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Real-time NMR monitoring of intermediates and labile products of the bifunctional enzyme UDP-apiose/UDP-xylose synthase

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ABSTRACT

The conversion of UDP-α-D-glucuronic acid to UDP-α-D-xylose and UDP-α-D-apiose by a bifunctional potato enzyme UDP-apiose/UDP-xylose synthase was studied using real-time nuclear magnetic resonance (NMR) spectroscopy. UDP-α-D-glucuronic acid is converted via the intermediate uridine 5′-β-L-threo-pentapyranosyl-4″-ulose diphosphate to UDP-α-D-apiose and simultaneously to UDP-α-D-xylose. The UDP-α-D-apiose that is formed is unstable and is converted to α-D-apio-furanosyl-1,2-cyclic phosphate and UMP. High-resolution real-time NMR spectroscopy is a powerful tool for the direct and quantitative characterization of previously undetected transient and labile components formed during a complex enzyme-catalyzed reaction.

1. Introduction

The branched sugar 3-*C*-(hydroxymethyl)-D-*glycero*-tetrose or D-apiose (see Fig. 1) is a component of several plant flavonoids[1–4] and is present in the plant cell-wall pectic polysaccharides[5], apiogalacturonan[6,7], and rhamnogalacturonan II (RG-II)[8,9] Two of the apiosyl residues present in separate RG-II molecules are crosslinked together by a borate diester that results in the formation of a dimer of RG-II. The formation of this dimer is believed to have an important role in regulating the chemical and physical properties of the primary cell wall[8] and in normal plant growth and development.[10]

Plants are believed to use uridine 5′-diphosphate- α-D-apiofuranose (UDP-apiose, see Fig. 1) to donate apiosyl residues in the biosynthesis of polysaccharides and flavonoids. Previous work has proposed that UDP-apiose is formed from UDP-α-D-glucuronic acid (UDP-GlcA) by the β-NAD$^+$-dependent, bifunctional enzyme UDP-D-apiose/UDP-D-xylose synthase (abbreviated herein as UAXS).[11–15] Despite clear evidence of a biosynthetic route from UDP-GlcA to apiose, it has only been inferred that UDP-apiose is the direct product of UAXS, and by extension, the activated sugar donor for subsequent pathways.[16] In fact it was never observed or isolated, but rather detected as α-D-apio-furanosyl-1,2-cyclic phosphate (apiofuranosyl-1,2-cyclic phosphate). Biochemically synthesized UDP-apiose was shown to be unstable, and it spontaneously converts to apiofuranosyl-1,2-cyclic phosphate,[17] which can also be made chemically.[18]

Characterization of some of the proposed products formed by UAXS has led to the suggestion that uridine 5′-β-L-*threo*-pentopyranosyl-4″-ulose diphosphate (UDP-4-keto-xylose) is an intermediate in the reaction, and to a proposal of a mechanism for the rearrangement of the ring carbons[19,20] as outlined in Fig. 1. However, unambiguous structural evidence supporting the formation of the intermediate is lacking.[20]

NMR spectroscopy is a powerful technique for monitoring chemical[21] and enzymatic reactions.[22–26] At high magnetic fields there is usually sufficient frequency resolution to follow the fate of each molecular species, even if they have closely related or isomeric structures. Moreover, the linear relationship between resonance intensity and molar quantities of each component of the reaction makes measuring the time course of enzyme-catalyzed conversions relatively straightforward.

Here, we describe the use of real-time ^{1}H NMR spectroscopy to examine the formation of products and intermediate when a cloned recombinant potato UAXS is reacted with UDP-GlcA. We show that enzyme kinetics and the conversion of substrate into products, including those that are labile and thus difficult to isolate and characterize, can be continuously monitored during the course of the enzyme-catalyzed reaction.

2. Results and discussion

2.1. Functional identification of UAXS and initial enzymatic characterization

Enzymes from diverse organisms are known to convert UDP-glucuronic acid (UDP-GlcA) into different products.[27–29] We

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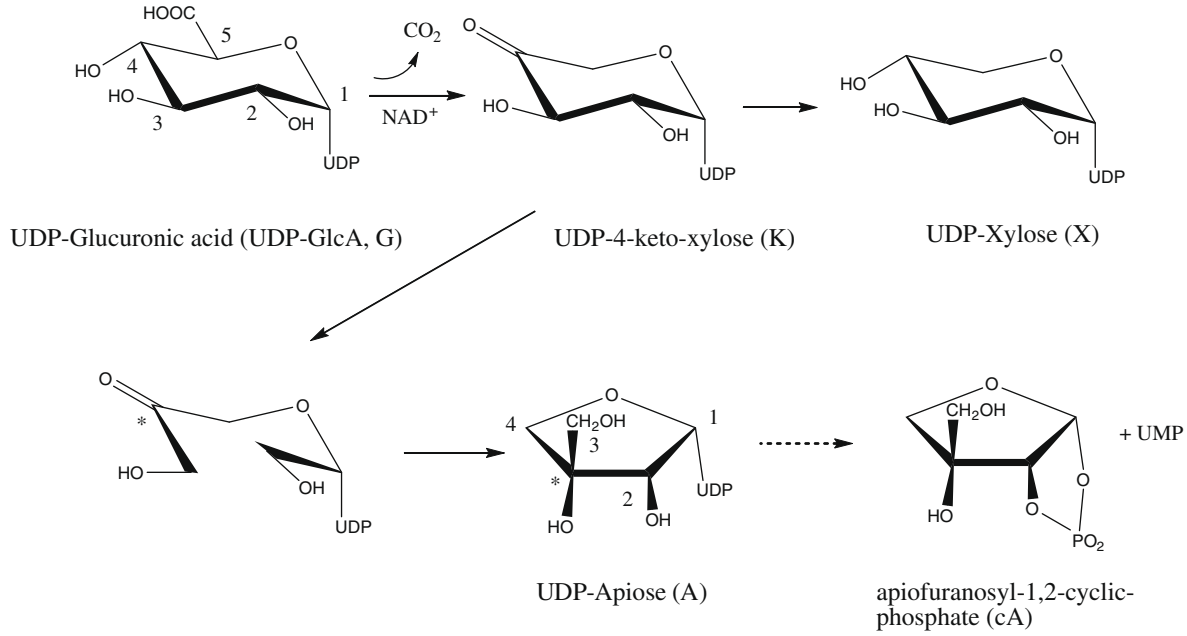

Figure 1. Proposed mechanism for the conversion of UDP-GlcA to UDP-xylose and UDP-apiose catalyzed by UAXS. The UDP-4-keto-xylose structure is depicted in the keto form, but it is most likely in equilibrium with the hydrated *gem*-diol form. The numbering schemes of the UDP-GlcA and UDP-apiose are independent. Carbon 4 of UDP-GlcA (marked with an asterisk) becomes carbon 3 of UDP-apiose. Note that the ring protons have been left off for clarity.

isolated a potato (*Solanum tuberosum*) cDNA that encodes a protein with 28% amino acid identity (see Supplemental Fig. 2) to a fungal UDP-GlcA decarboxylase (UXs) that converts UDP-GlcA to UDP-xylose[28], and with 34% amino acid identity to a bacterial NAD^+-dependent decarboxylase, ArnA[29], that converts UDP-GlcA to UDP-4-keto-xylose. Motif analysis of the putative potato protein (GenBank ABC75032) revealed a conserved NAD^+-binding motif, GxxGxxG (Gly^{21} to Gly^{27}), and a conserved catalytic motif, YxxxK (Tyr^{182} to Lys^{186}), associated with interconversion of nucleotide sugars. Thus, we reasoned that the potato protein (referred to as UAXS) may have UDP-xylose and/or UDP-apiose synthase activity.

To obtain potato UAXS in amounts sufficient for kinetic and product formation studies the protein was expressed in *E. coli*. A unique protein band was detected in the crude protein extract of the UAXS-expressing cells (Fig. 2A, lane A, marked by arrow), but it was absent in cells expressing an empty vector (lane B). UAXS was column purified yielding a distinct 43 kDa protein (Fig 2A lane C) that is not produced by *E. coli* cells expressing an empty vector as control (lane D). The amino acid sequence of the purified recombinant protein was confirmed by MS/MS analyses of its trypsin digestion products (data not shown).

Recombinant potato UAXS is active only in the presence of NAD^+ (data not shown); therefore, all activity assays contained UDP-GlcA and NAD^+ as substrates. The enzymatic products of UAXS were analyzed by an anion-exchange column with UV detection (Fig. 2B panel 3) that showed the presence of two new products with retention times characteristic of UMP and UDP-xylose. These components were isolated, and their structures were confirmed by ^1H NMR spectroscopy and by analyses of authentic standards (data not shown). No UDP-apiose was detected, which is consistent with previous work.[14]

To determine whether UMP was a specific product of UAXS or formed by degradation of UDP-apiose, anion-exchange chromatography was performed with a CAD-Corona detector (a charged aerosol HPLC detector). A new peak with elution time of 12.9 min (Fig. 3A, arrow) was detected, collected, and shown to be apiofuranosyl-1,2-cyclic phosphate (Fig. 3B) by 1D- and 2D-^1H NMR spectroscopy (Fig. 3C). The chemical shift data (Supplemental Table 1) are comparable to those of a previous report of the NMR spectra of apiofuranosyl phosphates.[18] The signals for H1 and H2 are shifted downfield relative to apiose[30] or its methyl glucoside[31], and show scalar couplings to ^{31}P as expected for phosphate substitution at those positions.[18] The COSY spectrum (Fig. 3C) shows four-bond scalar couplings between H2, H3, and H4. Small molecules with appropriate geometry will have measurable couplings over multiple bonds.[32] In this case there is a relatively strong cross-peak between H2 (cA2) and one of the H4 protons (cA4), most likely due to a favorable 'W' orientation.[33] A similar argument can be made for the cross-peak between one of the H3s (cA3) and the other H4.

2.2. Real-time ^1H NMR spectroscopy establishes that UAXS converts UDP-GlcA to UDP-apiose

No evidence for the formation of UDP-apiose was obtained by HPLC analyses of the products formed by UAXS. Thus, we sought to further investigate the nature of these products in real time using ^1H NMR spectroscopy. Preliminary studies indicated that enzymatic reactions preformed in 80% D_2O gave an acceptable NMR signal-to-noise ratio and that D_2O buffer exchange was not required after recombinant protein was column purified.

The spectra shown in Figure 4 cover the full time course for the reaction at pH 7.8 (upper set 4A) and at pH 6.5 (lower set 4B). The data for the reaction at pH 7.8 show the disappearance of UDP-GlcA, and the formation of products, some of which are transient. A comparison of spectra collected at the beginning and the end of the time course confirms that UDP-GlcA (peaks labeled G1–G5) has been consumed, and that at least two products, UDP-xylose (peaks X1–X5) and apiofuranosyl-1,2-cyclic phosphate (peaks cA1, cA3, and cA4) have been formed. The signals were assigned based on comparison to authentic samples of apiofuranosyl-1,2-cyclic phosphate and UDP-xylose.[34] Signals labeled 'U' belong to the two H5 protons of ribose in UMP, which is generated when UDP-apiose is converted to the cyclic phosphate. Signals labeled A1–A4 that increase in amplitude from the earliest time point up to ~60 min and then decay, were assigned to UDP-apiose. The trip-