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Mechanism Study of dTDP-D-Glucose 4,6-Dehydratase: General Base in Active Site Domain

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dTDP-D-glucose 4,6-dehydratase as an oxidoreductase catalyzes the conversion of dTDP-D-glucose to dTDP-4-keto-6-deoxy-D-glucose, which is essential for the formation of 6-deoxysugars. dTDP-D-glucose 4,6-dehydratase shows remarkable stereochemical convergence in which displacement of the C-6 hydroxyl group by a C-4 hydrogen proceeds intramolecularly with inversion of configuration. The reaction mechanism is known to be oxidation, dehydration, and reduction by bases mediating proton transfer and NAD^+ cofactor. In this study, the bases in the active site domain are proposed to be His-79 and His-300 from a comparison of the peptides of the dehydratase and UDP-D-glucose epimerase. His-79 and His-300 were mutated to prepare the mutants H79L (mutation of histidine to leucine at the 79th amino acid) and H300A (mutation of histidine to alanine at the 300th amino acid) by site-directed mutagenesis. The H79L protein was inactive, showing that His-79 participates in the reaction mechanism.

Keywords: dTDP-D-glucose 4,6-dehydratase, Mechanism, Site-directed mutagenesis, UDP-D-glucose epimerase.

Introduction

The O-antigen is a repeating unit of four hexoses which consists of rhamnose, abequose, mannose, and galactose. The Rfb gene cluster from *Salmonella serovar* typhimurium LT2 was cloned and expressed to identify the function of each ORF by Reeves and colleagues (Jiang *et al.*, 1991; Romana *et al.*, 1991). Of these is the second gene in the rhamnose synthesis pathway. The rfbB gene encodes dTDP-D-glucose 4,6-dehydratase which catalyzes the conversion of dTDP-D-glucose to dTDP-4-keto-6-deoxy-D-glucose (Fig. 1). The expressed protein is a homodimer of 43 kDa subunits and highly specific for dTDP-D-glucose. In several labeling experiments, the stereochemical results suggested a reasonable model for the events in the active site of the enzyme during the catalytic process with the NAD^+ cofactor and two bases mediating proton transfer (Melo *et al.*, 1968; Snipes *et al.*, 1977; Floss and Beale, 1989). They allow the transfer of H-4 to C-6 via NAD^+ with the loss of water through oxidation, dehydration, and reduction steps (Fig. 1). In this paper, we report the active-site base by site-directed mutagenesis and then prove the reaction mechanism of dTDP-D-glucose 4,6-dehydratase suggested by Glaser and Floss (Melo *et al.*, 1968; Snipes *et al.*, 1977).

Materials and Methods

Material The *rfbB* gene (pPR1162) was obtained from Professor Peter R. Reeves, University of Sydney. The XLI-Blue MRF was purchased from Stratagene (La Jolla, USA). The Xpree™ protein expression system including pRSET plasmid

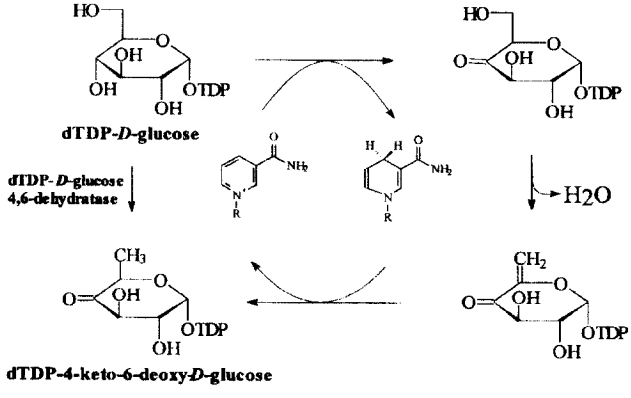

Fig. 1. dTDP-D-glucose 4,6-dehydratase.

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was purchased from Invitrogen Corporation (San Diego, USA). The Scuptor™ *in vitro* mutagenesis kit from Amersham (Cleveland, USA) was used for site-directed mutagenesis. All other chemicals were obtained from Sigma or United States Biochemical (Cleveland, USA). Restriction enzymes, DNA modified enzymes, and other enzymes were purchased from Promega Biotech. (Madison, USA).

Isolation of single-stranded template DNA and plasmid DNA General DNA manipulation was carried out following the methods of Sambrook *et al.* (1989). Plasmid DNA and bacteriophage RF DNA were isolated from XLI-Blue MRF by alkaline SDS extraction of cell lysate. Small and large-scale DNA purifications were performed using Qiagen resin (Chartworth, USA).

Site-directed mutagenesis The site-directed mutagenesis was performed with standard molecular biology procedures (Sambrook *et al.*, 1989) using the reagent in the Scuptor™ *in vitro* mutagenesis kit (Amersham) with single-stranded template. Mutagenesis was carried out according to the manufacturer's instructions. The mutated template was identified by DNA sequencing carried out directly on single-stranded templates by the dideoxy chain termination method (Sanger *et al.*, 1977).

Construction of plasmid The pPR1162 gene was digested with *Eco*RI and *Hin*dIII to cut the 1.5 kb *rfbB* gene fragment, and ligated at the *Eco*RI and *Hin*dIII restriction enzyme sites of M13mp18 and renamed pNH100. After site-directed mutagenesis of pHR100, the 1.5 kb *rfbB* gene fragment digested with *Eco*RI and *Hin*dIII was ligated at the *Eco*RI and *Hin*dIII restriction enzyme sites of the *E. coli* expression vector pRSET-B containing a strong T7 promoter to produce the pRS200 recombinant plasmid. *E. coli* BL21(DE3) was transformed with pRS200. *E. coli* BL21(DE3), which is a lysogen of bacteriophage DE3 and carries the T7 RNA polymerase gene under the control of the inducible *lac UV5* promoter was used as the host strain for the expression of *rfbB* and the mutated *rfbB* gene.

Expression and partial purification of dTDP-D-glucose 4,6-dehydratase Transformation of the host strain with the recombinant plasmid was performed as described by Sambrook *et. al.* (1989). Transformed cells were grown to an OD_{600} of 1.8–2.0 at 30°C in LB broth (1 l) containing carbenicillin (100 μg/ml) and then isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.4 mM. After a further 3.0 h growth at 30°C, cells were harvested by centrifugation at 5000 × *g* for 5 min, and resuspended in standard buffer (80 ml) consisting of 50 mM Tris·HCl (pH 7.6), 1 mM EDTA, 1 mM $MgCl_2$, and 1 mM dithiothreitol. The suspension was disrupted by ultrasonicator and then centrifuged at 15,000 × *g* for 30 min. To the supernatant (105 ml), ammonium sulfate powder was added to 25–60% saturation. The suspension was stirred for a further 20 min, and then the precipitate was collected by centrifugation and dissolved in the standard buffer solution (10 ml) and dialyzed against the same buffer.

Protein determination Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The protein concentration was measured at A_{280}.

Assay for dTDP-D-glucose 4,6-dehydratase Enzyme activity was determined as described by Vara *et. al.* (1988). The assay was carried out in a reaction mixture of 7.5 μl Tris (2 M, pH 7.6), 7.5 μl NAD^+ (10 mM), 15 μl dTDP-D-glucose (5 mM), and 75 μl cell-free extract. The mixture was incubated at 37°C for 30 min. At the end of the incubation period, the reaction was terminated by adding 750 μl 0.1 N NaOH and then incubated for a further 20 min at 37°C. The control sample contained the same reaction mixture without dTDP-D-glucose. Extinction differences at 318 nm were measured between all reaction mixtures and mixtures without dTDP-D-glucose. The amount of product formation was determined using an extinction coefficient (ε) of 6500 $M^{-1}L^{-1}$. One unit of enzyme activity corresponds to the formation of 1 μmol of product per hour under the given assay conditions, and the specific activities are reported as units per mg of protein.

Results and Discussion

Strategy of mutated sites dTDP-D-glucose 4,6-dehydratase and CDP-D-glucose 4,6-dehydratase show a remarkable stereochemical convergence in which the displacement of the C-6 hydroxyl group by a C-4 hydrogen proceeds intramolecularly with inversion of configuration (Snipes *et al.*, 1977; Floss *et al.*, 1989; Yu *et al.*, 1992). UDP-D-glucose epimerase is also a NAD^+-dependent oxidoreductase (Walsh, 1979). The reaction mechanism of UDP-D-glucose epimerase is suggested to be similar to that of dehydratase. The peptide sequences of dehydratase and epimerase have about 24–35% identity (Fig. 2). In this study, their high similarity from the peptide comparison between epimerase and dehydratase proposes the bases mediating proton transfer. Based on the known mechanism, the bases in the active-site domain were searched in the alignment of the deduced amino acid sequences from dTDP-D-glucose 4,6-dehydratase, CDP-D-glucose 4,6-dehydratase, and UDP-D-glucose epimerase (Fig. 2). His-79 is conserved perfectly in dTDP-, CDP-D-glucose 4,6-dehydratase and UDP-D-glucose epimerase. His-300 are conserved in dTDP- and CDP-D-glucose 4,6-dehydratase at proximal sites. The conserved histidines are proposed to mediate the proton transfers in these peptide sequences because histidine, having an imidazole group, can play the role of a general base. We prepared the mutated primers based on the *rfbB* DNA sequences (Fig. 3). The mutant H79L (mutation of histidine to leucine at the 79th amino acid) and H300A were constructed with the mutated primers (Fig. 4).

The activity of wild-type and mutant enzymes The mutagenic fragment was cloned into pRSET-B to prepare pRS200-H79L and overexpressed in *E. coli* BL21(DE3). The expressed protein was purified with ammonium sulfate precipitation. The partially purified enzymes were assayed. Table 1 shows the activities of the control and the mutated enzymes. The H79L mutant shows only background activity similar to the control. The H300L has the same activity as that of the wild-type.

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                                           79                                                                  300
RfbB    (54)...FEHADICDSA EITRIFEQYQ PDAVMHLAAE SHVDRSITGP AAFIETNIVG(103)  (287)......YREQ ITYVADRPGH DRRYAIDAGK ISRELGWKPL(320)
OxiI    (56)...FVRGDICDRE LLDRILPGH. .DAVVHFAAE SHVDRSLRSA SEFVRTNVAG(103)  (261).......WSR VRHVPDRKGH DLPYALDDSK IREEL.LRPA(282)
Orf7    (63)...LVRGDIAD.D TGDSLMAEA. .DQVVHFAAE THVDRSITAP GTFVRTNVLG(109)  (268).......WDV VDPVADRKGH DAGYALDCAK AADELGYRPP(300)
GraE    (57)...FVHGDICDRD LLDRVLPGH. .DAVVHFAAE SHVDRSLTGP GEFVRTNVMG(104)  (263).......WDM VRHVEDRLGH DFRYAIDDSK IREELGYAPR(295)
TylA2   (57)...LEFVRGDIAD HGWWRRLMEG VGLVVHFAAE SHVDRSTESS EAFVRTNVEG(106)  (264)......DRSA LRRVADRPGH DRRYSVDTTK IREELGYAPR(297)
Gdh     (59)...FVRGDICEWD VVSEVMREV. .DVVVHFAAE THVDRSILGA SDFVVTNVVG(106)  (265).......WSM VQPVTDRKGH DRRYSVDHTK ISEELGYEPV(297)
Stre    (56)...RYRFERGDIC DAPGRRVMAG QDQVVHLAAE SHVDRSLLDA SVFVRTNVHG(105)  (264).......WGS VEYVEDRKGH DRRYAVDSTR IQRELGFAPA(296)
RfbB1   (54)...FEHADICDAV AMSRIFAQHQ PDAVMHLAAE SHVDRSITGP AAFIETNIVG(103)  (287)......YREQ ITYVADRPGH DRRYAIDADK ISRELGWKPQ(320)
Gale1   (54)...FVEGDIRNEA LITEILHDHA IDTVIHFAGL KAVGNSVARP LEYYDNNVNG(103)  (287)......HFAP RDGDLPAYWA DASKADRELN WRVTRTLDEM(320)
Gale2   (54)...FVEGDIRNEA LMTEILHDHA IDTVIHFAGL KAVGESVQKP LEYYDNNVNG(103)  (288)......FAPR REGDLPAYWA DASKADRELN WRVTRTLDEM(321)
Gale3   (54)...FYQGDIRDCQ ILRQIFSEHE IESVIHFAGL KAVGESVAEP TKYYGNNVYG(103)  (288)......IQPR RAGDLACSYA DPSHTKQQTG WETKRGLQQM(321)
RfbG    (61)...SHIGDIRDFE KLRNSIAEFK PEIVFHMAAQ PLVRLSYEQP IETYSTNVMG(110)  (288)........SW LLDGENHPHE AHYLKLDCSK ANMQLGWHPR(319)
AscB    (61)...SEIGDIRDQN KLLEAIREFQ PEIVFHMAAQ PLVRLSYSEP VETYSTNVMG(110)  (288)........SW QLDGNAHPHE AHYLKLDCSK AKMQLGWHPR(319)
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Fig. 2. The base-meditated proton transfers in the active site domain were proposed from the alignment of the peptide sequences of the dehydratases and the epimerases. RfbB: dTDPDH from *Salmonella serovar* typhimurium LT2 (Jiang *et al.*, 1991); OxiI: dTDPDH from *S. antibioticus* Tü99 (Sohng and Yoo, 1996); Orf7: dTDPDH from *S. antibioticus* Tü99; GraE: dTDPDH from *S. violaceoruber* Tü22 (Bechthold *et al.*, 1995); Tyla2: dTDPDH from *Streptomyces fradiae* (Merson-Davies and Cundliffe, 1994); Gdh: dTDPDH from *Saccharopolyspora erythraea* (Linton *et al.*, 1995); StrE: dTDPDH from *S. griseus* (Pissowotzki *et al.*, 1991); RfbB1: dTDPDH from *Shigella flexneri* (Macpherson *et al.*, 1994); GalE1: UDPE from *Salmonella typhimurium* (Haung *et al.*, 1990); GalE2: UDPE from *E. coli* (Lemaire and Muller-Hill, 1986); GalE3: UDPE from *Neisseria gonorrhoeae* (Robertson *et al.*, 1993); RfbG: CDPDH from *Salmonella serovar* typhimurium LT2 (Jiang *et al.*, 1991); and AscB: CDPDH from *Y. pseudotuberculosis* (Kessler *et al.*, 1993).

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                V   M   H   L   A   A
rfbB        5'-CGGTGATGCATTTGGCTGC-3'
JKS-11:     5'-CGGTGATGCTTTTGGCTGC-3'

                D   R   P   G   H   D   R   R
rfbB        5'-GGATCGTCCGGGCCATGATCGTCGT-3'
JKS-14:     5'-GGATCGTCCAGGCGCTGATCGTCGT-3'
                        894th
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Fig. 3. Designed primers: JKS-11 [CAT (His-79) to CTT (Leu-79)], JKS14 [CAT (His-300) to GCT (Ala-300)]. G-894 was additionally replaced by A-894 to remove the *Nci*I site (CCGGG) for site-directed mutagenesis.

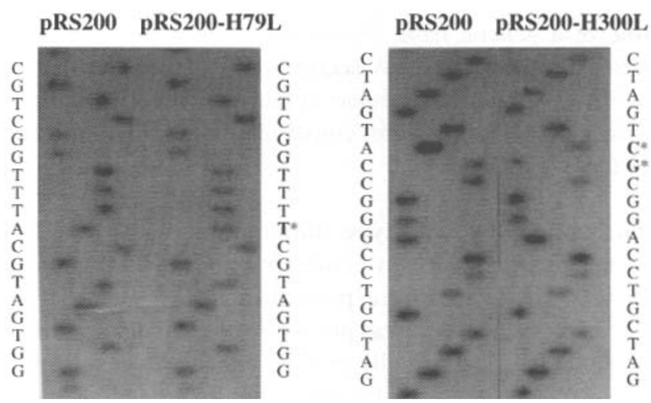

Fig. 4. DNA sequences of pRS200 (wide-type), pRS200-H79L, and pRS200-H300A.

Table 1. Enzyme activity of the wide-type and mutants.

	Total activity* (unit)	protein (mg)	Specific activity (unit/mg)
pRSETB (only vector)	5.43	48.5	0.1
pRS (wild-type)	82.9	40.4	2.1
pRS200-H79I	5.24	57.8	0.09
pRS200-H300A	85.3	45.5	1.9

*(1 unit = 1 *m*mol/h)

Glaser and Floss have proposed the reaction mechanism (Floss and Beale, 1989) and the model in the active site domain of the dehydratase from the stereochemical results by the feeding experiment on this enzyme (Melo *et al.*, 1968; Snipes *et al.*, 1977). In this model, the carbohydrate ring and NAD^+, and two bases aided to mediate proton transfer are organized as a sandwich-like arrangement. One of two bases is near the 4-hydroxyl groups and the other is the H-5 and 6-hydroxyl group of a glucose (Fig. 5A). In our study, the His-79 is regarded as an active-site base from the mutagenesis results. His-79 is proposed as the base near the 4-hydroxyl group, and is conserved in the epimerases and dehydratases. The reaction mechanism of the dTDP-D-glucose 4,6-dehydratase agree with the results of the mutagenesis and the mechanism proposed by Floss *et al.* (1989). Initial oxidation at C-4 with proton transfer to His-79 and hydride transfer to bound NAD^+ generates NADH and the 4-ketoglucose derivative. The adjacent C-5 acidic hydrogen is easily abstracted by another base, and

the proton of His-79 is returned to O-4 and forms the enol sugar intermediate. Subsequent loss of OH-6 completes the elimination of H_2O with His-85 and another base and then generates the 4-keto-5,6-glucoseen intermediate. The 5,6 double bond of this intermediate is reduced by delivery of hydride ion from NADH to C-6 of the glucoseen with the regeneration of NAD^+, and 4-keto-6-deoxy glucose is finally produced. UDP-D-glucose epimerase is also a NAD^+-dependent enzyme like the dehydratases and shows a high similarity of its peptide sequence. The mechanism of UDP-D-glucose epimerase is similar to that of dehydratase except for dehydration. The site-directed mutagenesis result of the dehydratase suggests that the active site base of the epimerase is His-79 because the His-79 is conserved in the epimerases and dehydratases (Fig. 5B).

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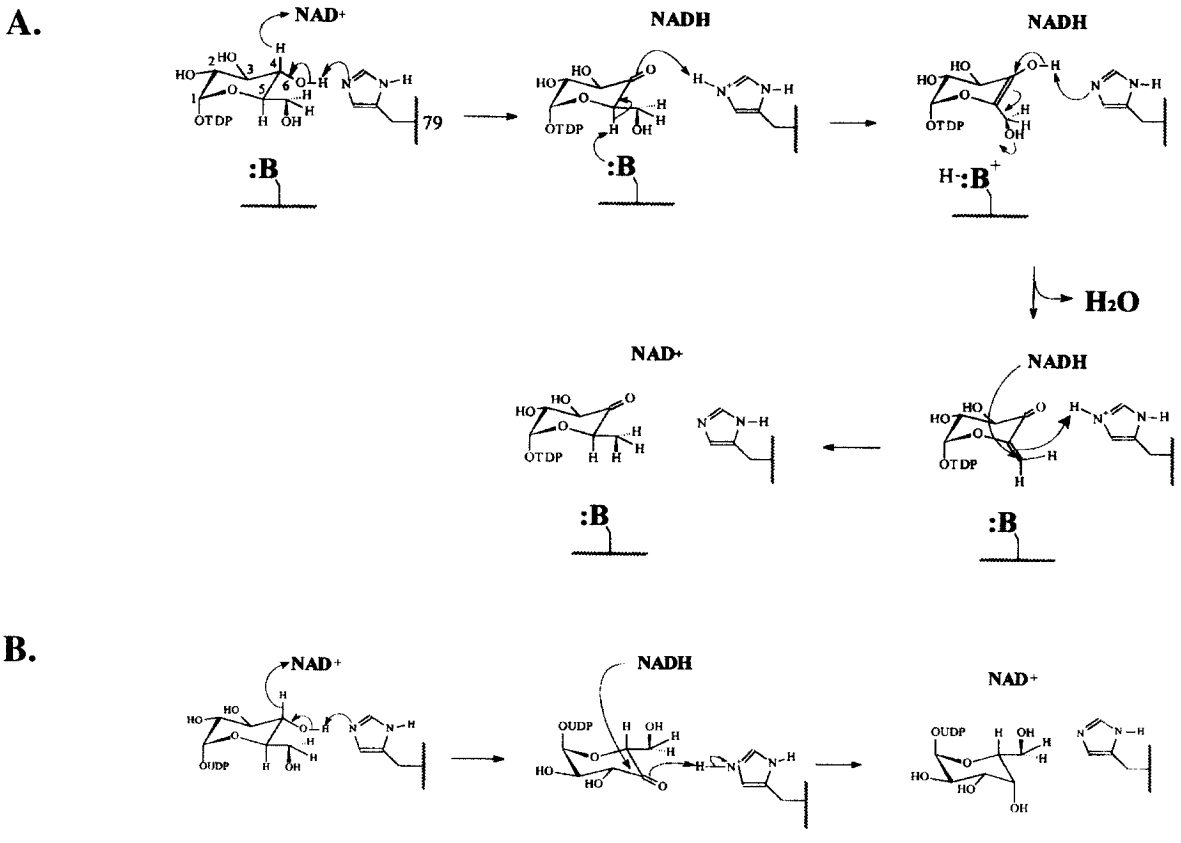

Fig. 5. A. The reaction mechanism of the dehydratase with NAD^+ cofactor and two bases (His-79 and another base). **B.** The reaction mechanism of the epimerase with NAD^+ cofactor and the proposed active-site base.

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