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Note

Probing of the reaction pathway of human UDP-xylose synthase with site-directed mutagenesis

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ABSTRACT

Uridine 5′-diphosphate (UDP)-xylose (UDP-Xyl) synthase (UXS) catalyzes the oxidative decarboxylation of UDP-glucuronic acid (UDP-GlcUA) to UDP-Xyl. The closely related UDP-glucuronic acid 4-epimerase (UGAE) interconverts UDP-GlcUA and UDP-galacturonic acid (UDP-GalUA) in a highly similar manner via the intermediate UDP-*xylo*-hexopyranos-4-uluronic acid (UDP-4-keto-GlcUA). Unlike UXS, however, UGAE prevents the decarboxylation. Human UXS (hUXS) and UGAE from *Arabidopsis thaliana* exhibit high structural similarity in the active site, but two catalytically important residues in hUXS (Glu^{120} and Arg^{277}) are replaced by Ser and Thr in the UGAE group. Additionally, Asn^{176}, which participates in substrate binding, is changed to Thr. We therefore analyzed single, double and triple mutants of hUXS carrying these substitutions to evaluate their significance for product specificity. All mutants showed considerably lower activities than wild-type hUXS (>1000-fold reduction). NMR spectroscopic analysis of the reaction products showed that UDP-β-L-*threo*-pentopyranos-4-ulose (UDP-4-keto-Xyl), UDP-Xyl or both, but no UDP-GalUA or UDP-4-keto-GlcUA were formed. Correlation of product characteristics, such as deuterium incorporation, with the amino acid replacements gave insights into structure–function relationships in UXS, suggesting that interaction between active site and overall enzyme structure rather than distinct conserved residues are decisive for product formation.

Uridine 5′-diphosphate (UDP)-xylose (UDP-Xyl) synthase (UXS) is responsible for the production of UDP-Xyl from UDP-glucuronic acid (UDP-GlcUA) in numerous organisms, from bacteria to mammals.[1] The UXS reaction is a complex oxidative decarboxylation in three distinct steps that proceeds via UDP-*xylo*-hexopyranos-4-uluronic acid (UDP-4-keto-GlcUA) and UDP-β-L-*threo*-pentopyranos-4-ulose (UDP-4-keto-Xyl) intermediates (Scheme 1).[2,3] Its product, UDP-Xyl, plays a central role in cell physiology.[4,5] In mammals, it is especially important in synthesis of extracellular matrix proteoglycans, as the glycosaminoglycan side chains of these macromolecules are attached to a core protein via a xylosyl residue in a common tetrasaccharide linker.[6,7] Loss of UXS activity leads to a defective extracellular matrix and malformation of various tissues, e.g. the bone.[8] The sequence of UXS is highly conserved and bacterial forms share 57% sequence identity with human UXS (hUXS).[8] The enzyme belongs to the diverse group of "extended short-chain dehydrogenases/reductases (SDR)" that have nucleotide sugar epimerization and dehydration as their main activities.[9] One remarkable feature of UXS is that the pyranoside ring of UDP-GlcUA is heavily distorted upon being bound by the enzyme ($^4C_1 \rightarrow B_{0,3}$). During the catalytic cycle, different conformations (^2S_O, $^2H_1$, $^4C_1$) are adopted by the sugar ring, which is strongly conducive to catalysis (Scheme 1).[3] This is achieved through six residues in the active site that are almost invariant in the UXS group of enzymes. Three of these residues (Thr^{118}, Tyr^{147} and Lys^{151} in hUXS) represent a typical SDR catalytic triad, while Ser^{119}, Glu^{120} and Arg^{277} are distinct features of UXS. Replacement of Tyr^{147} by Phe or Arg^{277} by Gln resulted in disruption of the catalytic cycle and release of UDP-4-keto-Xyl and NADH.[3]

Within the group of extended SDRs, UDP-glucuronic acid 4-epimerases (UGAEs) are closely related to UXS and catalyze the interconversion of UDP-GlcUA and UDP-galacturonic acid (UDP-GalUA) in a highly similar transformation via oxidation and reduction at *C*-4 (Scheme 1).[10–12] However, UGAE is able to prevent decarboxylation of UDP-4-keto-GlcUA, in contrast to UXS. Different accommodation of the glucuronic acid moiety in the active site was suggested as possible explanation, as the pattern of residue conservation in the active site of UGAE differs from that of UXS.[3] While the catalytic triad is essentially unchanged, Glu^{120} is replaced by Ser and Arg^{277} by Thr in the UGAE group. Additionally, Asn^{176}, which is not directly involved in catalysis in UXS, is altered to Thr. These changes are highly conserved among UGAE (Fig. 1A); however, the

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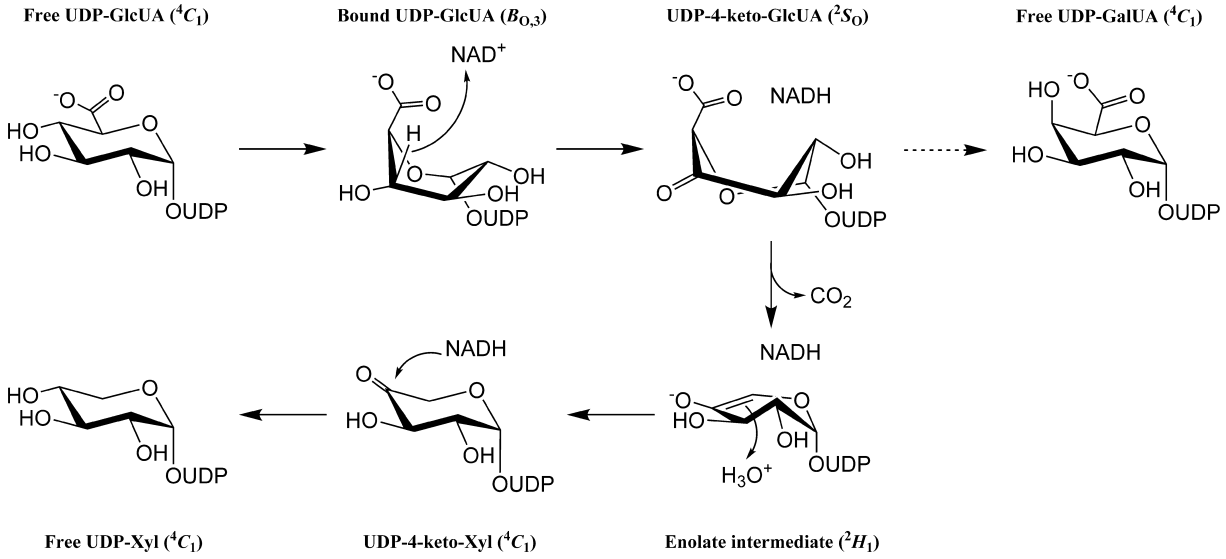

Scheme 1. Catalytic mechanism of hUXS. Distortion of the pyranoside ring during transformation strongly promotes catalysis by optimally aligning the reactive side groups of substrate/intermediate with the active site residues. In wild-type hUXS, deuterium incorporation at *C*-5 of UDP-Xyl originates from protonation of the enolate intermediate by a water molecule present in the active site. The reaction done by *At*UGAE is shown with a dashed arrow (note: the ^2S_O conformation of UDP-4-keto-GlcUA is only true for hUXS, it is unknown for *At*UGAE).

impact of these differences on the product pattern has not yet been investigated. As a crystal structure of UGAE was not available, we used the Phyre2 suite to create a homology model of the enzyme from *Arabidopsis thaliana* (*At*UGAE).[14] Comparison with the crystal structure of hUXS revealed very high structural similarity in the active site (Fig. 1B), although sequence identity between *At*UGAE and hUXS was only 22%. Therefore, it was suspected that replacement of Glu^{120}, Asn^{176} and/or Arg^{277} in the active site of hUXS by the respective amino acids found in *At*UGAE could result in the mutant enzymes producing UDP-GalUA or another product with altered stereochemistry at *C*-4. Evidence gained from analysis of these mutants could thus be helpful in identifying the origin of the unique features of UXS and getting a better understanding of structure–function relationships in the enzyme. Additionally, knowledge about the interesting residue conservation pattern within different SDR subgroups could be enhanced.[15]

Starting from wild-type hUXS1, we created Glu^{120}→Ser (E120S) and Arg^{277}→Thr (R277T) single mutants, a Glu^{120}→Ser/Arg^{277}→Thr (E120S/R277T) double mutant and a Glu^{120}→Ser/Asn^{176}→Thr/Arg^{277}→Thr (E120S/N176T/R277T) triple mutant using established protocols.[16] Wild-type and mutant enzymes were expressed in *Escherichia coli* BL21 Gold (DE3), and after His_6-tag affinity chromatography purification, SDS-PAGE showed one band at 39 kDa, in accordance with the expected molecular weight of 38.6 kDa.[3] Expression yields of the mutants were comparable to wild-type hUXS1; however, all mutants showed considerably lower initial activities (at least 1000-fold reduction). R277T, the double and the triple mutant showed formation of NADH upon incubation with UDP-GlcUA (Fig. 2), in contrast to E120S and wild-type enzyme (data not shown). Measurement of absorption spectra at the end of the reaction confirmed that indeed NADH was present and the increase in absorption did not only represent unspecific scattering, e.g. due to enzyme denaturation. As this result was indicative of a catalytic cycle that does not (or only partly) involve reduction of an intermediate, which is a prerequisite for formation of UDP-GalUA, a complete switch from oxidative decarboxylation to *C*-4 epimerization by exchange of the selected active site residues seemed unlikely. Analysis of the reactions by HPLC and capillary zone electrophoresis showed appearance of unknown peaks, but identification of the formed compounds was unsuccessful, as authentic standards for reaction intermediates and products were unavailable or could not be separated from the substrate, respectively.

^{1}H NMR analysis was therefore chosen to elucidate the product pattern of the mutant enzymes. Suspected compounds included UDP-GalUA, UDP-Xyl, UDP-4-keto-GlcUA and UDP-4-keto-Xyl. The results are summarized in Table 1. ^{1}H NMR signals of UDP-GlcUA, UDP-4-keto-Xyl and UXP-Xyl were assigned according to literature or by two-dimensional NMR spectroscopy (COSY, HSQC and HMBC).[17] As the signal of the anomeric proton (*H*-1) is very sensitive to changes within the sugar ring, it allowed to distinguish between the different products. E120S exclusively produced UDP-Xyl, not even traces of UDP-4-keto-Xyl could be detected, in contrast to a Glu^{120}→Ala mutant investigated in previous studies (Fig. 3).[3] Glu^{120}, although highly conserved in UXS, therefore does not seem to be an essential residue determining product specificity and is not necessary for formation of UDP-Xyl. Investigation of the R277T mutant showed that mainly UDP-4-keto-Xyl, but also small amounts of UDP-Xyl were formed, similar to an Arg^{277}→Gln (R277Q) mutant studied previously.[3] The products formed by R277T contained two deuterium atoms at *C*-5 when the reaction was done in D_2O (Fig. 3), in contrast to the wild-type enzyme, which showed single deuteration (from protonation of the enolate intermediate; see Scheme 1). In the R277Q mutant, this effect was ascribed to a water molecule in the active site, allowing for D/H exchange, which was excluded in the wild-type through a different hydrogen bonding network and space restrictions due to the longer Arg^{277} side group.[3] Therefore, a similar

Table 1
Comparison of product formation by hUXS1 wild-type and mutants (●, product detected; –, product not detectable; n.d., not determined)

UXS	UDP-Xyl	UDP-4-keto-Xyl	Degradation	*C*-5 deuteration
Wild-type	●	–	n.d.	Single
E120S	●	–	n.d.	Single
R277T	●	●	●	Double
E120S/R277T	–	●	●	Single
E120S/N176T/R277T	–	●	n.d.	Single