Potentiometric Analysis of UDP-Galactopyranose Mutase: Stabilization of the Flavosemiquinone by Substrate†

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ABSTRACT: UDP-galactopyranose mutase is a flavoprotein which catalyses the interconversion of UDP-galactopyranose and UDP-galactofuranose. The enzyme is of interest because it provides the activated biosynthetic precursor of galactofuranose, a key cell wall component of many bacterial pathogens. The reaction mechanism of this mutase is intriguing because the anomeric oxygen forms a glycosidic bond, which means that the reaction must proceed by a novel mechanism involving ring breakage and closure. The structure of the enzyme is known, but the mechanism, although speculated on, is not resolved. The overall reaction is electrically neutral but a crypto-redox reaction is suggested by the requirement that the flavin must adopt the reduced form for activity. Herein we report a thermodynamic analysis of the enzyme's flavin cofactor with the objective of defining the system and setting parameters for possible reaction schemes. The analysis shows that the neutral semiquinone ($FADH^{\bullet}$) is stabilized in the presence of substrate and the fully reduced flavin is the anionic $FADH^-$ rather than the neutral $FADH_2$. The anionic $FADH^-$ has the potential to act as a rapid 1-electron donor/acceptor without being slowed by a coupled proton transfer and is therefore an ideal crypto-redox cofactor.

The five membered ring sugar galactofuranose (Gal*f*)[1] is a constituent of a polymer linking the peptidoglycan and mycolic acid layer of the cell wall of *Mycobacterium tuberculosis* (*1*). The sugar is also found in the O-antigens of several Gram-negative bacteria, including *Klebsiella pneumoniea* (*2*) (a common noscomial pathogen); synthesis of the sugar is essential for the viability of *M. tuberculosis*, but it is not found in humans (*1*, *3*−*5*). Gal*f* biosynthesis is therefore a potential therapeutic target. The immediate precursor of gal*f* is UDP-Gal*f*, which is in turn synthesized from UDP-galactopyranose (UDP-Gal*p*), a six-membered carbohydrate ring (*3*). In free solution Gal*p* and Gal*f* interconvert via the open chain form of galactose. However, once the reducing oxygen is covalently bonded, as in UDP-Gal, the ring is locked and cannot interconvert by this means. The interconversion of UDP-Gal*p* and UDP-Gal*f* is catalyzed by an enzyme, UDP-galactopyranose mutase (mutase), which has also been called a contractase. The reported equilibrium constant for this interconversion varies slightly but is approximately 0.1 (10 UDP-Gal*p* to 1 UDP-Gal*f*) (*3*−*5*).

The structural gene for the enzyme (Glf) has been cloned from a variety of bacterial sources. UDP-galactopyranose mutase from *Klebsiella pneumonia* is reported to require the presence of NADH or NADPH for it activity. As noted by the authors, this requirement seems strange given that there is no net transfer of electrons (*3*). Further studies on the enzyme demonstrated that the transfer of hydride from the R face of NADH is rate limiting in aerobic conditions (*5*). Subsequent reports show that reduction by dithionite and anaerobiosis activates the enzyme and oxidation by $K_3(FeCN_6)$ inactivates it (*6*), although other studies had suggested that the oxidized enzyme retained some activity (*7*). Flavoproteins can undergo photoreduction, which could lead to small inconsistencies, and a review of the published work (*3*, *5*) strongly indicates that the reduced enzyme is active and the oxidized enzyme is not. Further insight into the catalytic mechanism comes from the demonstration that the phosphate group bound to the anomeric position statistically scrambled a labeled oxygen atom with the same rate as the reaction itself (*8*). This observation led to the conclusion that during turnover the glycosidic bond must be broken as part of the mechanism. The same researchers also showed that fluoro−deoxy analogues of substrates (substituted at the 2 and 3 positions) were substrates for the enzyme (although the 2-substituent was a poor substrate); however, neither eliminated HF (*9*). These results were confirmed by a subsequent study on the *Escherichia coli*

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[1] Abbreviations: EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid; UDP-Gal*f*, UDP-galactofuranose; UDP-Gal*p*, UDP-galactopyranose; UDP-Gal, UDP-galactose; Gal*f*, galactofuranose; Gal*p*, galactopyranose. Note: All redox potentials are given with respect to the standard hydrogen half cell (NHE).

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UDP-galactopyranose mutase, although in this case covalent intermediates were apparently formed between enzyme and substrate (*10*). An elegant mechanism was proposed, by both groups, which accommodates the requirement for rupture of the glycosidic bond during catalysis. In this model the O4 of galactose acts as a nucleophile to displace the UDP group, forming a bicyclic sugar and UDP. The mechanism does not require any transfer of electrons, but in this scenario reduced flavin could promote the reaction by stabilizing the oxocarbonium ion formed in the transition state.

When we determined the structure of the *E. coli* mutase enzyme we noted that the isoalloxazine ring of the flavin sat in an exposed position (*6*). Modeling studies based on the structure suggested that the galactose ring of UDP-gal*p* would be adjacent to this isoalloxazine ring. In this paper we report a thermodynamic analysis of the mutase, we show that the presence of substrate stabilizes a neutral flavosemiquinone radical and the fully reduced flavin is anionic. The anionic flavohydroquinone would allow rapid single electron transfer in a crypto-redox reaction. In the discussion we incorporate these findings, with previous reports, into considerations of the enzyme mechanism.

MATERIALS AND METHODS

Unless stated all chemicals were supplied by Sigma Chemicals, Poole, Dorset, U.K.

Protein Purification. E. coli strain BL21 DE3 was used to express the Histidine tagged *K. pneumoniae* UDP-galactopyranose mutase. The *GALF* gene from the pWQ66 plasmid (*3*) was cloned into a modified HIS-tagged expression vector containing a tobacco etch virus (TEV) protease cleavage site. The bacteria were grown aerobically at 37 °C at pH 7.5 in Terrific Broth (*11*) until an optical density of 0.6−0.8 at 600 nm was attained, at which point *GALF* expression was induced for 3 h with 1 mM isothiolgalactopyranoside (IPTG). Cells were harvested by centrifugation at 7500 rpm. A 50 g amount of cell pellet (wet weight) was resuspended to 100 mL in 50 mM HEPES buffer pH 7.0 containing 1 mM (phenylmethyl)sulfonyl fluoroside (PMSF) at 4 °C. A 0.1 mg·mL^{-1} concentration of lysozyme and 0.1 mg mL^{-1} DNAase I were added, and the suspension was stirred at room temperature for 30 min before sonication with a Sanyo soniprep 150, at 4 °C for 10 × 30 s. The sonicate was clarified by centrifugation at 20 000 rpm at 4 °C for 30 min. The supernatant was decanted and passed through a 0.45 μm filter (Acrodisc) before loading onto 10 mL Quiagen Ni−NTA metal chelate column at 4 °C. The column was precharged with 10 column volumes of 500 mM nickel sulfate and washed with 20 column volumes of 50 mM HEPES, pH 7.0. The supernatant was loaded onto the column at 5 mL min^{-1} and the column washed with 20 column volumes of 50 mM HEPES and 25 mM imidazole, pH 7.0, before elution of the protein with 50 mM HEPES and 250 mM imidazole, pH 7.0. The eluate (approximately 60 mL) was dialyzed in 5 × 30 min batches at 4 °C in 5 L of 50 mM HEPES, pH 7.0. TEV cleavage was carried out by the addition of 30 μg mL^{-1} TEV protease and 1 mM DTT (dithiothreitol) to the sample. The cleavage reaction was left stirring at room temperature for 3 h. The reaction mixture was dialyzed against 50 mM HEPES, pH 7.0, to remove excess DTT before further purification. The cleaved protein sample was passed through a metal chelate column as before and the cleaved protein collected in the unbound fraction. SDS−PAGE analysis (Coomassie staining) shows a single band at the correct molecular weight. MALDI TOF spectrometry of trypsin digests unequivocally confirmed identity. We obtained 15−20 mg of pure protein from 1 L of culture. The protein sample was concentrated using an Amicon ultra filtration cell (10 kDa cutoff) to 5 mg mL^{-1} and then frozen at −80 °C with 30% glycerol for storage. Concentration was determined using the absorbance of the flavin at 450 nm using extinction coefficient of 13500 M^{-1} cm^{-1} (*12*).

Redox Potentiometry. Redox titrations were carried out at 25 ± 2 °C under a nitrogen atmosphere in a Belle technology glovebox. Oxygen levels maintained at less than 5 ppm as described previously (*13*). Aliquots of 1.5 mL of 5 mg mL^{-1} UDP-galactopyranose mutase were run through a Bio-Rad 10 DG column equilibrated with 100 mM MOPS, pH 6.5, 7, and 7.5, to remove oxygen and glycerol. The resultant 3 mL aliquots of 50−100 μM UDP-galactopyranose mutase were titrated potentiometrically using sodium dithionite as reductant and potassium ferricyanide as oxidant in the presence of redox mediators. These included 1 μM pyocyanine, 10 μM hydroxynaphthaquinone, 2 μM FMN, 0.5 μM benzyl-viologen, and 2 μM methyl-viologen. Further titrations were conducted in the presence of UDP-galactose (200 μM), 2-fluoro-deoxy-UDP-Gal*p* (200 μM), and UDP (500 μM).

Data Manipulation and Analysis. The potentiometric titrations were analyzed by plotting absorbance at 450 and 580 nm against the electrode potential. Data were fitted simultaneously at both wavelengths to a Nernst function describing a two-electron redox process (*13*) using nonlinear regression analysis, eq 1 (Origin software, Microcal). Some analyses required subtraction of absorbance contributions caused by increases in turbidity. As turbidity causes greater scattering at lower wavelengths, it was possible to remove this effect by using $A_{600} - 1.5A_{670}$ instead of the absorbance at 580 nm. To estimate the single electron reduction potentials effectively in this case, the absorbance coefficients ϵ_{600} and ϵ_{670} for the semiquinone were fixed during the fitting process to values determined using the substrate-bound enzyme.

$$A = (a10^{(E-E'_1)/59} + b + c10^{(E'_2-E)/59})/(1 + 10^{(E-E'_1)/59} + 10^{(E'_2-E)/59}) \quad (1)$$

Substrate-Binding Titrations. UDP-galactopyranose mutase was prepared in an anaerobic glovebox as described above at a concentration of approximately 35 μM. Aliquots of sodium dithionite were added until the enzyme was approximately 50% reduced (characterized by loss of half the absorbance at 450 nm). UDP-Gal and UDP were titrated into the solution stepwise. The absorbance change ($A_{450} - A_{575}$) was plotted against substrate concentration and fitted to a single-binding site model using nonlinear regression analysis to determine an apparent dissociation constant (K_d).

Electron Paramagnetic Resonance (EPR) Studies. EPR spectra were obtained using a Bruker X-band EPR spectrometer (EMX) with spectrometer settings as reported in the appropriate figure legend. Sample temperature was regulated by a liquid nitrogen transfer system.

EPR spectra were obtained of UDP-galactopyranose mutase samples potentiometrically poised during redox titration and trapped by freezing. The titrations were performed according to ref *14*, and the mediating dye system was developed to give minimum free radical interference (*15*). The redox mediators were varied, but the final mix contained the following at 50 μM except 5-phenazine ethoxy sulfate, which was added to a final concentration of 0.5 μM: indigo tetrasulfonate ($E_{m7} = -47$ mV); duraquinone ($E_{m7} = +10$ mV); 2-hydroxynaphthaquinone ($E_{m7} = -146$ mV); 5-phenazine ethoxy sulfonate ($E_{m7} = +74$ mV); 1,4-naphthaquinone ($E_{m7} = +60$ mV). The mutase concentration was 40 μM and the buffer 100 mM HEPES, pH 7.0; when present, the substrate UDP-galactose was 150 μM. The ambient redox potential (E_h) was adjusted by titration of potassium ferricyanide and sodium dithionite and measured using a combination platinum (Ag/AgCl) reference electrode purchased from Russell pH Ltd (Auchtermuchty, Fife, U.K.). Redox titrations were conducted anaerobically; the redox titration vessel was continuously flushed with N_2 that had been passed through a Nil-Ox O_2-scrubbing apparatus (Jencons Scientific, Hemel Hempstead, Herts, U.K.). The samples were poised, transferred anaerobically to EPR tubes, quick frozen in an isopentane−cyclohexane freezing mixture, and stored under liquid N_2 until required.

The semiquinone signals were quantitated by double integration of the signal against a 10 μM 1,1-diphenyl-2-picrylhydrazyl free radical standard and a 1 mM $CuSO_4$ standard and analyzed as previously described (*15*).

RESULTS

Redox Potentiometry and Ligand Effects Observed in the Near-Ultraviolet and Visible Spectral Region. UDP-galactopyranose mutase is a typical yellow flavoprotein with absorption maxima at 457 and 380 nm. Figure 1 shows spectra taken during progressive reduction of the enzyme during a redox titration. Three different conditions are shown, the absence of substrate (panel A), the presence of UDP (panel B), and the presence of UDP-galactose (panel C). In the absence of substrate, reduction of the enzyme causes a decrease in the intensity of the visible absorption bands and no strong absorbance characteristic of either red anionic or blue neutral flavosemiquinone. In the presence of 500 μM UDP, the spectrum of the fully oxidized enzyme is altered. The most obvious effect is an apparent loss of fine structure in the band centered on 457 nm. Spectra obtained during reductive titration of the enzyme in the presence of UDP show the presence of a blue flavosemiquinone as a redox intermediate, with an increase in absorption between 500 and 650 nm. In the presence of 200 μM substrate (UDP-galactose), the spectrum of the fully oxidized enzyme is identical to that of the substrate-free enzyme. However, progressive reduction of the substrate-bound enzyme also leads to the formation of a stable blue flavo-semiquinone, which bleaches on further reduction. The semiquinone formed during titration in the presence of UDP-galactose has a clear absorption maximum at 580 nm and extends to longer wavelengths than that formed with bound UDP, with more spectral fine-structure.

Figure 2 shows plots of data taken from redox titrations. In Figure 2 (panel A), is an analysis of a redox titration of

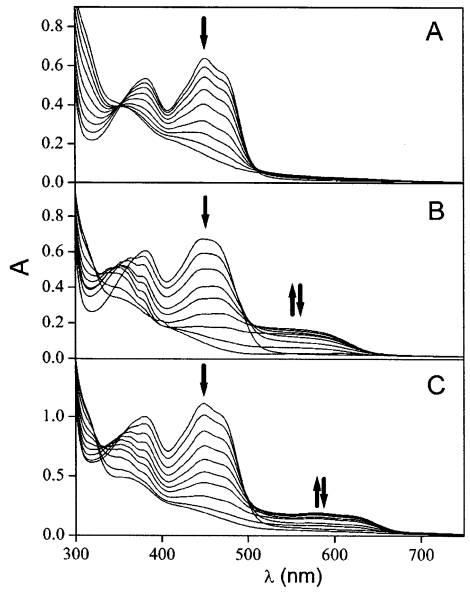

FIGURE 1: UV/visible absorption spectra taken during potentiometric redox titration. Spectra were recorded at different ambient redox potentials over the reductive phase of a potentiometric redox titration of UDP-galactopyranose mutase in the absence of ligand (A), in the presence of 500 μM UDP (B), and in the presence of 200μM UDP-galactose (C). The incubation was carried out as described in Materials and Methods, and the enzyme concentration was 50−100 μM in 100 mM MOPS at pH 7.0 in (A) and (B) and pH 6.5 in (C). Reduction of the enzyme causes a loss of absorbance at 450 nm (arrow) and an increase followed by a loss of absorbance between 520 and 620 nm (arrows).

the enzyme in the absence of substrate such as shown. Changes at 450 and 600 nm are plotted as a function of ambient redox potential. The 450 nm data show an $n = 2$ midpoint potential (pH 7.0) of −32 mV (with respect to the hydrogen half cell). Also shown in Figure 2 (panel A) is a plot of the change in absorbance at 600 nm corrected for baseline drift (magnified 10-fold). Although small, this effect indicates that some neutral blue semiquinone is formed during the titration. Using absorption coefficients calculated from the substrate-bound data (below), it is possible to estimate the potentials of the two one-electron redox couples by fitting the absorbance data at both wavelengths simultaneously. The two half-potentials are separated by approximately 137 mV (see Table 1).

Redox titrations were also conducted in the presence of 200 μM UDP-galactose at pH 6.5, pH 7.0, and pH 7.5. All titrations were reversible, generating the same spectral species at similar potentials in both oxidative and reductive directions. As shown in Figure 1 (panel C), the presence of substrate results in the stabilization of the blue semiquinone form of the FAD with a broad absorption band appearing around 580 nm as a redox intermediate. Midpoint reduction potentials were determined for the two single $n = 1$ transitions from analysis of the spectral changes at 450 and 580 nm at the different pHs (This shown in Figure 2, panel B). The datasets were fitted simultaneously at both wavelengths for each titration. At pH 7.0 the reduction potential of the two-electron couple is the same as in the absence of substrate (−31 mV; Table 1). However, the one-electron couples are shifted by 70 mV each leading to a stabilization of the neutral (blue) semiquinone. At pH 6.5 the oxidized/semiquinone (E_1) potential is shifted by 31 ± 5 mV relative to pH 7, whereas

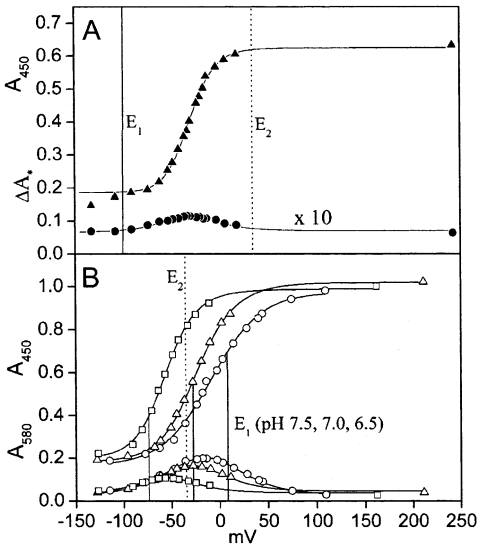

FIGURE 2: Analysis of the potentiometric titrations in the visible spectral region: determination of the reduction potentials. In the absence of substrate, panel A shows the absorbance changes at 450 nm (▲) and at 600 nm (●) (absorption difference calculated using ($A_{600} - 1.5A_{670}$) (ΔA_*)) plotted as a function of the ambient redox potential. The titration was performed in 100 mM MOPS, pH 7.0, as described in Materials and Methods. Note that the 600 nm change is multiplied by 10 in this case. In the presence of substrate, panel B shows normalized absorbance at 450 nm (upper set) and 580 nm (lower bell set) in the presence of 200 μM UDP-galactose in 100 mM MOPS at pH 6.5 (○), pH 7.0 (△), and pH 7.5 (□). All data points are shown fitted to a Nernst relationship as described in Materials and Methods. Values obtained for E_1, the quinone/semiquinone redox couple, and E_2, the semiquinone/hydroquinone redox couple, are given in Table 1. E_1 and E_2 are marked by solid and broken lines, respectively.

Table 1: Midpoint Reduction Potentials (vs NHE) for the FAD of UDP-Galactopyranose Mutase in the Presence and Absence of Substrate

	E_1 (mV)[a]	E_2 (mV)[b]	E_{2e} (mV)[c]
mutase (pH 7.0)[d]	-100 ± 3	$+37 \pm 3$	-32
mutase + UDP-Gal (pH 6.5)[d]	$+4 \pm 3$	-34 ± 4	-15
mutase + UDP-Gal (pH 7.0)[d]	-27 ± 2	-34 ± 3	-31
mutase + UDP-Gal (pH 7.5)[d]	-75 ± 3	-43 ± 3	-59

[a] Midpoint potential ($n = 1$) of the oxidized/semiquinone FAD redox couple. [b] Midpoint potential ($n = 1$) of the semiquinone/hydroquinone FAD redox couple. [c] Midpoint potential ($n = 2$) of the oxidized/hydroquinone FAD redox couple. [d] Conducted in 100 mM MOPS, pH 6.5, 7.0, or 7.5 (±0.1) as indicated. Experimental conditions are as for Figures 1 and 2. Where present, the UDP-galactose was at 200 μM.

the semiquinone/hydroquinone (E_2) potential is unchanged. For each proton coupled to an electron acquisition, a pH shift of 0.5 units should be accompanied by a reduction potential shift of 30 mV. This indicates that a single proton is accepted by the flavin on formation of the semiquinone, but no protons are associated with the subsequent formation of the hydroquinone; i.e., FAD is reduced to $FADH^{\bullet}$ which is reduced to $FADH^-$. pH dependency is also observed from pH 7.0 to pH 7.5, where there are shifts in the opposite direction of 48 ± 5 mV (E_1) and 9 ± 5 mV (E_2) (Table 1).

Redox titrations conducted in the presence of 2-flourodeoxy-UDP-gal*p* (gift of Laura L. Kiessling, University of Wisconsin) are identical to those conducted with UDP-galactose (results not shown). However, in the presence of UDP alone the titrations are not readily reversible; the hysteresis prevented accurate determination of reduction potentials. Comparison of spectra for UDP and UDP-galactose (Figure 1) shows that UDP perturbs the spectrum of the oxidized enzyme and UDP-galactose does not. The most probable explanation of this is that UDP binds to the oxidized enzyme leaving a hole next to the flavin which perturbs the spectrum.

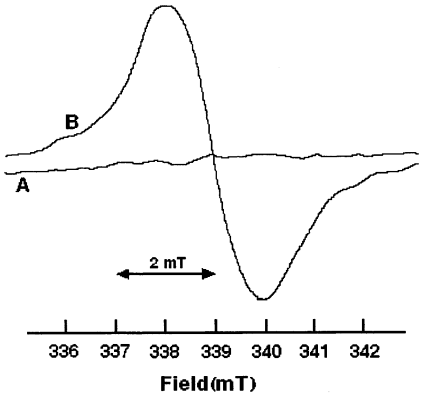

FIGURE 3: EPR spectra of the flavosemiquinone of UDP-galactopyranose mutase in the absence and presence of substrate: (A) EPR spectrum of a mutase sample poised at 55 ± 5 mV in the absence of substrate; (B) EPR spectrum of a sample poised at 55 ± 5 mV in the presence of 150 μM UDP-galactose. The redox titration was carried out as described in Materials and Methods. The EPR parameters were the following: temperature, 120 K; modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; microwave frequency, 9.512 GHz; microwave power, 0.2 mW. The zero crossing point of spectrum (B) is approximately $g = 2.005$, and the peak to trough line width is approximately 1.85 mT.

Measurement of the Apparent K_d for UDP-Galactose by Titration of the 580 and 457 nm Bands. To determine a dissociation constant of the substrate, the difference in the visible absorption spectrum caused by semiquinone stabilization was used as a measure of bound substrate. The enzyme was first reduced until only half of the 457 nm absorption band remained and was then titrated with substrate (UDP-gal). Addition of UDP-gal causes a decrease in absorbance at 457 nm and a concomitant increase at 580 nm, characteristic of blue semiquinone formation (not shown). Addition of excess substrate leads to saturation and no further spectral change. The change in the absorbance difference $A_{450} - A_{575}$ was fitted to a single binding-site model to give an apparent K_d of 28 ± 3 μM for UDP-galactose. A similar titration with UDP was also conducted, but the data did not conform to any reasonable model.

Redox Potentiometry and Ligand Effects Observed by Electron Paramagnetic Resonance Spectroscopy. Potentiometric redox titrations were performed in the presence and absence of substrate and samples measured by EPR spectroscopy, as described in Materials and Methods. EPR can only detect the semiquinone radical form of the enzyme. In the absence of substrate no significant radical signal is observed; in the presence of 150 μM UDP-galactose a radical signal is observed and its magnitude follows a bell-shaped redox-titration curve which is similar to the optical data under similar conditions (not shown). EPR spectra poised at approximately -55 mV, in the presence (B) and absence (A) of substrate, are shown in Figure 3. The radical observed has the line shape and line width expected of a neutral flavosemiquinone by comparison with known neutral and anionic flavosemiquinones. The presence of a strongly

coupled proton at the N(5) position causes broadening and a peak to trough line width of 1.85−2.0 mT, while an anionic flavosemiquinone gives a narrower peak to trough line width of 1.5 mT (*16*). The zero crossing point of the mutase flavosemiquinone spectrum is approximately $g = 2.005$, and the peak to trough line width is approximately 1.85 mT. Quantitation of the EPR spectrum qives the maximal radical concentration of 50−60% of the enzyme concentration.

DISCUSSION

The stabilization of a neutral semiquinone radical by substrate and the reduction of the flavin to an anionic hydroquinone is interesting in terms of the potential involvement of the flavin in catalysis. While it is possible that the flavin has no direct role in catalysis and serves only to stabilize the protein fold, this seems unlikely given the impact that its redox state has on the catalytic rate (reduction leading to at least 10^5 fold stimulation) (*6*). Our results are the first detailed evaluation of the thermodynamic redox properties of the flavin in a mutase of this type. These redox parameters limit what is and is not possible chemistry for the flavin during turnover. They establish that the reduced flavin is found in the anionic hydroquinone form at pH 7.0, that substrate binding causes a stabilization of the neutral, blue semiquinone form of the FAD, and that the single-electron oxidation of hydroquinone to semiquinone is not coupled to proton transfer in the pH range studied. The results also suggest that the carbohydrate moiety of the substrate is positioned close in space to the isoalloxazine ring of the FAD on binding. Any proposed mechanism requires that the glycosidic bond is broken, i.e., UDP is transiently separated from the galactose, to account for the observed scrambling of the oxygen atoms attached to the glycosidic phosphate group during turnover (*8*). This seems to force the conclusion that a step in the reaction is the formation of an oxocarbonium anion, even if only at the transition state. Combining this requirement with our results, we discuss possible mechanisms, involving the transfer of two, one, or no electrons.

The published proposal that the turnover mechanism progresses through a bicyclic intermediate is attractive for its simplicity (*7*−*10*) and is consistent with the oxygen scrambling data (*8*). Our data provide some support for this mechanism. The carbohydrate moiety of UDP-Gal*p* is close in space to the flavin and the fully reduced hydroquinone is anionic. Therefore, the negatively charged isoalloxazine ring could stabilize the carbocation transition state involved in forming the bicyclic sugar. Such an interpretation could explain the effect of redox state of the flavin on activity. However, for simple charge stabilization this is an extremely large effect. The mechanism requires an intermediate state consisting of two molecules: UDP and a bicyclic sugar (*8*). The bicyclic sugar has been made and is kinetically stable in water (R. A. Field, University of East Anglia, personal communication). UDP is also chemically stable. Therefore, this mechanism appears to involve formation of two stable intermediates, neither of which are observed as byproducts of catalytic turnover. Although it does not conclusively rule out this mechanism, the presence of a kinetic trap coupled to a requirement for the flavin to be reduced does provide difficulty for this mechanism. Without structural data we cannot exclude the possibility of another nucleophile (other than O4) attacking the oxocarbonium anion and thus preventing formation of the stable bicyclic sugar. However, unless $FADH^-$ is directly involved in activating this nucleophile, it is difficult to see why the oxidation state of flavin is so important for activity.

An attractive alternative to this mechanism is a cryptoredox process involving transient transfer of electrons. However, we have been unable to observe any changes in the spectral properties of the flavin during stopped flow rapid turnover experiments. This negative result indicates that either electron transfer does not occur or is not part of the rate-determining step. This latter is entirely reasonable if, for example, electron transfer results in the formation of an unstable species. For electron transfer to be feasible the potential of electron donor and electron acceptor must be closely matched, since the electron(s) require movement back and forth without forming stable intermediates. Second, the substrate must be close enough in space for such a redox process to be feasible. Our data quantitate the driving force for electron transfer from the FAD hydroquinone for a one- and two-electron process. There are no estimates in the literature for the reduction potentials of oxocarbonium ions. However, they may have an appropriately accessible redox state. The results comparing the UDP and UDP-galactose titrations suggest that, indeed, the carbohydrate ring is close in space to the isoalloxazine ring of the FAD. Flavins commonly transfer or accept 2 electrons during turnover. The fact that both 2- and 3-fluoro-deoxy-UDP-galactose are viable substrates for the mutase presents formidable difficulties for any mechanism involving formation of a carbanion at C1, C2, C3, or C4. In each case, the β-elimination of F^- would be highly favored, but no flouride ions were detected (*9*, *10*). We are unable to formulate a mechanism which involves two-electron transfer from FAD to substrate but does not involve carbanion formation at C1, C2, C3, or C4. A two-electron process therefore seems unlikely. For one electron-transfer to be viable, the semiquinone form of the donor must be thermodynamically accessible. The results of this study show that the semiquinone form of flavin is indeed significantly stabilized in the substrate bound enzyme. On the available data, we propose a mechanism involving a single-electron transfer to the oxocarbonium to produce an anomeric radical. Single-electron transfer from the anionic hydroquinone results in formation of the neutral blue semiquinone so need not be coupled to proton transfer. This would allow the reaction to be both rapid and reversible. By generation of a carbohydrate radical transition state in this direct manner, stable intermediates would also be avoided. The formation of a radical at the C1 position of sugars has precedent in the chemistry of both furanosyl and pyranosyl rings (*17*). Such anomeric radicals are highly reactive and have been harnessed synthetically by many laboratories (*17*, *18*). Radical formation would facilitate ring contraction by inducing nucleophilic attack by O4 at the C1 position, with cleavage of the C1−O5 bond (Figure 4). The electron could then be transferred from the anomeric position of the carbohydrate back to the FAD semiquinone. The probability of this mechanism depends on the accessibility of the carbohydrate radical transition state. It has the attractions of avoiding carbanion formation, which is largely ruled out on the basis of experiments with fluoro substrates and of introducing a redox role for the flavin, which seems

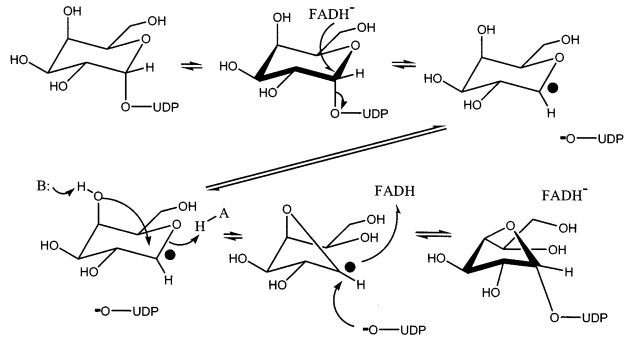

FIGURE 4: Proposal for the outline mechanism of ring contraction. The key intermediate in this proposed mechanism is the anomeric radical, which is formed by single-electron transfer from the anionic hydroquinone $FADH^-$ to the oxocarbonium ion. The radical-based mechanism avoids some of the pitfalls of other proposals and rationalizes chemical data. The data show that the semiquinone form of the flavin is thermodynamically accessible under conditions of turnover.

likely in view of the dependence of activity on its reduction state. Our results demonstrate that the FAD is ideally suited to fulfilling its role in this particular mechanism.

CONCLUSION

The mechanism of ring contraction performed by the mutase enzyme provides a formidable challenge to the chemist and biologist. Our work provides important quantitative measurements of the chemistry that is possible at the flavin cofactor of the enzyme. We have advanced a testable mechanism consistent with all the published data, which involves a single-electron crypto-redox process.

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