−Rate Profiles for S124A-GalE. Plots of log k_{cat} and log k_{cat}/K_m for epimerization by S124A-GalE are shown in Figure 2. The rates are about 1/2900 of those for wild-type GalE, and the profiles for both log k_{cat} and log k_{cat}/K_m are pH-dependent. The profile for k_{cat} in Figure 2A when fitted to eq 3 (half-bell) gives a pK_a value of 6.35 ± 0.10. The rate data could not be extended below pH 6.0 because of the instability of this mutated GalE. The fit of data to the pK_a value of 6.35 is based on the assumption that the protonated form of the Michaelis complex would display no significant activity. In practical terms, its activity would be less than 10% of that of the unprotonated form. Inasmuch as the deuterium kinetic isotope effect indicates that hydride transfer is rate-limiting throughout the pH range, it appears

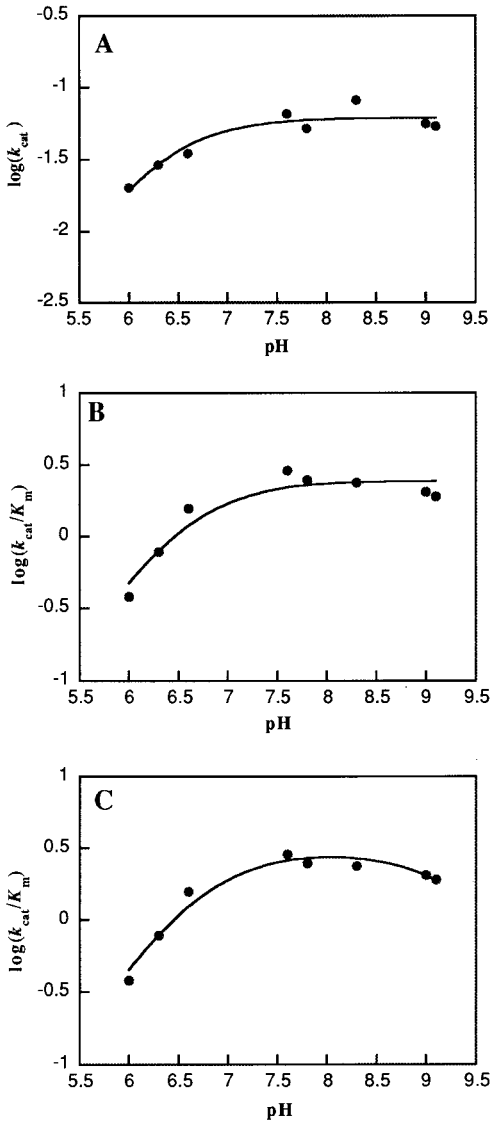
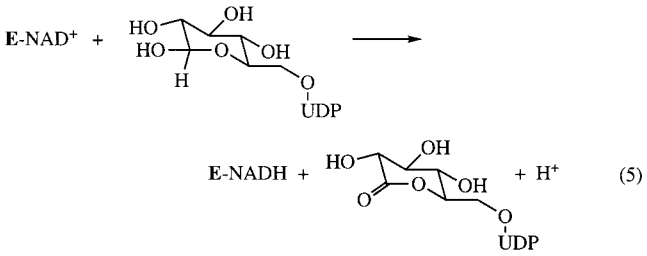


FIGURE 2: pH−rate profiles for S124A-GalE. Values of k_{cat} and K_m were determined in the pH range of 6.0−9.0 as described in Experimental Procedures. Panel A is a plot of log k_{cat} vs pH. The solid line represents the fit of data points to eq 3 with a pK_a value of 6.35 ± 0.10. Panel B is a plot of log k_{cat}/K_m vs pH with the data fitted to eq 3. The solid line represents the fitted value of 6.62 ± 0.11 for pK_a. Panel C is also a plot of log k_{cat}/K_m, but the data are fitted to eq 4. The values of pK_a arising from fitting to eq 4 are 6.76 ± 0.09 and 9.32 ± 0.21.

that a functional group with a pK_a of 6.35 participates in acid−base catalysis of hydride transfer in the Michaelis complex.

The plot of log k_{cat}/K_m versus pH turns downward at lower pHs and also at higher pHs. It is fitted to eq 3 (half-bell) in Figure 2B, with a value of 6.6 ± 0.1. The fit is to eq 4 for a bell-shaped profile in Figure 2C is much better, with a pK_{a1} of 6.7 ± 0.1 on the acid side and a pK_{a2} of 9.3 ± 0.2 on the alkaline side, and this is probably the appropriate fit. In either case, the value of pK_a on the acid side for the catalytically significant ionization of free S124A-GalE may be assigned as 6.7.

Although the data points for S124A-GalE do not extend above pH 9.0, and they show only a small drift downward, there is likely to be a catalytically significant ionization of the enzyme with a pK_a of 9.1−9.2. This conclusion is supported by the alkaline side of the pH−rate profile for the reduction of wild-type GalE according to eq 5 with UDP-6-glucose, an analogue of UDP-glucose



(5)

in which glucose is bonded to UDP through O6 of glucose (*20*). The reaction is irreversible because the strongly reducing carbon of UDP-6-glucose donates a hydride to NAD^+, and the pH−rate profile breaks downward at high pHs with an estimated pK_a of 9.1−9.2 (*20*). Inasmuch as hydride transfer is rate-limiting for epimerization by S124A-GalE throughout the pH range, the pH−rate profile for epimerization is likely to be analogous to that for GalE in reaction 3.

Activity of S124A/Y149F-GalE. The structure of the doubly mutated epimerase is similar to that of wild-type GalE except for the absence of the oxy groups of Tyr 149 and Ser 124 (*6*). Evaluation of the doubly mutated epimerase by the standard continuous assay with coupling of UDP-glucose dehydrogenase reveals a discernible increase in A_{340} at an extremely low rate at very high protein concentrations. The coupled assay is impractical for evaluating kinetic parameters of Y149F/S124A-GalE because of the long reaction times required to observe any epimerization. In an end-point version of this assay, Y149F/S124A-GalE at 5 mg/mL is first incubated with UDP-galactose for up to 5 h before the addition of UDP-glucose dehydrogenase and NAD^+ to oxidize the UDP-glucose that is produced. As measured by this method at 27 °C and pH 8.5, the values of k_{cat}, K_m, and k_{cat}/K_m are $(1.3 \pm 0.4) \times 10^{-5}$ s^{-1}, 0.20 ± 0.11 mM, and $(6.5 \pm 1.9) \times 10^{-5}$ mM^{-1} s^{-1}, respectively. Comparison with the corresponding values for the wild-type epimerase (760 s^{-1}, 0.225 mM, and 3380 s^{-1} mM^{-1}, respectively) makes it clear that the K_m value for the doubly mutated enzyme is similar to that of the wild-type enzyme, but the activity is 7 orders of magnitude lower. The relatively large standard deviation of the kinetic parameters that were obtained represents the difficulty of monitoring the extremely low rates.

In cases such as this, the possibility of adventitious traces of the wild-type enzyme had to be addressed. Mutation of Tyr 149 to phenylalanine increased the temperature sensitivity of epimerase (*7*) which would likely be true of the doubly mutated enzyme as well. Therefore, to determine whether the extremely low activity could have been due to wild-type contamination, we compared the thermal lability at 50 °C of the activity displayed by S124A/Y149F-GalE with that of wild-type GalE and obtained the results shown in Figure 3. The activity exhibited by the double variant was clearly much more thermally labile than that of the wild-type epimerase. Therefore, the residual enzymatic activity observed in preparations of S124A/Y149F-GalE could not have been due to wild-type contamination and was attributed to the doubly mutated enzyme.

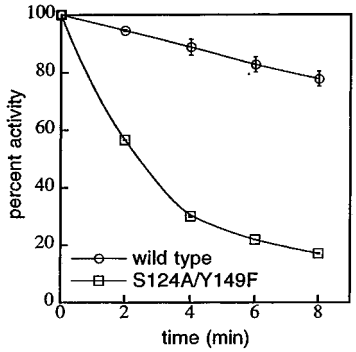


FIGURE 3: Heat denaturation of GalE and Y149F/S124A-GalE at 50 °C. Stock solutions of epimerase (20−30 mg/mL) were heated at 50 °C for various time intervals. After centrifugation to remove the precipitate of the denatured enzyme, the residual epimerase activity was assayed as described in the text.

Table 2: Kinetic Parameters and pK_a Values for Wild-Type and Mutated GalE

epimerase	k_{cat}[a] (s^{-1})	K_m[a] (mM^{-1})	pK_{a1} (kinetic)	pK_{a2} (kinetic)	pK_a (thermo)
GalE	760 ± 19	0.2 ± 0.1	6.1[b]	9.2[b]	6.1[c]
S124A-GalE	0.26 ± 0.5	0.023 ± 0.009	6.7[d]	9.3[d]	6.7[c]
Y149F-GalE	0.073 ± 0.004	0.021 ± 0.004	7.1[d]	ND[e]	ND[e]

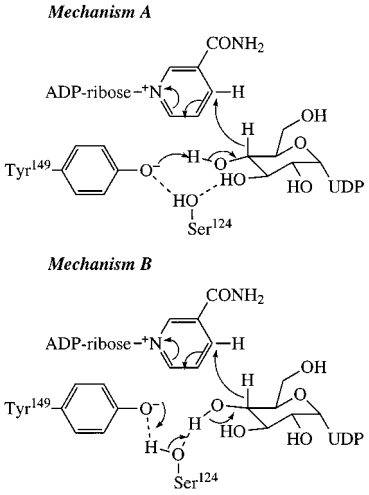
[a] Values first reported in ref *7* at pH 8.5 and 27 °C. [b] From ref *20*. [c] Spectrophotometrically measured value of pK_a for Tyr 149 reported in ref *7*. [d] This work. [e] Not detected.

DISCUSSION

Correlation of Kinetic and Thermodynamic Values of pK_a in Acid−Base Catalysis by Tyr 149. Values of kinetic parameters and kinetically and thermodynamically determined values of pK_a for wild-type and mutated GalE are collected in Table 2. Values of pK_{a1} refer to the acid side of the pH−rate profiles and pK_{a2} to the alkaline side. In the work presented here, no significant pH dependence is detected for wild-type GalE; therefore, the kinetically determined values of pK_a in the first line of Table 2 are those previously deduced from the kinetics of the reduction of GalE-NAD^+ by UDP-6-glucose according to eq 5 (*20*). Thermodynamic values of pK_{a1} for Tyr 149 in GalE and S124A-GalE are derived from the pH dependence of the intensity of the charge transfer band between Tyr 149 and NAD^+ (*7*). The kinetically measured value of 6.1 for pK_{a1} of free wild-type GalE in line 1 of Table 2 is essentially identical to the thermodynamic pK_a of Tyr 149 in wild-type GalE. Furthermore, the kinetically measured pK_a of 6.7 for epimerization by free S124A-GalE in line 2 is essentially identical to the thermodynamic value for Tyr 149 in this variant of GalE. These identities support the assignment Tyr 149 as the acid−base catalyst of hydride transfer. The previous postulation of Tyr 149 as the acid−base catalyst, based on structural and spectrophotometric evidence, is consolidated by the kinetic data.

While Tyr 149 is the driving force for acid−base catalysis, Ser 124 is likely to participate in hydride transfer as well. Mutation of Ser 124 to alanine decreases activity by almost 3000-fold. Moreover, double mutation of Tyr 149 and Ser 124 to produce Y149F/S124A-GalE leads to extremely low epimerization activity, $\sim 10^{-7}$ times that of wild-type GalE. The overall structural consequences of double mutation are modest, apart from the ablation of the two hyroxy groups of Tyr 149 and Ser 124 at the active site (*6*). Therefore, the catalytic actions of Tyr 149 and Ser 124 are synergistic and likely to be exerted in the same steps rather than in sequential steps. This function is most likely acid−base catalysis of hydride transfer. Two mechanisms for the participation of both Tyr 149 and Ser 124 in hydride transfer are outlined in Scheme 1. In mechanism A, Tyr 149 abstracts the proton from C4(OH) of the galactosyl or glucosyl group and catalyzes hydride transfer to NAD^+. The role of Ser 124 is to position the pyranosyl C3(OH) and the phenolate of Tyr 149 properly for the proton transfer. In mechanism B, Ser 124 plays a more direct role in proton transfer by relaying a proton between Tyr 149 and pyranosyl C4(OH).

Scheme 1



Because the published structures of GalE have been those of abortive complexes and not Michaelis complexes, and the orientations of Ser 149 and Tyr 149 would differ little in the two mechanisms, it has not been possible to decide between them. However, structural information about the homologous human epimerase has been interpreted to favor mechanism A, direct proton transfer between Tyr 149 and pyranosyl C4(OH) (*21*).

Mutation of Tyr 149 to phenylalanine has interesting mechanistic consequences. Y149F-GalE retains detectable but very low activity, with most of the difference being a 10000-fold decrease in k_{cat}. The value of K_m is $\sim 1/10$ of that for wild-type GalE.

pH Dependence and the Rate-Limiting Conformational Change in Y149F-GalE. Hydride transfer is not rate-limiting between pH 5.5 and 9.3 for wild-type GalE. Because hydride transfer is the only chemical step in the mechanism, the rate-limiting process must be a pH-independent process such as diffusion or a conformational change. Because small but definite tritium kinetic isotope effects are observed for epimerization of UDP-[4-^{3}H]glucose and dTDP-[4-^{3}H]glucose by the wild-type GalE (*12*, *13*), hydride transfer is likely to proceed within an order of magnitude of the rate of the limiting process.

Mutation of GalE to the 10000-fold less active Y149F-GalE might be expected to make hydride transfer rate-limiting. However, the pH-dependent deuterium kinetic isotope effects prove that hydride transfer is not rate-limiting above pH 7.3 (Table 1). A physical process such as diffusion

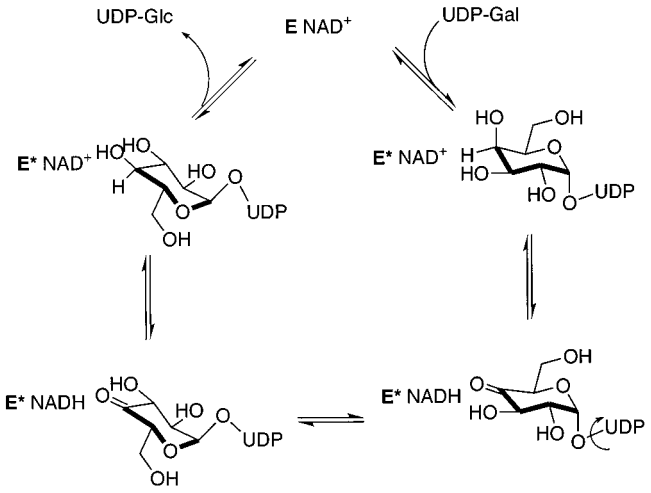


FIGURE 4: Conformational changes in the mechanism of epimerization by GalE. The first step of the epimerization process is the binding of UDP-Gal to the free enzyme, E−NAD$^+$, a process that is accompanied by a conformational change of the enzyme to E*−NAD$^+$. Hydride transfer produces E*−NADH and UDP-4-ketohexopyanoside as a tightly bound intermediate. The 4-ketopyranoside is free to undergo a conformational change within the site by torsion about bonds linking the anomeric oxygen to the nucleotide in such a way as to project either face of the 4-keto group toward the *si* face of NADH. Hydride transfer to produce UDP-Glc forms the epimeric product, which dissociates to regenerate E−NAD$^+$.

or a conformational change must be rate-limiting above pH 7.3, as it is for wild-type GalE. Diffusion rates are very unlikely to be affected by the mutation, yet a physical process remains rate-limiting. The roles of two conformational changes known to be important in the mechanisms of action of GalE are illustrated in Figure 4. Binding of the substrate induces an enzyme conformational change from E in free enzyme to E* in the enzyme−substrate complex. This change enhances the reactivity and reduction potential of NAD$^+$ in hydride transfer (*4*, *22*−*24*). The second essential conformational change is that of the UDP-4-ketohexopyanosyl intermediate, in which the 4-ketohexopyranosyl ring is reoriented to place opposite faces of the 4-keto group in position for hydride transfer from NADH (*22*, *25*). This conformational change potentiates nonstereospecific hydride transfer, an essential mechanistic process in epimerization.

Both conformational changes are likely to be pH-independent in the pH ranges studied here. In principle, either conformational change could be rate-limiting in the action of wild-type GalE. It is significant that a conformational change persists in limiting the rate of epimerization by Y149F-GalT. Mutation of Tyr 149 to Phe 149 decreases the extent of catalysis 10000-fold, a factor much larger than the difference between the rates of hydride transfer and conformational change in the action of wild-type GalE. Therefore, the mutation must have decreased the rate of the conformational change as well as that of hydride transfer. Because this mutation would decrease the level of hydrogen bonding between the enzyme and substrate, it is unlikely to decrease the rate of conformational change in UDP-4-ketohexopyranoside. It seems much more likely that the mutation decreases the rate of the enzyme conformational change, E → E*, in parallel with the decrease in the rate of hydride transfer. This may well involve Tyr 149, which interacts closely in charge complexation with NAD$^+$ (*7*), so that mutation of this residue may decrease the rate of conformational change. Consequently, Tyr 149 may play the dual role of catalyzing hydride transfer between the substrate and NAD$^+$ and mediating the effect of the conformational change on the reactivity and reduction potential of NAD$^+$.

pH Dependence and the Kinetic Value of pK_{a1} in Y149F-GalE. A classic theory of pH dependence in kinetic isotope effects explains the physical significance of values of pK_a derived from pH−rate profiles as follows (*18*, *19*). When there is a kinetic isotope effect on the acidic side and none on the plateau of the plot of log k_{cat}/K_m versus pH, the break represents a change from rate-limiting conformational change at high pH to a rate-limiting chemical process at low pH. The kinetic pK_a bears a specific relationship to the thermodynamic value of pK_a for the group that catalyzes the chemical step. Because the pH-independent conformational change controls the rate at high pH, the normal position of the break for the pH dependence of the chemical step is shifted toward the acid side of the pH−rate profile. Therefore, the thermodynamic pK_a of the acid−base catalytic group must be higher than the kinetically measured pK_a (*18*, *19*).

In the case of Y149F-GalE, hydride transfer limits the rate of epimerization at pH 6.3, whereas a conformational change limits the rate above pH 7.1. Therefore, Y149F-GalE displays classic behavior, in which a break in a pH−rate profile arises from a change in the rate-limiting step. The kinetically measured value of pK_a differs in a predictable way from the thermodynamic value for the controlling functional group (*18*, *19*). The pH-independent ceiling imposed on the observable rates by the rate-limiting conformational change displaces a break in the log plot of rate versus pH to a value of pK_a lower than the thermodynamic pK_a of the ionizing group. In the case of Y149F-GalE, the plot of log k_{cat}/K_m breaks at pH 7.1 (Figure 1B), with hydride transfer limiting the rate below this pH and a conformational change limiting the rate at higher pHs. Therefore, the pK_a of an ionizing group that controls the rate must be higher than 7.1 in the free enzyme.

The question of the identity of a group controlling acid−base catalysis in the action of Y149F-GalE is worth taking into consideration. Acid−base catalysis is required for hydride transfer; however, Tyr 149 is absent and cannot perform this function. A base that takes over for Tyr 149 must be nearby and must display a pK_a of >7.1. The only nearby enzymatic group is Ser 124 (*7*). Acid−base catalysis would not normally be expected for a serine residue because of the high value of the pK_a for the 3-hydroxyl group. The pK_a of *N*-acetylserinamide, a model for a seryl residue, is 13.4 (*26*). However, Ser 124 in Y149F-GalE is a special case. In the structure of wild-type GalE, the hydroxyl groups of Ser 124 and Tyr 149 are hydrogen bonded to each other, and both are near pyranosyl C4(OH) of UDP-glucose in the structure of the abortive complex. Both are in position to function as bases in abstracting a proton from C4(OH) and catalyze hydride transfer. However, in wild-type GalE, only Tyr 149 is effective as a base because of its low pK_a of 6.1, which allows it to exist and function in the basic phenolate form above pH 6.

The low pK_a of Tyr 149 is attributed to the positive electrostatic field created by the ϵ-aminium ion of Lys 153 and nicotinamide N1 of NAD$^+$, both of which are within 3.7 Å of the phenolic group in Tyr 149 (*7*). The structure of

Y149F-GalE is very similar to that of wild-type GalE except for the absence of the phenolate oxygen of Tyr 149. Therefore, in the absence of the negatively charged phenolate of Tyr 149, the 3-hydroxyl group of Ser 124 should fall under the influence of the positive electrostatic field from Lys 153 and the nicotinamide ring. The pK_a of Ser 124 in Y149F-GalE should, therefore, be depressed to a degree similar to that experienced by Tyr 149 in wild-type GalE. In the wild-type enzyme, the pK_a of Tyr 149 is depressed from 10.2 to 6.1, and a similar electrostatic depression of the pK_a of Ser 124 from 13.4 would lead to a pK_a of 9.4. This value is low enough to allow Ser 124 to serve as a base catalyst. At the same time, it is higher than the kinetic pK_a of 7.1, as is required by the pH-dependent deuterium kinetic isotope effect. We believe that Ser 124 is likely to be the base catalyst in epimerization by Y149F-GalE.

Acid−Base Catalysis in Epimerization by Y149F/S124A-GalE. Neither Tyr 149 nor Ser 124 is available as an acid−base catalyst in Y149F/S124A-GalE. This is presumably the reason for its activity being so much lower than that of either of the singly mutated epimerases. The lyate species of water and the acid and base components of buffers are the catalysts available to facilitate hydride transfer. The activity of Y149F/S124A-GalE is so low that a systematic study of acid−base catalysis by various buffers and as a function of pH would require massive amounts of enzyme and time. Such a study might be interesting but would not be likely to resolve outstanding mechanistic questions about this enzyme.

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