Accelerated Publications

Characterization of Enzymatic Processes by Rapid Mix−Quench Mass Spectrometry: The Case of dTDP-glucose 4,6-Dehydratase†

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Received August 17, 2000; Revised Manuscript Received September 26, 2000

ABSTRACT: The single-turnover kinetic mechanism for the reaction catalyzed by dTDP-glucose 4,6-dehydratase (4,6-dehydratase) has been determined by rapid mix−chemical quench mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was employed to analyze quenched samples. The results were compatible with the postulated reaction mechanism, in which NAD^+ initially oxidizes glucosyl C4 of dTDP-glucose to NADH and dTDP-4-ketoglucose. Next, water is eliminated between C5 and C6 of dTDP-4-ketoglucose to form dTDP-4-ketoglucose-5,6-ene. Hydride transfer from NADH to C6 of dTDP-4-ketoglucose-5,6-ene regenerates NAD^+ and produces the product dTDP-4-keto-6-deoxyglucose. The single-turnover reaction was quenched at various times on the millisecond scale with a mixture of 6 M guanidine hydrochloride and sodium borohydride, which stopped the reaction and reductively stabilized the intermediates and product. Quantitative MALDI-TOF MS analysis of the quenched samples allowed the simultaneous observation of the disappearance of substrate, transient appearance and disappearance of dTDP-hexopyranose-5,6-ene (the reductively stabilized dTDP-4-ketoglucose-5,6-ene), and the appearance of product. Kinetic modeling of the process allowed rate constants for most of the steps of the reaction of dTDP-glucose-d_7 to be evaluated. The transient formation and reaction of dTDP-4-ketoglucose could not be observed, because this intermediate did not accumulate to detectable concentrations.

dTDP-glucose-4,6-dehydratase[1,2] catalyzes the transformation of dTDP-glucose into dTDP-4-keto-6-deoxyglucose (*1*). This and other 4,6-dehydratases catalyze the first committed step in 6-deoxysugar biosynthesis, and they are frequently employed in the production of components used in bacterial secondary metabolism and in bacterial lipopolysaccharide production (*2*). We have begun an investigation of the mechanism of catalysis by the 4,6-dehydratase encoded by the *rffG* gene from the *Escherichia coli* enterobacterial common antigen operon (*3*).

The 4,6-dehydratase is structurally related to but mechanistically distinct from UDP-galactose 4-epimerase[3] (*4*, *5*). The *E. coli* 4-epimerase has been studied in great detail, but little has been reported on the 4,6-dehydratase. Both enzymes have been identified as members of the extended short-chain dehydrogenase/reductase enzyme superfamily (*6*) that use nicotinamide dinucleotides in the catalysis of oxidoreductase chemistry. Primary amino acid sequence alignments show 25% identity between 4-epimerase and 4,6-dehydratase, and their three-dimensional structures exhibit similar folds (*7*; PDB 1BXK). The 4,6-dehydratase and 4-epimerase are homodimers, and 4-epimerase contains one irreversibly

† This work was supported by Grants GM30480 (P.A.F.) and GM20552 (J.W.G.) from the National Institute of General Medical Sciences. National Science Foundation award 9520868 to the Department of Chemistry was used to purchase the Bruker REFLEX II mass spectrometer.

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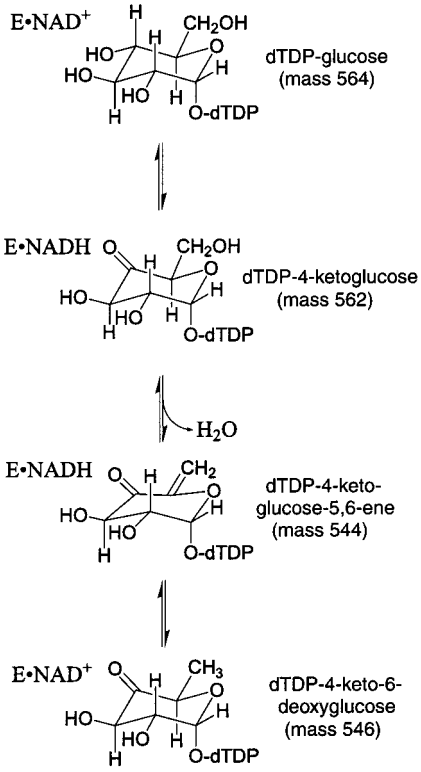
[1] Abbreviations: 4,6-dehydratase, dTDP-glucose 4,6-dehydratase; 4-epimerase, UDP-galactose 4-epimerase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; dTDP-glucose, thymidine 5′-diphospho-α-D-glucose; NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); NADD, [4-$^2H_1$]nicotinamide adenine dinucleotide (reduced); UDP, uridine 5′-diphosphate; PDB, Protein Data Bank; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; DTT, dithiothreitol; ORF, open reading frame; dTTP, thymidine 5′-triphosphate; ATP, adenosine 5′-triphosphate; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; ATCC, American Type Culture Collection; EDTA, ethylenediaminetetraacetic acid; OD_{600}, optical density at 600 nm; dTDP-glucose-d_7, dTDP-α-D-[1,2,3,4,5,6,6-$^2H_7$]glucose; dTDP-4-ketoglucose-d_6, dTDP-[1,2,3,5,6,6-$^2H_6$]-4-ketoglucose; dTDP-glucose-d_6, dTDP-[1,2,3,4,6,6-$^2H_6$]glucose; dTDP-4-ketoglucose-d_5, dTDP-[1,2,3,6,6-$^2H_5$]-4-ketoglucose; dTDP-4-ketoglucose-5,6-ene-d_5, dTDP-[1,2,3,6,6-$^2H_5$]-4-ketoglucose-5,6-ene; dTDP-4-keto-6-deoxyglucose-d_6, dTDP-[1,2,3,6,6,6-$^2H_6$]-6-deoxyglucose; dTDP-hexopyranose, mixture of hexopyranose C4 epimers resulting from sodium borohydride reduction of dTDP-4-ketoglucose; dTDP-6-deoxyhexopyranose, sodium borohydride reduced dTDP-4-keto-6-deoxyglucose; dTDP-hexopyranose-5,6-ene, sodium borohydride reduced dTDP-4-ketoglucose-5,6-ene.

[2] dTDP α-D-glucose 4,6-hydro-lyase, EC 4.2.1.46.

[3] UDP α-D-galactose 4-epimerase, EC 5.1.3.2.

10.1021/bi001963d CCC: $19.00 © 2000 American Chemical Society
Published on Web 10/21/2000

Scheme 1



bound NAD^+ cofactor in each subunit (*8*). Catalysis by both enzymes is initiated by the same mechanistic step, where tightly bound NAD^+ oxidizes carbon-4 in the hexopyranose moiety of the nucleotide−sugar substrate. The two mechanisms diverge following that step.

The mechanism postulated for the 4,6-dehydratase, as shown in Scheme 1, includes substrate, product, and two chemically unique intermediate species connected by three chemical steps (*9*). The dTDP-glucose is oxidized at glucosyl C4 by enzyme-bound NAD^+, forming dTDP-4-ketoglucose and NADH. Water is eliminated between glucosyl C5 and C6, forming dTDP-4-ketoglucose-5,6-ene. Finally, the second intermediate is reduced by the NADH at glucose C6, forming the product dTDP-4-keto-6-deoxyglucose and regenerating NAD^+. The hypothetical 4,6-dehydratase mechanism is consistent with available results; however, the reaction intermediates have heretofore not been directly observed.

A direct and quantitative analysis of reaction intermediates would test the chemical mechanism and allow the determination of the rate constants that describe the interconversion of intermediates. Rapid mix−chemical quench experiments can be used to terminate reactions at early time points for the analysis of reaction components. Such experiments require methods to isolate and quantify those components. Inasmuch as the chemical identities of the substrate, product, and two intermediates in the reaction of 4,6-dehydratase are distinguished by unique mass values (Scheme 1), a quantitative mass spectrometric analysis of chemically quenched 4,6-dehydratase reactions would provide a simultaneous measure of all reaction components. Mass spectrometric techniques have been used for the analysis of transiently formed intermediates in enzyme-catalyzed reactions (*10*, *11*; reviewed in ref *12)*. In the work presented here, rapid mix−quench MALDI-TOF mass spectrometry has been used to characterize the single-turnover conversion of dTDP-glucose to the dTDP-4-ketoglucose-5,6-ene intermediate and finally to dTDP-4-keto-6-deoxyglucose, as catalyzed by 4,6-dehydratase.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases and other DNA-modifying enzymes were purchased from Promega; pTZ18U plasmid DNA was purchased from Bio-Rad. All deoxyoligonucleotides were obtained commercially and further purified by urea denaturing acrylamide gel electrophoresis. DNA sequencing was performed by dideoxy chain termination using a kit (United States Biochemicals) and [^{35}S]ATPαS (600 Ci/mmol, Amersham). dTDP-α-D-glucose was synthesized from dTMP-morpholidate and α-D-glucose 1-phosphate as previously described (*13*). Phosphoglucomutase, hexokinase, and inorganic pyrophosphatase were purchased from Boehringer Mannheim; UDP-glucosepyrophosphorylase was purchased from Sigma. ATP, dTTP, dTMP-morpholidate, α-cyano-4-hydroxycinnamic acid, and α-D-glucose 1-phosphate were purchased from Aldrich. D-Glucose-d_7 (98%) was obtained from either Isotech or Cambridge Isotopes.

The pTZ18U vector was modified to include the translation initiation sequence of the *E. coli gal* operon (*14*). This was accomplished by cloning annealed 36-base deoxyribooligonucleotides (SD2sen, 5′-CATACCATAAGCCTAATGGAGGATATCTAGTGGTAC-3′, and SD2anti, 5′-CACTAGATATCCTCCATTAGGCTTATGGTATGAGCT-3′) into the *Kpn*I and *Sst*I sites of pTZ18U. The resulting expression vector was named pTZ18UgalSD. The *E. coli* K12 *rffG* ORF (ORF o355, Genbank accession number M87049) was amplified by PCR from the *E. coli* K12-derived strain W3110 (ATCC number 27325) using oligonucleotide primers: o355ECsen, 5′-GTCTGAGGCCTATGAGAAAAATTCTGATAACAGG-3′; and o355ECanti, 5′-TGTTTGGATCCGCTGAAAATTAGCCTTTCAGACC-3′. The PCR product was cleaved by *Bam*HI and *Stu*I endonucleases, purified by agarose gel electrophoresis, and ligated to the linearized, dephosphorylated (*Bam*HI and *Eco*RV, calf alkaline phosphatase) pTZ18UgalSD expression vector. The ORF and flanking sequences of the resultant vector, pTZ18UgalSDo355, were confirmed by DNA sequencing.

dTDP-glucose-d_7. dTDP-glucose-d_7 with deuterium at all of the nonexchangeable hydrogen positions of the glucose moiety was synthesized. The synthesis was accomplished enzymatically in a single reaction mixture. The 50 mL solution containing 4.7 mM D-glucose-d_7, 4 mM dTTP, 6 mM ATP, 37.5 units of hexokinase, 20 units of phosphoglucomutase, 50 units of UDP-glucose pyrophosphorylase, 4 units of inorganic pyrophosphatase, 50 mM potassium phosphate at pH 7.8, 5% glycerol, 13 mM $MgCl_2$, and 1 mM DTT was incubated at 4 °C for 4 days. Formation of dTDP-glucose-d_7 was assayed enzymatically using the spectrophotometric assay described below. Enzymes were removed by ultrafiltration, and the solution was diluted to 350 mL and divided into two fractions. Each fraction was loaded onto a Mono-Q anion-exchange column (HR 16/10, Pharmacia) and eluted with a 300 mL linear gradient of NH_4OAc increasing from 10 mM to 1 M. Fractions containing dTDP-glucose-d_7 were pooled and then concentrated by rotary evaporation to 2 mL. dTDP-glucose-d_7 was desalted on a

Toyopearl HW-40S gel filtration column (2.6 × 40 cm) equilibrated and eluted with water. The chemical identity of the lyophilized product was confirmed enzymatically; the isotopic distribution was determined by MALDI-TOF MS.

Methods. Standard UV/visible measurements were made using a Shimadzu UV-1601PC dual beam spectrophotometer. Pre-steady-state spectrophotometric traces were collected using an OLIS Inc. RSM-1000 stopped-flow spectrophotometer. All mass spectral data were collected using a Bruker REFLEX II MALDI-TOF mass spectrometer equipped with delayed extraction in the reflectron-negative ion mode. The 4,6-dehydratase extinction coefficient was calculated by performing quantative amino acid analysis (AAA Laboratory, Mercer Island, WA) on triplicate samples with measured A_{280} values.

Purification of 4,6-Dehydratase. BL21(DE3)pLysS cells (Novagen) were transformed with pTZ18UgalSDo355 and grown at 37 °C in 2× YT medium supplemented with 50 μg/mL ampicillin and 37 μg/mL chloramphenicol. Cells were induced (OD_{600} = 1.2) with 0.2% lactose, harvested by centrifugation 12 h postinduction, and frozen as pellets in liquid nitrogen. For purification, cells were thawed and resuspended to 0.33 g/mL in ice-cold lysis buffer (100 mM MOPS, pH 7.0, 5 mM EDTA, 100 mM NaCl, 1 mM DTT, 1 mM PMSF) and were broken by sonication. All subsequent steps were carried out on ice, or at 4 °C, and in buffers containing 1 mM DTT. Cell debris was removed by centrifugation, and the supernatant was brought to 3% streptomycin sulfate. The precipitate was removed by centrifugation, and the supernatant was subjected to ammonium sulfate fractionation at 38% and 52% saturation. The pellet from the 52% ammonium sulfate precipitation was resuspended in 200 mL of 20 mM potassium phosphate at pH 7.4 and was loaded directly onto a Bio-Gel HTP hydroxyapatite (Bio-Rad) column (675 mL, 5 cm diameter) equilibrated and eluted with the same buffer. The 4,6-dehydratase, which emerged in the void volume, was pooled and loaded directly onto a 300 mL, 2.5 cm diameter Q-Sepharose fast-flow (Pharmacia) column equilibrated with 20 mM MOPS, pH 7.0. The 4,6-dehydratase emerged at 250 mM sodium chloride in a 2 L, 10−300 mM NaCl gradient. Enzyme was exchanged into 20 mM MOPS, pH 7.0, and 1 mM DTT and concentrated to 20 mg/mL by ultrafiltration prior to freezing. The 4,6-dehydratase was delivered dropwise into liquid nitrogen and stored at −80 °C.

Dehydratase Assay. dTDP-4-keto-6-deoxyglucose production was monitored in a stopped-point assay by the formation of a 318 nm chromophore following incubation in 0.1 M sodium hydroxide at 37 °C (*1*). Because several different extinction values for the chromophore have been reported (*4*, *15*), the extinction coefficient was redetermined in this laboratory and found to be 6500 M^{-1} cm^{-1}. This assay allowed the accurate determination of k_{cat}, but the practical detection limit for product, 25 μM, was greater than K_M, and so accurate K_M values could not be determined.

Rapid Mixing. Enzyme and substrate solutions were combined and thoroughly mixed using an Update Instruments Inc. (Madison, WI) rapid mixer prototype interfaced with a model 745 syringe ram controller. Both reactant solutions and apparatus were equilibrated to 18 °C. The final mixture contained an excess of enzyme (300 μM) over dTDP-glucose-d_7 (200 μM), with both solutions in 100 mM Tris-HCl at pH 7.5 and 1 mM DTT. Duration of the reaction was controlled by varying the length of the common outlet tube (aging tube), or the flow rate, and quenching was accomplished by spraying 100 μL of the reaction mixture through a nozzle into the quench solution.

Quench. The 4,6-dehydratase catalyzed reactions were quenched in 6 M guanidine hydrochloride, buffered with 50 mM potassium bicinate at pH 8.0 and containing 20 mM sodium borohydride (or borodeuteride). The efficacy of the quench was confirmed by observing no change in the amount of NADH formed at 52 ms as a function of quench temperature. Reduction of dTDP-4-ketoglucose-5,6-ene occurred selectively at the keto functionality; mass peaks corresponding to the doubly reduced intermediate were not observed.

Reduction was critical for stabilizing dTDP-4-ketoglucose-5,6-ene. This intermediate was not observed in quenched samples that lack the reducing agent, the intermediate having degraded during the 4−6 h postquench purification (see below). A progressive (0−15 min) delay between the quench and the addition of reductant and a 1−100 mM variation in the reductant concentration were introduced into the quench procedure to test whether the reduction was fast enough to quantitatively stabilize the dTDP-4-ketoglucose-5,6-ene. In both experiments the levels of dTDP-hexopyranose-5,6-ene detected did not vary; this was accepted as prima facie evidence that the rate of reductive stabilization was significantly faster than that of degradation.

Mass Spectrometry Sample Preparation. Preparation of samples for MALDI-TOF mass spectrometric analysis was done to enrich the nucleotide portion of the reaction while removing contaminants that would interfere. Protein in the sample was removed by ultrafiltration in Centricon 30 filtration units (Amicon); then samples were subjected to gel filtration (TOSOHAAS, Toyopearl HW-40S; 2.6 × 40 cm) to separate reaction intermediates from the denaturant and buffer. All post gel filtration glassware was acid washed. Pooled fractions were lyophilized to dryness and then resuspended in a minimal volume of water (2−40 μL) prior to application to the MALDI target.

Matrix was prepared as a saturated solution of α-cyano-4-hydroxycinnamic acid in 70% MeOH and 30% 10 mM NH_4OAc, at pH 7.5, treated with Dowex 50WX8-400 resin in the NH_4^+ form. Aliquots (0.5 μL) of matrix and analyte solutions were mixed directly on the MALDI target, which, when triturated, rapidly formed a fine homogeneous precipitate. Minor variations in sample composition in the spot were minimized by summation of data collected by sampling randomly across the spot. Duplicate spectra confirmed the homogeneity.

Data Handling. Substrate, intermediate, and product peaks from MALDI-TOF MS spectra were integrated using the program Peak Fit (SPSS Science Inc.). Peak volumes at *m*/*z* 569 and 570 were corrected for overlapping primary and ±1 isotope peaks; the −1 isotope peak was due to incomplete deuterium enrichment in D-glucose-d_7. For the complete set of analyte peaks, concentrations were derived by assigning percent-of-total-integrated-area values to each peak and by multiplying the percentage value for each species by the initial substrate concentration (200 μM) to normalize time points. All data sets were subjected to the standard Q-test

with 90% confidence limits to look for significant deviation from the population to justify exclusion. If a point was excluded, the other three mass values calculated in conjunction with that point were also removed from the analysis because of the normalization technique. Single-turnover progress curve data were fitted to enzymatic mechanistic schemes using the program DYNAFIT by Petr Kuzmic (*16*).

RESULTS AND DISCUSSION

Stoichiometry of 4,6-Dehydratase NAD$^+$/NADH and Spectrophotometric Properties. Since the late 1960s, 4-epimerase and 4,6-dehydratase from *E. coli* were thought to contain 1 equivalent of tightly bound NAD^+ per homodimer (*5*, *17*). Recent assessment of this stoichiometry for 4-epimerase using an amino acid analysis derived protein extinction coefficient showed that one NAD^+ binds per subunit (*8*). Using a similarly derived extinction coefficient for 4,6-dehydratase [$\epsilon_{280} = 2.05$ ($\pm$0.12) mL mg^{-1} cm^{-1} (pH 7.0, 25 °C)] and spectrophotometric quantitation of NAD^+ released upon denaturation, the stoichiometry of the 4,6-dehydratase NAD^+ complex was found to be one NAD^+ molecule per 4,6-dehydratase subunit. Chemical reduction of tightly bound NAD^+ to NADH was attempted using $NaBH_4$, $NaCNBH_3$, and the dimethylamine−borane complex. Treatment of 4,6-dehydratase NAD^+ with the 200 mM aqueous dimethylamine−borane complex resulted in the formation of a broad spectral feature at 355 nm that did not increase with additional borane. The 355 nm band was assigned to NADH bound to 4,6-dehydratase red shifted 15 nm from the solution λ_{max} of 340 nm. A similar red shift was observed previously for 4,6-dehydratase (*4*) and 4-epimerase (*8*, *17*). Addition of dTDP-4-keto-6-deoxyglucose to the reduced 4,6-dehydratase resulted in a rapid bleaching of the 355 nm band and formation of dTDP-6-deoxyglucose (*4*). From the 355 nm absorbance the extinction coefficient for NADH bound to 4,6-dehydratase was calculated to be $\epsilon_{355} = 6$ mM^{-1} cm^{-1} at pH 7.0 and 25 °C. Interestingly, unlike the epimerase, both $NaBH_4$ and $NaCNBH_3$ treatment produced, in addition to the 355 nm band, a broad 425 nm band that did not diminish upon addition of dTDP-4-keto-6-deoxyglucose. Although this feature has not been characterized, it may indicate reduction of NAD^+ partly at the 2 or 6 position of the nicotinamide instead of the biologically relevant carbon-4 (*18*).

Rapid Mix−Quench MALDI-TOF MS. Rapid mix−quench MALDI-TOF mass spectrometry employs traditional techniques for preparing pre-steady-state reaction samples but replaces chromatographic, or other, analysis with MALDI-TOF MS. For small molecule analysis, high-resolution MALDI-TOF mass spectrometers attain baseline separation between isotopes of a single compound. Thus 4,6-dehydratase reaction intermediates, differing by 2 and 18 mass units between adjacent species, are easily resolved. The MALDI-TOF MS analysis has several advantages over chromatographic analysis. First, because detection is made by mass alone, simultaneous quantitation of chemically similar molecules of distinct masses is facile. Second, mass spectral quantification obviates the need to collect radiochemical or spectrophotometric data in order to monitor the reaction. Finally, the mass spectrometric analysis provides atomic detail about the chemical composition of the intermediates, which also allows additional information to be gathered through stable isotopic labeling. The drawbacks of rapid quench MALDI-TOF analysis include sensitivity to salts and a requirement for the ionizability of samples. Also, intermediates must be sufficiently stable to survive the quenching and purification procedures.

Table 1: Atomic Weights of Unreduced and Reduced Substrate, Intermediates, and Products[a]

	unreduced	reduced	
	g/mol	[H], g/mol	[D], g/mol
dTDP-glucose	564 (563)	564 (563)	564 (563)
dTDP-4-ketoglucose	562 (561)	564 (563)	565 (564)
dTDP-4-ketoglucose-5,6-ene	544 (543)	546 (545)	547 (546)
dTDP-4-keto-6-deoxyglucose	546 (545)	548 (547)	549 (548)
dTDP-glucose-d_7	571 (570)	571 (570)	571 (570)
dTDP-4-ketoglucose-d_6	568 (567)	570 (569)	571 (570)
dTDP-4-ketoglucose-5,6-ene-d_5	549 (548)	551 (550)	552 (551)
dTDP-4-keto-6-deoxy-glucose-d_6	552 (551)	554 (553)	555 (554)
dTDP-glucose-d_6	570 (569)	570 (569)	570 (569)
dTDP-4-ketoglucose-d_5	567 (566)	569 (568)	570 (569)

[a] Masses detected by MALDI-TOF MS in the negative ion mode are for the monoanionic species, which are the mass of the neutral species minus the mass of one proton; these values are given parenthetically next to the mass for the neutral species.

Reductive Stabilization and dTDP-glucose-d_7. The need for reductive stabilization of 4,6-dehydratase intermediates and the advantages of using dTDP-glucose-d_7 were revealed in preliminary experiments. Initial investigations of quenching conditions showed that extremes of acid, base, or temperature (boiling ethanol) stopped the catalysis but degraded the analytes. Other denaturing conditions such as 8 M urea and boiling acetonitrile, acetone, or methanol failed to stop the reaction at an adequate rate. Reduction by sodium borohydride, when combined with the 6 M guanidine hydrochloride quench, provided quenching conditions that reproducibly stabilized the dTDP-4-ketoglucose-5,6-ene, allowing its detection as a mixture of reductively trapped dTDP-hexopyranose-5,6-enes by MALDI-TOF MS (data not shown). The reductive stabilization prevents the unique detection of dTDP-4-ketoglucose, because its reduction generates a mixture of dTDP-hexopyranoses (C4 epimers), both having a mass identical to that of dTDP-glucose. Reduction with sodium borodeuteride shifts the mass of the dTDP-hexopyranoses one mass unit above that of dTDP-glucose (Table 1), so that the dTDP-hexopyranoses are detected coincident with the large +1 isotope peak of dTDP-glucose. By using dTDP-glucose-d_7 as the substrate and reducing with sodium borohydride, the mass of the reduced dTDP-4-ketoglucose-d_6 is shifted to a position one mass unit below that of the dTDP-glucose-d_7, eliminating interference from the dTDP-glucose-d_7 and its heavy isotopic species. Consequently, all steady- and pre-steady-state analyses were performed with dTDP-glucose-d_7. The masses of the sodium borohydride and borodeuteride reduced dTDP-glucose and dTDP-glucose-d_7-derived chemical species are outlined in Table 1.

A concern about using dTDP-glucose-d_7 in reactions run in H_2O is an enzyme-catalyzed deuterium to hydrogen exchange at the glucosyl C5 of the substrate. This exchange may result from the reversibility of the water elimination step (as depicted in Scheme 1), which is accompanied by

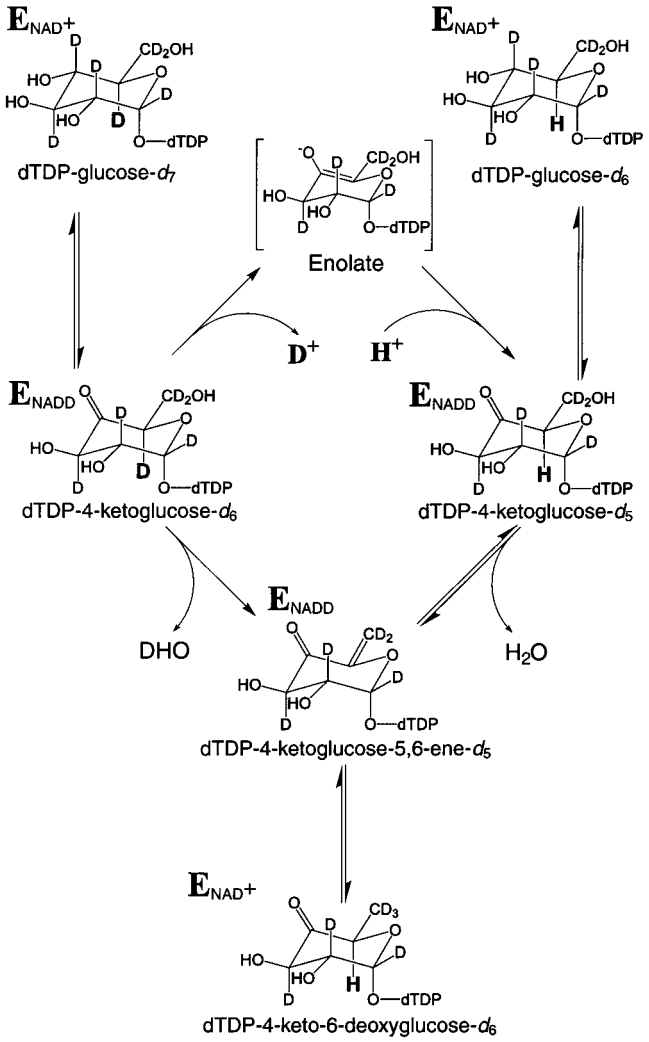


FIGURE 1: 4,6-Dehydratase branched mechanism. A branched mechanism describes glucosyl C5 hydrogen exchange into dTDP-glucose-d_7 and dTDP-4-ketoglucose-d_6, a consequence of using dTDP-glucose-d_7 as substrate for reaction in H_2O. dTDP-glucose-d_6 and dTDP-4-ketoglucose-d_5 species (right branch) have been added to the original mechanism. The enolate species has been added to posit an alternative exchange mechanism.

exchange of the deuteron abstracted in that step with solvent hydrogen. Exchange at the glucosyl C5 takes place in the overall reaction (*9*, *19*). The C5 deuterium to hydrogen exchange can then be propagated back through the reaction, forming dTDP-glucose-d_6. Alternatively, formation of a dTDP-4-ketoglucose enolate followed by solvent exchange and re-formation of the ketone would also allow glucosyl C5 solvent hydrogen exchange without preforming the dTDP-4-ketoglucose-5,6-ene (Figure 1). The glucosyl C5 exchange results in a branched mechanism, where in one branch dTDP-glucose-d_7 and dTDP-4-ketoglucose-d_6 have deuterium at C5 and in the other branch protium occupies this position (Figure 1). Now, the mass of the exchanged dTDP-glucose-d_6 is identical to that of the borohydride-reduced dTDP-4-ketoglucose-d_6 (Table 1). The masses of the borohydride- and borodeuteride-reduced dTDP-glucose-d_7-derived, glucosyl C5 exchanged species are outlined in Table 1.

Quantitative MALDI-TOF MS. Despite a series of reports detailing its use in quantitative applications (*20*, *21*), MALDI-TOF MS is frequently held to be a nonquantitative analytical

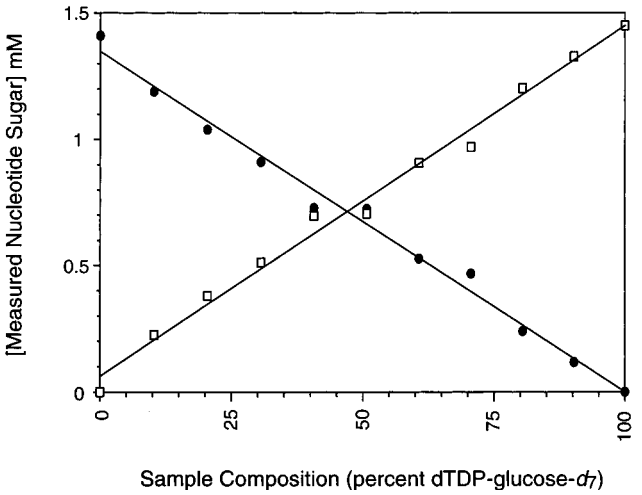


FIGURE 2: Linear detection of dTDP-glucose-d_7 and sodium borohydride treated dTDP-4-keto-6-deoxyglucose-d_6. MALDI-TOF MS quantitation of mixtures of dTDP-glucose-d_7 (□) and $NaBH_4$-reduced dTDP-4-keto-6-deoxyglucose-d_6 (●). Samples were prepared from stock solutions (1.45 and 1.41 mM, respectively) that were mixed at ratios from 0% to 100%, in 10% increments. Results are plotted as the percent of dTDP-glucose-d_7 in the sample vs the measured nucleotide concentration. Linear regressions gave slopes 0.014 and −0.013, *y* intercepts 0.062 and 1.348, and r^2 0.990 (both) for dTDP-glucose-d_7 and $NaBH_4$-reduced dTDP-4-keto-6-deoxyglucose-d_6, respectively.

technique. This belief appears to center on two topics. The first concern is that the chemically distinct analytes will likely be detected with different efficiency. This problem is easily overcome by including internal standards or by constructing standard curves. The second concern centers on the homogeneity of the sample on the MALDI target. It has been observed (*21*, *22*) that the cocrystallization of complex samples with matrix results in the segregation of analytes while the sample is drying on the target. Analysis of different regions on the target will provide distinct, and inaccurate, representations of the sample composition. Electrospray deposition (*20*, *23*) has been used to ensure homogeneous sample application, a technique that we have repeated. While there was little or no improvement over our empirically determined sample application conditions, it served to verify the validity of our procedure.

We performed a series of calibration experiments using dTDP-glucose-d_7 and sodium borohydride-treated dTDP-4-keto-6-deoxyglucose-d_6 to determine the efficiency of analyte detection. Linear plots (Figure 2) for both species show that ionization and detection are independent of the other analyte's concentration. Because only minor chemical differences distinguish the substrate, product, and intermediates, segregation of analytes during drying and/or unequal ionization or detection do (does) not appear to affect quantitation.

Mass Spectral Pileup. Rapid mix−quench experiments were performed in triplicate using enzyme (300 μM) and dTDP-glucose-d_7 (200 μM), with borohydride in the quench. Samples were collected over a range of time points and subjected to workup and MALDI-TOF MS analysis, as described in Experimental Procedures. Spectra are collected in the negative ion mode; thus analytes are detected as their corresponding monoanionic species, one mass unit below that of the neutral species. A representative series of spectra is shown in Figure 3. In the MALDI-TOF MS spectra, we

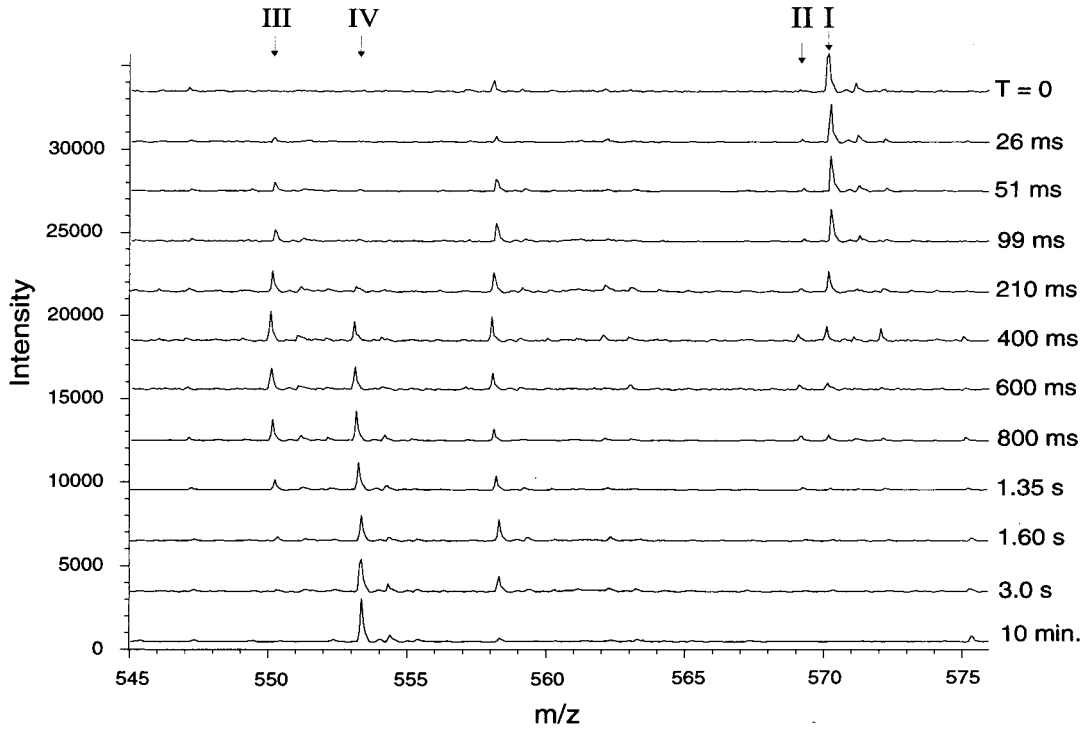


FIGURE 3: MALDI-TOF MS spectra of 4,6-dehydratase single turnover. Enzyme (300 μM) and dTDP-glucose-d_7 (200 μM) were mixed and quenched at the indicated times; sodium borohydride was included for the reductive stabilization of intermediates. MALDI-TOF MS spectra, collected in the negative ion mode, showed the time dependence of four signals: dTDP-glucose-d_7 at *m*/*z* 570 (I); the sum of dTDP-glucose-d_6 plus the reduced dTDP-4-ketoglucose-d_6 at *m*/*z* 569 (II); the reduced dTDP-4-ketoglucose-5,6-ene-d_5 at *m*/*z* 550 (III); and the reduced dTDP-4-keto-6-deoxyglucose-d_6 at *m*/*z* 553 (IV).

observe the time-dependent decrease in dTDP-glucose-d_7 at *m*/*z* 570 (I), the transient accumulation of signal at *m*/*z* 569 (II, sum of the dTDP-glucose-d_6 plus the reduced dTDP-4-ketoglucose-d_6), the transient and significant accumulation of the reduced dTDP-4-ketoglucose-5,6-ene-d_5 at *m*/*z* 550 (III), and the gradual appearance of dTDP-6-deoxyhexopyranoses-d_6 at *m*/*z* 553 (IV). Peaks that do not originate from dTDP-sugar species are also detected in the MALDI spectra. These derive from matrix and other components of the reaction (i.e., NAD^+ and NADH) and do not interfere with the analysis. The peak at *m*/*z* 568 is observed only when NAD^+ or NADH is present and is considered a degradation product of the cofactor. NAD^+ and NADH were interconverted during MALDI-TOF MS analysis, preventing their identification by this technique.

To resolve the composition of the species at *m*/*z* 569 (dTDP-glucose-d_6 and borohydride-reduced dTDP-4-ketoglucose-d_6), the experiments were repeated using sodium borodeuteride for the reductive stabilization, shifting the observed mass of reduced dTDP-4-ketoglucose-d_6 from 569 to 570. As expected, the masses of reduction products dTDP-hexopyranose-5,6-ene-d_5 and dTDP-6-deoxyhexopyranose-d_6 were shifted to *m*/*z* 551 and 554, respectively (data not shown). Moreover, the peaks at *m*/*z* 570 (now dTDP-glucose-d_7 plus dTDP-4-ketoglucose-d_6 after borodeuteride reduction) and 569 (now only dTDP-glucose-d_6) were not shifted. All peaks were identical in relative area to those in the reactions reduced with sodium borohydride. We conclude that the peak at *m*/*z* 569 is predominantly dTDP-glucose-d_6.

Detection of dTDP-4-ketoglucose. While the position of dTDP-4-ketoglucose-d_6 is always coincident with that of a more abundant species, its transient formation can be calculated from differences between several independent data sets. In each case it was impossible to detect statistically meaningful differences between data sets that included and excluded dTDP-4-ketoglucose-d_6. There were, however, two cases where a dTDP-4-ketoglucose peak would not have overlapped other signals, but it was still not observed. In experiments in which proteo-dTDP-glucose was the substrate and there was no reductive stabilization, dTDP-4-ketoglucose would be located two mass units below dTDP-glucose; it was not detected. A dTDP-4-ketoglucose-d_5 is predicted in the C5 exchanged branch, with its reductively stabilized adduct uniquely detectable at *m*/*z* 568 (Table 1), yet none was seen (data not shown). Because the dTDP-4-ketoglucose-d_6 does not accumulate during the reaction to a level detectable within the sensitivity limits of this technique (conservatively $\sim$1.5 μM), it is concluded that the net conversion of dTDP-4-ketoglucose-d_6 to dTDP-4-ketoglucose-5,6-ene-d_5 is considerably faster than the net conversion of dTDP-glucose-d_7 to dTDP-4-ketoglucose-d_6.

Identification and Competence of dTDP-4-ketoglucose-5,6-ene-d_5. The observation of the reduced dTDP-4-ketoglucose-5,6-ene-d_5 provides the first detection of this reaction intermediate. It or analogous species have been proposed in several other enzymatic systems including CDP-glucose 4,6-dehydratase, GDP-mannose 4,6-dehydratase, and UDP-sulfoquinovose synthase (*24*−*26*). Its competence is confirmed by both the temporal nature of its formation and the mass values at which it is detected. As described below, its detected mass is dependent upon the isotopic composition of both the substrate and the reductant. The mass indicates that dTDP-4-ketoglucose-5,6-ene-d_5 forms upon abstraction of a deuteride, a corresponding proton, and a water molecule (DHO) from dTDP-glucose-d_7. The accumulation of the intermediate is concomitant with the decay of dTDP-glucose-

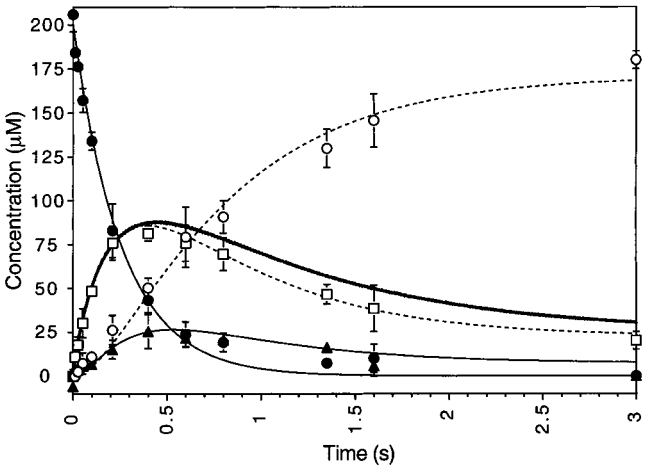


FIGURE 4: Single-turnover progress curves and stopped flow. The complete set of rapid mix−quench ([H]- and[D]-reduced) MALDI-TOF MS data [dTDP-glucose-d_7 (●), dTDP-4-ketoglucose-5,6-ene-d_5 (□), dTDP-glucose-d_6 (▲), and dTDP-4-keto-6-deoxyglucose-d_6 (○)] is shown with error bars and lines from fitting. The thicker trace shows the triplicate stopped-flow data (error < line thickness).

d_7, and its disappearance is accompanied by formation of dTDP-4-keto-6-deoxyglucose-d_6. Its complete disappearance, at a rate (1.85 s^{-1}) comparable or faster than k_{cat} (0.5 s^{-1}, 18 °C, dTDP-glucose-d_7), supports its kinetic competence. In summary, this intermediate is substrate-derived, dehydrated, and reducible, and its formation is both transient and linked to the stage of catalysis.

Confirmation of Analyte Identities by Isotopic Substitution. The structures of the 4,6-dehydratase ligands were probed by substitution of isotopically enriched analogues; each combination of natural abundance dTDP-glucose or dTDP-glucose-d_7 reduced with sodium borohydride or sodium borodeuteride was tested. Masses of detectable species proposed to contain a ketone functionality (dTDP-4-keto-glucose-5,6-ene and dTDP-4-keto-6-deoxyglucose) were shifted the expected amount by reduction with sodium borohydride and borodeuteride. dTDP-glucose, which does not contain a keto moiety, was not modified. Using dTDP-glucose-d_7 as the substrate results in a three mass unit difference between reduced dTDP-4-keto-6-deoxyglucose-d_6 and dTDP-4-ketoglucose-5,6-ene-d_5, a difference that is only two mass units with proteo-dTDP-glucose. This change reflects the formation of NADD and NADH from d_7 and proteo-dTDP-glucose, respectively, and the participation of NADD in the reduction of the dTDP-4-ketoglucose-5,6-ene-d_5. The mass values at which substrate, reduced intermediate, and reduced product were detected are all entirely consistent with and fully supportive of the proposed mechanism and intermediates (Figure 1, Table 1).

Results from the single-turnover experiments with dTDP-glucose-d_7 as substrate show that dTDP-4-keto-6-deoxyglucose-d_6 contains only six deuterium atoms. This extends the findings of Melo and co-workers (*19*), who demonstrated that the glucosyl C5 hydrogen of dTDP-glucose completely exchanges with hydrogen from solvent during steady-state turnover. The C5 deuterium is completely exchanged with bulk solvent, presumably by a rapidly exchanging active site residue that is responsible for the general base catalysis of water elimination.

Progress Curves and Kinetic Model. MALDI-TOF MS data were integrated and normalized as described in Experimental Procedures. Single-turnover progress curves showing disappearance of dTDP-glucose-d_7, formation of borohydride-reduced dTDP-4-keto-6-deoxyglucose-d_6, and the transient formation of the dTDP-glucose-d_6 and the borohydride-reduced dTDP-4-ketoglucose-5,6-ene-d_5 are presented in Figure 4. Rate constants were determined using the kinetic mechanism shown in Figure 5; curves generated from that fit are included in Figure 4. The dTDP-4-ketoglucose-d_6 and dTDP-4-ketoglucose-d_5 were not included in this mechanism because these species were not detected. The dTDP-glucose-d_7 to dTDP-4-ketoglucose-5,6-ene-d_5 transition was made irreversible by the infinite dilution of DHO in bulk H_2O. The reversibility of the dTDP-4-ketoglucose-5,6-ene-d_5 to dTDP-4-keto-6-deoxyglucose-d_6 transition was dictated by the evaluation of residuals (data not shown).

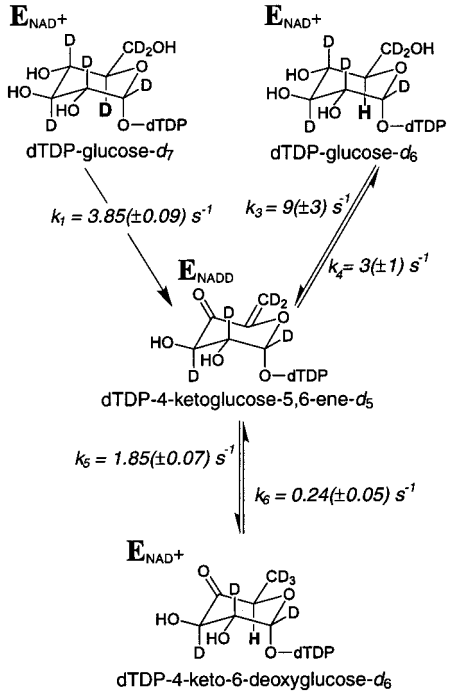


FIGURE 5: Kinetic model for the action of 4,6-dehydratase. Rate constants are derived from fitting data to this model. dTDP-4-ketoglucose is not included in our model because it is not detected, but it must still be included in the 4,6-dehydratase chemical mechanism.

Stopped Flow. Corroboration of the rapid mix−quench MALDI-TOF MS results was accomplished by comparison to stopped-flow spectrophotometric traces. Raw absorbance data from the stopped-flow experiments were converted into NADD concentration using the ϵ_{355} value of 6 mM^{-1} cm^{-1} for 4,6-dehydratase NADH described in Experimental Procedures. Stopped-flow data were collected in triplicate, with the same reaction conditions and concentrations used in the rapid mix−quench experiments (Figure 4). An uncharacterized, very low intensity chromophore appears approximately coincident with product, which explains the minor discrepancy between the dTDP-hexopyranose-5,6-ene-d_5 progress curve and the stopped-flow trace during the latter portions of the reaction. The weak chromophore, which decays within 1 min, has not been included in our kinetic models. The otherwise tight agreement between these two curves confirms the accuracy of our rapid mix−quench MALDI-TOF pre-steady-state analysis of 4,6-dehydratase.

Conclusion. Rapid mix−quench MALDI-TOF MS is a new technique for pre-steady-state analysis of enzymatic reactions. In applying this technique to study the mechanism of 4,6-dehydratase, we have been able to validate the proposed mechanism by characterizing the transient formation of dTDP-4-ketoglucose-5,6-ene as an intermediate. New information about hydrogen exchange and the relative rates for each part of this complex chemical mechanism have been obtained that will be important for understanding the precise mechanistic details of catalysis. We are excited about the potential for interfacing rapid mixing techniques with the analytical power of mass spectrometry. Rapid mix−quench MALDI-TOF MS will be generally useful for the pre-steady-state analysis of other enzymes, particularly in cases where required reaction conditions interfere with other types of mass spectral analysis.

NOTE ADDED IN PROOF

Following the acceptance of this paper, a paper detailing the use of MALDI-TOF MS for the pre-steady-state analysis of phosphotransfer by protein−tyrosine phosphatase came to our attention (*27*)

ACKNOWLEDGMENT

We thank Professor Brian Fox for use of his stopped-flow spectrophotometer. We also thank Roger L. Clausen, Instrument Innovator at the Mass Spectrometry Laboratory Chemistry Instrument Center, for his efforts in creating the electrospray deposition apparatus and also for help with the MALDI-TOF mass spectrometer. Finally, we thank Professor Dexter B. Northrop and Dr. Frank B. Simpson for helpful conversations and encouragement.

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BI001963D