Reduction Precedes Cytidylyl Transfer without Substrate Channeling in Distinct Active Sites of the Bifunctional CDP-Ribitol Synthase from *Haemophilus influenzae*†

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ABSTRACT: CDP-ribitol synthase is a bifunctional reductase and cytidylyltransferase that catalyzes the transformation of D-ribulose 5-phosphate, NADPH, and CTP to CDP-ribitol, a repeating unit present in the virulence-associated polysaccharide capsules of *Haemophilus influenzae* types a and b [Follens, A., et al. (1999) *J. Bacteriol. 181*, 2001]. In the work described here, we investigated the order of the reactions catalyzed by CDP-ribitol synthase and conducted experiments to resolve the question of substrate channeling in this bifunctional enzyme. It was determined that the synthase first catalyzed the reduction of D-ribulose 5-phosphate followed by cytidylyl transfer to D-ribitol 5-phosphate. Steady state kinetic measurements revealed a 650-fold kinetic preference for cytidylyl transfer to D-ribitol 5-phosphate over D-ribulose 5-phosphate. Rapid mixing studies indicated quick reduction of D-ribulose 5-phosphate with a lag in the cytidylyl transfer reaction, consistent with a requirement for the accumulation of K_m quantities of D-ribitol 5-phosphate. Signature motifs in the C-terminal and N-terminal sequences of the enzyme (short chain dehydrogenase/reductase and nucleotidyltransferase motifs, respectively) were targeted with site-directed mutagenesis to generate variants that were impaired for only one of the two activities (K386A and R18A impaired for reduction and cytidylyl transfer, respectively). Release and free diffusion of the metabolic intermediate D-ribitol 5-phosphate was indicated by the finding that equimolar mixtures of K386A and R18A variants were efficient for bifunctional catalysis. Taken together, these findings suggest that bifunctional turnover occurs in distinct active sites of CDP-ribitol synthase with reduction of D-ribulose 5-phosphate and release and free diffusion of the metabolic intermeditate D-ribitol 5-phosphate followed by cytidylyl transfer.

CDP-ribitol synthase from *Haemophilus influenzae* is a 474-amino acid bifunctional enzyme with both reductase and cytidylyltransferase activities that catalyzes the following overall reaction (*1*):

D-ribulose 5-phosphate + NADPH + CTP → CDP-ribitol + $NADP^+$ + pyrophosphate

The product of this reaction, CDP-ribitol, is thought to be the activated form of D-ribitol 5-phosphate, a repeating unit present in the virulence-associated polysaccharide capsules of *H. influenzae* types a and b (*2, 3*). The polyol phosphate, D-ribitol 5-phosphate, is a repeating monomer in the capsules of a variety of bacteria (*4*−*6*) as well as in cell wall teichoic acids and lipoteichoic acids of Gram-positive bacteria (*7*).

Almost forty years ago, cytidylyl transfer from CTP to D-ribitol 5-phosphate was demonstrated in bacterial extracts (*8*). Since then, however, the genetics and biochemistry of CDP-ribitol synthesis have remained relatively obscure until the discovery of a gene encoding a CDP-ribitol synthase in the capsulation locus of *H. influenzae* (*9*). That finding led to recent groundwork in expression, purification, and functional characterization of CDP-ribitol synthase (*1*).

The bifunctionality of *H. influenzae* CDP-ribitol synthase is consistent with sequence similarities that are evident in the primary structure of the protein. The protein's C-terminal sequence is similar to those of a variety of short chain dehydrogenase/reductase proteins, and its N-terminal sequence is similar to those of a number of nucleotidyltransferases (*1*). Protein sequences for CDP-ribitol synthases from *H. influenzae* strains a and b (here termed Acs1 and Bcs1, respectively) are 96% identical but currently have no convincing orthologues in the public databases. The CDP-ribitol synthase from *H. influenzae* therefore currently represents an accessible prototype for biochemical study with probable relevance to so far undiscovered enzymes that activate D-ribitol 5-phosphate for bacterial polysaccharide synthesis.

The bifunctionality of the *H. influenzae* enzyme raises mechanistic issues that are peculiar to bifunctional enzymes in metabolism. Among these are the order imposed by the enzyme in bifunctional catalysis and the question of substrate

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channeling. Substrate channeling, the process of direct transfer of an intermediate between active sites that catalyze sequential reactions in a biosynthetic pathway (*10*−*12*), is thought to be a significant driving force for gene fusion in evolution (*13, 14*) and remains an engaging possibility for enzymes such as CDP-ribitol synthase.

In this work, we present kinetic studies aimed primarily at rigorously examining the order of the reductase and cytidylytransferase reactions catalyzed by CDP-ribitol synthase and at investigating the possibility that substrate channeling occurs in this bifunctional enzyme. The results indicate that CDP-ribitol synthase catalyzes reduction of D-ribulose 5-phosphate to yield the D-ribitol 5-phosphate, prior to nucleotidyl transfer. Our studies also argue strongly against substrate channeling and are most consistent with a model of bifunctional catalysis at distinct active sites with release of the metabolic intermediate D-ribitol 5-phosphate to the bulk solvent.

MATERIALS AND METHODS

Materials. *H. influenzae* type b was obtained from the Clinical Microbiology Laboratory, Health Sciences Centre, McMaster University, and maintained on chocolate agar plates (PML Microbiologicals, Wilsonville, OR). The following compounds were obtained from BioShop Ltd. (Burlington, ON): 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),[1] ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), ampicillin (Amp), imidazole, and isopropyl thiogalactoside (IPTG). 2-Amino-6-mercapto-7-methylpurine riboside (MESG) was obtained from P. Berti (Department of Chemistry, McMaster University). All other reagents were obtained from Sigma-Aldrich (Oakville, ON).

Synthesis of Ribitol 5-Phosphate. D-Ribitol 5-phosphate was synthesized as previously described in refs *15* and *16*. Briefly, the synthesis involved the incubation of 180 mg of D-ribose 5-phosphate and 23 mg of $NaBH_4$ in 4 mL of water at room temperature for 16 h. The reaction was stopped with 300 μL of glacial acetic acid and the pH increased to 8−9 with approximately 0.5 M ammonium hydroxide. The reaction mixture was passed through a Dowex 1X8-400 (Sigma-Aldrich) strongly anionic exchanger column (1 cm × 13.5 cm) equilibrated with water and eluted with 1 N ammonium formate. The eluted fractions were completely dried to crystals using a Speed Vac concentrator (Savant, Holbrook, NY) set on the lowest heat setting. To remove all traces of borate, the sample was dissolved in approximately 50 mL of methanol and then evaporated to dryness on a rotoevaporator; this process was repeated four times. The dried product was resuspended in 50 mL of deionized water and allowed to evaporate on the Speed Vac apparatus at low heat. The product was resuspended in water and then added to a 50 mL solution containing 100 g of Dowex 50WX8-400 (Sigma Aldrich), an H^+ form resin, stirred vigorously for 1 h, and vacuum filtered. The flow through was collected and evaporated on the Speed Vac apparatus. The product was verified to be D-ribitol 5-phosphate by positive ion electrospray mass spectrometry (weight = 232.1) and by NMR in D_2O. ^{1}H chemical shifts (and coupling constants, in hertz) for protons 1, 1′, 2, 3, 4, 5, and 5′ were 3.963 ($^3J_{1,2}$ = 2.9, $^2J_{1,1'}$ = −10.9), 3.864 ($^3J_{1',2}$ = 6.3), 3.788 ($^3J_{2,3}$ = 6.3), 3.586 ($^3J_{3',4}$ = 6.1), 3.693 ($^3J_{4,5}$ = 3.1, $^3J_{4,5'}$ = 7.1), 3.642 ($^3J_{5,5'}$ = −11.9), and 3.496 ppm, respectively. ^{13}C chemical shifts (and ^{13}C−^{31}P coupling constants, in hertz) for carbons 1−4 were 66.9, 70.9 ($J_{c,p}$ = 6.7), 71.7, 72.2, and 62.5 ppm, respectively. The ^{31}P chemical shift was 0.956 ppm.

Overexpression of CDP-Ribitol Synthase. The gene for CDP-ribitol synthase of *H. influenzae* type b (*bcs1*) was cloned from chromosomal DNA using the polymerase chain reaction. Amplification was performed using VENT DNA polymerase (New England Biolabs, Beverly, MA) and oligonucleotide primers with the following sequences: 5′-GGGCTTGGCATATGAATAAAAATAAAAACATAGG-AATCAT-3′ and 5′-AGCTGATGCGAGCTCTTATTTAT-AGAGATCAGCTAA-3′. The underlined sequences denote the restriction sites for *Nde*I, which incorporate the start codon, and *Sac*I, which flanks the stop codon. This gene was also amplified using the first primer (with the *Nde*I site) and an alternate 3′-end primer with a *Xho*I site that removes the stop codon (5′-GGTGCTCGAGTCCTTTATAGAGAT-CAGCTAAAAT-3′) to fuse this gene with the C-terminal His_6-encoding sequence in the pET22b vector (Novagen, Madison, WI). PCR-amplified DNA was ligated into *Srf*I-digested PCRscript (Stratagene, La Jolla, CA) and the nucleotide sequence of the cloned gene confirmed by sequencing (MOBIX, McMaster University). The gene was excised from the PCRscript vector by digesting it with *Nde*I and *Sac*I (untagged version) or *Nde*I and *Xho*I (C-terminal His-tagged version) and ligated into *Nde*I-, *Sac*I-, or *Xho*I-digested pET22b. The resulting plasmid constructs were transformed into *Escherichia coli* BL21(DE3) cells (Novagen) encoding native [BL21(DE3)/pET22b-*bcs1*] and C-terminally His-tagged [BL21(DE3)/pET22b-*bcs1*-His] Bcs1 proteins.

Site-Directed Mutagenesis. Site-directed variants of His-tagged Bcs1 encoded in pET22b-*bcs1*-His were created using the protocol from the QuikChange (Stratagene) mutagenesis kit. Oligodeoxynucleotides were as follows: R18A, 5′-GGTGGTGTTGGCTCTGCCATGGGATTGGGCTAC-3′; and K386A, 5′-GCTATTTACTCTTCTGCAGCAGCAGCT-GTGGTAAAC-3′. The underlined sequences highlight the mutation that has been created. Mutagenesis was confirmed by DNA sequence analysis (MOBIX, McMaster University).

Enzyme Purification. Native untagged Bcs1 was purified from *E. coli* BL21(DE3) cells, freshly transformed with pET22b-*bcs1*. Cells were grown (23 °C) in Luria-Bertani media (2 L) supplemented with 50 μg/mL ampicllin to an optical density (595 nm) of 0.8. The culture was then induced with isopropyl β-thiogalactoside to a final concentration of 1 mM, grown for an additional 16 h at 23 °C, and harvested by centrifugation. All subsequent purification steps were performed at 4 °C. Cells were resuspended in 20 mL of lysis buffer containing 50 mM HEPES (pH 8.0), 5 mM EDTA, 5 mM DTT, 20% glycerol, 0.1 mg of DNase, 0.1 mg of RNase, and 215 mg of protease inhibitor cocktail [Sigma catalog no. P8465, 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, and bestatin]. Cells were disrupted with four passes through a French press at 20 000 psi and centrifuged at

[1] Abbreviations: Amp, ampicillin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl thiogalactoside; MESG, 2-amino-6-mercapto-7-methylpurine riboside; PPase, inorganic pyrophosphatase; PRPase, purine ribonucleoside phosphorylase.

20000*g* for 2 h before being loaded onto a Q-Sepharose Fast Flow (Amersham-Pharmacia, Baie d'Urfé, PQ) column (2.7 cm × 13.5 cm) equilibrated with 25 mM HEPES (pH 8.0), 5 mM EDTA, 1 mM DTT, and 10% glycerol. The column was developed at a flow rate of 3 mL/min, and proteins were eluted with an 800 mL gradient of 25 to 500 mM NaCl in the running buffer. SDS−polyacrylamide gel electrophoresis of column fractions indicated that Bcs1 (≈50 kDa) was eluted with approximately 80−100 mM NaCl. Pooled fractions were dialyzed for 12 h against 5 mM potassium phosphate (pH 6.2), 1 mM DTT, and 10% glycerol loaded onto a Macro-Prep ceramic hydroxyapatite type I (Bio-Rad) column (2.7 cm × 12.5 cm) equilibrated with 5 mM potassium phosphate (pH 6.2), 1 mM DTT, and 10% glycerol. The column was developed at a flow rate of 0.5 mL/min with a gradient of 10 to 500 mM potassium phosphate over 500 mL. Analysis (SDS−polyacrylamide gel electrophoresis) of column fractions indicated that Bcs1 eluted in two regions of the chromatogram, the least contaminated of which (eluting at about 100 mM potassium phosphate) were pooled, concentrated, and chromatographed on a Superdex 200 (Amersham-Pharmacia′) column (1.8 cm × 62 cm) equilibrated with 25 mM HEPES (pH 8.0), 1 mM DTT, and 50 mM NaCl. The purified enzyme was stored at −80 °C.

His-tagged Bcs1 was purified from the overproducing strain [BL21(DE3)/pET22b-*bcs1*-His] according to the following procedure. Cells (500 mL) were grown, induced, harvested, and lysed as described above with the following exceptions. Cells were resuspended in lysis buffer containing 20 mM Na_2PO_4 (pH 7.2), 500 mM NaCl, 5% glycerol (v/v), 0.1 mg of DNase, and 0.1 mg of RNase. The cell lysate was clarified at 20000*g* for 2 h and loaded onto a 1 mL Hi Trap affinity nickel column (Amersham Pharmacia′) equilibrated with 20 mM sodium phosphate (pH 7.2) and 500 mM NaCl at a flow rate of 0.5 mL/min. After the mixture was washed with 10 column volumes in the same buffer, Bcs1 was eluted with 200 mM imidazole and loaded onto a Superdex 200 column (1.8 cm × 62 cm) equilibrated with 25 mM HEPES (pH 8.0), 1 mM DTT, 50 mM NaCl, and 10% glycerol (0.5 mL/min). Pooled fractions were dialyzed for an additional 12 h against 25 mM HEPES (pH 8.0), 1 mM DTT, and 10% glycerol and stored in aliquots at −80 °C. Protein concentrations of the purified Bcs1 enzyme were determined according to the method of Gill and von Hippel (*17*).

Enzyme Assays. All assays were carried out at 25 °C and were performed in triplicate. D-Ribulose 5-phosphate reductase activity was assayed continuously in microwell plates by monitoring the absorbance at 340 nm, which was an indication of the depletion of the level of NADPH (extinction coefficient of 6.22 mM^{-1} cm^{-1}). The standard 250 μL assay reaction mixture contained 25 mM HEPES (pH 7.2), 1 mM DTT, 0.01 mg/mL bovine serum albumin, and 85 μM CTP.

Cytidylyltransferase activity was monitored in a continuous coupled assay for the detection of inorganic pyrophosphate, modified from protocols previously described (*18*, *19*). Pyrophosphate production from the cytidylyltransferase reaction was monitored with excess inorganic pyrophosphatase (PPase), while purine ribonucleoside phosphorylase (PRPase) catalyzes the phosphorylysis of 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) with a resulting change in the extinction coefficient at 360 nm of 11.0 mM^{-1} cm^{-1}. In addition to substrates, the standard 250 μL reaction mix contained 25 mM HEPES (pH 7.2), 1 mM DTT, 0.01% Triton X-100, 10 mM $MgCl_2$, 200 μM MESG, 0.5 unit of PRPase, and 5 units of PPase. When CTP and D-ribitol 5-phosphate were added to saturating concentrations, they were at 500 and 600 μM, respectively.

Assay of the overall reductase and cytidylyltransferase activity of Bcs1 was achieved with a discontinuous assay using radioactive detection of [5-^{3}H]CTP depletion and [5-^{3}H]CDP-ribitol formation. The standard 200 μL assay mixture contained 25 mM HEPES (pH 7.2), 1 mM DTT, 0.01% Triton X-100, 10 mM $MgCl_2$, 0.6 mM CTP, 1.0 mM D-ribulose 5-phosphate, 0.6 mM NADPH, and 1 μCi of [5-^{3}H]CTP. Reactions were initiated by adding Bcs1, stopped by the addition of 600 μL of 8 M urea, and analyzed by anion exchange (MonoQ 5/5, Amersham-Pharmacia) HPLC in a 15 mL gradient of 0.5 M triethylamine bicarbonate using a Waters 600 chromatography system with Millenium analysis software (Milford, MA). Detection was accomplished using a Packard in-line radioactivity detector (Flow Scintillation Analyzer 150TR, Meriden, CT). At least three time points were used for each rate determination.

In evaluation of K_m and k_{cat} values, substrate concentrations ranged from approximately 0.5−5 times the K_m. In all cases, apparent steady state parameters for a given substrate were determined in the presence of saturating amounts of cosubstrate, at concentrations between 5 and 10 times the K_m. Steady state kinetic parameters (k_{cat} and K_m) were generated by nonlinear regression of data using the equation $V = k_{cat}[E][S]/(K_m + [S])$ using the program GraFit (Erithacus Software, Surrey, U.K.).

Rapid Mixing Experiments. Rapid mixing experiments were performed to explore reaction order and the approach to steady state turnover of bifunctional Bcs1 using a Biologic SFM-400/S mixer and MOS-250 optical unit (Molecular Kinetics, Pullman, WA). In a typical experiment, Bcs1 was mixed rapidly with substrates to a final volume of 250 μL [25 mM HEPES (pH 7.2), 1 mM DTT, 0.01% Triton X-100, 10 mM $MgCl_2$, 0.75 mM CTP, 1.0 mM D-ribulose 5-phosphate, 0.46 mM NADPH, 5 μCi of [5-^{3}H]CTP, and 600 ng of Bcs1]. Depletion of the level of NADPH was monitored by absorbance at 340 nm in stopped-flow mode. Similarly, CDP-ribitol formation was followed by quenching the reaction with 6 M urea. Quenched flow reactions were analyzed by HPLC with in-line radioactivity detection as described above.

RESULTS

Figure 1 depicts an SDS−polyacrylamide gel of the overexpressed and purified Bcs1 protein and variants investigated in this study: native full-length Bcs1, C-terminally His-tagged Bcs1, R18A variant of C-terminally His-tagged Bcs1, and K386A variant of C-terminally His-tagged Bcs1. Previous work on the CDP-ribitol synthase from *H. influenzae* type a, Acs1 (*1*), had established that low-temperature expression of the native protein was necessary to overcome solubility problems that were evident at 37 °C. Not surprisingly, we also found that the majority of Bcs1 was insoluble (90−95%) after induction at 37 °C, and purified the enzyme from cells induced for 16 h at 23 °C (solubility increased to 50%). We modified the purification procedure from that used

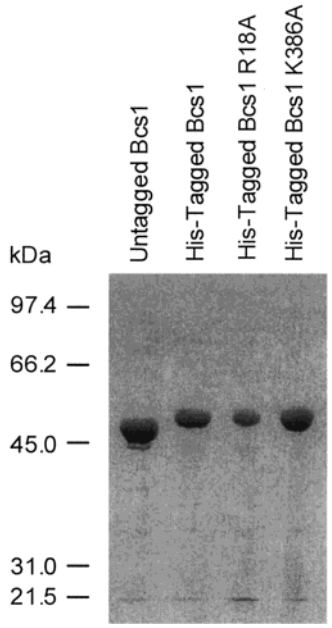


FIGURE 1: SDS−polyacrylamide gel (11.5% acrylamide, w/v) of Bcs1 and variants used in this work. Shown are native Bcs1 with no His tag (31 μg), C-terminally His-tagged Bcs1 (22 μg), the R18A mutant of C-terminally His_6-tagged Bcs1 (18 μg), and the K386A variant of C-terminally His_6-tagged Bcs1 (27 μg). Samples were denatured by boiling in Laemmli (*27*) sample buffer containing 8% 2-mercaptoethanol prior to electrophoresis. Apparent molecular weights were approximately 52 000 for native Bcs1 and 53 000 for His-tagged Bcs1.

previously (*1*) to include hydroxyapatite and gel-filtration chromatography instead of the Blue Sepharose affinity step which, in our hands, proved to ineffective in removing key contaminants. Overall, our purification of untagged Bcs1 protein (1.1 mg from a 2 L fermentation) was achieved with low recovery of CDP-ribitol synthase (0.4%), likely due to the proteolytic degradation of the protein, despite the use of protease inhibitors in the initial purification steps. A C-terminally His-tagged version of the Bcs1 protein was created which was purified cleanly (Figure 1) and in good yield (15 mg from 0.5 L). Site-directed variants of His-tagged Bcs1 were purified with similar results (Figure 1).

Substrate Specificity and Reaction Order. Table 1 depicts the results of steady state kinetic analysis of both reductase and cytidylyltransferase activities of C-terminally His-tagged Bcs1 with a variety of substrates. Steady state kinetic parameters for the His-tagged enzyme were very similar to those of the native, purified, untagged protein [for native reductase, $k_{cat} = 26\ s^{-1}$, $K_{m(D-ribulose\ 5-phosphate)} = 42\ \mu M$, and $K_{m(NADPH)} = 25\ \mu M$; for native cytidylyltransferase, $k_{cat} = 12\ s^{-1}$, $K_{m(D-ribitol\ 5-phosphate)} = 75\ \mu M$, and $K_{m(CTP)} = 74\ \mu M$]. Therefore, because of the ease of purification, the His-tagged form of the Bcs1 enzyme was used for the all of the experiments described in detail herein.

The reductase reaction proceeded slightly faster than the cytidylyltransferase reaction with a k_{cat} of 22 s^{-1} compared to a k_{cat} of 13 s^{-1}. Positive ion electrospray mass spectrometry confirmed the identities of the products of reduction (D-ribitol 5-phosphate, weight = 232.1) and cytidylyl transfer (CDP-ribitol, weight = 553.3) following purification by anion exchange HPLC as described in Materials and Methods (data not shown).

Table 1: Steady State Kinetic Parameters Describing Substrate Specificity of Reductase and Cytidylyltransferase Activities of Bcs1

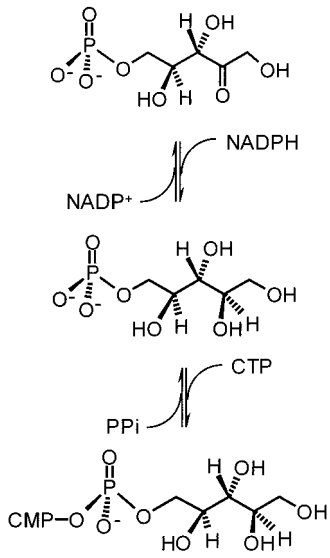
	K_m[a] (μM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m ($\times 10^{-5}\ M^{-1}\ s^{-1}$)
(1) reductase			
D-ribulose 5-phosphate	76 ± 5.2	22 ± 0.6	2.9
D-arabinose 5-phosphate	3000 ± 570	2.3 ± 0.3	0.0077
D-erythrose 4-phosphate	38 ± 3.8	0.5 ± 0.02	0.13
D-ribose 5-phosphate	2200 ± 280	4.1 ± 0.3	0.019
D-ribulose	1100 ± 180	3.6 ± 0.3	0.033
D-xylulose 5-phosphate	>3500	3.6 ± 0.6[2]	<0.010
NADPH	44 ± 4.8	22 ± 0.6	5.0
NADH	>600	0.064[b]	<0.0011
(2) cytidylyltransferase			
D-ribitol 5-phosphate	130 ± 12	13 ± 0.5	1.0
D-erythrose 4-phosphate	>5000	0.15[b]	<0.00030
D-galactose 6-phosphate	>5000	0.21[b]	<0.00042
D-ribose 5-phosphate	>5000	0.52[b]	<0.0010
D-ribulose 5-phosphate	>5000	0.77[b]	<0.0015
CTP	99 ± 9.1	13 ± 0.5	1.3

[a] Errors are standard deviations of three separate determinations. [b] Values are not k_{cat}. These values are turnover of CDP-ribitol synthase at a substrate concentration of 3.5 mM.

Of the substrates tested, D-ribulose 5-phosphate and NADPH were the preferred substrates for the reductase reaction, with apparent K_m values of 76 and 44 μM, respectively. Follens et al. (*1*) previously described a stimulatory effect of CTP (100 μM) on the reductase reaction (3-fold increase in V_{max} and 8-fold decrease in K_m). We found that inclusion of CTP and $MgCl_2$ (85 μM and 10 mM, respectively) decreased the noise in our assay and increased k_{cat} (from 11 to 22 s^{-1}) without having an effect on K_m (data not shown). We interpreted this to mean that CTP increased the specific activity of the enzyme by stabilizing it in our assay system, presumably through a specific association with the enzyme since the reductase reaction was not stimulated by the presence of 100 μM CDP, ATP, GTP, or UTP (10 mM $MgCl_2$).

The reductase reaction was specific for NADPH and demonstrated little activity with NADH present (Table 1). Alternate sugar substrates were examined in the reductase assay, and all demonstrated a significantly lower turnover and higher K_m values. The highest specificity constant (k_{cat}/K_m) of the alternate sugar substrates that were tested was observed for the four-carbon aldose D-erythrose 5-phosphate ($0.13 \times 10^5\ M^{-1}\ s^{-1}$), reduced 22-fold from that observed for D-ribulose 5-phosphate ($2.9 \times 10^5\ M^{-1}\ s^{-1}$). Pentose phosphate substrates with C-5 aldehyde groups, such as D-ribose 5-phosphate and D-arabinose 5-phosphate, had 5-fold lower turnover rates (k_{cat} = 4.1 and 2.3 s^{-1}, respectively) and 30-fold lower apparent substrate affinities (K_m = 2.2 and 3.0 mM, respectively). Reductase activity was undetectable (turnover < 0.001 s^{-1} at 3.5 mM sugar) with the following substrates: D-fructose 6-phosphate, D-fructose 1,6-diphosphate, D-galactose 6-phosphate, D-galactose 6-sulfate, D-glucose 6-phosphate, D-glucose 1-phosphate, D-glucose 6-sulfate, D-glucose 1,6-diphosphate, D-mannose 6-phosphate, D-ribulose 1,5-diphosphate, and D-ribose 1-phosphate (data not shown). D-Xylulose 5-phosphate, a C-3 epimer of D-ribulose 5-phosphate, was shown to have an at least 6-fold lower reductase activity (turnover = 3.6 s^{-1} at 3.5 mM) and was at least 46-fold lower in apparent affinity (K_m > 3.5 mM) compared to D-ribulose 5-phosphate. Experiments with D-ribulose demonstrated a 90-fold kinetic preference for the

Scheme 1: Reductase Precedes Cytidylyltransferase in Bcs1

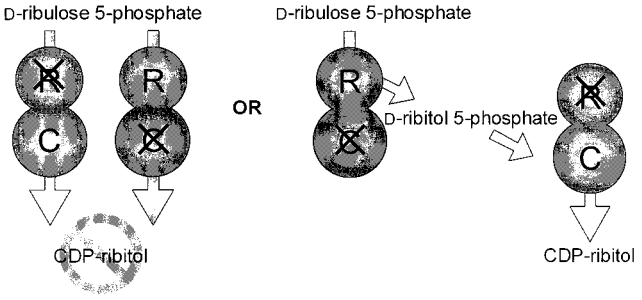


phosphorylated ketose D-ribulose 5-phosphate (k_{cat}/K_m = 0.033×10^5 and 2.9×10^5 M^{-1} s^{-1}, respectively). The reductase of Bcs1 on D-ribulose 5-phosphate was unaffected by the presence of any of the above alternative substrates, D-ribitol 5-phosphate, or $NADP^+$ at 1 mM in the reaction mixture.

In the cytidylyltransferase reaction, CTP and D-ribitol 5-phosphate were preferred substrates with apparent K_m values of 99 and 130 μM, respectively, and a turnover rate of 13 s^{-1} (Table 1). Cytidylyltransferase activity was specific for CTP since no activity was detected in the presence of ATP, GTP, UTP, ADP, or CDP (data not shown). The Bcs1 protein showed a kinetic preference of more than 650-fold for cytidylyl transfer to D-ribitol 5-phosphate over D-ribulose 5-phosphate ($k_{cat}/K_m = 1.0 \times 10^5$ and $<0.0015 \times 10^5$ M^{-1} s^{-1}, respectively), indicating that ribulose 5-phosphate is a very poor substrate for cytidylyl transfer and supporting the reaction order depicted in Scheme 1. Other aldose phosphate sugar substrates that were tested (D-erythrose 4-phosphate, D-galactose 6-phosphate, and D-ribose 5-phosphate) similarly demonstrated little cytidylyltransferase activity (Table 1). Cytidylyltransferase activity was undetectable (turnover < 0.001 s^{-1} at 3.5 mM sugar) with the following substrates: D-fructose 6-phosphate, D-fructose 1,6-diphosphate, D-galactose 6-sulfate, D-glucose 6-phosphate, D-glucose 1-phosphate, D-glucose 6-sulfate, D-glucose 1,6-diphosphate, D-mannose 6-phosphate, D-ribulose 1,5-diphosphate, sorbitol 6-phosphate, and D-ribitol (data not shown). The cytidylyltransferase reaction was unaffected by the presence of 1 mM D-ribulose 5-phosphate or 0.1 mM CDP.

Test for Substrate Channeling in CDP-Ribitol Synthase. Having established the reaction order of CDP-ribitol synthase, we were interested in determining whether the metabolic intermediate D-ribitol 5-phosphate was channeled from reductase to cytidylyltransferase active sites or released to the bulk solvent. Scheme 2 illustrates our approach to distinguishing channeling from release of D-ribitol 5-phosphate. We endeavored to create two monofunctional variants in Bcs1 that were impaired primarily in k_{cat} for the reductase and cytidylyltransferase reactions. Assays of bifunctional catalysis by equimolar mixtures of these monofunctional variants in the presence of saturating D-ribulose 5-phosphate,

Scheme 2: Active Site-Directed Variants of Bcs1 Distinguish Substrate Channeling from Free Diffusion



CTP, and NADPH would be diagnostic of channeling or release of the D-ribitol 5-phosphate. Channeling of the metabolic intermediate would be indicated if the mixture of monofunctional variants were incompetent for the production of CDP-ribitol, while release would be suggested by bifunctional catalysis by the same mixture. Indeed, this approach would be particularly unambiguous if the Bcs1 protein existed in solution as a monomer, where D-ribitol 5-phosphate could not be channeled among subunits in a complex. We therefore tested the oligomeric state of Bcs1 with gel-filtration chromatography (Superdex-200, Amersham-Pharmacia Biotech) by comparing its elution (in the presence of 100 μM CTP and 10 mM $MgCl_2$) to those of globular proteins of varying molecular weight (molecular weight gel-filtration standards, Sigma). Both the His-tagged and native Bcs1 enzymes eluted with an apparent molecular weight of 50 000, indicating that Bcs1 was a monomer in solution.

Bcs1 monofunctional variants were created by site-directed mutagenesis on the basis of amino acid sequence alignments of Bcs1 with homologues. The C-terminal region of CDP-ribitol synthase has been previously shown to be approximately 25% identical to members of the short chain dehydrogenase/reductase family (*1*). We chose to create the K386A variant in the conserved YXXXK motif (residues 382−386 in Bcs1), where the tyrosine and lysine are understood to play a critical and coordinated role in proton abstraction in catalysis (*20*). Indeed, the K386A variant was significantly impaired in k_{cat} compared to the wild-type enzyme for reduction (0.32 and 22 s^{-1}, respectively) with relatively little impact on K_m for either D-ribulose 5-phosphate or NADPH (Table 2).

The N-terminal region of CDP-ribitol synthase is significantly similar to a number of sugar−phosphate nucleotidyltransferases (*1*) and contains, in particular, a motif ($G^{111}GGTRLPK^{123}$) which has recently been identified as a signature sequence involved in sugar−nucleotide interaction in the active site of *E. coli N*-acetylglucosamine-1-phosphate uridylyltransferase, GlmU (*21*). Thus, the R18A variant constructed in this work was inspired by the k_{cat} effect on uridylyl transfer of the analogous substitution in GlmU (*21*). Table 2 indicates that the R18A variant in Bcs1 was reduced nearly 100-fold in k_{cat} compared to the wild-type enzyme for cytidylyl transfer (0.14 and 13 s^{-1}, respectively), with notable increases in K_m relative to that of the wild type for both D-ribitol 5-phosphate (7.7-fold) and CTP (4-fold).

It is noteworthy that the reductase-impaired variant, K386A, was 60- and 170-fold reduced in k_{cat}/K_m for NADPH and D-ribulose 5-phosphate, respectively, but demonstrated little change in the specificity constants for the cytidylyl-

Table 2: Steady State Kinetic Parameters Describing the Reactions Catalyzed by Bcs1 Mutants

	R18A	wild type/R18A ratio	K386A	wild type/K386A ratio
(1) reductase[a]				
k_{cat} (s^{-1})	6.8 ± 0.4	3.2	0.32 ± 0.0002	68.8
D-ribulose 5-phosphate				
K_m (μM)	270 ± 36	0.3	190 ± 18	0.4
k_{cat}/K_m ($\times 10^{-5}$ M^{-1} s^{-1})	0.25	11.6	0.017	171
NADPH				
K_m (μM)	17 ± 0.48	2.6	39 ± 5.0	1.1
k_{cat}/K_m ($\times 10^{-5}$ M^{-1} s^{-1})	4.0	1.3	0.082	61.0
(2) cytidylyltransferase[a]				
k_{cat} (s^{-1})	0.14 ± 0.007	92.9	9.7 ± 0.47	1.3
D-ribitol 5-phosphate				
K_m (μM)	1000 ± 150	0.1	120 ± 9.9	1.1
k_{cat}/K_m ($\times 10^{-5}$ M^{-1} s^{-1})	0.0014	714	0.81	1.2
CTP				
K_m (μM)	390 ± 47	0.3	92 ± 8.9	1.1
k_{cat}/K_m ($\times 10^{-5}$ M^{-1} s^{-1})	0.0036	361	1.1	1.2

[a] Errors are standard deviations of three separate determinations.

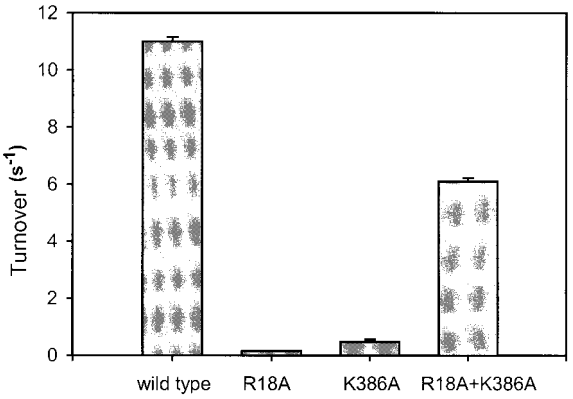


FIGURE 2: Rate of CDP-ribitol formation in bifunctional catalysis by Bcs1 and variants to probe the release of D-ribitol 5-phosphate. Plotted are the turnover values for the wild type, the R18A variant, the K386A variant, and a mixture of the R18A and K386A variants. Substrate concentrations in the assay were as follows: 1.2 mM D-ribulose 5-phosphate, 600 μM NADPH, and 600 μM CTP. Values of turnover were based on the protein concentration in each reaction mixture, except in the variant mixture experiment where that concentration was halved (i.e., 177 μM/s with 15.2 μM wild type, 3.2 μM/s with 22.3 μM R18A, 10.5 μM/s with 22.3 μM K386A, and 130 μM/s with 21.3 μM R18A and 21.3 μM K386A). The reaction was monitored by the extent of [5-^{3}H]CDP-ribitol formation following separation on an anion exchange (MonoQ) HPLC system using a triethylammonium bicarbonate gradient.

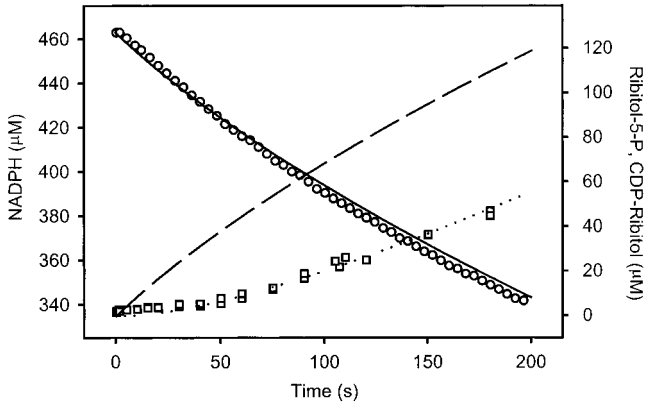


FIGURE 3: Bifunctional catalysis following rapid mixing of CDP-ribitol synthase with saturating amounts of D-ribulose 5-phosphate, NADPH, and CTP. Shown are data indicating the time course of NADPH depletion (○) and CDP-ribitol formation (□), monitored by stopped flow absorbance (340 nm) spectroscopy and by rapid quench flow followed by HPLC to measure the amount of [5-^{3}H]-CDP-ribitol, respectively. Duplicate data are presented for rapid quench experiments and an average of eight trials for the stopped flow. Substrate concentrations were as follows: 1 mM D-ribulose 5-phosphate, 463 μM NADPH, and 750 μM CTP (5 μCi/mol of [5-^{3}H]CTP). Curves are theoretical assuming reductase precedes cytidylyltransferase and that ribitol 5-phosphate is released from the enzyme. Solid, dashed, and dotted lines represent theoretical NADPH, D-ribitol 5-phosphate, and CDP-ribitol concentrations, respectively, calculated by numerical integration at 1 s intervals using the steady state kinetic parameters determined in this work for Bcs1. The CDP-ribitol concentration was equal to $k_{cat}t$[E][S]/(K_m + [S]), where the enzyme concentration [E] was 0.045 μM, t was the time, k_{cat} was 13 s^{-1}, [S] was the concentration of D-ribitol 5-phosphate at time t, and K_m was 130 μM. The D-ribitol 5-phosphate concentration was equal to $k_{cat}t$[E][S]/(K_m + [S]), where k_{cat} was 22 s^{-1}, [S] was the concentration of NADPH at time t − 1 s, and K_m was 44 μM. The NADPH concentration was 463 μM lower than the concentration of D-ribitol 5-phosphate formed at time t.

transferase reaction (Table 2). Similarly, the cytidylyltransferase-impaired variant, R18A, was reduced 360- and 714-fold in k_{cat}/K_m for CTP and D-ribitol 5-phosphate, respectively, without significant effects on these parameters for the reductase function. These findings are consistent with the hypothesis that reductase and cytidylyl transfer functions of Bcs1 occur at distinct and independent active sites.

Having characterized the monofunctional variants of Bcs1, R18A and K386A, we were interested in addressing the question of substrate channeling as discussed above by monitoring the bifunctional reaction of the enzyme. Figure 3 illustrates the overall turnover numbers seen for wild-type Bcs1 (11 s^{-1}) and the R18A and K386A variants (0.13 and 0.47 s^{-1}, respectively) compared to an equimolar mixture of the R18A and K386A variants (6.1 s^{-1}). Though reduced by about 50% relative to that of the wild type, the overall turnover of the mixture indicates that the metabolic intermediate is released to the bulk solvent, not channeled from reductase to cytidylyltransferase active sites.

Approach to Steady State in Bifunctional Turnover. We were interested in following the approach to steady state in bifunctional catalysis since both the order of the bifunctional reaction and the fate of a metabolic intermediate would be manifested in such a time course. Figure 3 illustrates the outcome of rapid mixing of the Bcs1 enzyme with saturating amounts of all substrates (NADPH, D-ribulose 5-phosphate,

and [^{3}H]CTP) followed by monitoring the depletion of the level of NADPH by stopped flow and appearance of [^{3}H]-CDP-ribitol by rapid quench. Also shown are theoretical curves for NADPH, D-ribitol 5-phosphate, and CDP-ribitol calculated using the steady state parameters for Bcs1 in Table 1, and assuming that the reductase reaction precedes cytidylyl transfer with release of the metabolic intermediate D-ribitol 5-phosphate. The lag in the appearance of CDP-ribitol lasts more than 50 s and contrasts with the rapid approach to steady state for the disappearance of NADPH. This is consistent with the need for accumulation of K_m levels of D-ribitol 5-phosphate to drive the cytidylyltransferase reaction and is evident in the calculated time course for D-ribitol 5-phosphate (200 s to reach 120 μM). In fact, the calculated time courses closely approximate those measured experimentally and provide further support for the model of reduction before cytidylyl transfer in distinct active sites without channeling.

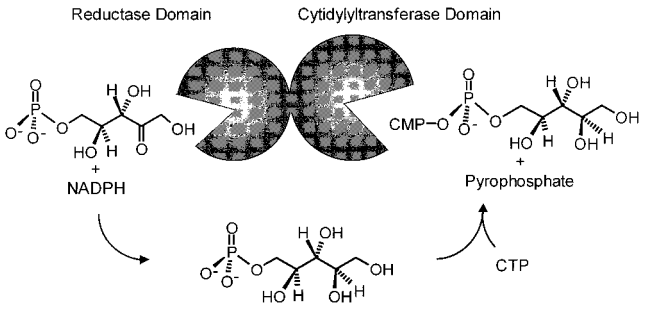
DISCUSSION

CDP-ribitol synthase was recently shown to be bifunctional, catalyzing both reductase and cytidylyltransferase reactions in the transformation of D-ribulose 5-phosphate to CDP-ribitol (*1*). In this work, we present kinetic evidence that establishes that the order imposed by the bifunctional enzyme is not random, but that Bcs1 first catalyzes the reduction of D-ribulose 5-phosphate and subsequently cytidylyl transfer to D-ribitol 5-phosphate to produce CDP-ribitol. Steady state kinetic experiments indicated a more than 650-fold kinetic preference of D-ribitol 5-phosphate over D-ribulose 5-phosphate for cytidylyl transfer. Further, rapid mixing experiments in the presence of saturating amounts of all substrates demonstrated rapid steady state oxidation of NADPH with a significant lag in the approach to steady state cytidylyl transfer.

We have also shown that reduction of D-ribulose 5-phosphate and cytidylyl transfer to D-ribitol 5-phosphate occur in distinct active sites. With the C-terminal sequence being similar to that of short chain dehydrogenase/reductase proteins and the N-terminal sequence being similar to that of nucleotidyltransferases (*1*), we targeted signature motifs in the putative reductase (*20*) and nucleotidyltransferase (*21*) active sites to create the variants K386A and R18A, respectively. The K386A variant was largely impaired for reduction of D-ribulose 5-phosphate with little effect on cytidylyl transfer, and the R18A variant was principally inactivated with respect to cytidylyl transfer without an impact on reduction. These findings are consistent with the conclusion that Bcs1 contains distinct and chiefly independent reductase and cytidylyltransferase folds that fall into existing short chain dehydrogenase/reductase (*20*) and nucleotidyl transfer (*21*) protein families.

Experiments in this work also indicate that the metabolic intermediate D-ribitol 5-phosphate is released without substrate channeling. Substrate channeling, the process of direct transfer of an intermediate between active sites that catalyze sequential reactions in a biosynthetic pathway, is thought to be advantageous physiologically where the intermediate (i) is chemically labile, (ii) can react in competing enzymatic reactions, or (iii) reacts with a diffusion-controlled rate constant (*10*−*12*). Further, metabolite channeling is also

Scheme 3: Two-Active Site Model for Catalysis by Bcs1



thought to provide kinetic advantages unrelated to the limits of diffusion in the achievement of steady state turnover in multienzyme systems (*22*). The metabolite D-ribitol 5-phosphate is not especially labile, might well be a substrate for other bacterial enzymes, and does not undergo cytidylyl transfer with a diffusion-controlled rate constant (k_{cat}/K_m from this work = 1.0×10^5 M^{-1} s^{-1}). We found that an equimolar mixture of reductase-impaired (K386A) and cytidylyl transfer-impaired (R18A) variants was capable of efficient catalysis of the overall two-step reaction. That finding is consistent with bifunctional turnover in distinct active sites of Bcs1 with the release and free diffusion of the metabolic intermediate D-ribitol 5-phosphate (Scheme 3). In a unique but related strategy, Geck and Kirsch (*23*) excluded substrate channeling between the enzymes aspartate aminotransferase (AAT) and malate dehydrogenase after observing efficient two-step catalysis for these enzymes in the presence of a large excess of inactive variants of AAT. To our knowledge, the approach used in this work on CDP-ribitol synthase is the first of its kind in addressing the question of substrate channeling in a bifunctional protein.

The sugar substrate specificity for both reduction and cytidylyl transfer by Bcs1 has been probed in this work with a variety of compounds. The reductase reaction, which was highly specific for D-ribulose 5-phosphate, tolerated some pentose phosphates, the aldotetrose phosphate, D-erythrose 4-phosphate, and D-ribulose. The enzyme, however, demonstrated no reductase activity with six carbon sugars. An important issue in these specificity studies is the composition of the sugar substrates, cyclic or acylic. Many monosaccharides exist in several tautomeric forms in solution at equilibrium, and knowledge of the relative abundance of these forms is necessary to fully understand their enzymatic reactivities. The alternate substrates, aldopentose phosphates and D-ribulose, tested in this work exist predominately in the cyclic furanose form, while the substrate D-ribulose 5-phosphate is exclusively an acyclic molecule (*24*−*26*). It is tempting to suggest that Bcs1 has a preference for acyclic substrates since the specificity constants (k_{cat}/K_m) for the predominantly cyclic alternative substrates were at least 100-fold lower than that for D-ribulose 5-phosphate. Further, the predominantly acyclic D-erythrose 4-phosphate (*24*) was only 20-fold reduced relative to D-ribulose 5-phosphate. The stereochemistry at C-3 was also shown to be a key determinant since the predominantly acyclic D-xylulose 5-phosphate was more than 290-fold reduced. In the cytidylyltransferase reaction, a strong preference was demonstrated for the alditol substrate, D-ribitol 5-phosphate, over all aldose and ketose phosphate substrates in cyclic and acyclic forms. No activity could be demonstrated with

D-ribitol, indicating a requirement for the phosphate group in cytidylyl transfer.

Bifunctional enzymes are thought to be distinctive and highly conserved products of relatively infrequent gene fusion events that link two proteins with separate yet related functions (*13*, *14*). The selective pressure causing certain genes to become fused over the course of evolution is thought to be due to particular advantages of integrating protein functions in a common metabolic pathway that include those offered by substrate channeling (*10*, *11*). The experiments described in this work are consistent with a two-active site model for Bcs1 where the two functional domains catalyze independent reactions but are tethered together into one polypeptide chain. The logic behind the fusion of these functions therefore remains elusive but may have its origins in genetic or biochemical mechanisms of regulation of the functions of CDP-ribitol synthase.

Experiments aimed at a fundamental understanding of the reactions catalyzed by the Bcs1 protein will be important to a complete understanding of the enzyme's role in surface carbohydrate biosynthesis in bacteria. In particular, little mechanistic information exists regarding the nucleotidyltransferase reactions catalyzed by proteins in the family, including CDP-ribitol synthase and the GlmU protein of *E. coli*, for which high-resolution structural information is currently available (*21*). With the understanding that distinct domains of CDP-ribitol synthase are responsible for reduction and cytidylyl transfer, future work may concentrate on the fine points of these respective functions in isolation.

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