BIFUNCTIONAL CDP-RIBITOL SYNTHASE FROM *H. INFLUENZAE*

REDUCTION PRECEDES CYTIDYLYLTRANSFER WITHOUT SUBSTRATE CHANNELING IN DISTINCT ACTIVE SITES OF THE BIFUNCTIONAL CDP-RIBITOL SYNTHASE FROM *HAEMOPHILUS INFLUENZAE*

By

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TITLE: Reduction precedes cytidylyltransfer 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* type a and b (Follens *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 cytidylyltransfer to D-ribitol 5-phosphate. Steady state kinetic measurements revealed a 650-fold kinetic preference for cytidylyltransfer 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 cytidylyltransfer 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 cytidylyltransfer, 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, release and free diffusion of the metabolic intermeditate D-ribitol 5-phosphate followed by cytidylyltransfer.

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❖ TABLE OF CONTENTS:

❖ LIST OF TABLES:

❖ LIST OF FIGURES:

❖ LIST OF SCHEMES:

❖ LIST OF APPENDICIES:

❖ PREFACE:

This thesis has been prepared for the purpose of publication for the journal *Biochemistry* under the authorship of Michela Zolli, Daniel J. Kobric and Eric D. Brown (*Biochemistry*. 2001; 40(16):5041-8). The clones used in this work (pET22b-*bcs1* and pET22b-3'-*bcs1*) were created by a project student at the time, Daniel J. Kobric. Positive ion electrospray mass spectrometry was performed by Kirk Greene (McMaster University) and NMR was performed by Don Hughes (McMaster University). All other research not mentioned above was performed exclusively by Michela Zolli. The manuscript was originally written by Michela Zolli and edited by Eric D. Brown.

❖ CHAPTER 1:

CDP-RIBITOL SYNTHASE: A MODEL FOR ENZYMES CATALYZING THE FORMATION OF NUCLEOTIDE ACTIVATED D-RIBITOL 5-PHSOPHATE PRECURSORS TO BACTERIAL POLYSACCHARIDE BIOSYNTHESIS SUCH AS *STAPHYLOCCOCUS AUREUS* AND *HAEMOPHILUS INFLUENZAE*

1.1 BACKGROUND:

1.1.1 *Staphylococcus aureus* Resistance in the Clinical Setting:

Gram positive microorganisms of the *Staphylococcus* species are known to be one of the major causes of infections in humans occurring in both the community and hospital setting in Canada, Europe and United States (Hryniewicz 1999; Tenover and Gaynes 2000, Perl and Golub 1998, Archer 1998). Every year, several hundred thousand patients are hospitalized in the United States and Canada due to serious infections caused by *Staphylococcus aureus* (Emori and Gaynes 1993; Tenover and Gaynes 2000, Perl and Golub, 1998). Of these hospitalized cases, it is estimated that approximately 60 000 to 80 000 infected patients die every year of *Staphylococcus aureus* infections. The wide spectrum of infections and syndromes caused by *Staphylococcus aureus* include abscesses, bacteremia, central nervous system infections, endocarditis, osteomyelitis, pneumonia, urinary tract infections, bullous impetigo, food poisoning, scalded skin syndrome and toxic shock syndrome (Emori and Gaynes 1993; Lowy 2000; Tenover and Gaynes 2000).

Treatment of infections caused by *S. aureus* usually involves the administration of antibiotics. However the abundant use of antibiotics has been correlated with the spread of antibiotic resistance of *S. aureus* (Albrich, Angstwurm et al. 1999; Hryniewicz 1999; Kresken and Hafner 1999; Tenover and Gaynes 2000). For example, in the 1940s, almost all strains of *S. aureus* were susceptible to penicillin which became the antibiotic treatment for that time period. Presently, almost 99% of all strains of *S. aureus* are resistant to penicillin (Dennesen et al. 1998, Hryniewicz 1999). In the 1960s, following the introduction of another antibiotic, methicillin, a methicillin resistant strain of *S. aureus* (MRSA) had emerged (Hryniewicz 1999). Since then many reports have consistently shown that MRSA prevalence in the hospital setting infections in the United States has increased from ~5% in 1981, to ~14% in 1987 and to over 40% today (Hryniewicz 1999; Lister 2000; Tenover and Gaynes 2000). More recently, the immediate concern of many in the medical community has been focused on the emergence of a strain of *S. aureus* that is resistant to all clinically used antibiotics, including vancomycin which is used as a last resort to control the spread of MRSA in health care settings (Hryniewicz 1999; Smith, Pearson et al. 1999; Tenover and Gaynes 2000). Although only clinical isolates of *S. aureus* with intermediate resistance to vancomycin has been demonstrated to date, the spread of a completely resistant strain of *S. aureus* would have catastrophic effects in the clinical environment to control the spread of MRSA (Tenover, Lancaster et al. 1998; Hryniewicz 1999; Smith, Pearson et al. 1999; Tenover and Gaynes 2000).

This clinical significance of *S. aureus* infections has demonstrated the immediate need to further study and understand the molecular mechanisms of *S. aureus* in order to determine novel therapeutic target sites for structure-based drug design of potential antimicrobial agents. This thesis focuses on the enzymes that catalyze the formation of nucleotide activated precursors used in cell wall biosynthesis as a novel therapeutic target site.

1.2 The Bacterial Cell Wall – Gram Positive versus Gram Negative

The bacterial cell wall is an essential structure that is intimately involved in cell growth and morphogenesis, cell division, genome segregation, interaction between the cell and its environment and movement of materials into and out of the cell (Archibald, Hancock et al. 1993; Brock, Madigan et al. 1994). The cell wall consists of polymers of carbohydrate and amino acids that form rigid, cross-linked structures that are critical to these important functions of the cell (Archibald, Hancock et al. 1993; Brock, Madigan et al. 1994). The uniqueness and indispensability of the cell wall to bacteria implicates this structure and its biosynthetic machinery as a potential therapeutic target for antimicrobial agents.

Two major groups of bacterial cell walls are Gram positive and Gram negative. Both types of bacterial cell walls primarily consist of a carbohydrate-based polymer of *N*-acetylmuramic acid-pentapeptide-β(1-4)-*N*-acetylglucosamine, the peptidoglycan layer, that is cross-linked to its peptide structures on the cell membrane. Gram negative bacteria, such as *Haemophilus influenzae*, contain a less substantial peptidoglycan layer

than Gram positive bacteria that is further surrounded by a lipopolysaccharide and protein layer referred to as the outer membrane (Archibald, Hancock et al. 1993). Gram positive bacteria do not have a second lipid bilayer structure surrounding the peptidoglycan layer as found in Gram negative bacteria. However the peptidoglycan layer of Gram positive bacteria, like *Staphylococcus aureus*, is more abundant than in Gram negative bacteria and is covalently attached to large amounts of the polymer teichoic acid (Brock, Madigan et al. 1994). Teichoic acids are diverse groups of hydrophilic, anionic polymers of polyols and/or sugars approximately 20-40 residues long. Most of the Gram positive bacteria studied have teichoic acids consisting of either poly(glycerol 3-phosphate) or poly(ribitol 5-phosphate) (Pooley and Karamata 1994). The teichoic acid layer of *Staphylococcus aureus* consists of poly(ribitol-5-phosphate) (Armstrong, Baddiley et al. 1958; Morse 1962; Nagel, Sheagren et al. 1977; Archibald, Hancock et al. 1993).

1.3 The Teichoic Acid Layer of *Staphylococcus Aureus*:

1.3.1 Teichoic Acid Structure:

The teichoic acid polymer is anchored to the cell wall by covalent attachment via a phosphodiester bond between the linkage unit of the teichoic acid and the 6-hydroxyl of the *N*-acetylmuramic acid of the peptidoglycan layer (Figure 1). The composition of the teichoic acid linkage unit is conserved in Gram positive bacteria consisting of a hexosamine-containing disaccharide attached to a series of one to three glycerol phosphate units (Archibald, Hancock et al. 1993; Pooley and Karamata 1994). In *S. aureus*, this linkage unit consists of a three glycerolphosphate residues attached to *N*-

acetylmannosaminyl-*N*-acetylglucosaminyl-phosphate. The remaining linear chain consists of 1,3-linked poly(glycerol phosphate) or 1,5-linked poly(ribitol phosphate) that may contain various substituents at the 2-position of glycerol or the 2-, 3- or 4- positions of ribitol (Archibald, Hancock et al. 1993; Pooley and Karamata 1994). Teichoic acid from *S. aureus* consists of ribitol phosphate polymers with D-alanyl ester substitutions at C-2 and β-glycosylated at C-4 (Archibald, Hancock et al. 1993).

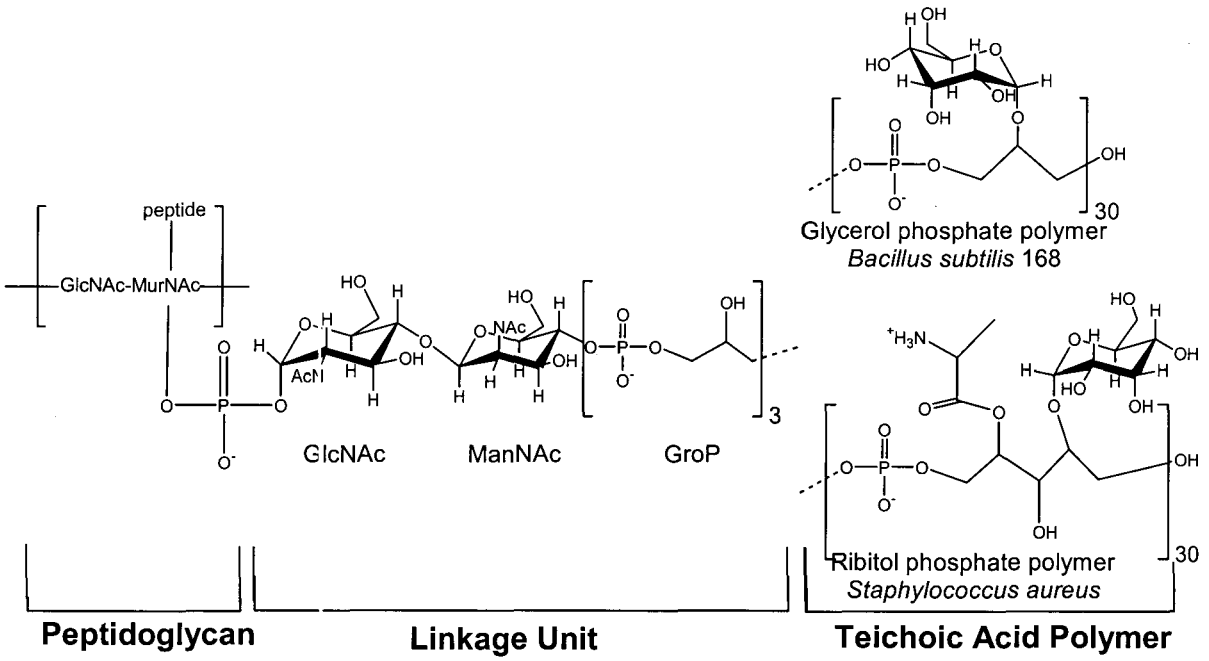
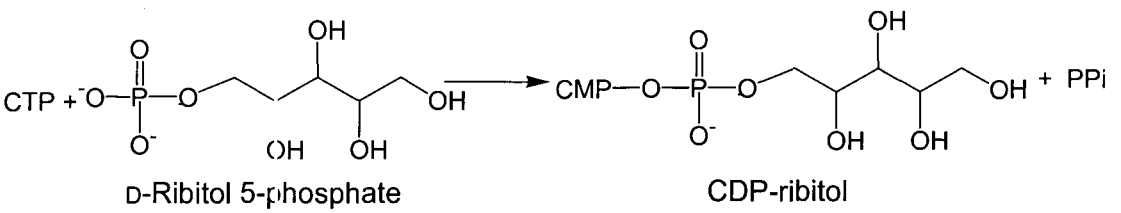


Figure 1: Teichoic Acid Structure of *B. subtilis* and *S. aureus*.
Teichoic acids are linked to the peptidoglycan layer through a phosphodiester bond between 6-hydroxyl of the *N*-acetylmuramic acid of the peptidoglycan layer and the linkage unit of the teichoic acid. The conserved linkage unit consists of a disaccharide and three glycerol 3-phosphate (GroP) units. The repeating polyol consists of either glucosylated D-ribitol 5-phosphate in *S. aureus* or glycerol 3-phosphate in *B. subtilis*.

1.3.2 Teichoic Acid Biosynthesis:

Teichoic acid biosynthesis of the ribitol teichoic acid in *S. aureus* is illustrated in Figure 2. This process can be categorized into three groups of reactions. First, the linkage unit is assembled onto a undecaprenolphosphate lipid anchor (Yokoyama, Mizuguchi et al. 1989; Archibald, Hancock et al. 1993). Second, polymerization and glucosylation reactions occur whereby nucleotide activated monomers synthesized in the cytoplasm are added onto the linkage unit that make up the teichoic acid polymer (Glaser and Burger 1964; Archibald, Hancock et al. 1993). In *S. aureus*, CDP-ribitol is the nucleotide activated monomer which is thought to be formed from CTP and D-ribitol 5-phosphate (Figure 2 and Scheme 1) in a cytidylyltransferase reaction (Glaser 1964; Kroll and Moxon 1988; Archibald, Hancock et al. 1993). However, an enzyme catalyzing this reaction has not been identified in *S. aureus* to date. The final step in biosyntheis occurs when the teichoic acids are exported and transferred from the prenol group to the peptidoglycan layer (Tenover, Lancaster et al. 1998).

Scheme 1: CDP-ribitol Synthesis Reaction



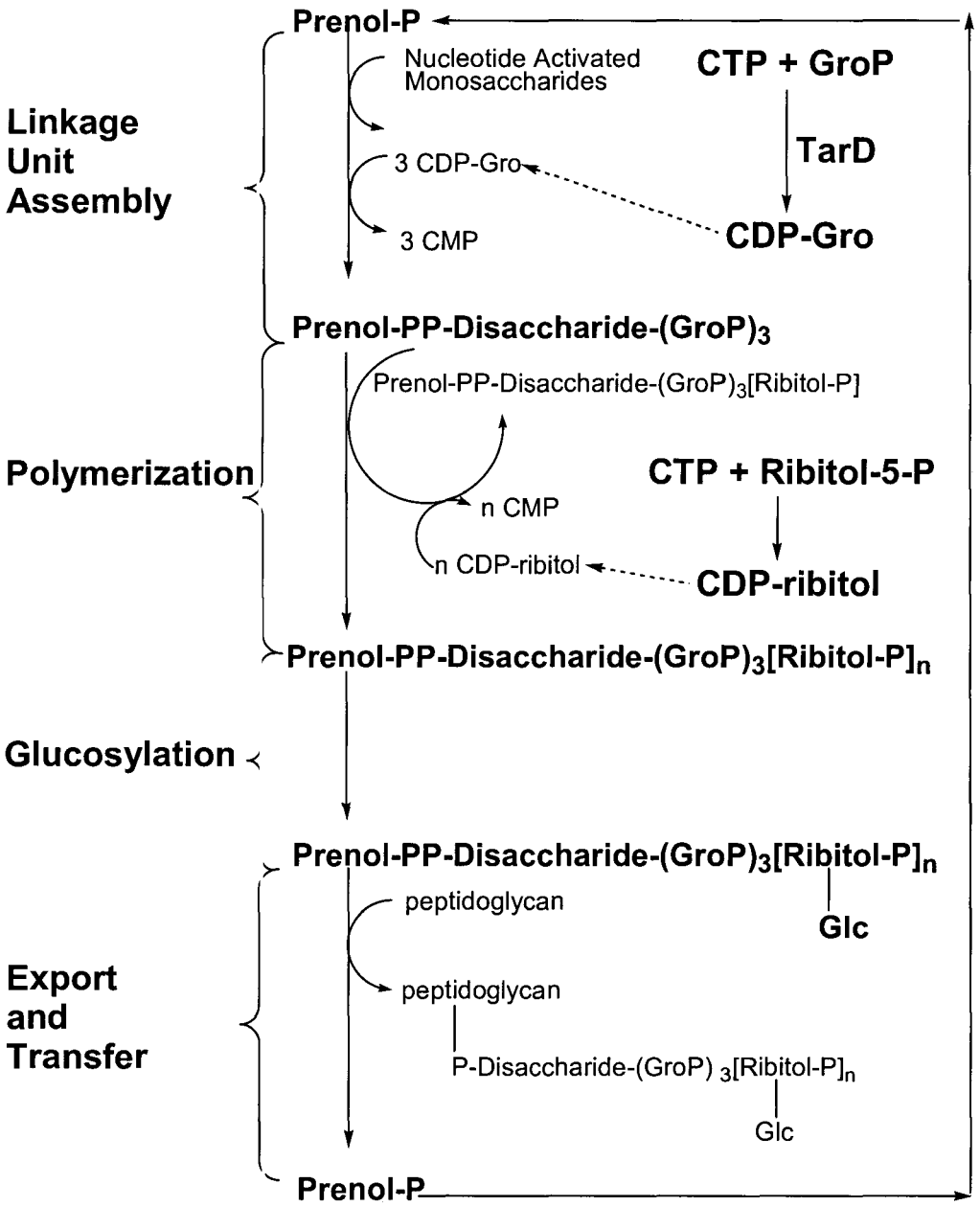


Figure 2: Proposed Teichoic Acid Biosynthesis in *S. aureus*
Adapted from teichoic acid biosynthesis in *B. subtilis* 168. Note that the polymer consists of a chain of D-ribitol 5-phosphate in *S. aureus* formed from the nucleotide activated precursor CDP-ribitol. The enzyme catalyzing the formation of CDP-ribitol has not been determined to date but the pyrophosphorylase reaction to form CDP-ribitol from CTP and ribitol has been shown in *S. aureus* crude cell lysate. (Archibald, Hancock et al. 1993; Glaser 1964)

1.4 *HAEMOPHILUS INFLUENZAE* CDP-RIBITOL PYROPHOSPHORYLASE MODEL FOR CDP-RIBITOL FORMATION IN *S. AUREUS*:

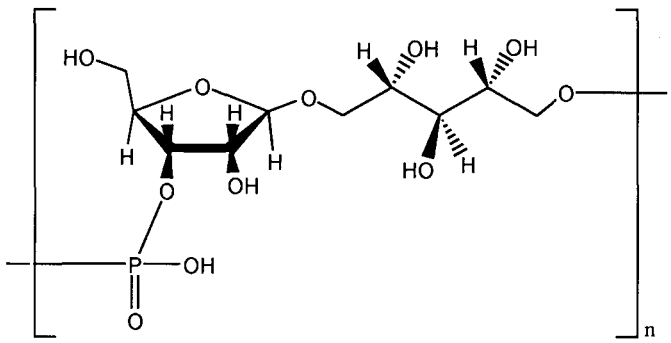
To further study teichoic acid biosynthesis in *S. aureus*, the goal of this project was to further investigate an enzyme model that could potentially catalyze the formation of CDP-ribitol through a cytidyltransferase reaction. Although a specific enzyme catalyzing this reaction in *S. aureus* has not been previously identified, a hypothetical CDP-ribitol pyrophosphorylase (named for the reverse reaction) was identified on the public database[i]. This hypothetical protein was found in the gene cluster involved in capsule biosynthesis of types a and b *Haemophilus influenzae* (Kroll, Zamze et al. 1989). As will be discussed in the ensuing sections, the poly(ribitol)-phosphate polymer of *S. aureus* teichoic acids bears resemblance to the structure of the capsule polymer of the Gram negative bacteria *H. influenzae* types a and b, and are thought to have some similarities in their biosynthetic pathways (Argaman 1973; Whitfield and Valvano 1993).

1.4.1 *Haemophilus influenzae* Serotypes and Pathogenicity:

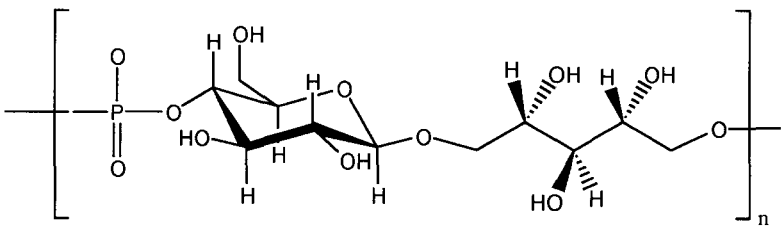
Haemophilus influenzae is a Gram negative rod that cause serious life-threatening invasive infections, such as meningitis, especially in children (Kroll and Moxon 1988; Moxon 1992; Follens, Veiga-da-Cunha et al. 1999; Santosham 2000). Although *H. influenzae* does not have a teichoic acid layer, its pathogenicity is contributed by a capsular polysaccharide that consists of polymers of repeating units of one to several different saccharides (Moxon and Kroll 1990). There are six different

[i] Public database searched December 1998.

encapsulated serotypes of *H. influenzae*, labeled a to f (Pittman 1931), where types a and b contain of D-ribitol 5-phosphate in their polymers (Figure 3 (Egan, Schneerson et al. 1982)). Before the widespread use of a vaccine for *H. influenzae* type b in the 1990s, it was the type b strain that contributed over 95% of all invasive infections caused by *H. influenzae* (Kroll, Hopkins et al. 1988; Kroll and Moxon 1988). However more recently, as the number of type b strains infections decrease due to type b vaccinations, there has been an increase in the number of infections caused by *H. influenzae* type a strains (Kroll, Moxon et al. 1994; Follens, Veiga-da-Cunha et al. 1999).



H. influenzae Type b capsular repeating units:
-3-[beta-D-ribose-(1-1)-D-ribitol-5-phosphate]



H. influenzae Type a capsular repeating units:
-4-[beta-D-glucose-(1-4)-D-ribitol-5-phosphate]

Figure 3: Structure of Capsule Polymers of *Haemophilus influenzae*: Type a (bottom) and Type b (top).

1.4.2 Structure and Synthesis of *H. influenzae* types a and b Capsule:

Investigations performed over three decades earlier demonstrated that the capsular polysaccharide of *H. influenzae* type b could serologically cross-react with extracts of the Gram positive bacteria *S. aureus* (Argaman 1973). This cross reactivity indicated the presence of a polyribitol-phosphate moiety in *H. influenzae* type b capsule that resembles the polyribitol-phosphate repeats in the teichoic acid polymer of *S. aureus* (Argaman 1973). More recently, the structure of the capsular polysaccharide of *H. influenzae* type b has been shown to consist of repeating units of 3-β-D-ribose-(1-1)-D-ribitol-5-phosphate while type a contains –4-β-D-glucose-(1-4)-D-ribitol-5-phosphate, as shown in Figure 3 (Egan, Schneerson et al. 1982). The repeating units of both types a and b capsule consists of D-ribitol 5-phosphate in its backbone connected by a phosphodiester bond with a 1,5 linkage that resembles the teichoic acid polymer of *S. aureus*. Further resembling teichoic acids of Gram positive bacteria, the type b capsular polysaccharide has been shown to be covalently anchored to the cell wall through a phosphodiester bridge. Although teichoic acids are linked to the peptidoglycan layer, the capsule polymer is hypothesized to be attached to a phospholipid in the outer membrane (Kuo, Doelling et al. 1985; Whitfield and Valvano 1993; Rick and Silver 1996).

Although little is known about capsule biosynthesis in *H. influenzae*, this complex process also slightly resembles the biosynthesis of teichoic acids. The formation of nucleotide activated precursors are used to successively transfer the monomers of the repeating units onto a large polymer that must be transported and anchored to the cell surface (Whitfield and Valvano 1993; Van Eldere, Brophy et al.

1995; Rick and Silver 1996; Follens, Veiga-da-Cunha et al. 1999). The first step in *H. influenzae* capsule biosynthesis involves the formation of nucleotide activated precursors in the cytoplasm (Whitfield and Valvano 1993; Van Eldere, Brophy et al. 1995; Rick and Silver 1996; Follens, Veiga-da-Cunha et al. 1999). In *H. influenzae* types a and b, the CDP-ribitol nucleotide activated precursor that is found in teichoic acid biosynthesis of *S. aureus*, has also been demonstrated to be the donor of D-ribitol 5-phosphate residues for capsular polysaccharide biosynthesis (Whitfield and Valvano 1993; Van Eldere, Brophy et al. 1995; Follens, Veiga-da-Cunha et al. 1999). Similar to the reaction in Gram positive bacteria, the formation of CDP-ribitol in *H. influenzae* types a and b is catalyzed by a CDP-ribitol pyrophosphorylase (also named for the reverse reaction) which transforms D-ribitol 5-phosphate into CDP-ribitol through a cytidylyltransfer (Van Eldere, Brophy et al. 1995). Following formation of the nucleotide activated precursors, the second step in capsule biosynthesis in *H. influenzae* involves polymerization of the growing capsular polysaccharide chain while attached through a phosphodiester linkage to a diglyceride anchor (Whitfield and Valvano 1993; Van Eldere, Brophy et al. 1995). In the final stage of capsule biosynthesis, the polysaccharide polymer is exported to the cell surface, which in the Gram negative bacteria, involves traversing the cytoplasmic membrane, periplasm and outer membrane (Whitfield and Valvano 1993; Rick and Silver 1996).

1.4.3 Capsule Biosynthetic Genes of *H. influenzae* Type a and b:

The chromosomal capsulation locus (*cap*) in *H. influenzae* contains the genetic information that is essential for the expression for the capsular polysaccharide and can be functionally divided into three distinct regions (Hoiseth, Moxon et al. 1986; Kroll and Moxon 1988; Van Eldere, Brophy et al. 1995; Follens, Veiga-da-Cunha et al. 1999). The biosynthetic genes are proposed to be found in the central serotype-specific region, referred to as Region II, which is flanked on both sides by serotype-non-specific regions that contain genes functioning in the more general aspect of capsule biosynthesis, called Regions I and III (Whitfield and Valvano 1993; Van Eldere, Brophy et al. 1995; Rick and Silver 1996; Follens, Veiga-da-Cunha et al. 1999). Region I contains four open reading frames, named *bexDCBA*, which encode an ATP-driven polysaccharide export apparatus (Kroll, Hopkins et al. 1988; Van Eldere, Brophy et al. 1995; Follens, Veiga-da-Cunha et al. 1999). The function of region III has not yet been determined but it is hypothesized to be involved in postpolymerization events (Kroll, Hopkins et al. 1988; Rick and Silver 1996; Follens, Veiga-da-Cunha et al. 1999). The genes in Region II contain information for specific sugar synthesis, activation and polymerization functions. Therefore this region is different in each of the serotypes in *H. influenzae* (Whitfield and Valvano 1993; Van Eldere, Brophy et al. 1995; Rick and Silver 1996; Follens, Veiga-da-Cunha et al. 1999). In types a and b, Region II consists of four open reading frames (*orf*) whereby *orf1* and *orf3* that have been shown to be essential for capsular polysaccharide biosynthesis (Van Eldere, Brophy et al. 1995; Follens, Veiga-da-Cunha et al. 1999). Of these four open reading frames, *orf3* and *orf4* are dissimilar between *H. influenzae* types

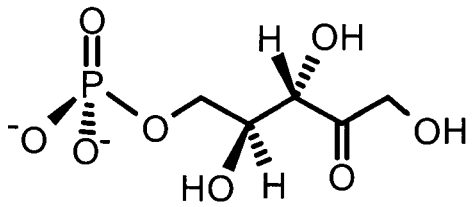
a and b, however, the first two open reading frames, *orf1* and *orf2*, revealed 96% and 67% homology, respectively, in amino acid sequence between the two serotypes (Follens, Veiga-da-Cunha et al. 1999). Although little is known about the function of the last three open reading frames of Region II, *orf1* from *H. influenzae* type a has been shown to encode a bifunctional protein that catalyzes the formation of CDP-ribitol which was later called Acs1 (named after type **a** **c**apsule **s**ynthesis *orf1*) (Follens, Veiga-da-Cunha et al. 1999).

1.4.4 Acs1 – A CDP-Ribitol Synthase:

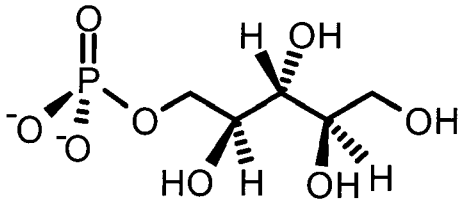
Acs1 consists of 474 amino acid residues and has a molecular mass of approximately 53 kDa. Alignments with the amino acid sequence of Acs1 against the public databases has revealed two distinct regions of the protein. The *C*-terminal region of Acs1 was found to be approximately 25% identical to members of the short-chain dehydrogenase/reductase (SDR) protein family (Follens, Veiga-da-Cunha et al. 1999). This homology was restricted to the 230 *C*-terminal residues of Acs1 and encompassed conserved SDR sequences including an active site motif ($Y^{382}XXXK^{386}$) as well as the nucleotide binding site motif ($G^{261}XXXGIG^{267}$) (Jornvall, Persson et al. 1995; Follens, Veiga-da-Cunha et al. 1999). The *N*-terminal region showed 21-33% homology to a number of unknown proteins as well as several less well-characterized sugar-phosphate nucleotidyltransferases, including *N*-acetylglucosamine 1-phosphate uridylyltransferase (GlmU1) from *Escherichia coli* (Follens, Veiga-da-Cunha et al. 1999). This bifunctional nature of Acs1 was confirmed by Follens *et al* (1999) whereby both D-ribulose 5-

phosphate reductase and D-ribitol 5-phosphate cytidylyltransferase activities were demonstrated (Follens, Veiga-da-Cunha et al. 1999). These results were consistent with the reaction depicted in Scheme 2.

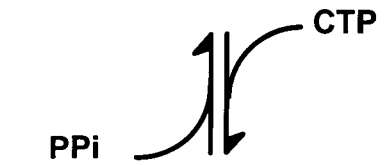
Scheme 2: Proposed Reaction for CDP-Ribitol Synthase

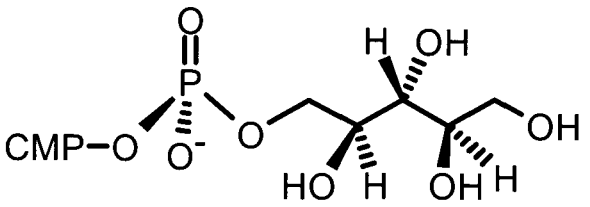


D-Ribulose 5-phosphate



D-Ribitol 5-phosphate





CDP-Ribtol

1.4.5 Bcs1 – A CDP-Ribitol Synthase Model:

The groundwork in expression, purification and basic functional characterization of the CDP-ribitol synthase from *H. influenzae* type a was accomplished by Follens *et al.* (Follens, Veiga-da-Cunha et al. 1999). The type b version of the CDP-ribitol synthase, or Bcs1 (type **b** capsule **s**ynthesis *orf1*), is the focus of this investigation. Protein sequences for CDP-ribitol synthases from *H. influenzae* types a and b demonstrate 96% sequence identity however currently have no convincing orthologues in the public databases. The CDP-ribitol synthase from *H. influenzae* therefore represents an available model for biochemical study with potential relevance to undiscovered enzymes that activate D-ribitol 5-phosphate for bacterial polysaccharide synthesis in organisms such as *S. aureus*.

The bifunctionality of *H. influenzae* CDP-ribitol synthase is consistent with sequence similarities evident in the primary structure. However, the bifunctionality raises mechanistic issues unique to bifunctional enzymes, including reaction order and substrate channeling. Substrate channeling is the process of direct transfer of an intermediate between active sites that catalyze sequential reactions in a biosynthetic pathway (Srere and Ovadi 1990; Miles, Rhee et al. 1999). Gene fusion events in evolution have been hypothesized to be driven by substrate channeling and could be a possibility for enzymes such as Bcs1. This thesis work presents kinetic studies to examine reaction order of the reductase and cytidylyltransferase reactions as well as address the possibility that substrate channeling occurs in this enzyme.

1.5 THESIS OBJECTIVES:

The specific objectives for this investigation of Bcs1, a CDP-ribitol synthase, involves the following:

i) Cloning of Bcs1 from *H. influenzae* type b into an overexpression vector (with and without a His_6-tag);

ii) Overexpression and purification of native and tagged forms of Bcs1 as well as mutant Bcs1 variants;

iii) Development of assays to monitor both reductase and cytidylyltransferase activities of Bcs1;

iv) Steady state kinetic characterization of Bcs1 (native and His_6-tagged forms) and comparing these values to the previously published steady state kinetic parameters defined by Follens *et al* (1999) for Acs1;

v) Determination of substrate specificity of Bcs1;

vi) Elucidation of the bifunctional reaction order of Bcs1 using kinetic data as well as examining the approach to steady state;

vii) Study to determine the release of a metabolic intermediate (ribitol-5-phosphate) and test the issue of substrate channeling.

Although Acs1 was previously studied by Follens *et al* (1999), these authors did not examine the issues of reaction order, release of an intermediate or an in-depth substrate specificity study. Comparisons of results from this investigation with Bcs1 and literature values previously determined for Acs1 (Follens, Veiga-da-Cunha et al. 1999) have been made where applicable.

❖ CHAPTER 2:
CLONING, OVEREXPRESSION AND PURIFICATION OF BCS1

2.1 MATERIALS:

Haemophilus influenzae type b was obtained from the Clinical Microbiology Laboratory at the Health Sciences Centre in McMaster University and grown on chocolate agar plates with enrichment (PML Microbiologicals, Wilsonville OR). Restriction enzymes, DNA ligase and VENT polymerase were from New England Biolabs (Beverly MA). Novablue supercompetent cells, pET22b overexpression vector and *Escherichia coli* BL21(DE3) overexpressing cells were obtained from Novagen (Madison CA). PCRscript subcloning kit, with vector and SrfI restriction enzyme was purchased from Stratagene (LaJolla CA). The following compounds were obtained from BioShop Ltd (Burlington ON): 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), ampicillin (Amp), imidazole and isopropyl β-thiogalactoside (IPTG). All other compounds and enzymes were obtained from Sigma-Aldrich (Oakville ON).

2.2 METHODS:

2.2.1 *Haemophilus influenzae* Growth and Genomic Preparation:

Haemophilus influenzae sample was streaked on Chocolate agar plates and allowed to grow overnight at 37°C. Colonies were collected by the addition of 2 mL Luria-Bertani (LB) media onto the plate and smearing the colonies to resuspend them into solution. These cells were pelleted by centrifugation at 5000 *g* for 5 minutes. Cells were resuspended in TE buffer (50 mM Tris (pH 8.0) and 50 mM EDTA). 250 μg of lysozyme in 0.25 M Tris (pH 8.0) was added to the solution and incubated on ice for 45 minutes. STEP solution (0.5% SDS, 50 mM Tris pH 7.4, 0.4 M EDTA and 1 mg/mL Proteinase K) was added to the solution and incubated at 50°C for 60 minutes. A phenol extraction was performed. 3 M sodium acetate (pH 5.2) was added to the solution followed by ice cold absolute ethanol. The chromosomal DNA was spooled, dried and resuspended in TE buffer with 0.2 μg/mL Rnase A. Genomic preparation was stored at –20°C.

2.2.2 Plasmid Construction and Subcloning:

The gene for CDP-ribitol synthase of *Haemophilus influenzae* type b (*bcs1*) gene was amplified from chromosomal DNA using the polymerase chain reaction (PCR). Amplification was performed using Vent DNA polymerase (New England Biolabs, Beverly MA) and the oligonucleotide primers with the following sequences: 5'-GGGCTTGGCATATGAATAAAAATAAAAACATAGGAATCAT-3' and 5'-AGCTGATGCGAGCTCTTATTTATAGAGATCAGCTAA-3'. The underlined

sequences denote the restriction sites for *NdeI*, which incorporates the start codon, and *SacI* which flanks the stop codon. This gene was also amplified using the first primer (with the *NdeI* site) and an alternate 3'-end primer with a *XhoI* site that removes the stop codon (5'-GGTGCTCGAGTCCT TTATAGAGATCAGCTAAAAT-3') in order to fuse this gene with a *C*-terminal His_6-tagged sequence in the pET22b vector (Novagen, Madison WI). The blunt ended PCR amplified DNA was inserted into the blunt cut *SrfI* site of PCRscript, a subcloning vector (Stratagene, LaJolla CA). The nucleotide sequence of the clone was confirmed by sequencing (MOBIX, McMaster University, Hamilton ON). The gene was excised from the PCRscript-*bcs1* vector by digesting it with *NdeI* and *SacI* (untagged version) or *NdeI* and *XhoI* (*C*-terminal His_6-tagged version) and ligated into *NdeI*, *SacI*- or *XhoI*-digested pET22b. The constructed plasmids were amplified in *E. coli* Novablues (Novagen, Madison CA), checked by restriction analysis and further confirmed by sequence analysis (MOBIX, McMaster University). The final plasmid constructs were transformed into *E.coli* BL21 (DE3) (Novagen) for protein overexpression, resulting in strains [BL21(DE3)/pET22b-*bcs1*] containing the native *bcs1* and [BL21(DE3)/pET22b-3'-*bcs1*] containing the *bcs1* with a *C*-terminal His_6-tag sequence.

2.2.3 Overexpression of the Recombinant Protein Bcs1 in *E. coli*:

Single colonies of *E. coli* BL21 (DE3) harbouring the pET*bcs1* plasmid were picked from cells plated on Luria-Bertani (LB) agar plates containing 50 μg/mL ampicillin and used to inoculate 10 mL cultures. The 10 mL cultures were grown

overnight at 30°C. These cells were then pelleted by centrifugation and resuspended in fresh LB-amp which were used to inoculate 1 L of LB with 50 μg/mL amp. 1 L cultures were grown at 30°C for 5 hours to an OD at 595 nm of ~0.8. The culture was then induced with IPTG to a final concentration of 1 mM. The induced cells were grown for an additional 16 hours at 23°C. The cells were harvested by centrifugation at 5000 x *g* for 10 minutes. The cell pellet was washed with ice cold 0.85% NaCl and further centrifuged at 5000 x *g* for 5 minutes. Cell pellets were stored at –20°C.

2.2.4 Enzyme Purification:

Native untagged Bcs1 was purified from the overproducing strain [BL21(DE3)/pET22b-*bcs1*] as described in the following procedure. All purification steps were performed at 4°C. The stored cell pellet (from a 2 L culture) was thawed on ice and resuspended in lysis buffer containing 50 mM HEPES (pH 8.0), 5 mM EDTA, 5 mM DTT, 20% glycerol, 0.1 mg DNase, 0.1 mg RNase and protease inhibitor cocktail Sigma #P8465 (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) and bestatin). The cells were disrupted by making four passes through the French press at 20 000 psi. The cell lysate was centrifuged at 20000 x *g* for 2 hours to remove the insoluble fraction, including cell debris and inclusion bodies. The supernatant was loaded onto a Q-Sepharose Fast Flow (Amersham-Pharmacia, Baie d'Urfé QB) column (2.7 cm x 13.5 cm) equilibrated with 25 mM HEPES (pH 8.0), 5 mM EDTA, 1 mM DTT and 10% Glycerol. The column was run 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. Bcs1 eluted off the column with approximately 80-100 mM NaCl present in the running buffer which was confirmed by 11.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (BioRad) at 200 volts and the gel was subsequently stained with Coomassie brilliant blue. The pooled fractions were dialyzed over 12 hours against 5 mM potassium phosphate (pH 6.2), 1 mM DTT and 10% glycerol and loaded onto a Macro-Prep ceramic hydroxyapatite Type I (BioRad) column (2.7 cm x 12.5 cm) equilibrated with 5 mM potassium phosphate (pH 6.2), 1 mM DTT and 10% glycerol. The column was run at a flow rate of 0.5 mL/min against a gradient of 10-500 mM potassium phosphate over 500 mL. Bcs1 eluted at two peaks, the least contaminated fractions (eluting at approximately 100 mM potassium phosphate) were pooled and used for further purification. The 75 mL pool was concentrated down to 9 mL using a Millipore concentrator in a table top centrifuge. The concentrated pool was loaded on a Superdex 200 (Amersham-Pharmacia, Baie d'Urfé QB) column (1.8 cm x 62 cm) equilibrated with 25 mM HEPES (pH 8.0),1 mM DTT and 50 mM NaCl. The column was run at a flow rate of 0.5 mL/min. The Bcs1 protein appears to elute in two separate peaks. These protein samples were concentrated and stored at –80°C.

The *C*-terminal His_6-tagged Bcs1 was purified from the overproducing strain [BL21(DE3)/pET22b-3'-*bcs1*] according to the following procedure. All purification steps were performed at 4°C. The stored cell pellet (from a 0.5 L culture) was thawed on ice and resuspended in lysis buffer containing 20 mM sodium phosphate pH 7.2, 500 mM NaCl, 5% glycerol (v/v), 0.1 mg DNase and 0.1 mg RNase. Note that no DTT or protease inhibitors were added. The cells were disrupted by making two passes through

the French press at 20 000 psi. The cell lysate was centrifuged at 20 000 x *g* for 2 hours to remove the insoluble fraction, including cell debris and inclusion bodies. The supernatant was run over a 1 mL Hi Trap Affinity Nickel Column (Amersham Pharmacia, Baie d'Urfé QB) equilibrated with 20 mM sodium phosphate pH 7.2 and 500 mM NaCl. After washing with 10 column volumes with the initial buffer at a flow rate of 0.5 mL/min, Bcs1 was eluted with 200 mM imidazole and was confirmed by 11.5% SDS-PAGE at 200 volts and stained with Coomassie brilliant blue. The eluted Bcs1 protein was then loaded on a Superdex 200 (Amersham-Pharmacia, Baie d'Urfé) column (1.8 cm x 62 cm) equilibrated with 25 mM HEPES (pH 8.0), 1 mM DTT, 50 mM NaCl and 10% Glycerol. The column was run at a flow rate of 0.5 mL/min. Eluted peaks were dialyzed for an additional 12 hours against 25 mM HEPES (pH 8.0), 1 mM DTT and 10% glycerol. The sample was then stored in aliquots at –80°C.

2.2.5 Protein Concentration:

Protein concentrations were determined using Bradford method with bovine gamma globulin as a standard (Bradford 1976). These concentrations were confirmed by a second method using ultra violet (UV) spectrophotometry. This second method involved denaturing the protein sample in 6M guanidinium hydrochloride and 0.02 M sodium phosphate at pH 6.5 and then measuring the absorbance at 280 nm. Protein concentrations were then determined from the absorbance reading using Beer's Law (*Absorbance = concentration x extinction coefficient x pathlength*). Extinction coefficient

calculations of the protein in 6 M guanidium hydrochloride were determined as outlined in Gill and von Hippel (Gill and von Hippel 1989).

2.3 RESULTS and DISCUSSION:

2.3.1 Overexpression and Purification of Bcs1:

Previous work on the CDP-ribitol synthase from *H. influenzae* type a, Acs1 (Follens, Veiga-da-Cunha et al. 1999), had established that low temperature expression of the native protein was necessary to overcome solubility difficulties evident at 37°C. In this study, the majority of the overexpressed Bcs1 was also found to be insoluble (90-95%) following a 5 hour induction at 37°C as depicted in lanes 1 and 3 of Figure 4. Instead, Bcs1 was purified from cell extracts that were induced for 16 hours at 23°C where solubility had increased to approximately 50% (Figure 4, lanes 2 and 4). The purification stages of the native Bcs1 protein using the protocol developed in this study are depicted in the SDS-polyacrylamide gel shown in Figure 5, including the supernatant, Q-sepharose pool, hydroxyapatite pool and the final purified protein sample. Modifications to this purification protocol from that previously used (Follens, Veiga-da-Cunha et al. 1999) included hydroxyapatite and gel filtration chromatography instead of the Blue Sepharose affinity column since this step proved ineffective in removing key contaminants.

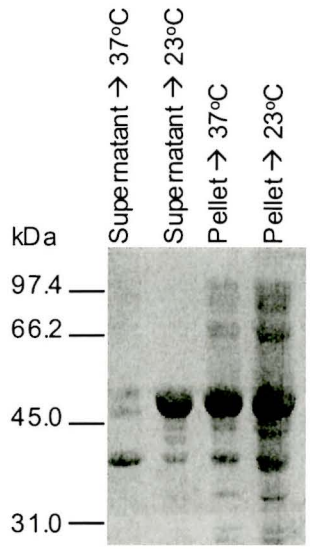


Figure 4: SDS-polyacrylamide gel (11.5% w/v) of solubility study of Bcs1. Shown are the supernatant from cultures grown at 37°C (lane 1) and 23°C (lane 2) as well as the pellet from cultures grown at 37°C (lane 3) and 23°C (lane 4). Note the presence of Bcs1 (~53 kDa) in the supernatant grown at 23°C (lane 2) but absent in the supernatant grown at 37°C (lane 1). Samples were denatured by boiling in Laemmli sample buffer containing 8% 2-mercaptoethanol prior to electrophoresis (Laemmli 1970).

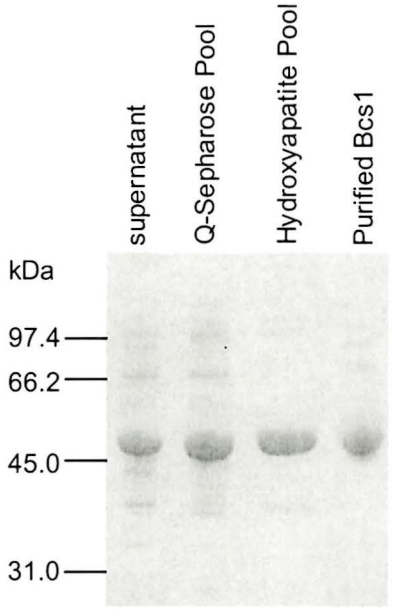


Figure 5: SDS-polyacrylamide gel (11.5% w/v) of the purification of native, untagged Bcs1.
Shown are : Supernatant (~40 μg), Q-Sepharose pool (~50 μg), Hydroxyapatite pool (~50 μg) and purified Bcs1 (~40 μg). Samples were denatured by boiling in Laemmli sample buffer (Laemmli 1970).

Table I presents the purification statistics for this Bcs1 purification protocol indicating approximately 12-fold purification of reductase activity and 4-fold purification of the cytidylyltransferase activity. This 3-fold lower cytidylyltransferase activity compared to the purification of reduction activity may be attributed to degradation of Bcs1 by proteases. Overall, the purification of the untagged, native Bcs1 protein resulted in low recovery of 0.4% (1.1 mg from 2 L fermentation preparation) that may be attributed to the significant proteolytic degradation of the protein despite the use of protease inhibitors in the initial purification steps.

Table 1: Purification Table of Native, Non-tagged Bcs1 from *E. coli* BL21 DE3/pET bcs1

Purification Step	Volume (mL)	Protein Concentration (mg/mL)	Specific Activity (units/mg)		Purification Fold		% Recovery
			Reductase Activity	CTase[a] Activity	Reductase Activity	CTase[a] Activity	
Supernatant	76.0	3.8	34	720	1.0	1.0	100
Hydroxyapatite Load	83.0	2.4	59	520	1.7	0.7	69
S-200 Load	9.0	13.0	91	500	2.7	0.7	41
Second S-200 Load	35.0	1.0	110	880	3.3	1.2	12
Final Bcs1 Pool	1.5	0.7	400	2900	12.9	4.0	0.4

[a]CTase: Cytidylyltransferase

To facilitate purification of Bcs1 in order to obtain higher yields of enzyme for this investigation, a *C*-terminal, His_6-tagged version of the protein was created and was purified in an efficient two step purification protocol. The reasonable quantities of His_6-

tagged Bcs1 purified resulted in an overall 20% recovered (or 15 mg of protein from a 0.5 L fermentation preparation) which demonstrates a 150-fold increase in the amount of Bcs1 recovered from the non-tagged purification protocol used previously. Figure 6 depicts an SDS-polyacrylamide gel of the overexpressed and purified untagged and His_6-tagged Bcs1 protein. The purified untagged Bcs1 protein appeared to have a contaminating degradative product (approximately 45 kDa lane 1 of Figure 6) that became progressively apparent when stored at 4°C and –20°C. However no degradative product was apparent in purified, untagged Bcs1 when stored at –80°C. The purified His_6-tagged Bcs1 protein samples were purified rapidly and stored at –80°C to prevent degradation from occurring.

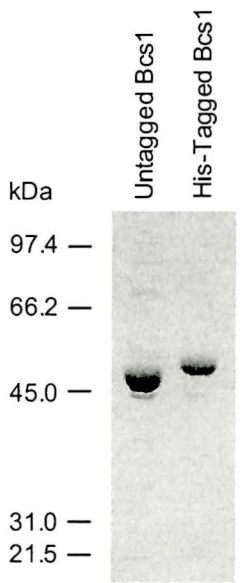


Figure 6: SDS-polyacrylamide gel (11.5% w/v) of purified native untagged and His_6-tagged Bcs1 variants used in this work.
Shown are: native Bcs1 with no His_6-tag (31 μg) and *C*-terminal His_6-tagged Bcs1 (22 μg). Samples were denatured by boiling in Laemmli sample buffer (Laemmli 1970). Apparent molecular weights were approximately 52 000 for native Bcs1 and 53 000 for His_6-tagged Bcs1.

2.4 SUMMARY:

The protocol developed for the purification of untagged Bcs1 resulted in extremely low yields of protein. To increase yield and efficiency of Bcs1 purification for this investigation, a *C*-terminal His_6-tagged Bcs1 protein was created and its purification demonstrated a 50-fold increase in yield compared to untagged Bcs1. Therefore, the *C*-terminal His_6-tagged Bcs1 protein was used in this investigation once steady state kinetic analysis of the tagged and untagged versions of Bcs1 were confirmed to be similar.

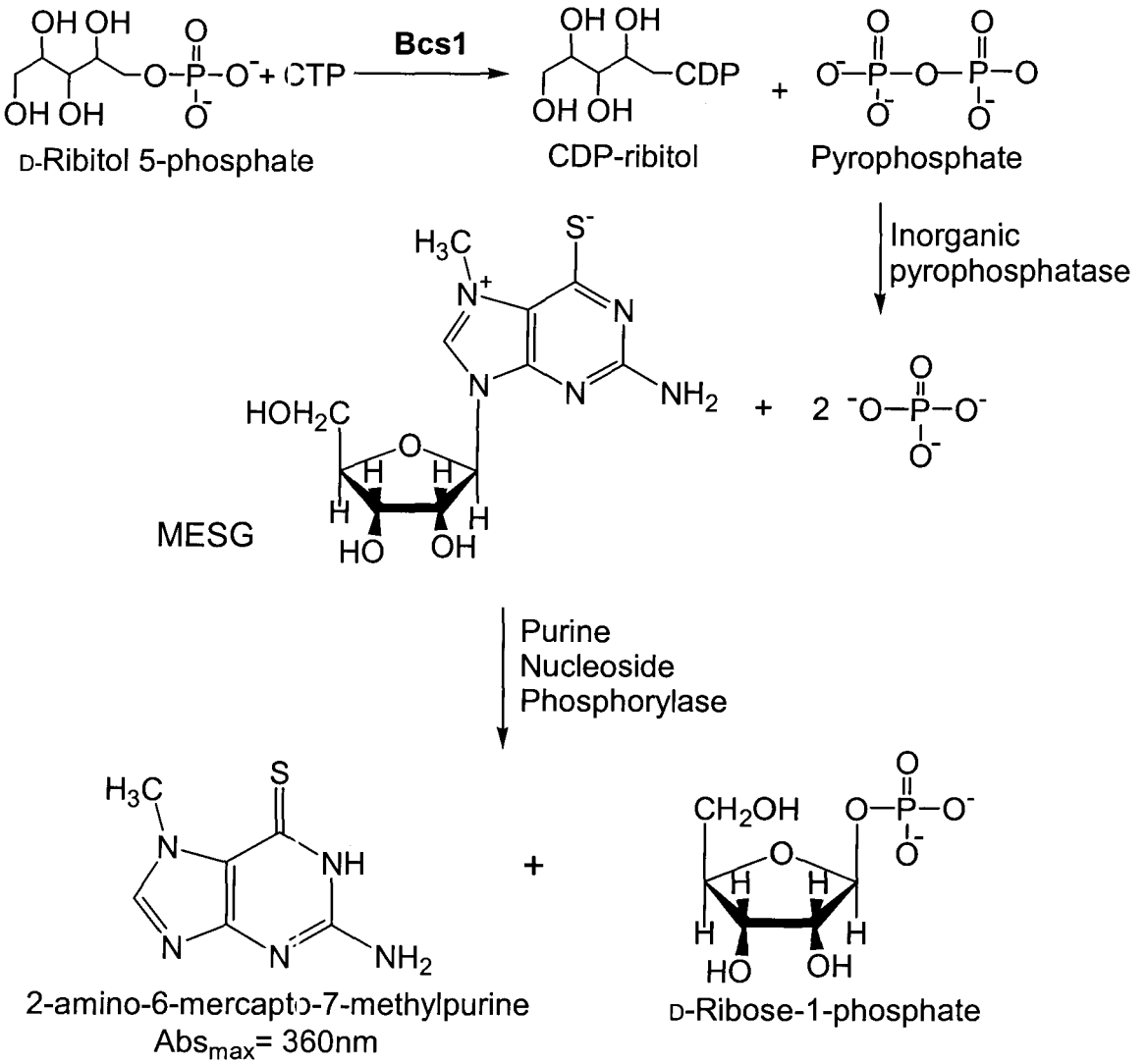
❖ CHAPTER 3: CDP-RIBITOL SYNTHASE SPECIFICITY AND REACTION ORDER

3.1 LITERATURE REVIEW:

3.1.1 Approach to Monitoring Activity:

The bifunctional reaction assumed to be catalyzed by Bcs1 is depicted in Scheme 2. To monitor the separate reactions catalyzed by the bifunctional Bcs1 enzyme two assays were used in this investigation. First, the reductase activity of Bcs1 was suspected to catalyze the reduction of D-ribulose 5-phosphate to form D-ribitol 5-phosphate (Scheme 2). This reaction was assayed continuously by monitoring the oxidation of NADPH to $NADP^+$ by measuring the decrease in absorbance at 340 nm. Second, the cytidylyltransferase activity of Bcs1 was expected to form CDP-ribitol and pyrophosphate from CTP and D-ribitol 5-phosphate (Scheme 2). Monitoring this reaction was possible using a coupled enzyme assay with MESG (2-amino-6-mercapto-7-methylpurine riboside) that continuously measured the formation of pyrophosphate as depicted in Scheme 3. As pyrophosphate is formed by the cytidylyltransferase reaction of Bcs1, it is cleaved by inorganic pyrophosphatase into two phosphates. These phosphate molecules are used by purine nucleoside phosphorylase (PRP) to convert MESG to D-ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine which can be monitored at an absorbance of 360 nm (Webb 1992; Upson, Haugland et al. 1996).

Scheme 3: MESG-PRPase/Pyrophosphatase Assay



3.2 MATERIALS:

The following compounds were obtained from BioShop Ltd (Burlington ON): 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT). [5-^{3}H]CTP was obtained from Amersham Pharmacia (Baie d'Urfé QB). All other compounds and enzymes were obtained from Sigma-Aldrich (Oakville ON). 2-amino-6-mercapto-7-methylpurine riboside (MESG) was obtained from Dr. P. Berti, Department of Chemistry, McMaster University.

3.3 METHODS:

3.3.1 Synthesis of D-Ribitol 5-phosphate:

D-Ribitol 5-phosphate was synthesized as previously described in Baddiley *et al* (1956) and Glaser (1964) (Baddiley, Buchanan et al. 1956; Glaser 1964). Briefly, the synthesis involved the incubation of 180 mg of D-ribose 5-phosphate and 23 mg $NaBH_4$ in 4 mL water at room temperature for 16 hours. Reaction was stopped with 300 µL glacial acetic acid and then the pH raised to 8-9 with 0.5 M ammonium hydroxide. To remove cations from the solution, the reaction mixture was passed through a Dowex 1X8-400 (Sigma-Aldrich, Oakville ON) strongly anionic exchanger column (1 cm x 13.5 cm) equilibrated with water The phosphorylated sugar(s) were eluted from the column using a 1 N ammonium formate solution. The eluted fractions were completely dried to crystals using a Speed Vac Concentrator (Savant, Holbrook NY) set on the lowest heat setting. To remove the traces of formate that still remaining in the product, the sample was

dissolved in approximately 50 mL of methanol and then evaporated to dryness on a rotovaporator – this was repeated 4 times. The dried product was resuspended in 50 mL of deionized water then allowed to evaporate on the Speed Vac at low heat. The product was resuspended in water and then added to a 50 mL solution containing 100 g of Dowex 50W X8-400 (Sigma-Aldrich, Oakville ON), an H^+ form resin. The mixture was stirred vigorously for 1 hour and vacuum filtered. The unbound compounds were collected and evaporated on the Speed Vac. The product was verified to be D-ribitol 5-phosphate by NMR (proton, carbon and phosphorus) performed by Don Hughes, Department of Chemistry at McMaster University, as well as by mass spectrometry performed by Kirk Green, Department of Chemistry at McMaster University.

3.3.2 Enzyme Assays:

D-Ribulose 5-phosphate reductase activity was assayed spectrophotometrically at an absorbance of 340 nm that continuously monitors the depletion 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. When NADPH or D-ribulose 5 phosphate were added to saturating concentration, their concentrations were at 500 µM and 600 µM, respectively. All assays proceeded at 25°C and were performed in triplicate.

Cytidylyltransferase activity was monitored in a continuous coupled assay for the detection of pyrophosphate. This assay was modified from the protocol previously described by Upson *et al* (Upson, Haugland et al. 1996) and will be referred here as the

MESG-PRPase/pyrophosphatase assay. Pyrophosphate production from the cytidylyltransferase reaction was monitored with excess inorganic pyrophosphatase (PPase) (Sigma-Aldrich), which hydrolyzes 1 equivalent of pyrophosphate to 2 equivalents of inorganic phosphate. Purine ribonucleoside phosphorylase (PRPase) (Sigma-Aldrich) present in the reaction uses the produced inorganic phosphate to catalyze the phosphorolysis of MESG with a resulting change in the extinction coefficient at 360 nm of 11.0 $mM^{-1}cm^{-1}$. 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 units PRPase and 5 units PPase. When CTP or D-ribitol 5-phosphate were added to saturating concentrations, their concentrations were at 500 μM and 600 μM, respectively. All reactions proceeded at 25°C and were performed in triplicate.

3.3.3 Steady State Kinetic Characterization:

K_m and k_{cat} values were determined by varying the substrate concentrations ranging from approximately 0.5 to 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, usually at concentrations between 5 and 10 times the K_m. The K_m and V_{max} values were determined by the GraFit 4 spreadsheet program (Erithacus Software, Surrey UK) using the linear, steady state enzymatic Michaelis-Menton equation which is defined as follows: $v_o = \frac{V_{max}[S]}{K_m + [S]}$, where S=substrate. The turnover rate, k_{cat}, was determined

using the equation: $k_{cat} = \frac{V_{max}}{[E]}$, where E=enzyme. All data was confirmed using Eadie-Hoftsee double reciprocal plots.

3.4 RESULTS:

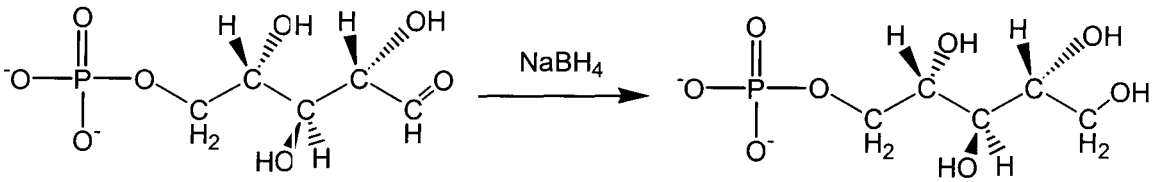
3.4.1 Assay Development:

Various assay conditions were assessed to ensure that Bcs1 reductase and cytidylyltransferase activities were effectively monitored. Control experiments were performed to ensure that coupled enzymes from the MESG-PRPase/ pyrophosphatase assay did not limit Bcs1 cytidylyltransferase activity (see Appendix I). Overall, assay conditions for both Bcs1 reductase and cytidylyltransferase reactions indicated that the presence of a stabilizer, such as 0.01 mg/mL BSA or 0.01% Triton X-100, was required for optimal Bcs1 activity (see Appendix II). Both Bcs1 reactions were also determined to be dependent on temperature with no activity at 37°C and optimal activity at 25°C. A linear relationship between the enzyme concentration and the rate of reaction was confirmed for both reductase and cytidylyltransferase activities using the defined assays (see Appendix III).

3.4.2 Synthesis of D-Ribitol 5-phosphate:

The D-ribitol 5-phosphate substrate used in these investigations was synthesized by reducing the aldehyde group of D-ribose 5-phosphate in the presence of sodium borohydride to give D-ribitol 5-phosphate, as summarized in Scheme 4.

Scheme 4: Synthesis of D-Ribitol 5-phosphate



D-ribose-5-phosphate

D-ribitol-5-phosphate

Purifying the synthesis product involved the removal of excess cations using a strongly anionic column, the removal of trace amounts of borate contaminating the product by washing with methanol followed by evaporation and the final removal of formate, by using a cationic (H^+ form) resin. Proton, carbon and phosphorous NMR confirmed the presence of D-ribitol 5-phosphate in D_2O: ^1H chemical shifts (and coupling constants, Hz) for proton 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; ^{13}C chemical shifts (and ^{13}C-^{31}P coupling constants, Hz) for carbon 1, 2, 3, and 4 were 66.9, 70.9 ($J_{c,p}$ = 6.7 Hz), 71.7, 72.2 and 62.5 ppm; ^{31}P chemical shift was 0.956 ppm (Don Hughes, McMaster University). Positive ion electrospray mass spectrophotometry also confirmed the mass of protonated D-ribitol 5-phosphate to be 232.1 (Kirk Green, McMaster University). Overall, the reaction seemed to have proceeded approximately 90% to completion with very little contamination.

3.4.3 Steady State Kinetic Characterization of Native and *C*-terminal His_6-tagged Bcs1:

Table 2 depicts the results of the steady state kinetic analysis of both reductase and cytidylyltransferase activities of native, non-tagged Bcs1 and *C*-terminal His_6-tagged Bcs1 compared with literature steady state kinetic values of Acs1. The kinetic parameters for the native Bcs1 and its His_6-tagged variant were similar in rates of reaction with slight deviations in K_m values with less than a two-fold difference in each case. These slight differences may be considered insignificant for the experimental objectives of this thesis. Therefore, the His_6-tagged version of Bcs1 was used for the remainder of this investigation unless otherwise noted.

Comparison of the Bcs1 enzymes (tagged and non-tagged) with the literature values of Acs1 presented by Follens *et al* (Follens, Veiga-da-Cunha et al. 1999) also demonstrated similar reaction rates with slight deviations in the K_m values. In Bcs1 and Acs1, the reductase reaction proceeds slightly faster than the cytidylyltransferase reaction with a turnover rate of 22 s^{-1} compared to 13 s^{-1}, respectively in His_6-tagged Bcs1, and a rate of 25 s^{-1} compared to 14 s^{-1}, respectively in Acs1. Follens *et al* (Follens, Veiga-da-Cunha et al. 1999) also previously described a stimulatory effect of CTP (100uM) on the reductase reaction that demonstrated a three-fold increase in k_{cat} and an eight-fold decrease in K_m. In this investigation, the inclusion of CTP and $MgCl_2$ (85 μM and 10 mM, respectively) decreased the noise in the assay and increased the turnover from 10 s^{-1} to 22 s^{-1} without a large effect on K_m (Table 2). This slight change in K_m and two-fold increase in k_{cat} was interpreted to mean that CTP increased the specific activity of the enzyme by stabilizing it in the assay system used in this investigation. Presumably this

Table 2: Steady State Kinetic Parameters Describing the Reactions Catalyzed by Native and His_6-Tagged Variants of Bcs1 Compared to Literature Values of Acs1(Follens, Veiga-da-Cunha et al. 1999)

	Native Bcs1	Tagged Bcs1	Acs1[a]
(1a) reductase (85μM CTP in reaction)			
k_{cat}[b] (s^{-1})	26 ± 1.2	22 ± 0.6	25
D-ribulose-5-phosphate			
K_m[b] (μM)	42 ± 3.6	76 ± 5.2	50
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	6.2	2.9	4.9
NADPH			
K_m[b] (μM)	25 ± 2.1	44 ± 4.8	10
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	11	5.0	25
(1b) reductase (no CTP present)			
k_{cat}[b] (s^{-1})	12 ± 1.8	10 ± 0.8	8.4
D-ribulose-5-phosphate			
K_m[b] (μM)	66 ± 6.1	120 ± 17	400
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	1.8	0.9	0.2
NADPH			
K_m[b] (μM)	29 ± 4.8	57 ± 9.3	10
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	1.8	1.5	8.4
(2) cytidylyltransferase			
k_{cat}[b] (s^{-1})	12 ± 0.9	13 ± 0.5	14
D-ribitol-5-phosphate			
K_m[b] (μM)	70 ± 5.7	130 ± 12	37
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	1.7	1.0	3.8
CTP			
K_m[b] (μM)	74 ± 7.2	99 ± 9.1	150
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	1.6	1.3	9.3

[a] Kinetic values for Acs1 were taken from Follens *et al.* (Follens, Veiga-da-Cunha et al. 1999)

[b] Errors are standard error of fit to data from three separate determinations.

stabilization occurs through a specific association with Bcs1 since the reductase reaction was not stimulated by the presence of 100 μM CDP, ATP, GTP or UTP in the presence of 10 mM $MgCl_2$.

3.4.4 Substrate Specificity and Reaction Order:

Table 3 depicts the results of steady state analysis of both reductase and cytidylyltransferase activities of *C*-terminal His_6-tagged Bcs1 with a variety of substrates. Positive ion electrospray mass spectrometry confirmed the identities of products of reduction (D-ribitol 5-phosphate, mass = 232.1) and cytidylyltransfer (CDP-ribitol, mass = 553.3) (Kirk Green, McMaster University) following purification by anion exchange HPLC using a triethylammonium bicarbonate gradient.

Of the substrates tested, the D-ribulose 5-phosphate and NADPH were the preferred substrates for the reductase reaction, with apparent K_m values of 76 μM and 44 μM, respectively. The reductase reaction was specific for NADPH and demonstrated little activity with NADH present (Table 3). Alternate sugar substrates were examined in the reductase assay and all tested substrates demonstrated a significantly lower turnover and higher K_m values (see Appendix IV for alternative sugar substrate structures). The highest specificity constant (k_{cat}/K_m) of the alternate sugar substrates tested was observed for the four carbon aldose D-erythrose 5-phosphate (0.13×10^5 $L \cdot mol^{-1} \cdot s^{-1}$), which demonstrated a 22-fold lower catalytic efficiency from that observed for D-ribulose 5-phosphate (2.9×10^5 $L \cdot mol^{-1} \cdot s^{-1}$). Pentose phosphate substrates with C-1 aldehyde groups, such as D-ribose 5-phosphate and D-arabinose 5-phosphate, were at least 5-fold

Table 3: Substrate Specificity of Reductase and Pyrophosphorylase (Cytidylyltransferase) Activities of Bcs1

	K_m [a] (μM)	k_{cat} [a] (s^{-1})	k_{cat}/K_m (x 10^{-5}) ($L \cdot mol^{-1} \cdot s^{-1}$)
(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 [b]	<0.010
NADPH	44 ± 4.8	22 ± 0.6	5.0
NADH	>600	0.064 [c]	<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 error of fit for 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.
[c] Value is not k_{cat}. This value is the turnover of CDP-ribitol synthase at a substrate concentration of 1 mM.

lower in turnover (k_{cat} values of 4.1 and 2.3 s^{-1}, respectively) and at least 30-fold lower in apparent substrate affinity (K_m values of 2.2 mM and 3.0 mM, respectively). Reductase activity was undetectable ($k_{cat} < 0.001$ 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-sulphate, D-glucose 6-phosphate, D-glucose 1-phosphate, D-glucose 6-sulphate, D-

glucose 1,6-diphosphate, D-mannose 6-phosphate, D-ribose 1-phosphate and D-ribulose 1,5-diphosphate. D-Xylulose 5-phosphate, a C-3 epimer of D-ribulose 5-phosphate, was shown to have at least a 6-fold lower reductase activity ($k_{cat} = 3.6\ s^{-1}$) 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 phosphorylated ketose D-ribulose 5-phosphate (k_{cat}/K_m were 0.033×10^5 and 2.9×10^5 $L \cdot mol^{-1} \cdot s^{-1}$, respectively). The reductase of Bcs1 on D-ribulose 5-phosphate was unaffected in the presence of 1 mM of the foregoing alternative substrates as well as 1 mM of D-ribitol 5-phosphate or $NADP^+$ in the reactions (see Appendix V).

In the cytidylyltransferase reaction, CTP and D-ribitol 5-phosphate were preferred substrates with apparent K_m values of 99 μM and 130 μM, respectively, and a turnover rate of 13 s^{-1} (Table 3). Cytidylyltransferase activity was specific for CTP since no activity was detected in the presence of ATP, GTP, UTP, ADP or CDP (turnover < 0.001 s^{-1} at 1 mM nucleotide di-/triphosphate). The Bcs1 protein showed a kinetic preference of more than 650-fold for cytidylyltransfer to D-ribitol 5-phosphate over D-ribulose 5-phosphate (k_{cat}/K_m were 1.0×10^5 and $<0.0015 \times 10^5$ $L \cdot mol^{-1} \cdot s^{-1}$, respectively), indicating that D-ribulose 5-phosphate is a very poor substrate for cytidylyltransfer and supporting the reaction order depicted in Scheme 2. Other aldose phosphate sugar substrates tested, D-erythrose 4-phosphate, D-galactose 6-phosphate and D-ribose 5-phosphate similarly demonstrated little cytidylyltransferase activity (Table 3). Cytidylyltransferase activity was undetectable ($k_{cat} << 0.001\ s^{-1}$ at 3.5 mM sugar) with the following substrates: D-fructose 6-phosphate, D-fructose 1,6-diphosphate, D-galactose 6-sulphate, D-glucose 6-

phosphate, D-glucose 1-phosphate, D-glucose 6-sulphate, 1,6-diphosphate, D-mannose 6-phosphate, D-ribulose 1,5-diphosphate, sorbitol-6-phosphate, D-ribitol and D-ribose 1-phosphate. The cytidylyltransferase reaction was unaffected by the presence of 1 mM D-ribulose 5-phosphate or 0.1 mM CDP as well as most of the above sugar substrates at 1 mM. However, the sensitivity of the cytidylyltransferase activity to the presence of 1mM D-glucose 1,6-diphosphate, D-glucose 1-phosphate, glycerol 3-phosphate and dihydroxyacetone phosphate was evident where a 62%, 65%, 52% and 61% decrease in activity, respectively, was noted (see Appendix VI).

3.5 DISCUSSION:

The CDP-ribitol synthase protein from *H. influenzae* type a, Acs1, was recently shown to be a bifunctional enzyme that catalyzes both reductase and cytidylyltransferase reactions in the transformation of D-ribulose 5-phosphate to CDP-ribitol (Follens, Veiga-da-Cunha et al. 1999). In the work reported here, Bcs1 a CDP-ribitol synthase with 96% amino acid sequence identity to Acs1, was shown to have very similar steady state kinetic parameters to those previously published for Acs1. This investigation presents further kinetic evidence that suggests the reaction order imposed by the bifunctional enzyme is not random, but instead that Bcs1 catalyzes first the reduction of D-ribulose 5-phosphate then subsequently cytidylyltransfer to D-ribitol 5-phosphate to produce CDP-ribitol. Steady state kinetic experiments indicated more than 650-fold kinetic preference for cytidylyltransfer to D-ribitol 5-phosphate over D-ribulose 5-phosphate. Similarly, the

observed catalytic efficiency for D-ribulose 5-phosphate for the reductase reaction indicates kinetic preference for this sugar phosphate substrate.

The sugar substrate specificity for both reduction and cytidylyltransfer 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, as well as D-ribulose. The enzyme however demonstrated no reductase activity with six carbon sugars. In light of these results, it should be mentioned that many monosaccharides exist in several tautomeric forms in solution at equilibrium (Swenson and Barker 1971; Que and Gray 1974). Since enzymes utilizing these sugars as substrates may not be able to use each of the forms, knowledge of the relative abundance and composition of cyclic and acyclic forms is necessary to fully understand their enzymatic reactivities (Gray and Barker 1970; Swenson and Barker 1971; Que and Gray 1974; Angyal 1984). The aldopentose phosphate and D-ribulose substrates tested in this work exists predominantly in the cyclic furanose form (>95% and 80%, respectively) while D-ribulose 5-phosphate does not exist in the furanose form but prefers an acyclic, ketal form (Serianni, Pierce et al. 1979; Angyal 1984; Buckingham and Macdonald 1996; Swenson and Barker 1971). Substrate specificity of Bcs1 reductase reaction decreased at least ten-fold for these compounds that exist predominantly in the cyclic forms suggesting a preference for the acyclic form. D-Erythrose 4-phosphate, also existing predominantly in the acyclic form (Serianni, Pierce et al. 1979), was shown to have less than two-fold difference in substrate specificity thereby further supporting the preference for the acyclic form of substrates by CDP-

ribitol synthase. Stereochemistry at C-3 was also shown to be fundamental for substrate specificity since the predominantly acyclic D-xylulose 5-phosphate demonstrated a 46-fold decrease in substrate specificity. In the cytidylyltransferase reaction, strong preference was demonstrated for the alditol substrate, D-ribitol 5-phosphate, over all aldose and ketose phosphate substrates in cyclic and acyclic forms. Also, no activity was demonstrated with D-ribitol, indicating the requirement of the presence of the phosphate group for cytidylyltransfer.

❖ CHAPTER 4: CDP-RIBITOL SYNTHASE: A CANDIDATE FOR SUBSTRATE CHANNELING?

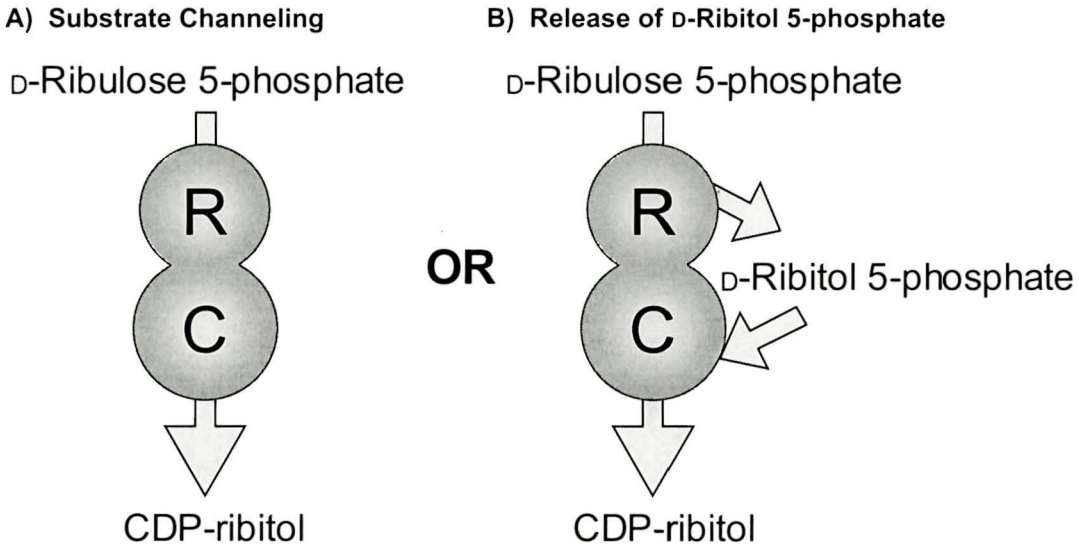
4.1 LITERATURE REVIEW:

4.1.1 Approach to Determining Substrate Channeling:

The dual functioning nature of Bcs1 raises mechanistic issues that are distinctive to bifunctional enzymes that include reaction order of the bifunctional catalysis and the question of substrate channeling. Kinetic evidence in Chapter 3 suggested that the reaction order for Bcs1 involves the reductase reaction preceding cytidylyltransfer. Substrate channeling is defined as the process of direct transfer of an intermediate between active sites that catalyze sequential reactions in a biosynthetic pathway without allowing free diffusion of the metabolite into bulk solvent (Anderson 1999; Miles, Rhee et al. 1999). It has been suggested that substrate channeling is a driving force for gene fusion in evolution (Enright, Iliopoulos et al. 1999; Marcotte, Pellegrini et al. 1999) and remains an engaging possibility for enzymes such as CDP-ribitol synthase.

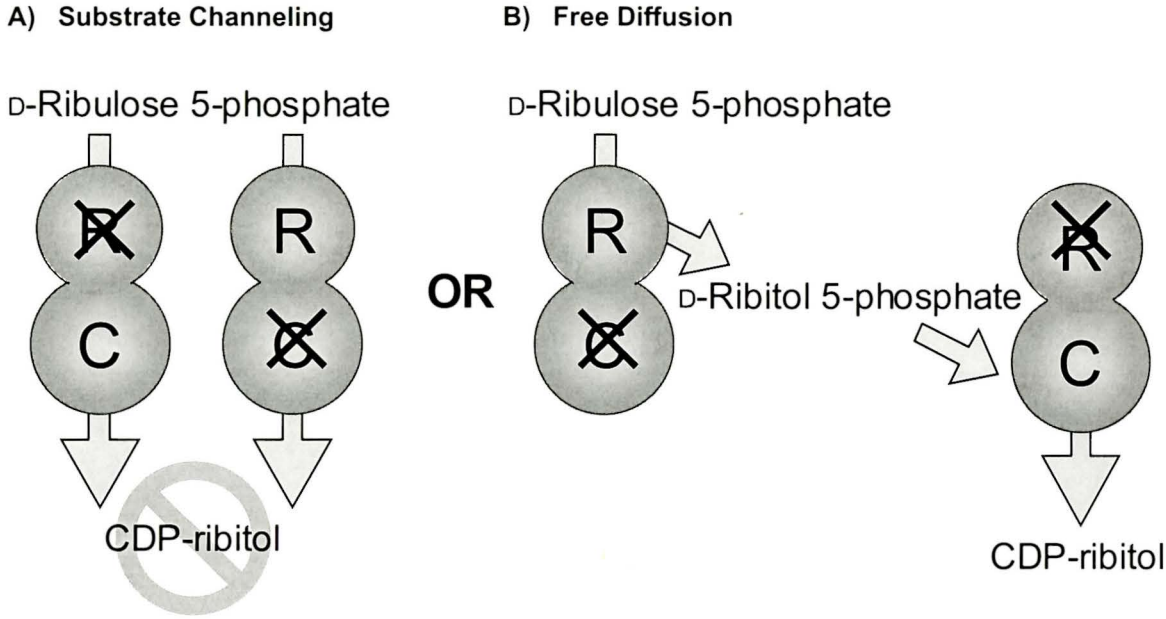
To test substrate channeling, this investigation attempted to determine whether the metabolic intermediate, D-ribitol 5-phosphate, was transferred from reductase to cytidylyltransferase active sites without direct exposure to the solution (Scheme 5A) or released to the bulk solvent (Scheme 5B).

Scheme 5: Substrate Channeling versus Release of D-Ribitol 5-phosphate in Wild Type Bcs1



The approach used in this investigation to distinguish between channeling versus the release of D-ribitol 5-phosphate is illustrated in Scheme 6. Two monofunctional Bcs1 variants were required for this approach where the k_{cat} values for the reductase or the cytidylyltransferase activities, respectively, were primarily impaired. Assays monitored the overall bifunctional catalysis of Bcs1 by equimolar mixtures of these k_{cat} variants in the presence of saturating D-ribulose 5-phosphate, CTP and NADPH which would be indicative of channeling or release of D-ribitol 5-phosphate. Channeling of the metabolic intermediate would be suggested if the mixture of k_{cat} variants were unable to produce CDP-ribitol (Scheme 6A) while release of the intermediate would be demonstrated by the bifunctional catalysis by the same mixture (Scheme 6B).

Scheme 6: Active site-directed variants of Bcs1 to distinguish channeling from free diffusion of metabolic intermediate.



4.1.2 Monofunctional Bcs1 Variants:

Bcs1 k_{cat} variants were created by site directed mutagenesis based on amino acid sequence alignments of Bcs1 with homologues. The *C*-terminal region of the CDP-ribitol synthase from *H. influenzae* type a has been previously shown to be approximately 25% identical to members of the short chain dehydrogenase/reductase (SDR) family (Follens, Veiga-da-Cunha et al. 1999). SDRs are well characterized proteins with an established mechanism of action as depicted in Figure 7 (Jornvall, Persson et al. 1995). A conserved YXXXK motif is present in SDR proteins where the tyrosine and lysine are understood to play a critical and coordinated role, respectively, in proton abstraction in catalysis (Jornvall, Persson et al. 1995). Alignments of the *C*-terminal region of Bcs1 with its SDR homologues also revealed the conserved YXXXK motif (residues 382 and

386 in Bcs1) as shown in Appendix VII. Both Y382F and K386A mutants of Bcs1 were created for this investigation.

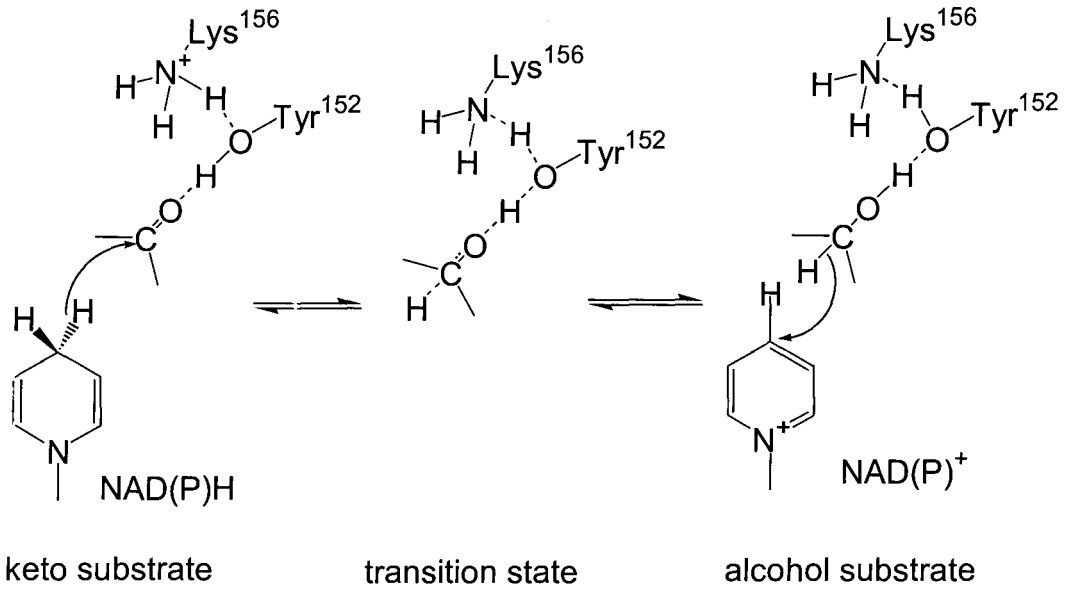


Figure 7: Reaction Mechanism of short-chain dehydrogenases involving the conserved Tyrosine and Lysine residues.
The mechanism was suggested by McKinley-McKee *et al.* (McKinley-McKee, Winberg et al. 1991) and supported by crystallographic interpretations (Ghosh, Weeks et al. 1991). Residue numbers refer to 3α/20β-hydroxysteroid dehydrogenase. (Jornvall, Persson et al. 1995)

The *N*-terminal region of Acs1 shows significant similarity to a number of sugar-phosphate nucleotidyltransferases (Follens, Veiga-da-Cunha et al. 1999). Recently, the motif ($G^{14}XGTRLPK^{25}$) has been identified as a signature sequence involved in sugar-nucleotide interaction in the active site of *Escherichia coli N*-acetylglucosamine 1-phosphate uridylyltransferase, GlmU (Brown, Pompeo et al. 1999). The GlmU arginine 18 residue from this motif was previously demonstrated to be essential to catalysis and shown to have a k_{cat} effect on uridylyltransfer when this residue was substituted with alanine. An aspartate 105 residue of GlmU that is conserved among sugar-phosphate

nucleotidyltransferases was also shown to be functionally important and involved in substrate coordination (Brown, Pompeo et al. 1999). Alignments of the *N*-terminal region of Bcs1 with sugar-phosphate nucleotidyltransferase, including GlmU, revealed the conserved arginine 18 and asparatate 105 residues of GlmU present in all homologues (arginine 18 and asparatate 110, respectively, in Bcs1) as shown in Appendix VIII. Both R18A and D110A mutants of Bcs1 were created for this investigation.

4.2 Materials:

QuikChange PCR amplified site directed mutagenesis kit was purchased from Stratagene (LaJolla CA). The following compounds were obtained from BioShop Ltd (Burlington ON): 4-(2-Hydroxyethyl)-1-peperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), ampicillin (Amp), imidazole and isopropyl thiogalactoside (IPTG). All other compounds and enzymes were obtained from Sigma-Aldrich (Oakville ON). [5-^{3}H]CTP was obtained from Amersham-Pharmacia (Baie d'Urfé QB).

4.3 Methods:

4.3.1 Site Directed Mutagenesis:

To create Bcs1 mutants, site directed mutagenesis was performed using the protocol from the QuikChange (Stratagene, LaJolla CA) PCR amplified site-directed mutagenesis kit. The pET22b-3'-*bcs1* construct containing the His_6-tagged sequence fused to the *C*-terminal of *bcs1* was used as the template. The oligodeoxynucleotides used as primers in this site directed mutagenesis protocol were synthesized at the MOBIX

(McMaster University). Sequences of the oligodeoxynucleotides were as follows:

R18A→ 5'-GGTGGTGTTGGCTCTGCCATGGGATTGGGCTAC-3';

D110A → 5'-CGAAATTAATCATTCATGCTGCTGTACGACCTTTACTAGCG-3';

Y382F → 5'-CGTCCATTCTATGCTATTTTGTCTTCTGCAAAAGCAGC-3';

K386A → 5'-GCTATTTACTCTTCTGCAGCAGCAGCTGTGGTAAAC-3'. The underlined sequences represent the mutation created. The amplified and transformed DNA fragments were examined by both restriction site analysis and confirmed by sequence analysis (MOBIX, McMaster University). For protein overproduction, the final plasmid constructs of the *bcs1* mutants were transformed into *E. coli* BL21(DE3) cells.

4.3.2 Overexpression and Purification of the Bcs1 Variants:

Overexpression and purification of the recombinant Bcs1 variant proteins was performed as previously described for His_6-tagged Bcs1 in Chapter 2.2.3 to 2.2.5.

4.3.3 Oligomeric State Determination:

Oligomeric state was determined by gel filtration using the MS-GF-200 kit provided by Sigma. The kit included Blue Dextran as well as the following calibration enzymes: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa). Superdex 200 column (Amersham-Pharmacia) (1.8 cm x 62 cm) was used. Standards were rerun for all running buffers used including: a) 50 mM HEPES (pH 7.5); b) 50 mM HEPES (pH 7.5), 1 mM

DTT, 100 μM CTP, 10 mM $MgCl_2$. In buffer a), Bcs1 appeared to run at ~158 kDa (apparent trimer) and in buffer b) Bcs1 appeared to run at ~50 kDa.

4.3.4 Enzyme Assays:

Overall reductase and cytidylyltransferase Bcs1 activity was monitored in a discontinuous assay by 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% TritonX-100, 10 mM $MgCl_2$, 0.6 mM CTP, 1.0 mM D-ribulose 5-phosphate, 0.6 mM NADPH and 1 μCi [5-^{3}H]CTP. Reaction samples were initiated by adding Bcs1 and were stopped by the addition of 600 μL of 8 M urea. Assay reactions were analyzed by high performance liquid chromatography (HPLC) whereby samples were loaded onto an anion exchange column (MonoQ 5/5, Amersham-Pharmacia) and run against a gradient of 0.5 M triethylamine bicarbonate using a Waters 600 chromatography system with Millenium^{TM} analysis software (Milford MA). Radioactivity detection was monitored by a Packard in-line radioactivity detector (Flow Scintillation Analyzer 150TR, Meridan CT) and further confirmed spectrophotometrically at absorbances of 271 nm. At least three time points were used for each rate determination and assays were performed in duplicate. All reactions proceeded at 25°C.

4.3.5 Rapid Mixing Experiments:

Rapid mixing experiments were performed to explore reaction order of bifunctional Bcs1 using Biologic SFM-400/S mixer and MOS-250 optical unit (Molecular Kinetics, Pullman WA). The standard 250 μL assay mixture contained 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, 0.6 mM NADPH, 5 μCi [5-^{3}H]CTP and 600 ng Bcs1. The reductase reaction was monitored continuously by NADPH depletion at an absorbance of 340 nm using stopped flow rapid mixing procedures. The final time course consisted of an average of 8 trials. The same standard reaction assay mix was used to monitor the cytidylyltransferase reaction which discontinuously measured CDP-ribitol formation using quench flow rapid mixing procedures. The quench flow reactions were initiated by the addition of Bcs1 and stopped using urea to a final concentration of 6 M. Assay reactions were analyzed by HPLC with in-line radioactivity described previously. Reactions were completed in duplicate.

4.4 RESULTS:

4.4.1 Monofunctional Bcs1 Variants:

Monofunctional Bcs1 variants were created by site directed mutagenesis based on amino acid sequence alignments of Bcs1 with homologues (as shown in Appendices VII and VIII). Figure 8 depicts an SDS-polyacrylamide gel of the overexpressed and purified Bcs1 protein and variants used in this substrate channeling investigation: native

full length Bcs1, *C*-terminal His_6-tagged Bcs1, R18A variant of *C*-terminal His_6-tagged Bcs1 and K386A variant of *C*-terminal His_6-tagged Bcs1.

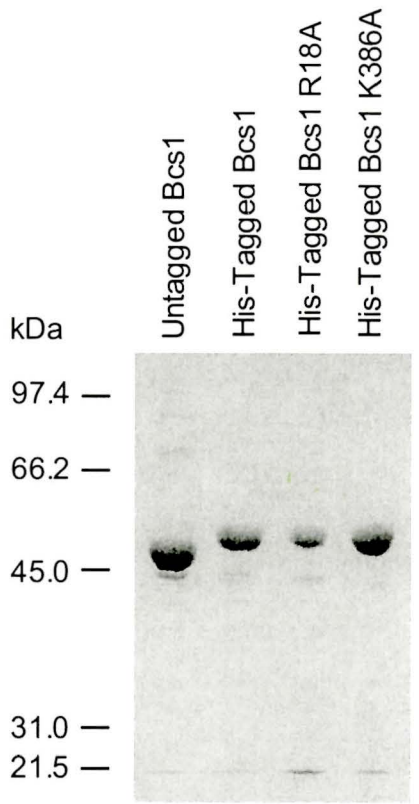


Figure 8: SDS-polyacrylamide gel (11.5 % w/v) of Bcs1 and variants. Shown are: native Bcs1 with no His-tag (31 μg); *C*-terminal His_6-tagged Bcs1 (22 μg); R18A mutant of *C*-terminal His_6-tagged Bcs1 (18 μg); K386A variant of *C*-terminal His_6-tagged Bcs1 (27 μg). Samples were denatured by boiling in Laemmli buffer (Laemmli 1970) prior to electrophoresis.

Both Y382F and K386A mutants of Bcs1 were made in attempts to create a monofunctional variant with only cytidylyltransferase activity. The K386A variant was significantly impaired in k_{cat} compared to the wild type enzyme for reduction reaction ($0.32\ s^{-1}$ and $22\ s^{-1}$, respectively) with relatively little impact on K_m for either D-ribulose 5-phosphate or NADPH (Table 4). However, the Y382F variant was defective in both

reductase and cytidylyltransferase activities as shown in Appendix IX. Therefore the Y382F mutant was not used further in this substrate channeling study.

Table 4: Steady State Kinetic Parameters Describing the Reactions Catalyzed by Bcs1 Mutants

	R18A	Ratio WT/R18A	K386A	Ratio WT/K386A
(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 (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	0.25	11.6	0.017	171
NADPH				
K_m (μM)	17 ± 0.5	2.6	39 ± 5.0	1.1
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	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 (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	0.0014	714	0.81	1.2
CTP				
K_m (μM)	390 ± 47	0.3	92 ± 8.9	1.1
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	0.0036	361	1.1	1.2

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

Attempts to create a monofunctional variant of Bcs1 with only reductase activity were accomplished by constructing both R18A and D110A mutants. Kinetic data for these variants are summarized in Table 4 and Appendix IX. The R18A variant of Bcs1 had a 100-fold decrease in k_{cat} compared to the wild type enzyme for cytidylyltransfer (0.14 s^{-1} and 13 s^{-1}, respectively) with notable increases in K_m relative to wild type for

both D-ribitol 5-phosphate (7.7-fold) and CTP (4-fold). However, as with the Y382F mutant, the D110A variant of Bcs1 was found to be defective in both reductase and cytidylyltransferase activities and thus was not used further for the substrate channeling study.

Proteolytic cleavage using chymotrypsin was used to determine whether these inactive Bcs1 variants, D110A and Y382F, were folded correctly. R18A and K386A mutants were found to cleave in a similar pattern as wild type. D110A was found to cleave in an irregular pattern compared to wild type and Y382F was completely degraded. The altered proteolytic cleavage patterns of D110A and Y382A Bcs1 variants indicated that these proteins may have been improperly folded; a likely explanation for the low rate for each activity.

Figure 9 depicts the cytidylyltransferase and reductase activities determined for the wild type and variant Bcs1 enzymes. The objective for the substrate channeling study was to create a reductase impaired variant, K386A, with decreased reductase activity and little or no effect on cytidylyltransferase activity, and a cytidylyltransferase impaired variant, R18A, which has defective cytidylyltransferase activity and little or no effect on reductase activity. In the reductase impaired variant, K386A, a 60- and 170-fold reduction in catalytic efficiency (k_{cat}/K_m) was observed for NADPH and D-ribulose 5-phosphate, respectively, but demonstrated little change in the specificity constants for the cytidylyltransferase reaction. Similarly, the cytidylyltransferase impaired variant, R18A decreased 360- and 710-fold in k_{cat}/K_m for CTP and D-ribitol 5-phosphate, respectively, without significant effects on these parameters for the reductase activity. These findings

support the hypothesis that reductase and cytidylyltransfer functions of Bcs1 occur at distinct active sites.

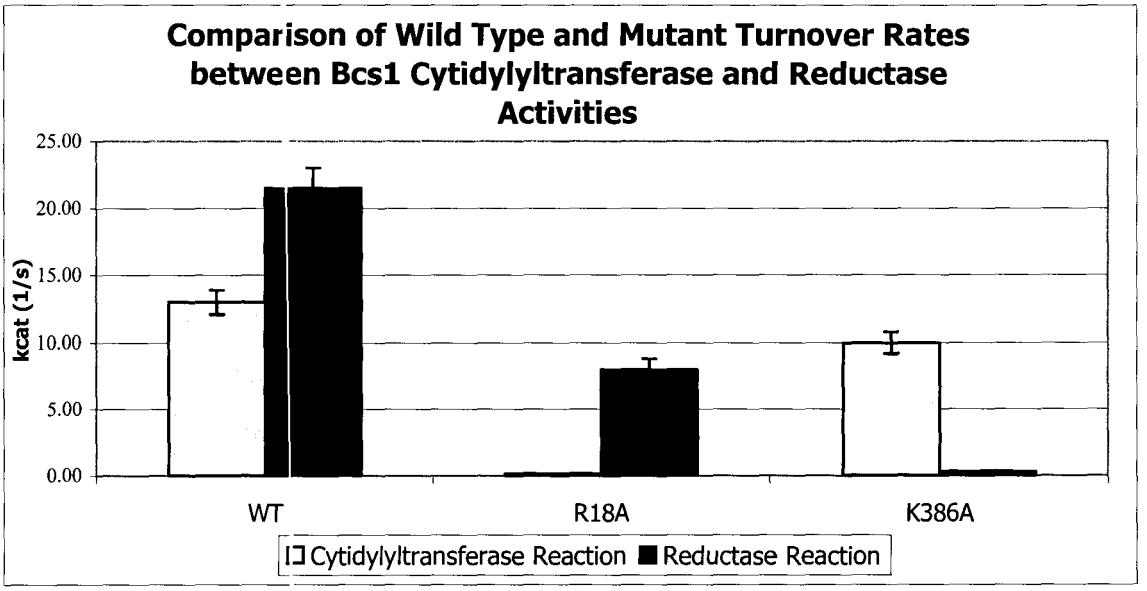


Figure 9: Comparison of mutant turnover rate between Bcs1 cytidylyltransferase and reductase activity.
R18A, the cytidylyltransferase impaired variant, maintained significant reductase activity but lost most cytidylyltransferase function. K386A, the reductase impaired variant, retained cytidylyltransferase activity but is defective in reductase function. Reactions contain saturating amounts of D-ribulose 5-phosphate (600 μM) and NADPH (500 μM) or D-ribitol 5-phosphate (600 μM) and CTP (500 μM) for reductase and cytidylyltransferase reactions, respectively. Reductase activity monitored NADPH depletion at 340 nm while cytidylyltransferase reaction was monitored using the MESG/PRPase-pyrophosphatase assay. Errors shown are standard deviations of triplicate determinations.

4.4.2 Test for Substrate Channeling:

Once the monofunctional characterization of Bcs1 variants, R18A and K386A, was completed, the issue of substrate channeling was addressed as previously discussed by monitoring the bifunctional reaction of the enzyme (Scheme 6). A linear relationship between the enzyme concentration and the rate of reaction was confirmed for the bifunctional reaction by monitoring the formation of the CDP-ribitol product in the

presence of saturating substrates (Appendix X). Figure 10 illustrates the overall turnover numbers seen for wild type Bcs1 (11 s^{-1}) and the R18A and K386A variants individually (0.13 s^{-1} and 0.47 s^{-1}, respectively) compared to an equimolar mixture of R18A/K386A variants (6.1 s^{-1}). Though reduced by 50% relative to wild type, the overall turnover rate of the R18A/K386A variant mixture indicates that CDP-ribitol product was formed. This result supports Scheme 6B thereby suggesting that the metabolic intermediate is released to the bulk solvent and not channeled from reductase to cytidylyltransferase active sites.

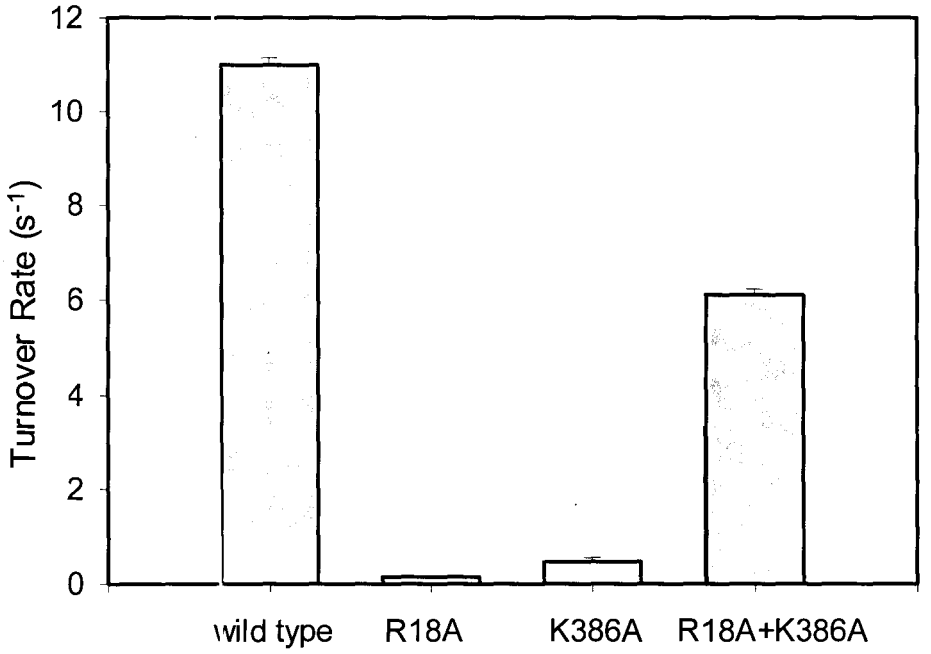


Figure 10: Rate of CDP-ribitol formation in bifunctional catalysis by Bcs1 and variants to probe the release of D-ribitol 5-phosphate.
Plotted are the turnover values for (a) wild type Bcs1, (b) R18A Bcs1 alone, (c) K386A Bcs1 alone and (d) R18A/K386A Bcs1 variant mixture. Substrate concentrations in assay were 1.2 mM D-ribulose 5-phosphate, 600 μM NADPH and 600 μM CTP. Values of turnover were based on the concentration of protein in each reaction, except in the variant mixture experiment where that concentration was halved (that is, 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; 130 μM/s with 21.3 μM R18A and 21.3 μM K386A). The reaction was monitored by [5-^{3}H]CDP-ribitol formation following separation on an anion exchange (MonoQ) HPLC using a triethylammonium bicarbonate gradient. Errors shown are standard errors of best fit to data from three separate experiments.

4.4.3 Approach to Steady State in Bifunctional Turnover:

The approach to steady state in bifunctional catalysis was studied using rapid mixing in order to examine both the order of the bifunctional reaction and the fate of a metabolic intermediate. Figure 11 illustrates the time course following the rapid mixing of Bcs1 enzyme with saturating amounts of all substrates for the bifunctional reaction (NADPH, D-ribulose 5-phosphate and [5-^{3}H]CTP) by monitoring both the depletion of NADPH (O) by stopped flow and the formation of [5-^{3}H]CDP-ribitol (□) by rapid quench. Also shown are theoretical curves (solid line, NADPH; dashed line, D-ribitol 5-phosphate; dotted line CDP-ribitol) calculated using the steady state parameters for Bcs1 in Table 3. These theoretical curves are based on the assumption that the reductase reaction precedes cytidylyltransferase reaction with the release of the metabolic intermediate, D-ribitol 5-phosphate. The distinct 50 s lag in the initial formation of CDP-ribitol contrasts with the rapid approach to steady state for the depletion of NADPH. This lag is consistent with the need for the metabolic intermediate, D-ribitol 5-phosphate to accumulate to K_m levels to drive the cytidylyltransferase reaction as shown in the calculated time course for D-ribitol 5-phosphate which reaches 120 μM after 200 s.

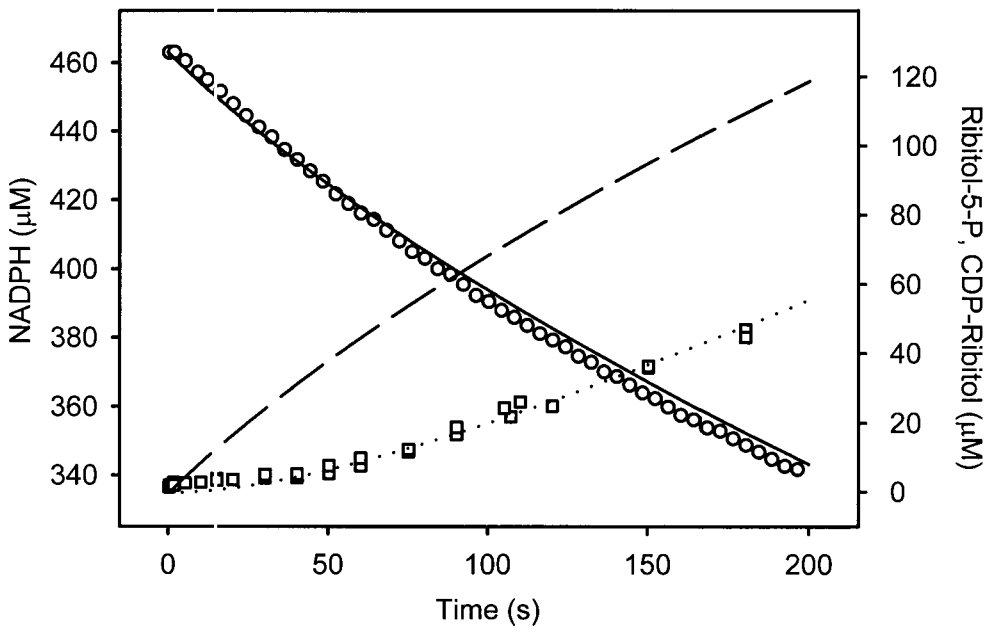
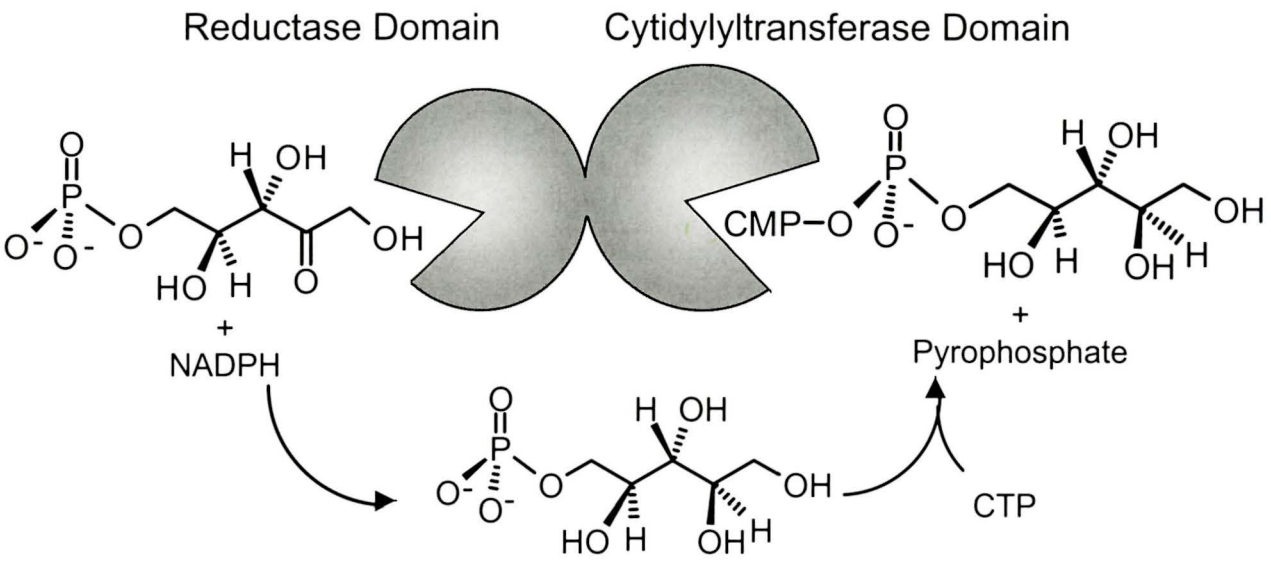


Figure 11: Bifunctional catalysis following rapid mixing of CDP-ribitol synthase with saturating amounts of D-ribulose 5-phosphate, NADPH and CTP. Shown are data indication the time course of NADPH depletion (O) and CDP-ribitol formation (□), monitored by stopped flow absorbance (340 nm) spectroscopy and by rapid quench flow followed by HPLC to measure [5-^{3}H]CDP-ribitol, respectively. Duplicate data are presented for rapid quench experiments and an average of 8 trials for the stopped flow. Substrate concentrations were 1 mM D-ribulose 5-phosphate, 463 μM NADPH and 750 μM CTP (5 μCi/mol [5-^{3}H]CTP). Curves shown are theoretical estimates assuming reduction precedes cytidylyltransfer and that D-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 second intervals using the steady state kinetic parameters determined in this work for Bcs1: CDP-ribitol concentration was $k_{cat}t[E][S]/(K_m + [S])$, where, 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; D-ribitol 5-phosphate was $k_{cat}t[E][S]/(K_m + [S])$, where k_{cat} was 22 s^{-1}, [S] was the concentration of NADPH at t = t – 1s and K_m was 44 μM; D-ribulose 5-phosphate was 1000 μM less the concentration of D-ribitol 5-phosphate formed at time t; NADPH 463 μM less the concentration of D-ribitol 5-phosphate formed at time t.

Since these calculated time courses closely approximate the experimentally measured values, these time courses supports the model depicted in Scheme 7 where the cytidylyltransferase reaction follows the reduction in distinct active sites without substrate channeling.

Scheme 7: Two active site model for catalysis by Bcs1.



4.5 DISCUSSIONS:

The mutagenesis study of CDP-ribitol synthase in this thesis work has shown that the reduction of D-ribulose 5-phosphate and cytidylyltransfer to D-ribitol 5-phosphate occurs in distinct active sites. With *C*-terminal sequence similarity with short chain dehydrogenase/reductase (SDR) proteins and *N*-terminal similarity to sugar phosphate nucleotidyltransferases (Follens, Veiga-da-Cunha et al. 1999), signature motifs in the putative reductase (Jornvall, Persson et al. 1995) and nucleotidyltransferase (Brown,

Pompeo et al. 1999) active sites were targeted to create the K386A and R18A variants, respectively. The K386A variant was largely impaired for reduction of D-ribulose 5-phosphate without impact on cytidylyltransfer and the R18A variant was mostly inactivated in cytidylyltransfer with little effect on reduction. These findings support the conclusion that Bcs1 contains distinct and predominantly independent reductase and cytidylyltransferase components that can be grouped into existing SDR (Jornvall, Persson et al. 1995) and nucleotidyltransfer (Brown, Pompeo et al. 1999) protein families.

Since the bifunctional activities of Bcs1 occur at separate active sites, experiments in this thesis work addressed the issue of substrate channeling. The physiological advantages of substrate channeling include (i) protection of chemically labile intermediates, (ii) prevention of intermediates to react in competing enzymatic reaction and/or (iii) increase catalytic efficiency by decreasing transit time of intermediates that reacts with a diffusion-controlled rate constant (Srere and Ovadi 1990; Ovadi 1991; Anderson 1999; Miles, Rhee et al. 1999). Accordingly, the Bcs1 metabolic intermediate, D-ribitol 5-phosphate, (i) is not especially labile, (ii) may be a substrate for other bacterial enzymes and (iii) does not undergo cytidylyltransfer with a diffusion-controlled rate constant (k_{cat}/K_m from this work is 1.0×10^5 $L \cdot mol^{-1} \cdot s^{-1}$). The foregoing may obscure an apparent physiological advantage for direct transfer of D-ribitol 5-phosphate since substrate channeling has also been suggested to provide kinetic advantages unrelated to the limits of diffusion in the achievement of steady state turnover (Welch and Easterby 1994).

Experiments in this work demonstrated that the metabolic intermediate D-ribitol 5-phosphate was released without substrate channeling in Bcs1. It was found in this investigation that an equimolar mixture of reductase-impaired (K386A) and cytidylyltransfer-impaired (R18A) variants was capable of efficient catalysis of the overall two-step reaction. This finding is consistent with the bifunctional turnover in distinct active sites of Bcs1 with the release and free diffusion of the metabolic intermediate, D-ribitol 5-phosphate (Scheme 7). In an alternate but related strategy, Geck and Kirsch 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 (Geck and Kirsch 1999). The approach used in this thesis work on CDP-ribitol is the first of its kind in addressing the question of substrate channeling in a bifunctional protein. Further examination of results outlining the approach to steady state turnover was also shown in this investigation to support the model of reduction occurring before cytidylyltransfer in distinct active sites without substrate channeling.

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 (Ahmad and Jensen 1988). The benefits of the selective pressure causing certain genes to become fused, and possibly allow channeling of substrates, over the course of evolution is suggested to physically integrate protein functions that are biologically coupled in the same metabolic pathways (Enright, Iliopoulos et al. 1999; Snel, Bork et al. 2000). In this work, the bifunctional nature of the CDP-ribitol synthase,

Bcs1, was proposed to fit a two active site model (Scheme 7) whereby 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 the benefits of genetic or biochemical co-regulation of functions of CDP-ribitol synthase.

❖ CHAPTER 5: SUMMARY AND CONCLUSIONS

In this investigation, untagged and His_6-tagged variants of CDP-ribitol synthase from *H. influenzae* type b were successfully cloned, overexpressed and purified. Steady state kinetic characterization and substrate specificity kinetic studies were aimed predominantly at rigorously examining the order of the reductase and cytidylyltransferase reactions catalyzed by CDP-ribitol synthase. These studies were also used for investigating the possibility that substrate channeling occurs in this bifunctional enzyme. The results from this thesis work indicate that CDP-ribitol synthase catalyzes reduction of D-ribulose 5-phosphate to yield the metabolic intermediate, D-ribitol 5-phosphate prior to cytidylyltransfer. This study also argues against substrate channeling for Bcs1 and is consistent with a model of bifunctional catalysis by CDP-ribitol synthase at distinct active sites with release of the metabolic intermediate, D-ribitol 5-phosphate, to the bulk solvent (Scheme 7).

Many investigations have examined gene fusions, and their suspected bifunctional gene products, to demonstrate by computational means that fusions resulting in one polypeptide gene product in a species may exist as separate proteins in another organism that are coupled in the same metabolic pathway (Marcotte, Pellegrini et al. 1999). The distinct active sites of Bcs1, indicated from the mutant study and the release

of the metabolic intermediate study, suggests that this bifunctional protein may exist as separate proteins in other organisms that may contain similar enzymes catalyzing the formation of CDP-ribitol. The Gram positive bacteria *Staphylococcus aureus* may be an example of an organism containing enzymes catalyzing similar reactions as CDP-ribitol synthase since this organism contains teichoic acids consisting of poly(D-ribitol 5-phosphate) polymers that are synthesized using the CDP-ribitol activated precursor. However a bifunctional CDP-ribitol synthase enzyme has not been detected in the *S. aureus* genome to date. To further explore the distinctive nature of the two active sites of CDP-ribitol synthase in *H. influenzae*, future work can focus on understanding the respective reactions in isolation, if possible. The *C*-terminus of Bcs1 was demonstrated to have functional similarities to the well-characterized class of SDR proteins for which detailed mechanistic and structural studies have already been performed for many members of this family (Jornvall, Persson et al. 1995). The *N*-terminal region of Bcs1 was shown to have functional similarities to a class of nucleotidyltransferases which include proteins such as GlmU for which basic kinetic data and a structure have also been presented (Brown, Pompeo et al. 1999). However, little mechanistic information has been elucidated for this class of nucleotidyltransferases despite an increasing number of proteins in this family in the public database. Therefore, experiments aimed at the mechanistic understanding of the reactions catalyzed by the CDP-ribitol synthase would be important to further understand the role of this enzyme in capsule biosynthesis in *H. influenzae* types a and b as well as in other bacteria that activate D-ribitol 5-phosphate, such as teichoic acid and lipoteichoic acid biosynthesis in some Gram positive bacteria, including *Staphylococcus aureus*.

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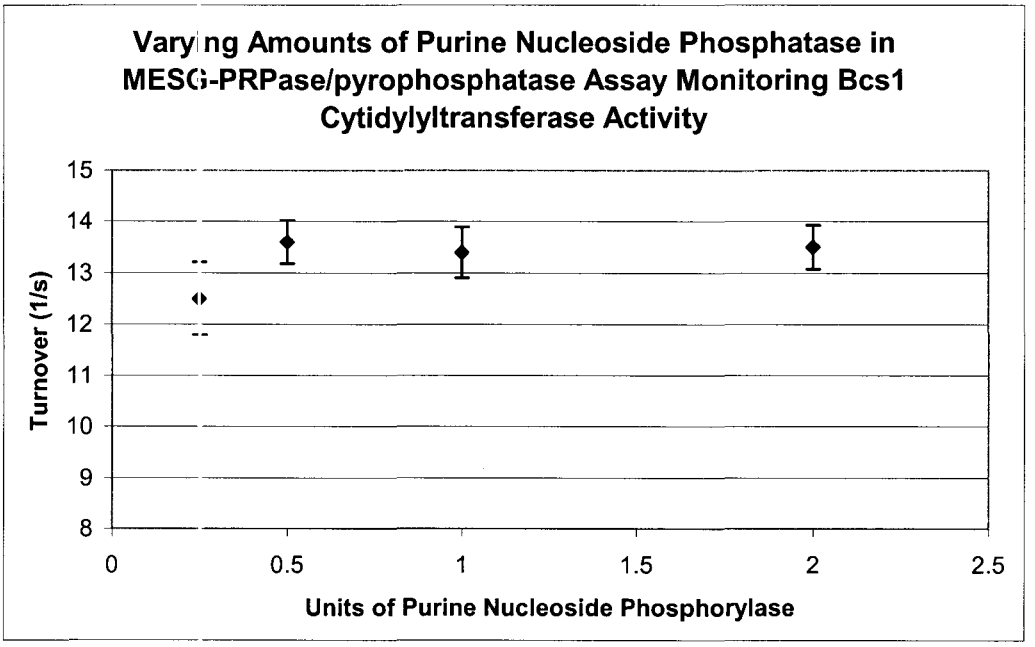
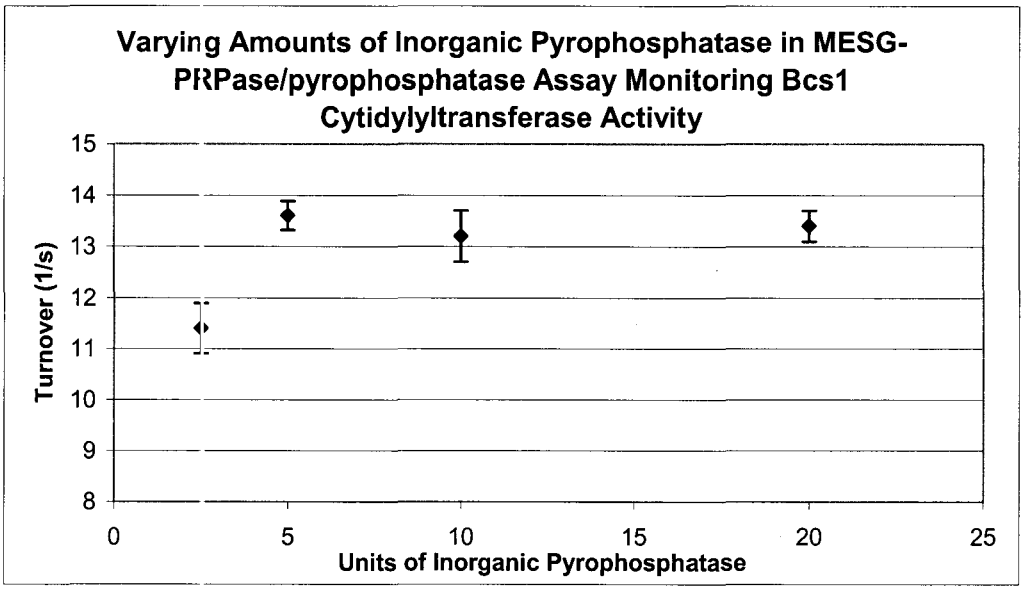
❖ APPENDIX I: MONITORING BCS1 CYTIDYLYLTRANSFERASE ACTIVITY AT VARYING AMOUNTS OF COUPLING ENZYMES

A)



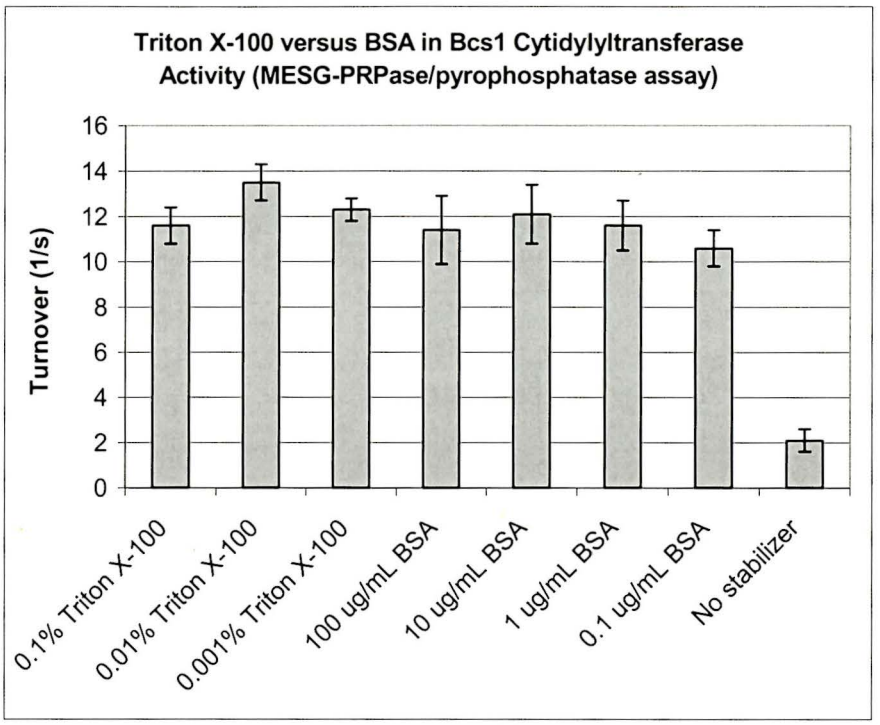
B)



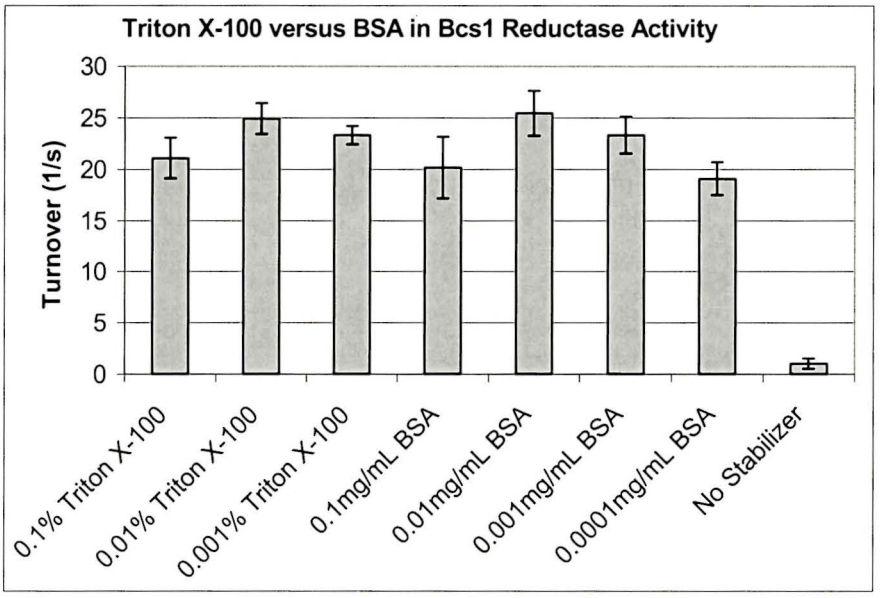
Plotted are the turnover values that monitored cytidylyltransferase activity of Bcs1 using MESG-PRPase/pyrophosphatase assay at varying amounts of (A) Purine Nucleoside Phosphatase and (B) Inorganic pyrophosphatase. Substrate concentrations in assay were 600 μM each of D-ribitol 5-phosphate and CTP. Coupled enzymes were added at concentrations indicated in the plot as well as 20 units of Inorganic Pyrophosphatase in (A) reactions and 5 units of purine nucleoside phosphatase in (B) reactions. Errors are standard deviations of three separate determinations.

❖ APPENDIX II: MONITORING BCS1 CYTIDYLYLTRANSFERASE AND REDUCTASE ACTIVITIES USING TRITON X-100 OR BSA

A)



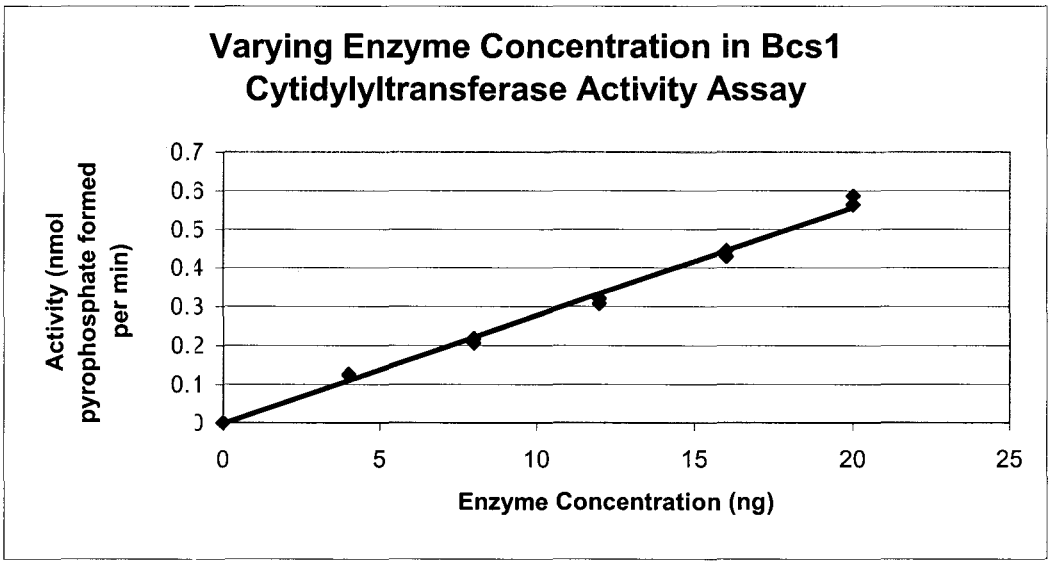
B)



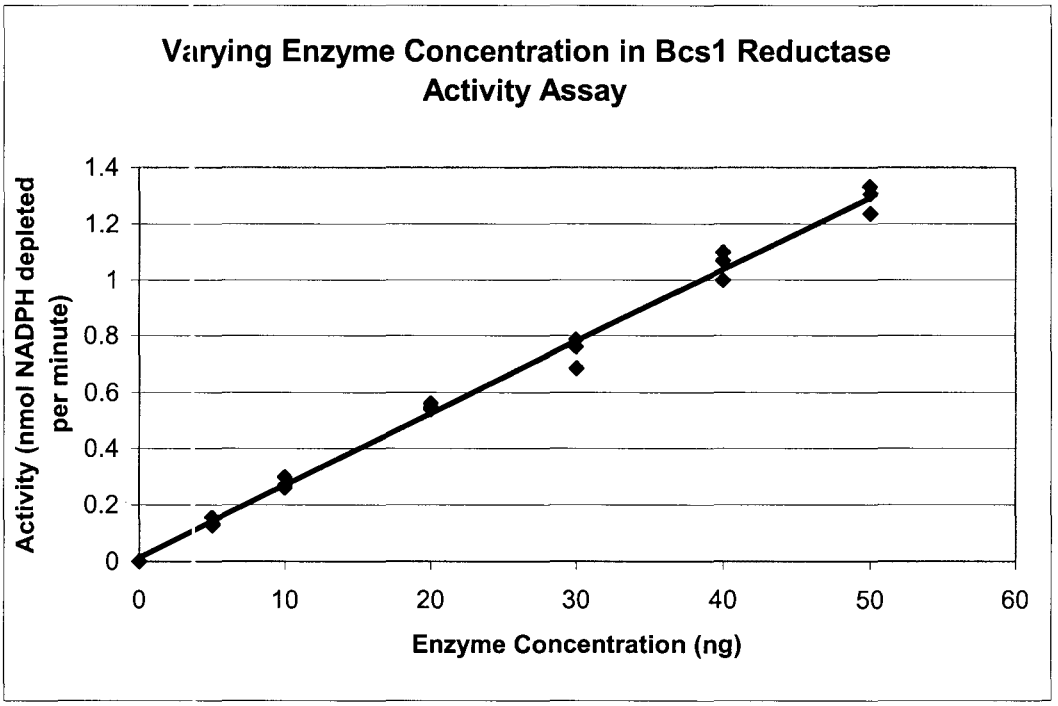
Plotted are the turnover values of Bcs1 (A) cytidylyltransferase activity and (B) reductase activity in the presence of varying concentrations of the stabilizers Triton X-100 or bovine serum albumin (BSA). Substrate concentrations in assays were (A) 600 μM each of D-ribitol 5-phosphate and CTP or (B) 600 μM NADPH and 1 mM D-ribulose 5-phosphate. Errors were standard deviations of three separate determinations.

❖ APPENDIX III: LINEAR RELATIONSHIP OF CYTIDYLYLTRANSFERASE AND REDUCTASE ACTIVITY ASSAYS AT VARYING ENZYME CONCENTRATIONS

A)



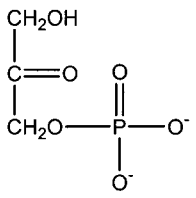
B)



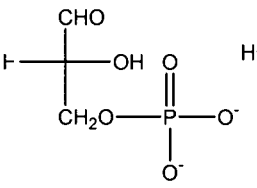
Plotted is the activity of Bcs1 (A) cytidylyltransferase and (B) reductase activities at varying enzyme concentrations to ensure a linear relationship. Substrate concentrations in assays were (A) 600 μM each of D-ribitol 5-phosphate and CTP or (B) 600 μM NADPH and 1 mM D-ribulose 5-phosphate. Reactions were performed in triplicate.

❖ APPENDIX IV: ALTERNATE SUGAR SUBSTRATE STRUCTURES

3 Carbon D-sugar phosphate:

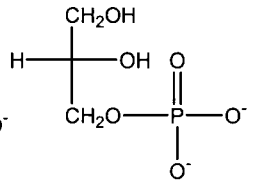


dihydroxyacetone phosphate



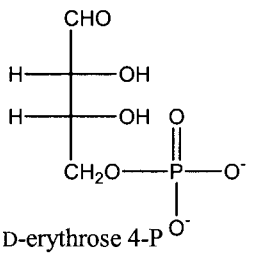
D-glyceraldehyde 3-P

3 Carbon alditol phosphate:



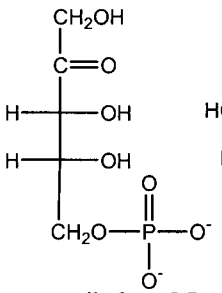
glycerol 3-P

4 Carbon D-sugar phosphate:

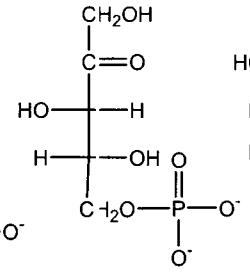


D-erythrose 4-P

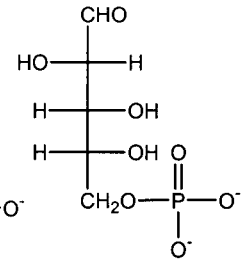
5 Carbon D-sugar phosphate:



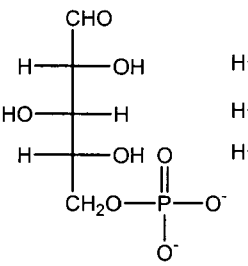
D-ribulose 5-P



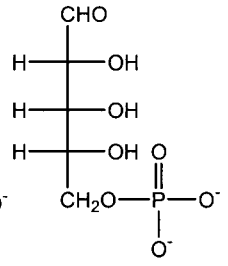
D-xylulose 5-P



D-arabinose 5-P

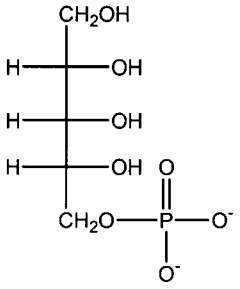


D-xylose 5-P



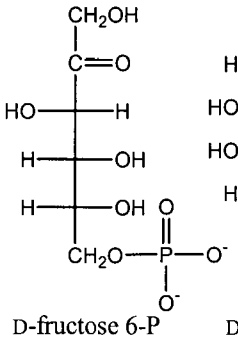
D-ribose 5-P

5 Carbon alditol phosphate:

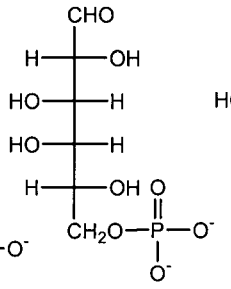


D-ribitol 5-P

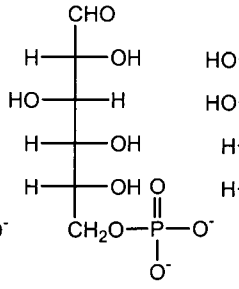
6 Carbon D-sugar phosphate:



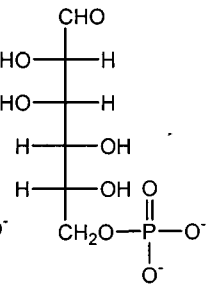
D-fructose 6-P



D-galactose 6-P

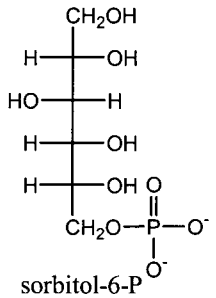


D-glucose 6-P



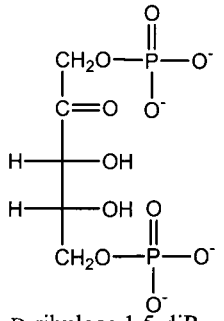
D-mannose 6-P

6 Carbon alditol phosphate:

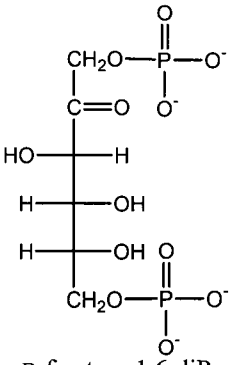


sorbitol-6-P
(D-glucitol 6-P)

Sugar Diphosphates:



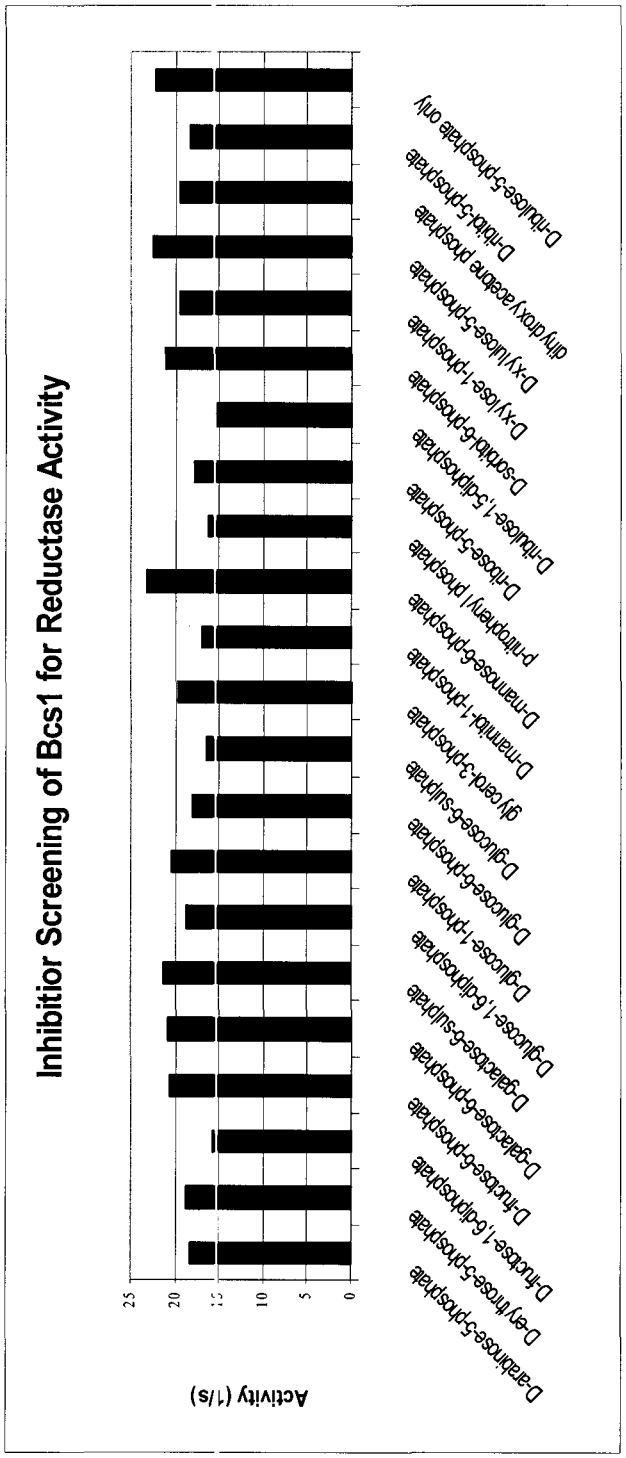
D-ribulose 1,5-diP



D-fructose 1,6-diP

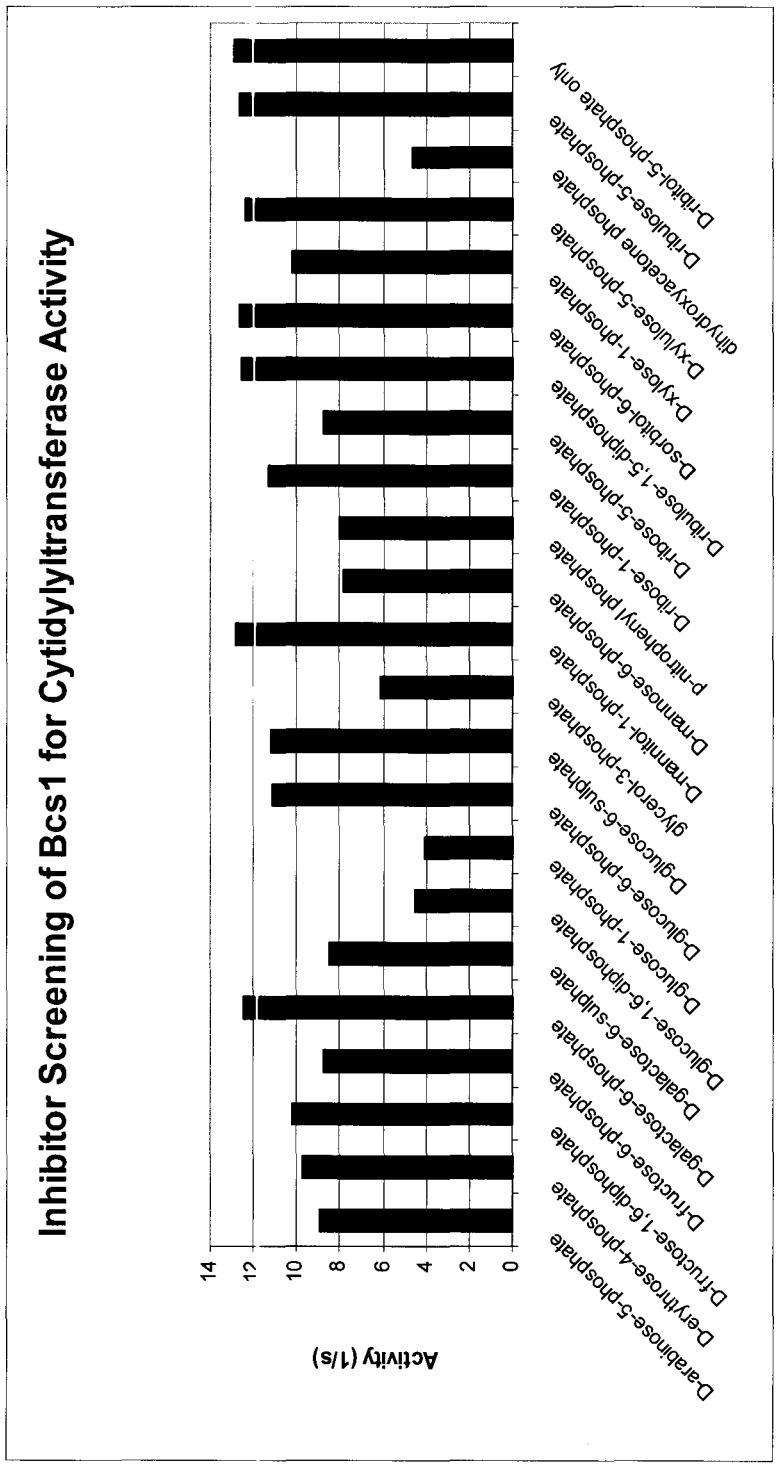
P = phosphate group

❖ Appendix V: Inhibition Studies of Reductase Activity of Bcs1



Plotted are the turnover values of the reductase activity of Bcs1 in the presence of saturating concentrations of substrates and 1 mM of the compounds listed above. Substrate concentrations in the assay were 1 mM D-ribulose 5-phosphate and 0.6 mM NADPH. Negative control is shown in "D-ribitol 5-phosphate only" where no additional compound was added. Samples were completed in duplicates.

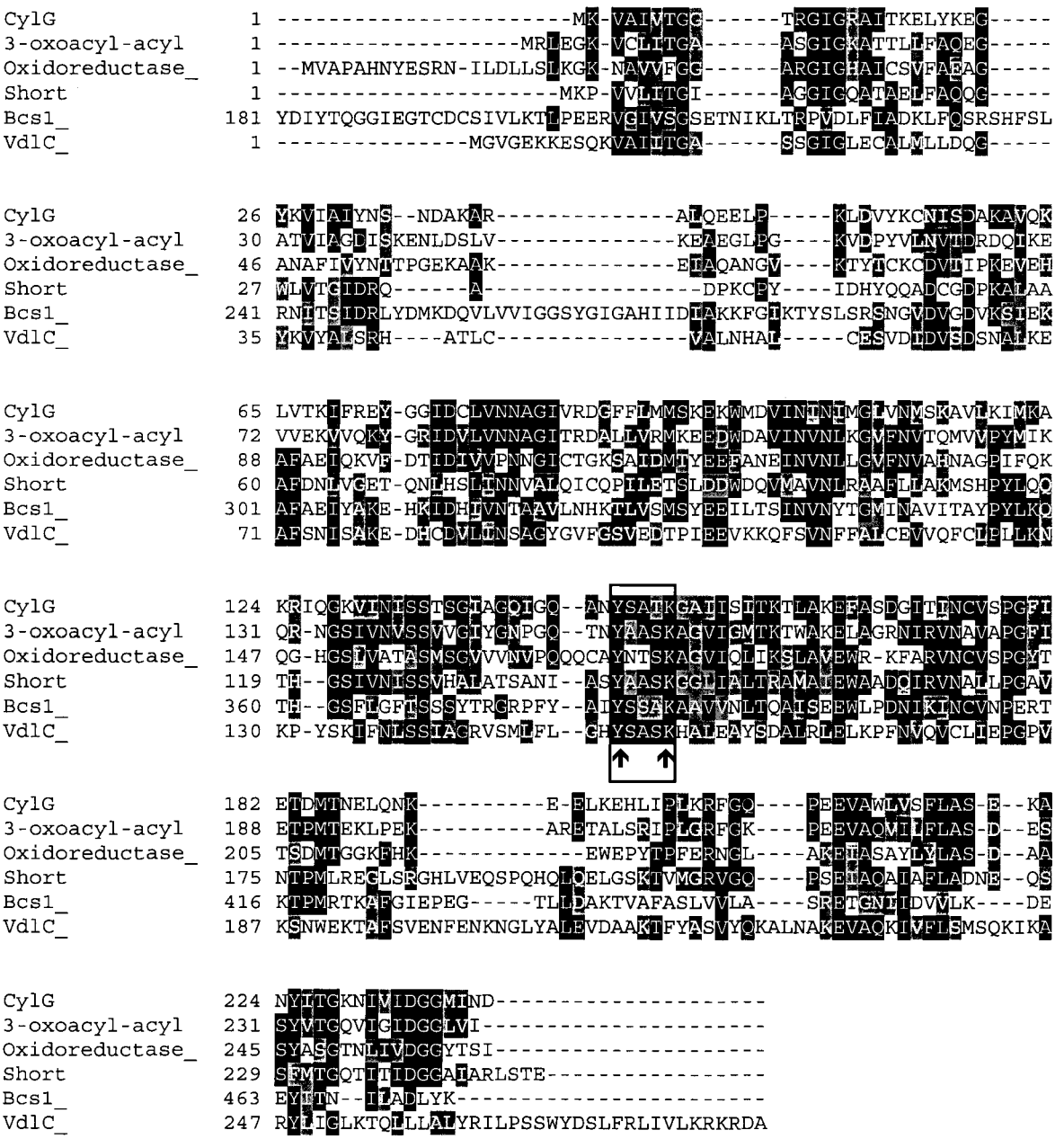
❖ Appendix VI: Inhibition Studies of Cytidylyltransferase Activity of Bcs1



Plotted are the turnover values of the cytidylyltransferase activity of Bcs1 in the presence of saturating substrate concentrations and 1 mM of the compounds listed above. Substrate concentrations in the assay were 1 mM D-ribitol 5-phosphate and 0.6 mM CTP. Negative control is shown in "D-ribulose 5-phosphate only" where no additional compound was added. Samples were completed in duplicates.

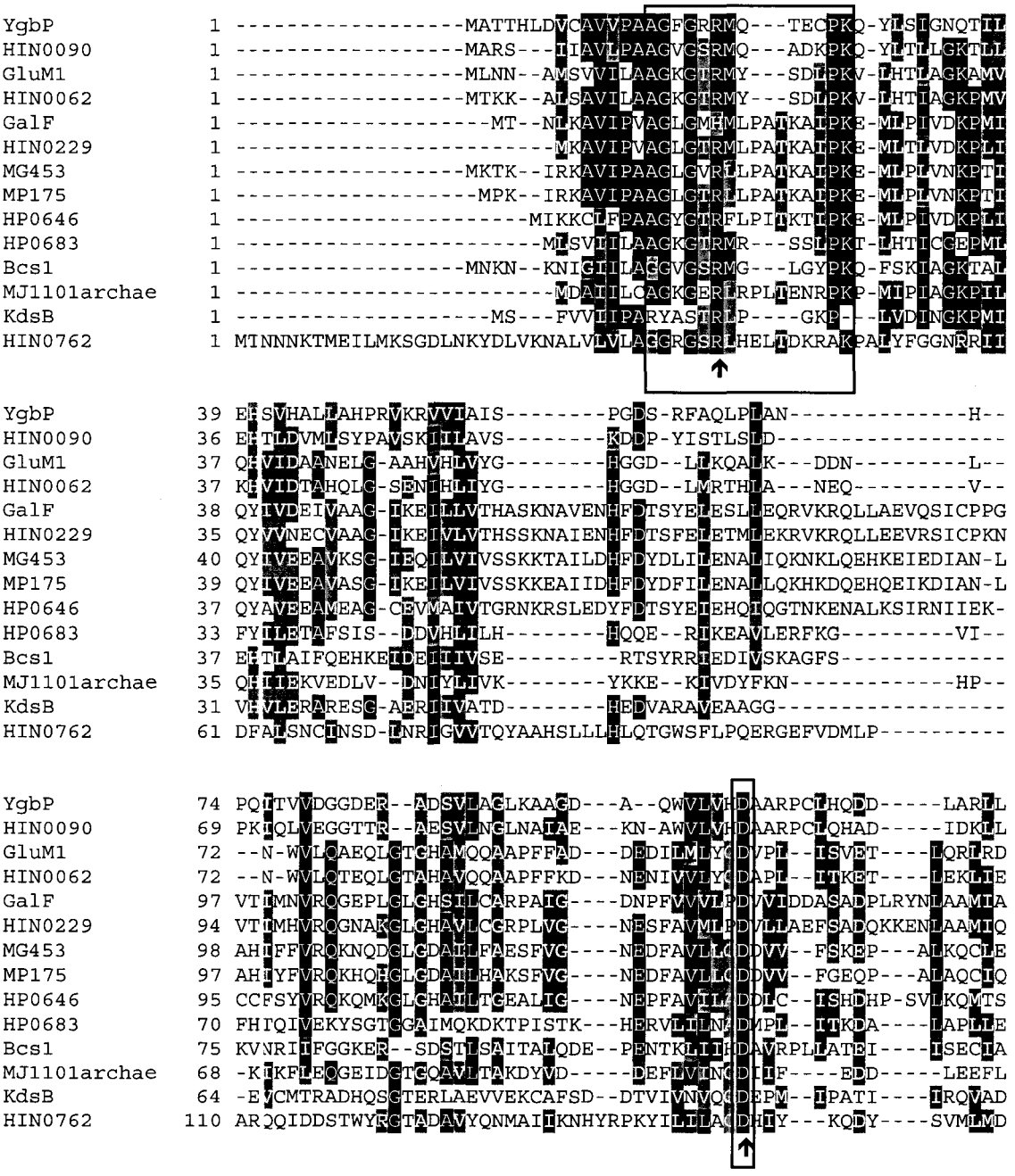
❖ APPENDIX VII: AMINO ACID SEQUENCE ALIGNMENT OF BCS1 WITH HOMOLOGOUS SHORT CHAIN DEHYDROGENASE/REDUCTASES

Conserved $Y^{382}XXXK^{386}$ motif in Bcs1 shown in Box.

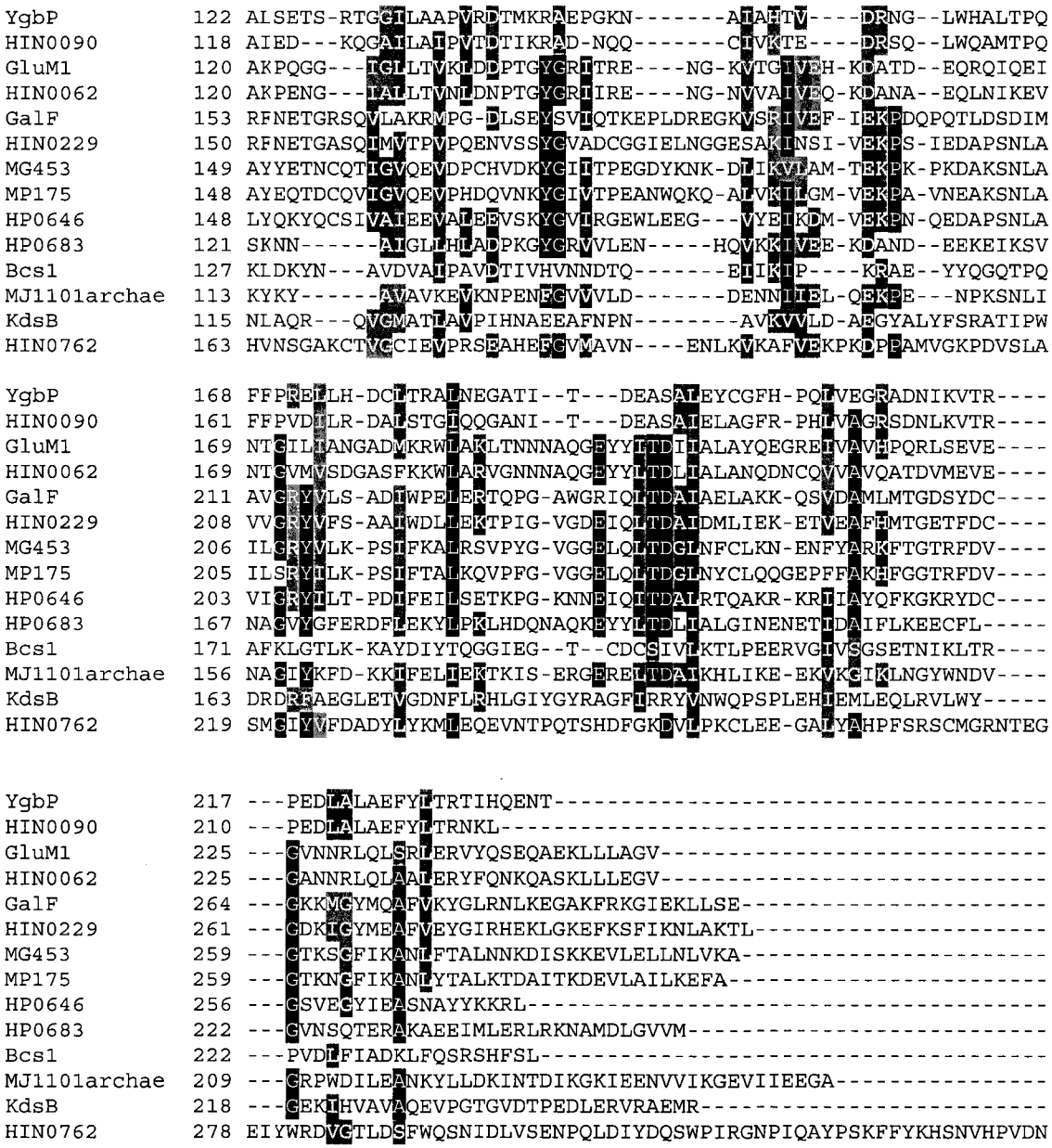


❖ APPENDIX VIII: AMINO ACID SEQUENCE ALIGNMENT OF BCS1 WITH HOMOLOGOUS SUGAR-PHOSPHATE NUCLEOTIDYLTRANSFERASES

Conservation of D^{105} and R^{18} of GlmU throughout sugar-phosphate nucleotidyltransferases including Bcs1 (D^{110} and R^{18}).



Bcs1 alignment with sugar-phosphate nucleotidylyltransferases conintued:



❖ APPENDIX IX: STEADY STATE KINETIC CHARACTERIZATION OF MUTANTS AND WILD TYPE BCS1

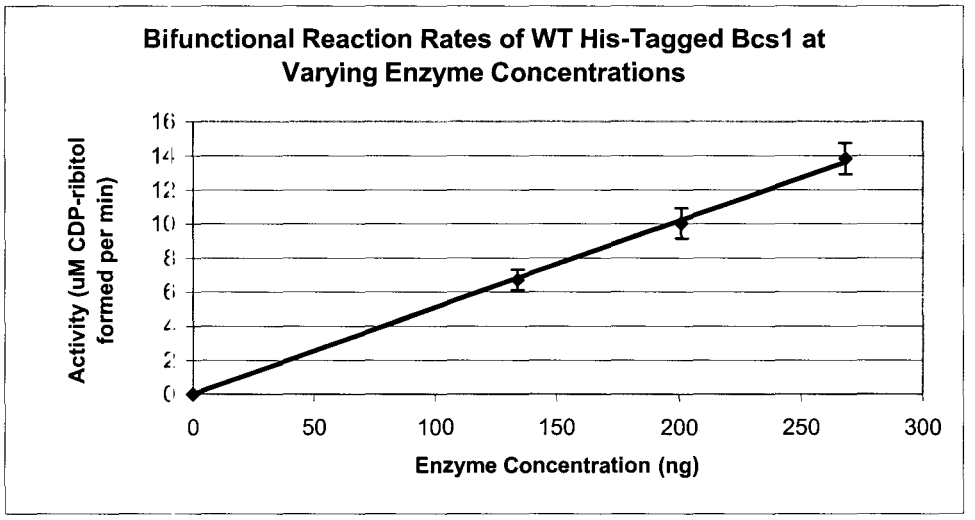
Steady State Kinetic Parameters Describing the Reactions Catalyzed by Bcs1 Mutants

	WT	R18A	D110A	Y382F	K386A
(1) reductase					
k_{cat} [a] (s^{-1})	22 ± 0.6	6.8 ± 0.4	0.12± 0.01	0.51 ± 0.04	0.32 ± 0.0002
D-ribulose 5-phosphate					
K_m [a] (μM)	76 ± 5.2	270 ± 36	140 ± 23	170 ± 21	190 ± 18
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	2.9	0.25	0.009	0.03	0.017
NADPH					
K_m [a] (μM)	44 ± 4.8	17 ± 0.5	43 ± 6.7	13 ± 1.7	39 ± 5.0
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	5.0	4.0	0.03	0.39	0.082
(2) cytidylyltransferase					
k_{cat} [a] (s^{-1})	13 ± 0.5	0.14 ± 0.01	0.006±0.001	0.07 ± 0.01	9.7 ± 0.47
D-ribitol 5-phosphate					
K_m [a] (μM)	130 ± 12	1000 ± 150	1200 ± 270	150 ± 20	120 ± 9.9
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	1.0	0.0014	4.9×10^{-5}	0.005	0.81
CTP					
K_m [a] (μM)	99 ± 9.1	390 ± 47	310 ± 52	210 ± 26	92 ± 8.9
k_{cat}/K_m (x 10^5) ($L \cdot mol^{-1} \cdot s^{-1}$)	1.3	0.0036	1.9×10^{-4}	0.003	1.1

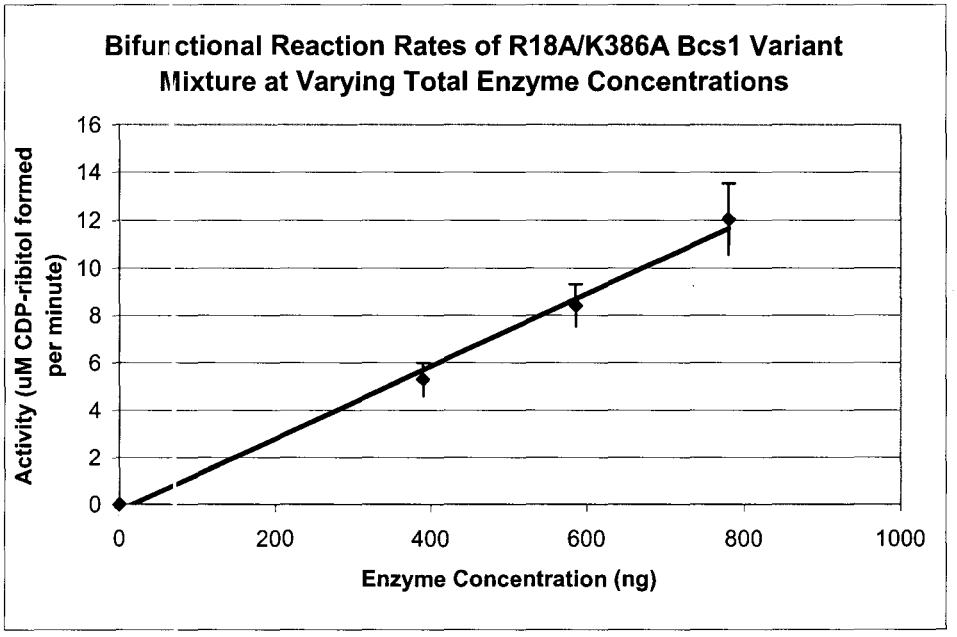
[a] Errors are standard errors of best fit for data from three separation determinations.

❖ APPENDIX X: LINEAR RELATIONSHIP OF BIFUNCTIONAL ACTIVITY ASSAYS AT VARYING ENZYME CONCENTRATIONS

A)



B)



Plotted are the turnover values of the bifunctional reaction of (A) wild type His_6-tagged Bcs1 and (B) R18A/K386A Bcs1 variant mixture at varying amounts of total enzyme concentrations to demonstrate linear relationship. Substrate concentrations in assay were 1.2 mM D-ribulose 5-phosphate, 600 μM NADPH and 600 μM CTP. The reaction was monitored by [5-^{3}H]CDP-ribitol formation following separation on an anion exchange (MonoQ) HPLC using a triethylammonium bicarbonate gradient. Errors are standard deviations of triplicate determinations.