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Preliminary Communication

# Reconstitution of UDP-galactopyranose mutase with 1-deaza-FAD and 5-deaza-FAD: analysis and mechanistic implications

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**Abstract**

The galactofuranose moiety found in many surface constituents of microorganisms is derived from UDP-D-galactopyranose (UDP-Gal*p*) via a unique ring contraction reaction catalyzed by a FAD-dependent UDP-Gal*p* mutase. When the enzyme is reduced by sodium dithionite, its catalytic efficiency increases significantly. Since the overall transformation exhibits no net change in the redox state of the parties involved, how the enzyme-bound FAD plays an active role in the reaction mechanism is puzzling. In this paper, we report our study of the catalytic properties of UDP-Gal*p* mutase reconstituted with deaza-FADs. It was found that the mutase reconstituted with FAD or 1-deazaFAD has comparable activity, while that reconstituted with 5-deazaFAD is catalytically inactive. Because 5-deazaFAD is restricted to net two-electron process, yet FAD and 1-deazaFAD can undergo concerted two-electron as well as stepwise one-electron redox reactions, the above results support a radical mechanism for the mutase catalyzed reaction. In addition, the activity of the mutase reconstituted with FAD was found to increase considerably at high pHs. These observations have allowed us to propose a new mechanism involving one-electron transfer from the reduced FAD to an oxocarbenium intermediate generated by C-1 elimination of UDP to give a hexose radical and a flavin semiquinone. Subsequent radical recombination leads to a coenzyme–substrate adduct which may play a central role to facilitate the opening and recyclization of the galactose ring. A deprotonation

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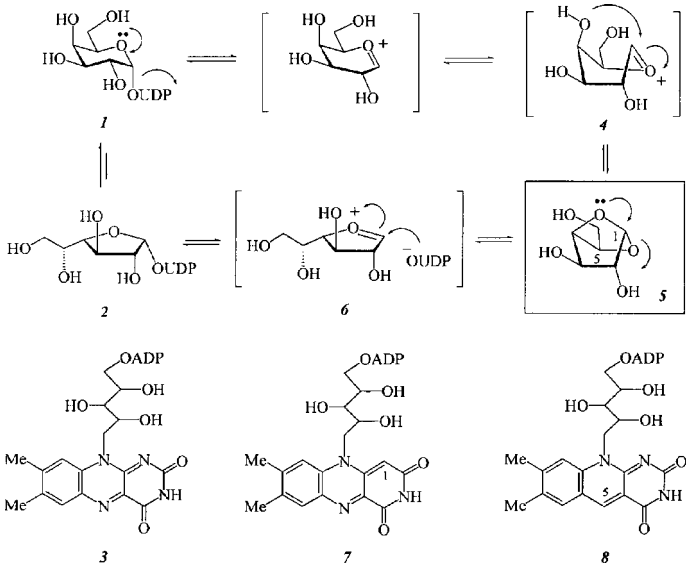
step, accompanied or followed the electron transfer step, to increase the nucleophilicity of the flavin radical anion may account for the activity enhancement at pH > 8.


The flavin-dependent enzymes are important catalysts involved in a wide variety of biological transformations [1,2]. Although the catalytic roles of flavoproteins are amazingly versatile, their functions rely primarily on the capability of the flavin coenzyme to transfer electrons in redox reactions, or to deliver reactive oxygen in mono- and dioxygenation reactions. While most reactions catalyzed by flavoproteins are redox reactions, exceptions are known in which the overall transformation catalyzed by a flavoprotein exhibits no net change in the redox state of the parties involved [3]. One recent example is the interconversion of UDP-D-galactopyranose (UDP-Gal*p*, **1**) to UDP-galactofuranose (UDP-Gal*f*, **2**) catalyzed by UDP-Gal*p* mutase [4]. This enzyme has been isolated from several bacterial sources [5] and all have been found to contain a flavin adenine dinucleotide (FAD, **3**) in the active site. Because galactofuranose units are found in many pathogens [6], but not in humans, UDP-Gal*p* mutase is an attractive target for therapeutic agents.

This unique enzyme has been the focus of considerable mechanistic and structural scrutiny in the last few years. Both 2-F and 3-F derivatives of UDP-Gal*f* are substrates for the mutase, which ruled out a mechanism involving the generation of a 2,3-enediol intermediate via redox recycling of the FAD coenzyme to facilitate the ring cleavage [7]. Since the reversible cleavage of the anomeric C-O bond of **1** was demonstrated to be part of catalysis in a positional isotope exchange experiment, a mechanism initiated by the elimination of UDP to form an oxocarbenium ion **4** followed by attack of the oxygen at C-4 on C-1 was proposed for the forward reaction (**1** → **2**, Scheme 1) [8]. The finding that the [2-F]-analogue of **2** is a poorer substrate than **2** for the mutase provided further support for the proposed mechanism [7b]. As illustrated in Scheme 1, ring opening between C-1 and the oxygen at C-5 of the bicyclo-acetal intermediate **5**, followed by the rebound of UDP at C-1 of **6** leads to the formation of **2** [8]. Interestingly, the catalytic efficiency of UDP-Gal*p* mutase is increased by more than two orders of magnitude under reduced conditions (20 mM dithionine) [9]. A comparable rate enhancement was also noted when the flavin coenzyme was selectively reduced by photo-reduction in the presence of 5-deaza-riboflavin under anaerobic conditions [9a]. In view of the fact that oxidation/reduction is not a requisite step in the above mechanism, how the enzyme-bound FAD plays an active role in catalysis is not immediately apparent.

One possibility is that reduction of FAD may induce a favorable conformational change in the enzyme, thereby making it more conducive to catalysis [7b,9a]. It is also conceivable that the reduced flavin, bearing a higher electron density at $N-1$ and/or $N-5$, may be used to stabilize the transiently formed oxocarbenium ion intermediates (such as **4** and **6**) to facilitate catalysis [7b,9a]. The involvement of a radical intermediate has also been speculated based on a recent finding that the neutral flavin semiquinone artificially generated in the active site of UDP-Gal*p* mutase is



Scheme 1.

stabilized in the presence of the substrate [10]. It is later suggested that the elimination of the UDP group may be facilitated by a single electron reduction of the substrate by the reduced FAD [3,10], a mode of bond activation used in the chorismate synthase reaction [11]. Whether structural effects, electronic effects, or a combination of both dictate the ability of FAD to enhance the rate of the mutase reaction is an interesting, albeit challenging, question. With a curious mind desiring to learn more of this intriguing transformation, we undertook a study of the catalytic properties of UDP-Gal*p* mutase reconstituted with deaza-FAD analogues having distinct redox behaviors.

The 1- and 5-deaza-riboflavin were prepared according to literature procedures [12] and were converted to the corresponding FAD derivatives (**7** and **8**) by the flavokinase/FAD-synthetase system of *Brevibacterium ammoniagenes* [13]. The apoenzyme was prepared by treating the mutase with acidic ammonium sulfate (pH 2.5) in the presence of 3 M potassium bromide [14], giving a 70–90% recovery yield of inactive protein. Binding of FAD to the enzyme must be relatively tight, since attempts to remove flavin by more gentle methods, such as dialysis against acidic buffer containing 2.2 M KBr [15] or gel filtration in the presence of 1 M KBr [16] were unsuccessful. The apoenzyme was separately reconstituted with FAD and its analogues and the excess free FAD was removed by centrifugal filtration using a Microcon YM-30 ultrafiltration unit (Amicon). The reduced form of the reconstituted mutase was prepared by the addition of excess of sodium dithionite in the incubation mixture. The equivalence of bound FAD per enzyme subunit after reconstitution was determined by quantifying the released FAD from a denatured mutase sample (boiling for 10 min) of known concentration. An extinction coefficient of 11,300 $M^{-1}$ $cm^{-1}$

was used for the calculation. Analysis showed a stoichiometry of 0.81 FAD/subunit for the FAD-reconstituted mutase, which is comparable with that found in the wild-type enzyme (0.81 FAD/subunit as isolated).

As shown in Fig. 1, the full restoration of enzyme activity occurred within 4 min at 37 °C when the apoenzyme was incubated with a 20-fold excess of oxidized FAD (**3**) in the presence of excess dithionite. Incubation for a longer time (30 min) resulted in activity loss, likely due to the instability of the mutase at 37 °C. In contrast, reconstitution of the apoprotein with FAD under the same conditions, but without dithionite, led to a holoprotein whose activity was barely detectable (Fig. 1). However, this inactive protein could be completely activated upon treatment with excess dithionite. These results clearly indicated that UDP-Gal*p* mutase in its fully oxidized form is inactive [9b]. It should be noted that the reduced mutase, when exposed to air, loses activity and becomes comparable to the mutase when isolated directly from the overproducing strain [9a]. It is likely that a small fraction of FAD remains reduced in the isolated enzyme contributing to the residual activity.

Reconstitution of apoenzyme with 1-deaza-FAD (**7**) and 5-deaza-FAD (**8**) was also carried out and a stoichiometry of nearly one coenzyme per protein subunit was achieved. The absorption spectra of the reconstituted enzymes are shown in Fig. 2. The well resolved spectral features of the flavin chromophore in both 1-deaza-FAD and 5-deaza-FAD reconstituted enzymes are characteristic for the coenzyme in a hydrophobic environment. Using UDP-Gal*f* as the substrate, the kinetic parameters of the wild-type and the reconstituted mutases were determined and the results are listed in Table 1. It was found that the activities of the mutase reconstituted with FAD (**3**) and 1-deaza-FAD (**7**) are comparable, while that reconstituted with 5-deaza-FAD (**8**) is catalytically inactive (Fig. 3). Since these flavin analogues will undergo a similar transformation upon reduction from a highly conjugated planar frame to a bent butterfly structure, any conformational changes of the active site induced by this transformation are expected to be similar for these flavin analogues. Thus, the fact that only 5-deaza-FAD has a detrimental effect on the mutase activity suggests a critical electronic difference, and not a conformational difference, between 5-deaza-FAD and the other two types of flavins.

To ensure that the observed phenomenon was not a result of improper binding of 5-deazaFAD in the active site, the affinities between mutase and deazaFADs (**7** and **8**) were determined and compared with that of FAD (**3**). The binding constants were deduced by measuring the change of the amount of free flavin in samples of known flavin concentration (6.3, 7.9, 9.4, and 11.0 μM) prior to and after the incubation with mutase (12 μM). The data were fitted to the equation $\Delta OD = \Delta OD_{max} - K_d \times \Delta OD/[FAD]$, where ΔOD is the change of absorbance of FAD solution before and after the incubation with mutase, $\Delta OD_{max}$ is the change in absorbance when all FAD is converted into complex, and $K_d$ is the dissociation constant [17]. The results, $K_d = 6.5, 6.8$, and 7.8 nM for FAD, 1-deazaFAD, and 5-deazaFAD, respectively, indicated that all three flavin coenzymes bind equally tightly in the active site and the structural variation of the deazaflavins apparently confers little change to their affinity for the enzyme. Thus, the aforementioned effects observed on the activity of the mutase are likely to be electronically induced and not physically influenced.

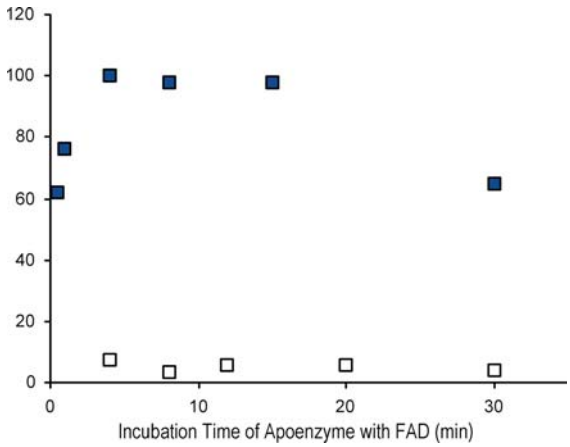



Fig. 1. The time course of the reconstitution of apo-mutase with FAD (**3**). A series of samples were prepared and each (40 μl) contained 0.8 μM apo-mutase and 15 μM FAD in 100 mM potassium phosphate buffer containing 15% glycerol, pH 7.5. The reaction was incubated at 37 °C and was tested at different time intervals for activity with 25 mM sodium dithionite (■) or without sodium dithionite (□). See Table 1 for the details of activity determination.

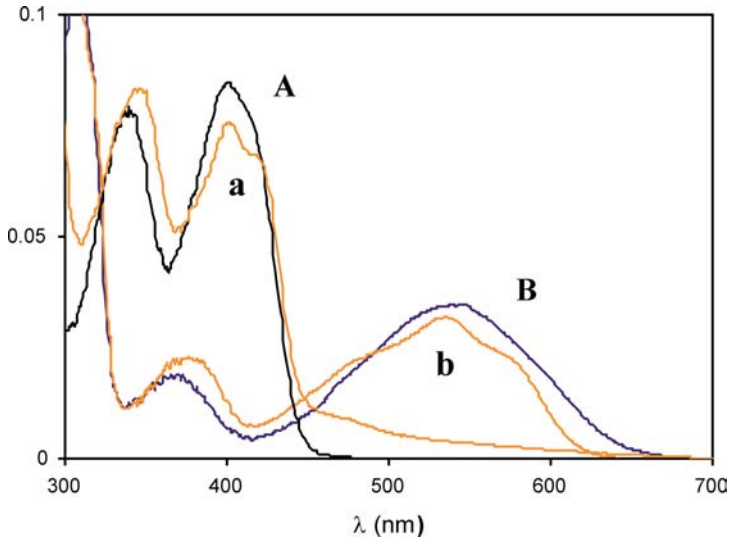



Fig. 2. The absorption spectra of 1-deazaFAD (**7**, 5.5 μM, curve A), 5-deazaFAD (**8**, 7.4 μM, curve B), mutase reconstituted with 1-deazaFAD (9.0 μM, curve a), and mutase reconstituted with 5-deazaFAD (8.4 μM, curve b) recorded in 100 mM potassium phosphate buffer containing 15% glycerol, pH 7.5.

Since 5-deaza-FAD is restricted to a net two-electron process, while FAD and 1-deaza-FAD can undergo concerted two-electron as well as one-electron redox reactions [18], our observations are consistent with the radical mechanism proposed

Table 1
Kinetic parameters of the wild-type and reconstituted mutase[a]

| Enzyme | | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}\,s^{-1}$) |
|---|---|---|---|---|
| Wild-type mutase | No $Na_2S_2O_4$ | 134 | 0.51 | 0.0038 |
| | $Na_2S_2O_4$ | 45 | 33 | 0.73 |
| Apo-mutase + FAD | $Na_2S_2O_4$ | 30 | 25 | 0.83 |
| Apo-mutase + 1-deazaFAD | $Na_2S_2O_4$ | 39 | 22 | 0.56 |

[a] The activity was determined by incubating the UDP-Gal*f* (**2**, 5–125 μM) and the purified mutase (0.76 μM) in 30 μl of 100 mM potassium phosphate buffer containing 15% glycerol (pH 7.5). For assays conducted under reducing conditions, 20 mM freshly prepared sodium dithionite was included in the mixture and less mutase (5.8 nM) was used. The reaction was carried out at 37 °C for 2 min and the resulting mixture was analyzed by HPLC using a $C_{18}$ column (4.6 × 250 mm) which was eluted with 1.5% acetonitrile in 50 mM triethylammonium acetate buffer, pH 6.8. The detector was set at 262 nm and the flow rate was 1.0 ml/min. The extent of conversion was determined by comparing the integration of the substrate and product peaks. The extent of conversion was typically controlled to be within 15% by properly adjusting the enzyme concentration. The kinetic parameters were determined by fitting the data to the Michaelis–Menten equation.

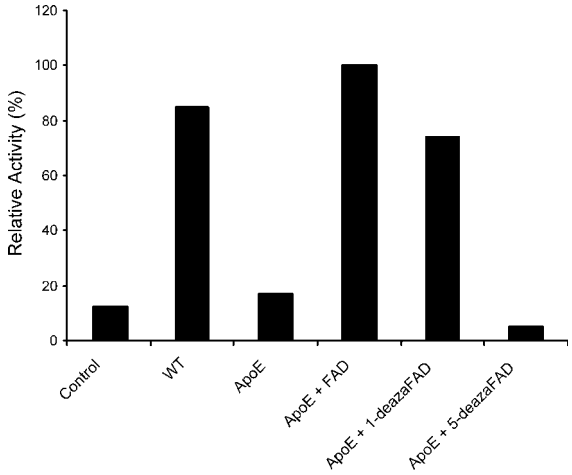



Fig. 3. Relative activity of the wild-type enzyme, apo-enzyme, and enzymes reconstituted with various FAD analogues. Column 1 (from left), no enzyme; column 2, wild-type mutase; column 3, apo-enzyme; column 4, mutase reconstituted with FAD; column 5, mutase reconstituted with 1-deazaFAD (**7**); and column 6, mutase reconstituted with 5-deazaFAD (**8**). See Table 1 for the details of activity determination.

for the UDP-Gal*p* mutase catalyzed reaction. To gain further insight into other factors affecting the ability of the reduced FAD to enhance the rate of the mutase reaction, the activity of mutase was examined over a pH range from 3.6 to 9.0. As illustrated in Fig. 4, mutase reconstituted with 5-deaza-FAD remained inactive throughout this range and little changes of the activity of mutase reconstituted with 1-deaza-FAD were observed. The elevated readings at low pHs are likely artifacts due to the hydrolysis of the sugar substrate to generate UDP, which has the same

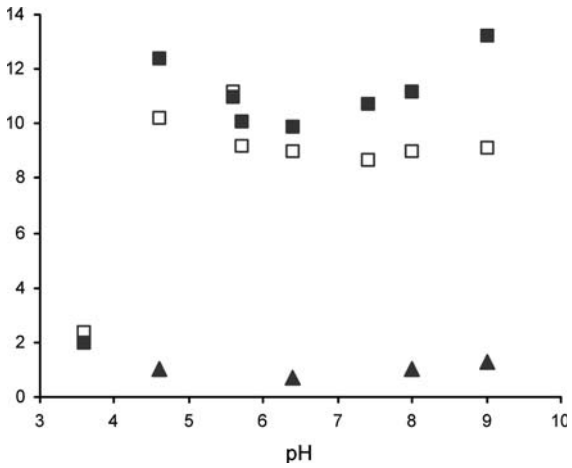
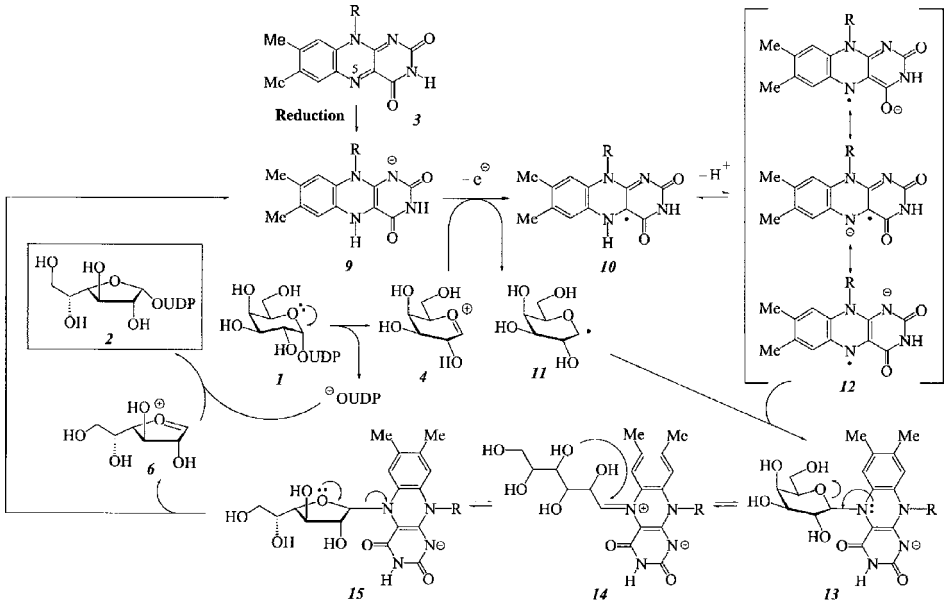



Fig. 4. The pH effect on the activity of mutase reconstituted with various FAD analogues: (■) mutase reconstituted with FAD (**3**); (□) mutase reconstituted with 1-deazaFAD (**7**); and (▲) mutase reconstituted with 5-deazaFAD (**8**). See Table 1 for the details of activity determination.

retention time as the product, UDP-Gal*p*, under the HPLC separation conditions [9a]. Interestingly, the enzyme activity of mutase reconstituted with FAD (the wild-type) increases considerably at high pHs.

Because the propensity of electron transfer is directly related to the potential difference between the donor and acceptor, the increase of activity of the wild type enzyme at high pH may be a result of lowering the redox potential of the flavin coenzyme within the experimental pH range. In fact, the midpoint potential of the semiquinone/hydroquinone FAD redox couple of UDP-Gal*p* mutase has been reported to decrease at a slightly higher pH (from $-34 \pm 3$ mV at pH 6.5 to $-43 \pm 3$ mV at pH 7.5). If such a trend holds beyond pH 7.5, our observation of activity enhancement at high pH would be a strong evidence supporting a radical mechanism. However, since the reported variation of the midpoint potential is minimal, it is uncertain that a significant potential change will take place at a high pH to facilitate the electron transfer. Thus, our data may instead suggest that a deprotonation step, accompanied or followed the electron transfer step, to increase the nucleophilicity of the flavin radical anion is important for enhancing the activity at pH $> 8$. Since the reduced flavin has been reported to be in the anionic hydroquinone form (such as **9**) at pH 7.0 [10], further deprotonation should occur at $N-5$, either during or after the electron transfer, to give a flavin semiquinone radical anion (such as **12**) as shown in Scheme 2. This prediction correlates well with a p$K_a$ of 8.3 estimated for $N-5$ of flavin semiquinone in solution [19].

The combination of these observations indicates that the UDP-Gal*p* mutase reaction likely proceeds by a radical mechanism and that the rate enhancements under reducing conditions are associated with the electronic properties of the flavin coenzyme, and not a conformational change. The most reasonable electron acceptor is the oxocarbenium intermediate (**4** and **6**) and the nascent product is neutral hexose




Scheme 2.

radical (**11** or the one electron reduced **6**). Our results further implicate that the $N-5$ of the FAD coenzyme is the likely site harboring the transiently formed unpaired electron during catalysis. As illustrated in Scheme 2, subsequent radical recombination between **11** and **12** should lead to a coenzyme–substrate adduct (such as **13** and **15**) which may play a central role to facilitate the opening and recyclization of the galactose ring [20]. A similar mechanism involving the formation of a Schiff base between an active site nucleophile and the sugar substrate has been proposed earlier by Blanchard [7a]. The current mechanistic proposal is consistent with our recent results that the chemically synthesized bicyclic intermediate **5** cannot be recognized by the mutase and several acyclic analogues of galactose are inhibitors for this enzyme [21]. Clearly, more research on the possible roles for the flavin coenzyme is needed to corroborate or debunk the current mechanistic proposal for the UDP-Gal*p* mutase catalyzed reaction. This knowledge is essential for future development of novel therapeutic agents directed against reactions involved in the formation of galactofuranose in many pathogens.

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[20] It should be noted that the intermediacy of a Schiff base formed between FAD and UDP-Gal*p* during catalysis in the active site of the mutase has recently been demonstrated (M. Soltero, E.E. Carlson, L.L. Kiessling, Poster Abstracts of the 29th Steenbock Symposium, Coenzyme, Cofactors and Catalysis, 2003, p. 87).
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