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Biochemical and mutational analysis of coenzyme B₁₂ biosynthesis

by

Chenguang Fan

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Biochemistry

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ABSTRACT

The B₁₂ coenzymes are required cofactors for at least 15 different enzymes that are widely distributed in nature and are essential for human health. Characterizing enzymes in each step of vitamin B₁₂ metabolism in humans will provide important information for human B₁₂-related disease diagnosis and therapy. Human ATP:cob(I)alamin adenosyltransferase (hATR) catalyzes the final step in the conversion of vitamin B₁₂ into the active coenzyme, adenosylcobalamin. Inherited defects in this gene result in a rare but life-threatening disease, methylmalonyl aciduria. We conducted a random mutagenesis of the hATR coding sequence. Fifty-seven missense mutations were isolated mapping to 30 positions, 25 of which had not previously been shown to impair enzyme activity. Kinetic analysis and *in vivo* tests for enzyme activity were performed on the hATR variants, and mutations were mapped onto a hATR structural model. These studies functionally defined the hATR active site and tentatively implicated three amino acid residues in facilitating the reduction of cob(II)alamin to cob(I)alamin which is a prerequisite to adenosylation.

Human cannot synthesize coenzyme B₁₂ *de novo* but assimilate B₁₂ complex precursors, which are only from certain prokaryotes. The biosynthesis of B₁₂ has been studied extensively in *Salmonella enterica*, however, the function of PduX gene involved in B₁₂-dependent propanediol degradation was still unknown. *In vitro* test including ³¹P NMR spectroscopy and enzyme assays established that purified PduX-His₆ catalyzed the conversion of L-threonine and ATP to L-threonine-*O*-3-phosphate and ADP. A series of *in vivo* growth studies showed that PduX is an L-threonine kinase used for AdoCbl synthesis. The PduX enzyme belongs to GHMP kinase family by sequence similarity. Kinetic analysis indicated an

ordered ternary complex mechanism in which ATP is the first substrate to bind. The lack of obvious solvent pH and isotope effects indicated that proton transfer is unlikely to be involved directly in the rate determining step of catalysis. Based on a multiple sequence alignment of PduX homologues and other GHMP family members, 14 PduX variants were constructed by site-directed mutagenesis. Comparison of the Circular Dichroism spectra and kinetic properties of the PduX variants to those of the wild-type enzyme functionally defined the L-threonine kinase active site.

CHAPTER 1 GENERAL INTRODUCTION

Introduction

Vitamin B₁₂ is the most chemically complex of all the vitamins. The structure of B₁₂ is based on a corrin ring, which is similar to the porphyrin ring found in heme, chlorophyll, and cytochrome. Biosynthesis of the basic structure of vitamin B₁₂ can only be accomplished by certain bacteria and archaea, but conversion between different forms of the vitamin can be accomplished in the human body. Vitamin B₁₂ is important for the normal functioning of the brain and nervous system, and for the formation of blood. It is normally involved in the metabolism of every cell of the body, especially affecting DNA synthesis and regulation, as well as fatty acid synthesis and energy production.

Vitamin B₁₂ is naturally found in foods of animal origin including meat and milk products. Animals, in turn, must obtain it directly or indirectly from bacteria, and these bacteria may inhabit a section of the gut which is posterior to the section where B₁₂ is absorbed. Vitamin B₁₂ can be supplemented in healthy subjects by liquid, strip, nasal spray, or injection and is available singly or in combination with other supplements.

Literature Review

I. History of Vitamin B₁₂

The early history of B₁₂ belongs entirely to medicine. In 1824, Combe recorded the first tentative description of pernicious anemia and mentioned that it could be related to a disorder of digestive system (1). The next hundred years witnessed a slow but steady increase in our knowledge of the signs of the disease and methods of diagnosis but a total absence of any advance in the treatment of the disease, which remained incurable and was usually fatal.

Then in 1925, Whipple showed that the regeneration of red blood cells in dogs made anemic by bleeding was stimulated by a diet containing liver, and in 1926 Minot and Murphy in Boston reported a remarkable improvement in patients fed a diet of raw liver. This clue started the search for the “liver factor” or “anti-pernicious anemia factor”, which last over twenty years. Whipple, Minot and Murphy were rewarded the Nobel Prize in Physiology and Medicine in 1934.

After the original impetus received from medicine in 1926 the main stimuli have come from the fields of microbiology and biochemistry, in which one can discern four successful and overlapping phases of activity (2).

The first phase (from 1926 to 1948) ended with successful isolation of B₁₂ in 1948. The isolation of crystalline vitamin B₁₂ was reported independently by two teams in 1948, first by Folkers and his colleagues at Merck Laboratories in the U.S. (3). They isolated a red crystalline compound from liver. This compound, designed as vitamin B₁₂, was found to produce hematological responses in three patients with pernicious anemia. Almost simultaneously, Smith and Parker at Glaxo Laboratories in the U.K. reported the purification of an anti-pernicious anemia factor from both nonproteolyzed and papain-treated liver (4, 5). In the following year, Ellis, Petrow and Snook at British Drug Houses in the U.K. reported the isolation of the crystalline anti-pernicious anemia factor from liver and showed its electronic spectrum (6).

The second phase (from 1948 to 1955) involved the development of methods for the large scale production B₁₂ by fermentation techniques through the screening and selection of microorganisms (7, 8). It soon became clear that B₁₂ was only one of a family of corrinoids occurring in nature. Immediately following the isolation crystalline vitamin B₁₂, studies were

initiated in several laboratories to determine the structure of this new vitamin. Early analysis showed that it contained both cobalt and phosphate. Brink carried out the first elemental analysis of the vitamin, yield a result of $C_{61-64}H_{86-92}N_{14}O_{13}PCo$, remarkably close to the now-known composition, $C_{63}H_{88}N_{14}O_{14}PCo$ (9). The elucidation of the structure of vitamin B_{12} and the identification of the unique corrin ring containing four reduced pyrrole rings with a direct connection between the A and D rings finally accomplished by X-ray crystallographic analysis in the laboratory of Hodgkin in 1954 and the next few years (10).

The third phase (from 1950 to 1962) may be considered as answering the question “which corrinoids occur in nature” (7, 8, 11). The most important single discovery under this heading was the isolation of the so-called “coenzyme” form of a corrinoid by Barker and his associates in California in 1958 (12). They reported a charcoal-absorbable substance was required as a cofactor for the glutamate mutase reaction and this yellow-orange cofactor was found to be light-sensitive. The relation between the cofactor and vitamin B_{12} was recognized when Weissbach noted the similarity between the spectra of vitamin B_{12} and the coenzyme in alkaline cyanide solution (13). In coenzyme form of vitamin B_{12} , the cyanide group is replaced by adenosine or methyl group. X-ray analysis, carried out by Professor Dorothy Hodgkin and her associates at Oxford, has played a very important part in the development of vitamin B_{12} chemistry. Their work revealed the presence of two structures hitherto unknown in nature, namely the corrin ring which is present in all corrinoids and cobalt-carbon bond which is present in the coenzymes (14). Professor Hodgkin’s elegant work in applying X-ray diffraction techniques to such complicated molecules was rewarded with the Nobel Prize for Chemistry in 1964.

Barker's discovery of the coenzyme can also be considered as the start of the fourth phase of activity (from 1958 to now and still continuing), that of enzymatic reactions, in which one aims to answer the question "what reactions do corrinoids catalyze in conjunction with proteins and how". To date, several adenosylcobalamin-dependent enzymes have been isolated including four carbon-skeleton mutases (glutamate mutase, methylmalonyl CoA mutase, 2-methyleneglutarate mutase, and isobutyryl CoA mutase), four eliminases (dioldehydrase, glycerol dehydrase, ethanolamine-ammonialyase, and ribonucleotide reductase), and three amino mutases (L- β -lysine mutase, D- α -lysine mutase and ornithine mutase). Methylcobalamin and methylcorrinoids have been shown to be cofactors for methionine synthase and for the synthesis of acetone and methane in several anaerobic bacteria and archaea (15-18).

The advances made during the past 60 years in B₁₂ research have been phenomenal and are certain to continue. In the short future, we can expect that the three-dimensional structure of all the B₁₂-dependent enzymes and binding proteins will be solved. At that time the mechanism of these enzymes will also be established.

II. Chemistry of Vitamin B₁₂

The various derivatives of vitamin B₁₂ belonging to the series of *cobalamins* are cobalt tetrapyrrole complexes having the structure shown on Figure 1.1. The central cobalt atom is coordinatively bound to the four nitrogens of the corrin ring, one of these bearing a negative charge. Attached to a carbon of the corrin ring is a nucleotide side chain terminated by a 5,6-dimethylbenzimidazole group (DMB), which may act as an axial ligand toward the cobalt atom. The side chain bears an additional negative charge on the phosphate group down to low pH values. Cobalamin complexes in which the DMB is bound to the cobalt atom are

usually named *base-on* forms, whereas if such coordination is absent or replaced by an exogeneous ligand the designation *base-off* is used. *Cobinamides* are obtained by hydrolytic cleavage of the nucleotide side chain at the phosphate group. The side chain was reduced, and then lost its potential liganding ability toward the cobalt atom. Complexes in which the side chain still possesses the α -D-ribofuranose 3-phosphate residue but not necessarily the terminal 5,6-dimethylbenzimidazole, which may be replaced by other imidazolic structures, are named *cobamides*. The various cobamides, cobalamins, and cobinamides differ one from the other by the nature of the axial ligands in the lower and upper positions: water, OH⁻, CN⁻, alkyl carbanions, giving rise respectively to aquo, hydroxo, cyano, and alkyl cobalamins (19).

The cobalt atom in cobalamins and cobinamides can exist under three main formal oxidation states, Co(III), Co(II), Co(I) (Figure 1.2): which display quite different chemical properties. Roughly speaking, Co(III) appears as an electrophile, Co(II) as a radical, and Co(I) as a nucleophile. Oxidoreductive conversions between the three oxidation states are thus of key importance in the chemistry of vitamin B₁₂. When the formal oxidation state of the cobalt atom decreases, its coordination number tends to decrease accordingly. In the thermodynamically predominating forms, the coordination number is generally 6 (two axial ligands) for Co(III), 5 (one axial ligand) for Co(II), and 4 (no axial ligand) for Co(I). In B₁₂ electrochemistry, electron transfer is therefore expected not to be a simple outer-sphere process. It is generally accompanied by the expulsion of one ligand when going to a lower oxidation state and by the formation of a coordination bond upon increasing the oxidation state. On the other hand, according to the presence of potential axial ligands in the reaction medium, several forms of a given oxidation state may coexist and compete in reduction or

oxidation processes. The electrochemistry of B₁₂ derivatives under both its thermodynamic and kinetic aspects is thus anticipated to depend heavily upon trans-ligandation factors.

The nomenclature for vitamin B₁₂ is complex. The rules for naming complete molecules and precursors have been published by the Commission on Biochemical Nomenclature (CBN) of the International Union of Pure and Applied Chemistry (IUPAC) and revised by the International Union of Biochemistry (IUB) (20). For the reader's benefit, I highlight below terminology used in this thesis.

Corrinoids. Molecules containing the cobalt-containing cyclic tetrapyrrole known as the corrin ring.

Incomplete corrinoids. Corrinoids lacking the lower (Co α) axial ligand base.

Cobamide (Cba). A complete corrinoid, that is, a corrinoid containing the nucleotide loop. The name of the Cba depends on the nature of the lower ligand.

Cobalamin (Cbl). The Cba whose lower (Co α) ligand base is 5,6-dimethylbenzimidazole (DMB).

Adenosylcobamide (AdoCba). The coenzymic form of a Cba whose lower (Co α) ligand is not DMB.

PseudoAdoCba. The coenzymic form of AdoCba with adenine as the lower (Co α) ligand.

Vitamin B₁₂, or cyanocobalamin. Cbl with a cyano group as the upper (Co β) ligand.

Coenzyme B₁₂ or AdoCbl. Cbl with 5'-deoxyadenosine (Ado) as the upper (Co β) ligand and DMB as the lower (Co α) ligand.

α -5,6-Dimethylbenzimidazole-ribosyl-5'-phosphate (α -ribazole-P). The nucleotide loop consisting of the DMB attached to ribose-5'-P. The N-glycosidic bond in this nucleotide is in the alpha configuration.

III. Biosynthesis of Vitamin B₁₂

Many authors have suggested that B₁₂ was synthesized prebiotically (21) and may have been important to catalysis in the “RNA world.” In some bacteria, B₁₂ biosynthesis begins with an aminoacyl-tRNA molecule, and an RNA molecule may play an intimate role in regulating expression of biosynthetic and transport genes. Porphyrins such as those contributing to the B₁₂ structure have been synthesized nonenzymatically in so-called primitive earth experiments (22). The cobalamin biosynthetic pathway, and the pathways common to heme, chlorophyll, and siroheme synthesis, may reflect the evolution of energy metabolism (Figure 1.3). It seems likely that this pathway first evolved to produce B₁₂, a view supported by considering the structure of uroporphyrinogen III (UroIII), the common precursor of heme, siroheme, chlorophyll, and cobalamin. UroIII is asymmetric in the sense that one of the porphyrin rings is reversed with respect to the others. The reversal may be important for the carbon elimination or ring contraction that occurs later in cobalamin synthesis. This arrangement indicates that the entire pathway developed initially to serve B₁₂ synthesis and later added the branches to siroheme, heme, and chlorophyll. It is suggested that the initial significance of B₁₂ was to support anaerobic fermentation of small molecules by generating internal electron sinks (23). Later, siroheme allowed inorganic molecules to be used as electron acceptors, which is seen in siroheme’s modern role as a cofactor for sulfite and nitrite reductases. Still later arrivals may have been chlorophyll and heme, which allowed

biological formation of molecular oxygen and use of oxygen as a respiratory electron acceptor.

According to a widely accepted historical view, B₁₂ synthesis is restricted to some Bacteria and Archaea. Many animals, including humans, and protists require B₁₂ but apparently do not synthesize it. Plants and fungi are thought to neither synthesize nor use B₁₂ in their metabolism. Figure 1.4 superimposes the distribution of B₁₂ on a prominent view of the evolutionary relationships between these life-forms. The distribution pattern, even with some exceptions, raises questions regarding the biochemical significance of B₁₂ and the metabolic differences among life-forms that allow some to escape the need for B₁₂. Such questions should be kept in mind as we review the general nature of B₁₂-dependent reactions.

Vitamin B₁₂ is manufactured by bacteria and archaea via two alternative routes that differ primarily in the early stages that lead to the contraction of the macrocycle and excision of the extruded carbon molecule and its attached methyl group. One pathway incorporates molecular oxygen into the macrocycle as a prerequisite to ring contraction, and has consequently been termed the aerobic pathway. The alternative, anaerobic, route takes advantage of a chelated cobalt ion, in the absence of oxygen, to set the stage for ring contraction. The intermediates of the aerobic biosynthetic route from UroIII to adenosylcobalamin have been determined by the isolation of the genes from *Pseudomonas denitrificans* (*cobA-cobW*) and the assignment of the functions of the encoded enzymes based on their catalytic activity (Figure 1.5) (24). Genes encoding proteins involved in the biosynthesis of cobalamin by the alternative anaerobic pathway have been isolated and sequenced from bacteria such as *Salmonella typhimurium* (25), *Bacillus megaterium* (26) and *Propionibacterium freudenreichii* (*shermanii*) (27) and, while catalytic activity has been

determined experimentally for a few of the gene products, most of their functions have been derived on the basis of sequence similarities.

The biosynthetic route to adenosylcobalamin from its five-carbon precursor, 5-aminolaevulinic acid (ALA) may be divided into three sections: (1) the biosynthesis of UroIII from ALA, which is shared by both pathways (Figure 1.5) ; (2) the conversion of UroIII into the ring-contracted, deacylated intermediate (precorrin 6 or cobalt-precorrin 6), wherein lies the primary differences between the two pathways (Figure 1.5) ; and (3) the transformation of this intermediate to adenosylcobalamin (Figure 1.6).

1. Biosynthesis of UroIII. UroIII is an intermediate not only of vitamin B₁₂ but also of haem, sirohaem, chlorophylls and factor F430. It is biosynthesized from ALA utilizing the enzymes ALA dehydratase (HemB), porphobilinogen deaminase (HemC) and UroIII synthase (HemD). The most recent advance in elucidation of the biosynthesis of UroIII has been the unveiling of the crystallographic structure of human UroIII synthase (28), which revealed an interesting barbell-shaped conformation, but which did not shed any further light on the mechanism of the enzyme, due to the lack of a structure with bound substrate or other ligand. The absence of any essential conserved residues, however, as revealed by site-directed mutagenesis, suggests that the function of the enzyme is to hold the substrate in the correct conformation, with catalysis then proceeding spontaneously.

2. Conversion of UroIII into precorrin 6 or cobalt-precorrin 6. In the aerobic pathway, this process, resulting in the formation of precorrin 6, requires five S-adenosyl-L-methionine-dependent methyltransferases for the introduction of six methyl groups and is characterized by the incorporation of molecular oxygen by a monooxygenase. First, precorrin 2 is formed from UroIII by methylation at C-2 and C-7 by CobA, followed by

methylation at C-20 by CobI to give precorrin 3. Incorporation of O₂, also at C-20, and the concomitant formation of a γ -lactone, both catalysed by CobG, to yield precorrin 3 hydroxylactone are prerequisites for contraction of the macrocycle. Ring contraction is then triggered by methylation at C-17 by CobJ to give precorrin 4, featuring amethylketone, derived from C-20 and its attached hydroxy and methyl groups, pendant to C-1. Precorrin 5 is formed by methylation of C-11 by CobM, and then, in the final step to form precorrin 6, the methylketone is lost as acetic acid upon methylation of C-1 by CobF.

The anaerobic pathway, resulting in cobalt-precorrin 6 rather than precorrin 6, also requires five methyltransferases, and is distinguished by the early incorporation of cobalt into the macrocycle. The biosynthesis of precorrin 2 is the same as in the aerobic pathway. After the synthesis of precorrin 2, the anaerobic route diverges from the aerobic route with the chelation of cobalt into the macrocycle to give cobalt-precorrin 2. In the anaerobic pathway, cobalt-precorrin 2 is methylated at C-20 by CbiL to afford cobalt-precorrin 3. The next known intermediate in the anaerobic pathway is cobalt-precorrin 4. CbiH is responsible for addition of a methyl group at C-17, which, as in the aerobic pathway, triggers contraction of the macrocycle. In contrast with precorrin 4 of the aerobic pathway, however, cobalt-precorrin 4 bears an unopened δ -lactone instead of a methylketone. Although the next intermediate of the anaerobic pathway, corresponding to precorrin 5 (cobalt-precorrin 5), has yet to be isolated, the C-11 methyltransferase is believed to be CbiF, based on its identity with CobM. The conversion of cobalt-precorrin 5 into cobalt-precorrin 6 requires opening of the δ -lactone ring, excision of acetaldehyde and C-1 methylation. Exchange with water in the carboxy group of the ring A acetate results from hydration during opening of the δ -lactone,

and the oxygen of the acetaldehyde is derived concomitantly from the ring A acetate during this process.

3. Conversion of precorrin 6 or cobalt-precorrin 6 into adenosylcobalamin. The known pathway and enzymes necessary for the conversion of precorrin 6 into adenosylcobalamin in the aerobic pathway are shown in Figure 1.6. The structures of the intermediates of the anaerobic pathway between cobalt-precorrin 6 and adenosylcobalamin are believed to be the same as those of the aerobic pathway, with the exception that, since the cobalt ion is already in place, dihydro-precorrin 6, precorrin 8 and hydrogenobyrrinic acid are replaced with the corresponding cobalt complexes. Thus, in the anaerobic pathway, hydrogenobyrrinic acid is replaced by cobyrinic acid, which then is the substrate for bisamidation to afford cob(II)yrinic acid a,c-diamide. All of the ensuing intermediates are identical in the aerobic and anaerobic pathways.

4. Control of the cob/pdu Regulon. The *cob* operon (encoding B₁₂ synthetic enzymes) maps adjacent to the *pdu* operon, which encodes enzymes for propanediol degradation. The two operons are both induced by propanediol using a single regulatory protein. These results indicate that the main role of B₁₂ in *S. typhimurium* is in supporting catabolism of propanediol. Two global regulatory systems (Crp/Cya and ArcA/ArcB) affect inducibility of the *cob* and *pdu* operons (29-31). Both operons are activated aerobically and anaerobically by Crp protein and anaerobically by ArcA protein. Maximum inducibility is seen during anaerobic respiration of a poor carbon source; under these conditions the Crp and ArcA proteins act additively. The control of the regulon depends on five promoters, all located in the central region between the *cob* and *pdu* operons; four of these promoters are activated by the PoxR protein (32).

The current model for control of this system includes the following features (Figure 1.7): The *cob* and *pdu* operons are transcribed divergently from promoters indicated at the far right and left in the figure. These promoters are activated whenever both the PocR protein and propanediol are present. Global control of the two operons is exerted by varying the level of PocR protein. The *pocR* gene is transcribed by three promoters controlled both by global regulatory proteins and by autoinduction (32). The shortest transcript (from Ppoc) appears to be regulated only by Crp/cAMP. The P2 transcript clearly is autoregulated by PocR and, in addition, requires the ArcA protein, which signals a reduced cell interior (32). Control of the P1 promoter is less certain but may involve either the Fnr protein (responding to a reduced cell interior) or the Crp protein (responding to a shortage of carbon and energy) in addition to the PocR activator. The involvement of these proteins in control of P1 is based heavily on the presence of appropriate binding sites upstream of that promoter.

It is proposed that the regulon has three states (23). In the *off* condition (during aerobic growth on glucose), all promoters are at their lowest level; PocR protein is produced by the basal expression levels of promoters P1, P2, and Ppoc. Cells enter a *standby* state when they grow under any set of global conditions appropriate for induction, but without propanediol. These conditions include aerobic growth on a poor carbon source and/or growth without oxygen. These conditions stimulate expression of both the PduF transporter and the PocR regulatory protein, but the *cob* and *pdu* operons remain uninduced. The *standby* expression of PocR can occur from the Ppoc and/or the P1 promoter without inducer. The two proteins induced in the *standby* state (PduF and PocR) are those needed to sense propanediol. If propanediol appears, the P1 and P2 promoters are induced, increasing the level of PocR

protein and placing the system in the on state. The resulting high level of PocR/propanediol complex induces expression of the *cob* and *pdu* promoters.

IV. Functions of Vitamin B₁₂

The original significance of B₁₂, and its remaining primary role in many modern bacteria, may support fermentation of small molecules by catalyzing molecular rearrangements that generate both an oxidizable compound and an electron sink for use in balancing redox reactions. In enteric bacteria, this role is seen in the B₁₂-dependent degradation of ethanolamine, propanediol, and glycerol (Figure 1.8). In these reactions, the B₁₂-mediated rearrangement generates an aldehyde that can be oxidized and provide ATP; the oxidation reactions can be balanced by reducing a portion of the aldehyde to an alcohol, which is excreted. The B₁₂-dependent reaction forms the reducible compound by a rearrangement, essentially an internal redox reaction. In nonenteric bacteria, the B₁₂-dependent amino mutases (specific for glutamic acid, lysine, leucine, or ornithine) catalyze mechanistically similar reactions that support fermentation of these amino acids.

Thus the earliest use of B₁₂ may have been to support anaerobic, fermentative growth at the expense of small molecules. Additional reactions (such as methionine synthesis and nucleotide reduction) appeared as secondary uses. After oxygen and aerobic respiration appeared on earth, many organisms no longer needed to perform fermentations and lost some of their original enzymatic capabilities. The secondary uses such as methyl transfer remained important and enforced a continued requirement for B₁₂. Obligate aerobes and animals appear to require B₁₂ to perform these nonfermentative functions. In humans, two B₁₂-dependent reactions are known. Methionine synthetase, a methyl transferase, is presumed to be

important primarily in recycling folate and secondarily in producing methionine (33). Methyl malonyl CoA mutase may serve mainly to remove toxic products of lipid breakdown (34).

1. B₁₂-Dependent Reactions in Enteric Bacteria

Propanediol Dehydratase, which converts 1,2-propanediol to propionaldehyde, is found in virtually all enteric bacteria tested except *Escherichia coli* (35, 36). Some bacteria ferment propanediol by oxidizing a portion of the propionaldehyde to provide carbon and energy while reducing the rest to provide an electron sink for balancing redox reactions (37). In *Salmonella* species this process provides energy but no carbon source. Propanediol is encountered frequently by bacteria, because it is produced during anaerobic catabolism of the common methylpentoses, rhamnose and fucose.

Ethanolamine Ammonia Lyase converts ethanolamine to acetaldehyde and ammonia (38, 39). Under some conditions, the produced acetaldehyde can serve as a carbon and energy source via acetyl-CoA. Ethanolamine is frequently encountered in nature as part of common lipids, phosphatidyl ethanolamine and phosphatidyl choline.

Glycerol Dehydratase converts glycerol to β -hydroxypropionaldehyde, which can be reduced to 1, 3-propanediol (40, 41). This reaction balances the reducing equivalents generated by glycerol dehydrogenase. Glycerol dehydratase is common in enterics but is absent from both *Salmonella* spp. and *E. coli* (35).

Epoxyqueuosine Reductase performs the last step in formation of the hypermodified tRNA base, queuosine, found in tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn}, and tRNA^{Asp} (42). The modified base is not essential for bacterial growth under laboratory conditions (43). Although the final reaction has been reported to require B₁₂ (42), it proceeds in anaerobically grown *E.*

coli cells, which do not make B₁₂ (35). We suggest that the reaction may be stimulated by B₁₂, perhaps indirectly, in vivo, but can be catalyzed without this cofactor.

2. B₁₂-Dependent Reactions in Human

Methionine Synthase transfers a methyl group from methyltetrahydrofolate to homocysteine as the final step in synthesis of methionine and is probably the best-known B₁₂-dependent reaction (Figure 1.9) (44, 45). During primary turnover (red arrows), a methyl group is transferred from CH₃-H₄folate to the enzyme-bound cob(I)alamin cofactor, forming H₄-folate and methylcobalamin. Methylcobalamin is then demethylated by Hcy, forming methionine and regenerating cob(I)alamin. In reactivation (green arrows), cob(II)alamin is reduced by one-electron transfer from flavodoxin and the resulting cob(I)alamin is methylated by AdoMet. The lack of this reaction underlies many aspects of human B₁₂-deficiency disorders (33). In *Salmonella* spp. and in *E. coli*, the B₁₂-dependent methyl transfer reaction is catalyzed by the MetH enzyme, and both organisms produce a second enzyme (MetE) that can catalyze the same reaction without B₁₂ (44, 46-48). The MetH enzyme is used preferentially when B₁₂ is available; the alternative MetE enzyme is induced in response to accumulated homocysteine when the MetH enzyme is inactive (49). In bacteria with an alternative, B₁₂-independent enzyme, methionine synthesis is unlikely to be the major selective force maintaining B₁₂ synthetic capacity.

Methylmalonyl-CoA Mutase interconverts (R)-methylmalonyl-CoA and succinyl-CoA (Figure 1.10) (50). The first step, homolysis of the Co-carbon bond, is observed upon addition of substrate and leads to the formation of cob-(II)alamin and the deoxyadenosyl radical (step i). This is followed by hydrogen atom (H-atom) abstraction from the substrate, methylmalonyl-CoA, by the deoxyadenosyl radical to generate a reactive primary substrate

radical (step ii). Rearrangement generates a secondary product-centered radical (step iii). Finally, a second H-atom transfer from deoxyadenosine yields product (step iv), and recombination of the cofactor-centered radical pair (step v) completes a turnover cycle. Higher animals require the mutase for degradation of odd-chain-length fatty acids and certain branched-chain amino acids (Figure 1.11). In humans, mutase deficiency results in an often fatal methylmalonic acidemia (34), and in certain neuropsychiatric symptoms. These symptoms may result from synthesis of abnormal myelin lipids in the presence of accumulated propionyl- and methylmalonyl-CoA (33, 51). In certain bacterial fermentations, succinate is converted to propionate via the mutase rather than being excreted. This pathway allows conservation of a biotin-activated CO₂ that is derived from succinate. In *Streptomyces cinnamomensis*, the mutase may also play a role in polyketide antibiotic synthesis (52).

3. Other Prominent B₁₂-Dependent Reactions

Acetyl-CoA Synthesis: In many anaerobic bacteria, methyl-corrinoids are involved in acetyl-CoA synthesis via the Wood/Ljungdahl pathway (53-55). In this pathway, a methyl group is transferred from methyltetrahydrofolate via a methyl-corrinoid/iron sulfur protein to CO-dehydrogenase, which synthesizes acetyl-CoA from this methyl group, CO, and coenzyme A (56). A corrinoid plays an analogous role in the energy-yielding metabolism of acetogenic bacteria (53), which synthesize acetate from 2 CO₂ as a means of generating a terminal electron sink.

Methyl Transfer in the methane-Producing Archaea: Methyl-corrinoids are essential for formation of methane by the strictly anaerobic methane-producing Archaea (54). Corrinoid proteins play a role in the transfer of methyl groups from methanogenic substrates to the thiol group of the methanogen-specific cofactor, coenzyme M. Different enzymes

mediate methyl transfer from alternative methanogenic substrates such as acetate (57), methylamines (58), methanol, pyruvate (59) and methyltetrahydromethanopterin, an intermediate of methanogenesis from formate and CO₂ (60). The latter reaction is analogous to methionine synthesis in that the methyl group is transferred from an intermediate pterin which is a heterocyclic compound composed of a pyrazine ring and a pyrimidine ring to a thiol group via methyl-B₁₂ (60). Considerable energy is released by transfer of a methyl group to coenzyme M from methyltetrahydromethanopterin (54, 61). The energy of the transfer to coenzyme M can be recovered by coupling the methyl transfer to extrusion of a sodium ion, which eventually leads to generation of a proton motive force (62). The transferase that achieves this feat is an integral membrane protein (63) composed of eight different subunits (61).

Ribonucleotide Reductases generate the deoxyribonucleotides needed for DNA synthesis. Four classes of this reductase are known, each with a different cofactor requirement and quaternary structure; this variability is unusual for enzymes that play such key metabolic roles. The Ado-B₁₂-dependent reductases belong to Class II and are found primarily in microorganisms (64, 65). The reaction mechanism and active site structure of the corrinoid-dependent reductase appears remarkably similar to that of the Ado-B₁₂-independent Class I enzyme of *E. coli* (66). In this reductase, Ado-B₁₂ serves as a free-radical generator. Other reductases form radicals by alternative means (67).

Degradation of β -Hydroxy Ethers and β -Hydroxy Amines: Corrinoids are implicated in the anaerobic degradation of several compounds thought to be generally recalcitrant to degradation in the absence of oxygen. The compounds include polyethylene glycol (68), triethanolamine (69), and possibly phenoxyethanol (70). The significant chemical feature of

these compounds is a hydroxyl group β to an ether, a tertiary amine, or a secondary amine. These reactions are related to those catalyzed by diol dehydratases in that they are intramolecular redox reactions that involve the migration of a hydroxyl group.

V. Vitamin B₁₂-Related Diseases

Till today, there are only two known B₁₂-dependent reaction in the human body: methionine synthase and methylmalonyl-CoA mutase. Thus, disorders of cobalamin transport and metabolism (Figure 1.12), whether inherited or acquired, manifest themselves as deficiencies in the activities of one or both of these enzymes and lead to accumulation in blood and urine of one or both of the substrates, homocysteine and methylmalonyl-CoA, respectively. The pathophysiology of these conditions varies with the severity and nature of the lesions and results from both the effects of substrates accumulation and products deficiency. From such studies, the following three subclasses of cobalamin disorders emerged: those resulting (i) in combined defects in MeCbl and AdoCbl synthesis (*cblF*, *cblC*, and *cblD*), (ii) in an isolated defect in MeCbl synthesis (*cblD*-variant 1, *cblE*, and *cblG*), and (iii) in an isolated defect in AdoCbl synthesis (*cblD*-variant 2, *cblA*, and *cblB*). The *mut* locus encodes methylmalonyl-CoA mutase. An additional locus, *cblH*, originally described as causing an isolated deficiency of methylmalonyl-CoA mutase (71), is now believed to be *cblD*-variant 2 (72).

Uptake and Transport of B₁₂. Cobalamin is a water-soluble vitamin and at physiological levels of intake (1–5 $\mu\text{g/day}$) is unable to traverse the plasma membrane unaided. Three B₁₂ binding proteins, intrinsic factor, haptocorrin, and transcobalamin II, bind B₁₂ with great avidity and mediate its uptake and transport (73). Intrinsic factor is a secretory glycoprotein that binds B₁₂ in the small intestine and in complex with a specific receptor,

cubulin, located on the brush border of enterocytes (73). Haptocorrin is a salivary glycoprotein that binds B₁₂ with higher affinity than intrinsic factor at the acidic pH of the stomach and liberates the cofactor in the proximal small intestine upon being digested by pancreatic enzymes. B₁₂ emerges from these cells into the portal circulation in complex with transcobalamin II (74). Haptocorrin is also found in the serum where it is derived from leukocytes. It appears to function as a major storage protein for cobalamin and may play a role in removal of degraded derivatives (75). All three proteins exhibit a very high affinity for B₁₂ with K_d values for aquocob(III)alamin of 1 pM (intrinsic factor), 0.01 pM (haptocorrin), and 5 fM (transcobalamin II) measured at pH 7.5 and 20 °C (76). The pathway (Figure 1.13) involves a series of binding proteins in different compartments and specific receptors for the transport of these proteins and the associated cobalamin into or across cells. The pathway takes advantage of changes in binding constants to provide both directionality and efficiency to the transport process, as cobalamin and its transport proteins are exposed to environments of different pH in the gastrointestinal system and subcellular organelles.

Inherited Defects Affecting Both AdoCbl and MeCbl Synthesis. B₁₂ is ferried to cells with a very high affinity transporter, transcobalamin II, which binds to a specific receptor on the cell surface (77). This complex is endocytosed and delivered to the lysosome (Figure 1.12). The *cblF* class of mutations impairs egress of B₁₂ from the lysosome and leads to the accumulation of the cofactor in this compartment (78). The affected gene in *cblF* patients remains to be identified.

The function lost or impaired in *cblC* patients was first suggested to be a cytoplasmic reductase that reduces cob(III)alamin to cob(II)alamin (79). In fact cob(III)alamin reductase activities associated with the microsomal and mitochondrial membranes that exhibited

diminished activity in *cblC* (and *cblD*) cell lines have been reported (80). However, both locations seem improbable for delivery of the resulting cob(II)alamin to the target cytoplasmic and mitochondrial matrix enzymes, methionine synthase and adenosyltransferase (that synthesizes AdoCbl), respectively. It is possible that a nonspecific reductase activity was measured in these studies since the redox potential for the cob(III)alamin/cob(II)alamin redox couple is relatively high, making this a relatively facile reduction. The gene responsible for methylmalonic aciduria and homocystinuria, of the *cblC* class has been identified recently (81) and based on its sequence, does not appear to be a reductase. Alternatively, an NADPH and FAD-dependent β -ligand transferase function has been ascribed to the *cblC* (or *cblD*) gene product that catalyzes the reductive exchange of the aquo or cyano substituent at the upper or β -axial position with glutathione to generate glutathionyl-cobalamin (80). However, the presence of glutathionyl-cobalamin in cells has not been established unequivocally since under conditions used for isolation (80), detection of sulfitocobalamin (a breakdown product of glutathionyl-cobalamin), may be expected. Furthermore, the uncatalyzed conversion of aquocobalamin to glutathionyl-cobalamin is predicted to be rapid and essentially irreversible under physiological conditions (82), and the very relevance of cyanocobalamin in biology is uncertain. Although cyanocobalamin is the cofactor form used in many vitamin supplements today, in the absence of a known biological source of this derivative, the question as to whether mammalian cells would have evolved a system to specifically catalyze its decyanation is an open one. Thus, while *cblC* is the most common of the inherited disorders in cobalamin utilization, the nature of its encoded function is presently unclear.

The *cblD* defect is enigmatic. Initially described in only two patients who were siblings, it was reported to result in a combined deficiency of methionine synthase and methylmalonyl-CoA mutase (83). As described above, limited biochemical characterization of these cell lines revealed similarities to *cblC* cells, albeit with deficits that were less severe (79). A more recent study on three additional patients revealed hetero-geneity within the *cblD* mutant class, which has consequently been divided into variants 1 and 2 respectively (72). Variant 1 is associated with an isolated deficiency in MeCbl synthesis and variant 2 with an isolated defect in AdoCbl synthesis (Figure 1.12). On the basis of these observations, it has been suggested that a single *cblD* gene product “presents” B₁₂ to methionine synthase and to the mitochondrial membrane or that alternative splicing results in cytosolic and mitochondrial variants of the resulting protein (72). Mutations in *cblD* affecting B₁₂ binding would result in a combined deficiency of both cofactors whereas other mutations could impair interactions with one or the other protein target.

Inherited Defects Affecting Only MeCbl Synthesis. The defects in the *cblE* and *cblG* cell lines have been localized to methionine synthase reductase (84, 85) and methionine synthase (86, 87), respectively. Methionine synthase belongs to the subfamily of B₁₂-dependent methyltransferases and the cofactor alternates between the MeCbl and cob(I)alamin oxidation states during the reaction cycle. Occasional oxidation of the reactive cob(I)alamin intermediate is the source of the inactive cob(II)alamin form of the enzyme, which is repaired via a reductive methylation reaction to MeCbl (88). The source of electrons for the reactivation reaction is NADPH, which is delivered via the dual flavoprotein oxidoreductase, methionine synthase reductase, to cob(II)alamin bound to methionine synthase (89). The activation reaction illustrates the sufficiency of the *cblG* and *cblE* loci for

converting cob(II)alamin to the active MeCbl cofactor form and precludes requirement for additional cytosolic reductases or factors for this reaction.

Inherited Defects Affecting Only AdoCbl Synthesis. The defect in the *cblA* cell line was postulated to be a mitochondrial cob(II)alamin reductase (90, 91). However, the biochemical rationale for a stand-alone cob(II)alamin reductase is weak, since its product, cob(I)alamin, is highly reactive and would rapidly succumb to side reactions. Furthermore, cob(II)alamin bound to the protein encoded by the *cblB* locus, adenosyltransferase, is reduced to cob(I)alamin *in situ*, in the first step of the reductive adenosylation reaction that generates AdoCbl (92). While the dual flavoprotein reductase, methionine synthase reductase, can serve as the reductase *in vitro* (93), the existence of a mitochondrial reductase dedicated for reduction of cob(II)alamin bound to adenosyltransferase appears unlikely based on the recent identification of all the mitochondrial specific *cbl* functions (93-95). Instead, a reductase that serves additional mitochondrial acceptors is likely used for the adenosyltransferase, explaining why it is not associated with a specific *cbl* defect. The recent identification of the genetic locus revealed a 418 residue long protein, designated MMAA (for causing methylmalonic aciduria type A) (95). It belongs to the G3E family of P-loop GTPases and contains the signature motifs associated with this superfamily. A bacterial ortholog of MMAA, MeaB, which is strongly conserved in operons encoding methylmalonyl-CoA mutase, is better characterized (96). MeaB exhibits low intrinsic GTPase activity that is activated >100-fold in complex with the mutase. MeaB in turn influences the mutase, protecting it from oxidative inactivation, but only in the presence of nucleotides. These results suggest that in addition to the mutase, MeaB interacts with adenosyltransferase and influences AdoCbl synthesis in mitochondria.

As expected from biochemical studies on *cblB* cell lines, the defect in them resides in the gene encoding adenosyltransferase also designated MMAB (for causing methylmalonic aciduria type B) (93, 94). Unlike methionine synthase that is the site for MeCbl synthesis in the presence of the auxiliary protein, methionine synthase reductase, inactive cofactor bound to methylmalonyl-CoA mutase cannot be converted to AdoCbl *in situ*. This activity is catalyzed by adenosyltransferase that converts cob(II)alamin or aquocobalamin in the presence of a reductant to AdoCbl. The human enzyme is trimeric and binds the various cobalamin derivatives with moderate affinity ($\sim 2\text{--}8\ \mu\text{M}$) (97). Spectroscopic analyses have revealed an unusual coordination environment for the cofactor bound to the enzyme, which is “base-off” in both the substrate cob(II)alamin and product AdoCbl oxidation states (97, 98). In the presence of ATP, four-coordinate cob(II)alamin with very unusual electron paramagnetic resonance and magnetic circular dichroism spectroscopic properties is observed (98).

Thesis Organization

This dissertation consists of five chapters. Chapter 1 includes an introductory review about the history of vitamin B₁₂ studies as well as the properties and functions of vitamin B₁₂. Chapter 2 is a paper that was published in the journal *Biochemistry*. Chapter 3 is a paper that was published in the journal *Journal of Biological Chemistry*. Chapter 4 is a paper to be submitted to the journal *Journal of Biological Chemistry*. Chapter 5 is a general concluding chapter, where the results and conclusions of the three papers are summarized. All of the papers were written by me with the editorial guidance and assistance from my major professors, Dr. Thomas Bobik.

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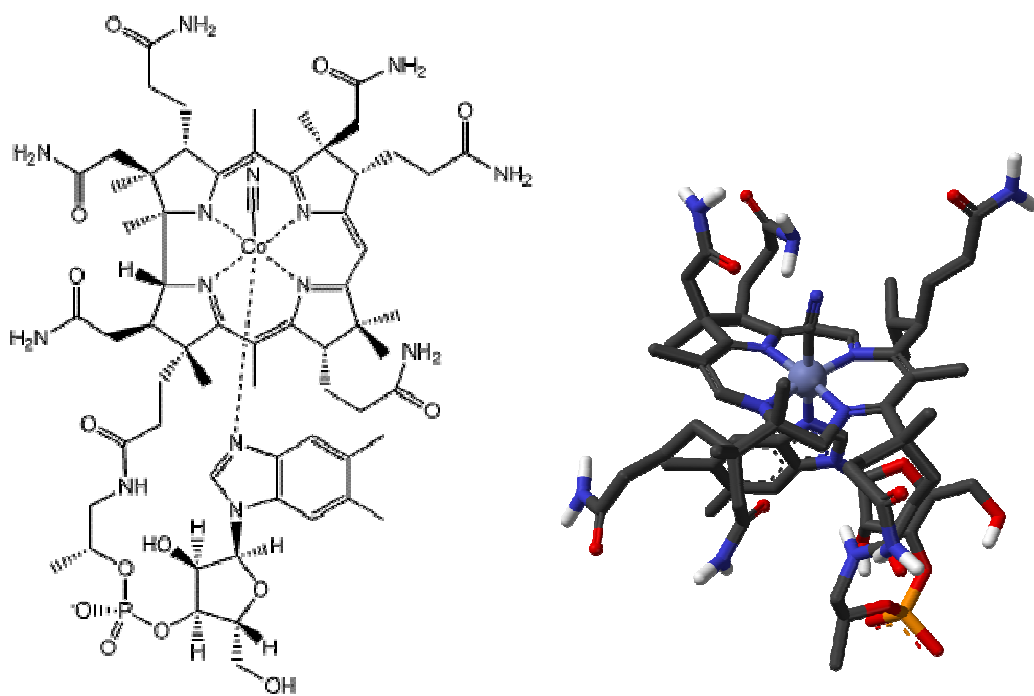


Figure 1.1 The structure of vitamin B₁₂. The left figure is cited from Wikimedia Commons. The right figure is the stick model of cyanocobalamin, based on 3DChem.com model.

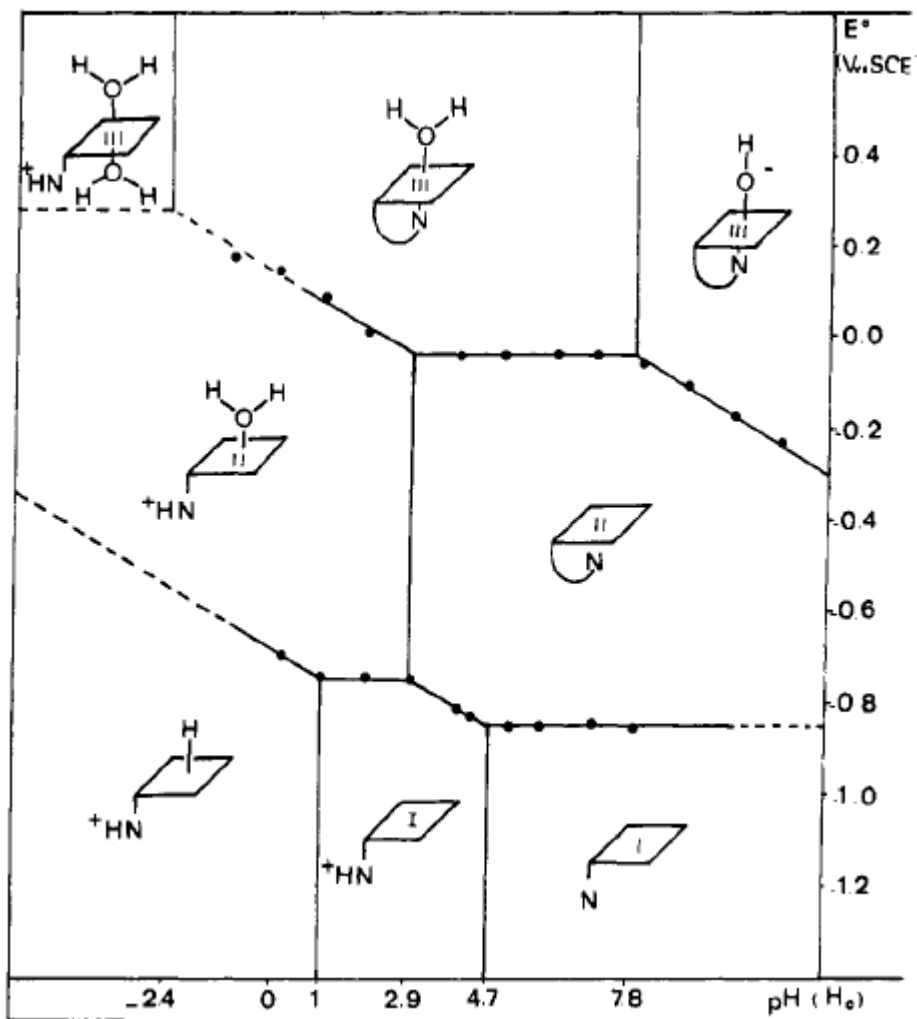


Figure 1.2 E° -pH diagram of Co(III)-Co(II)-Co(I) in water at 22 °C. Potentials are referred to the saturated calomel electrode (19).

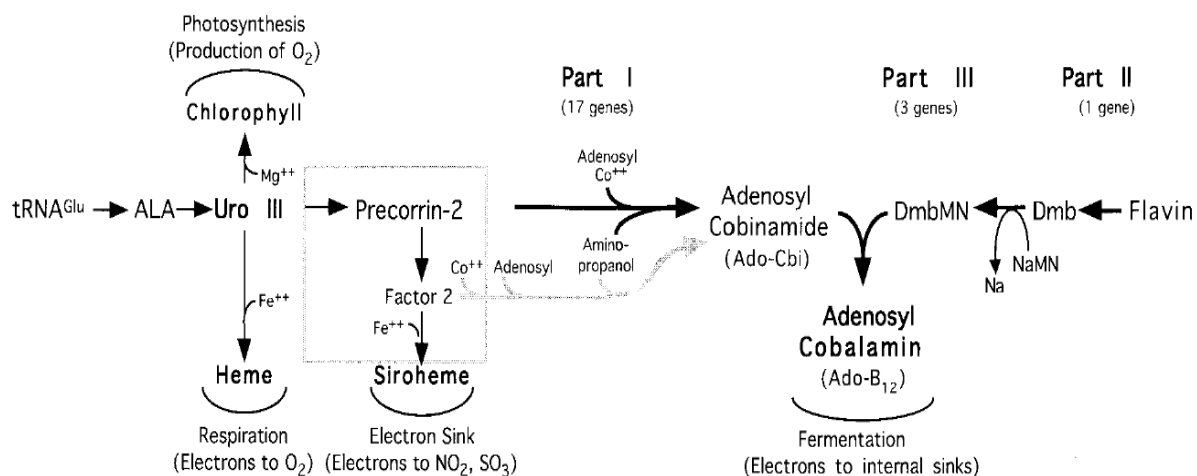


Figure 1.3 Overview of B₁₂ synthetic pathway (23). Parts I, II, and III are portions of the cobalamin biosynthetic pathway defined by nutritional behavior of mutants. The two pathways leading to Ado-Cbi are the aerobic pathway of *P. denitrificans* (top arrow) and the anaerobic pathway of *S. typhimurium* (bottom arrow). General functions of cofactors are indicated above and below the pathway.

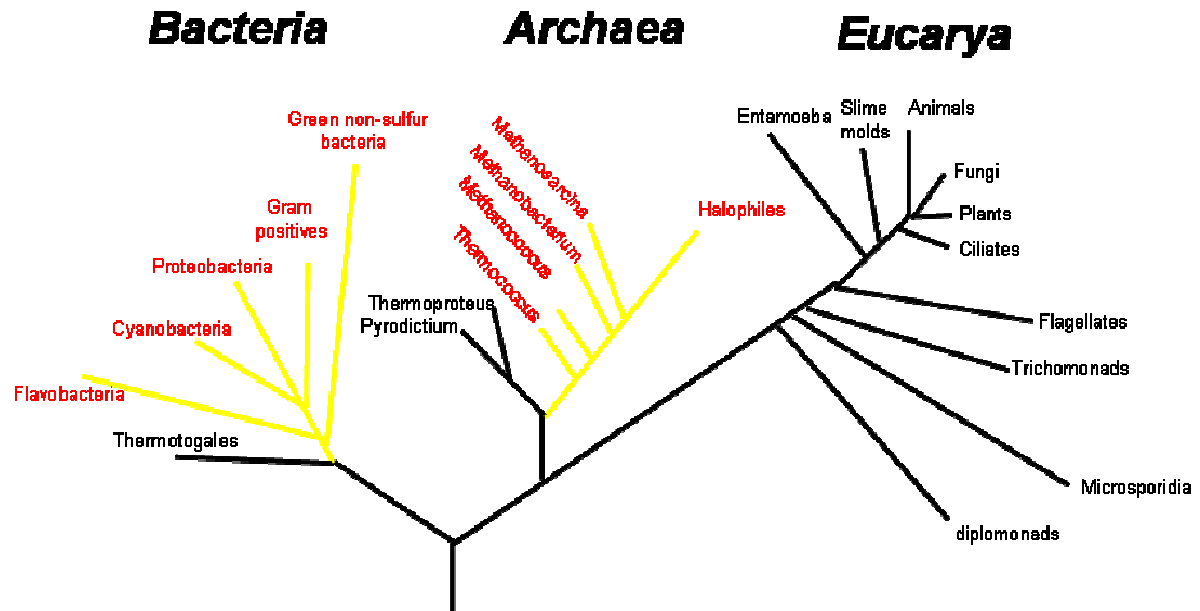


Figure 1.4 Distribution of vitamin B₁₂ synthesis. The lineages indicated in color have at least some members that synthesize B₁₂. The above tree was made primarily from computer analysis of 16s (prokaryotes) and 18s (eukaryotes) ribosomal RNA sequences (Wheelis *et.al*).

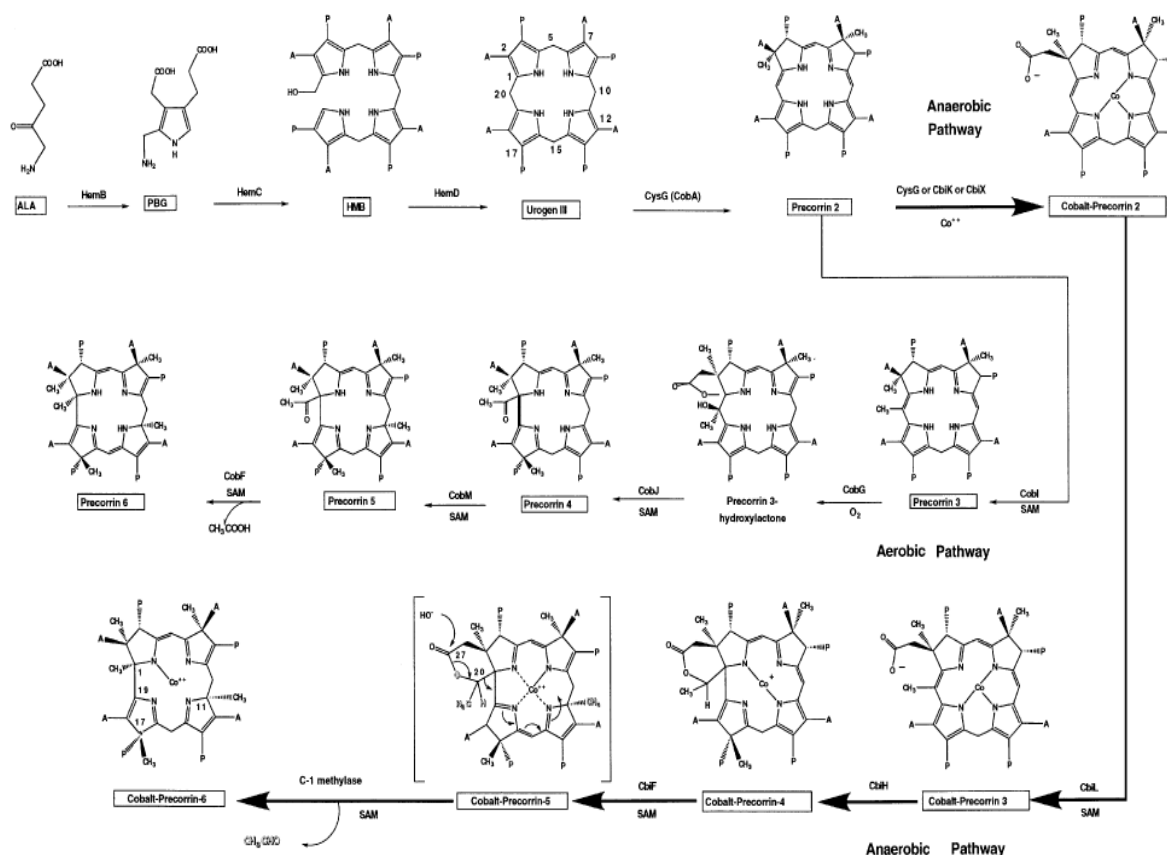


Figure 1.5 Biosynthesis of precorrin 6 (aerobic pathway; light arrows) and cobalt-precorrin 6 (anaerobic pathway; bold arrows) on the route from ALA to vitamin B₁₂ (24). The two pathways are identical from ALA to precorrin 2, at which point they diverge as shown. PBG, porphobilinogen; HMB, hydroxymethylbilane; SAM, S-adenosyl-L-methionine.

Figure 1.6 Complete aerobic pathway from UroIII to adenosylcobalamin (24). Vitamin B₁₂ is formed by replacing the adenosyl group of the final product of this pathway, adenosylcobalamin, with cyanide. SAM, S-adenosyl-L-methionine; Ado-, adenosyl.

Figure 1.7 Regulation of the *cob/pdu* regulon of *S. typhimurium* (23). The genetic map describes the region between *pdu* and *cob* operons whose transcripts start at the far sides of the figure. Boxes enclose structural genes. Black arrows designate transcripts. Gray arrows indicate regulatory influence; dashed gray arrows indicate the proposal that a higher level of PocR protein may be required to activate these promoters.

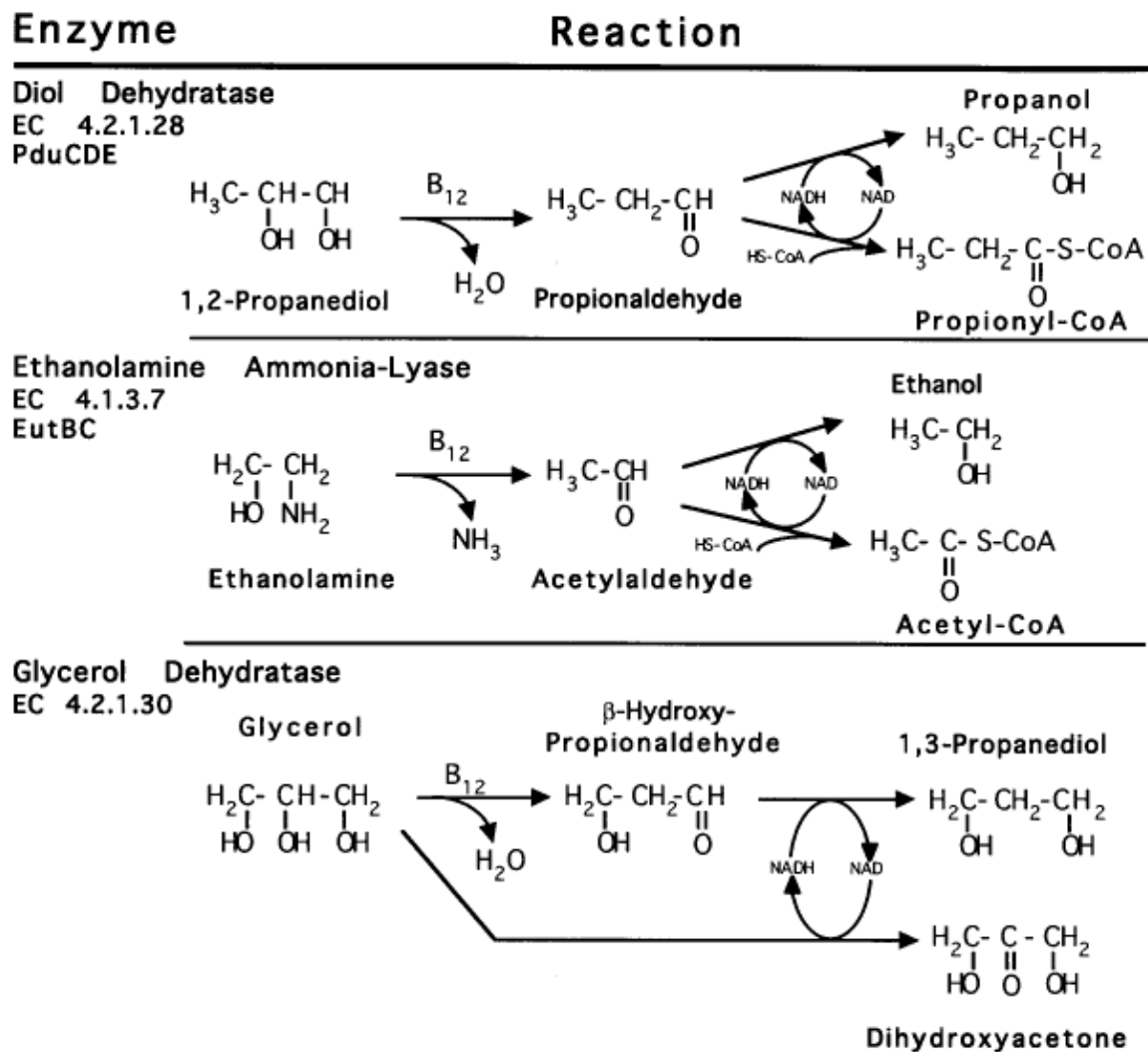


Figure 1.8 Cobalamin-dependent pathways known for enteric bacteria (23). Only the first reaction for each pathway employs cobalamin as a cofactor.

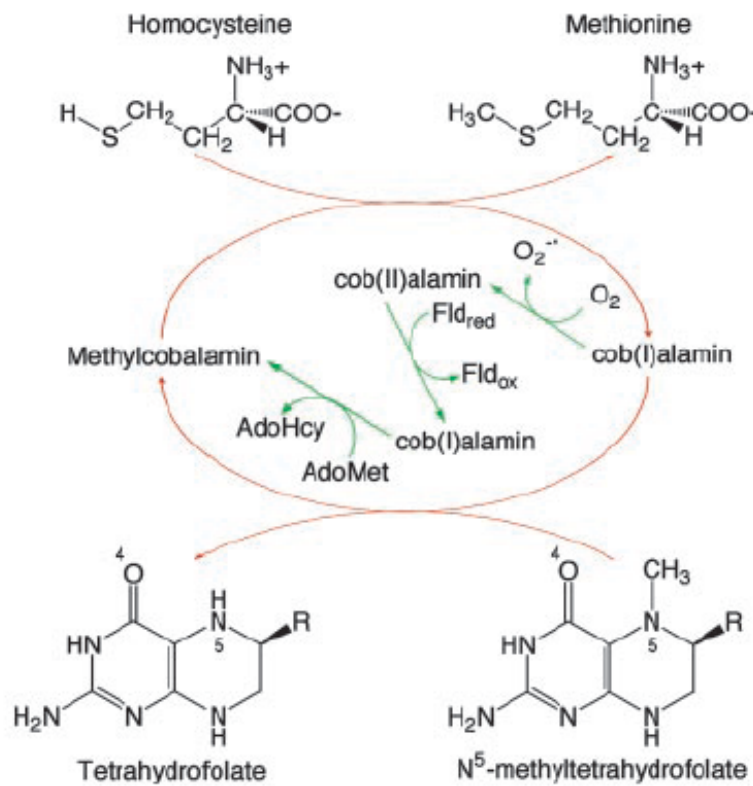


Figure 1.9 The reactions catalyzed by methionine synthase (44).

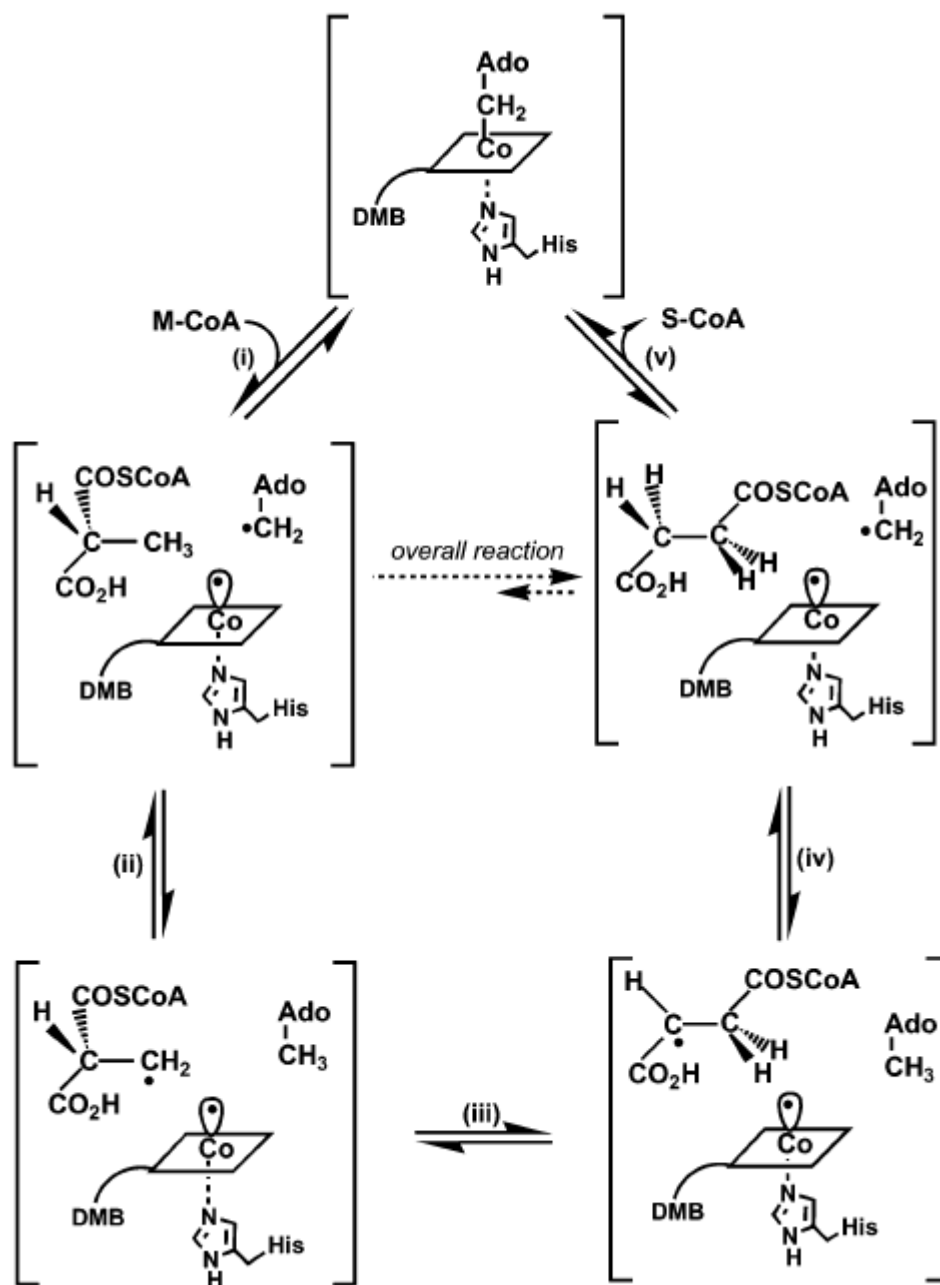
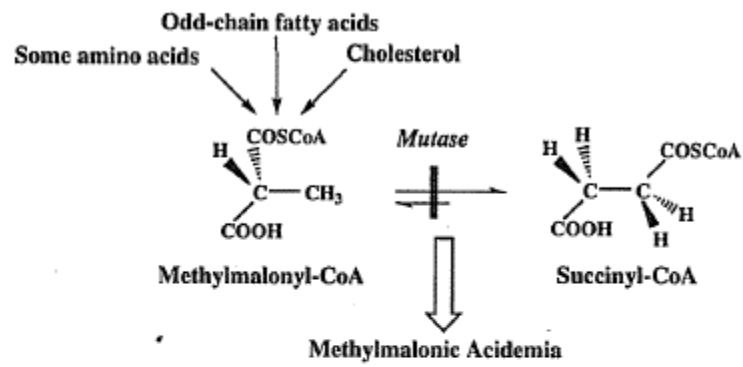


Figure 1.10 Postulated reaction mechanism of methylmalonyl-CoA mutase. M-CoA and S-CoA denote methylmalonyl-CoA and succinyl-CoA, respectively (50).

A. Animal



B. Bacterial

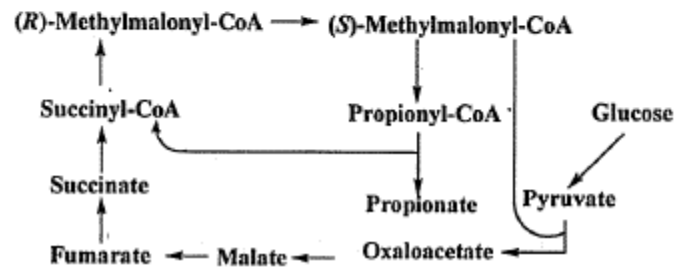


Figure 1.11 Role of methylmalonyl-CoA mutase in animal and bacterial physiology (34).

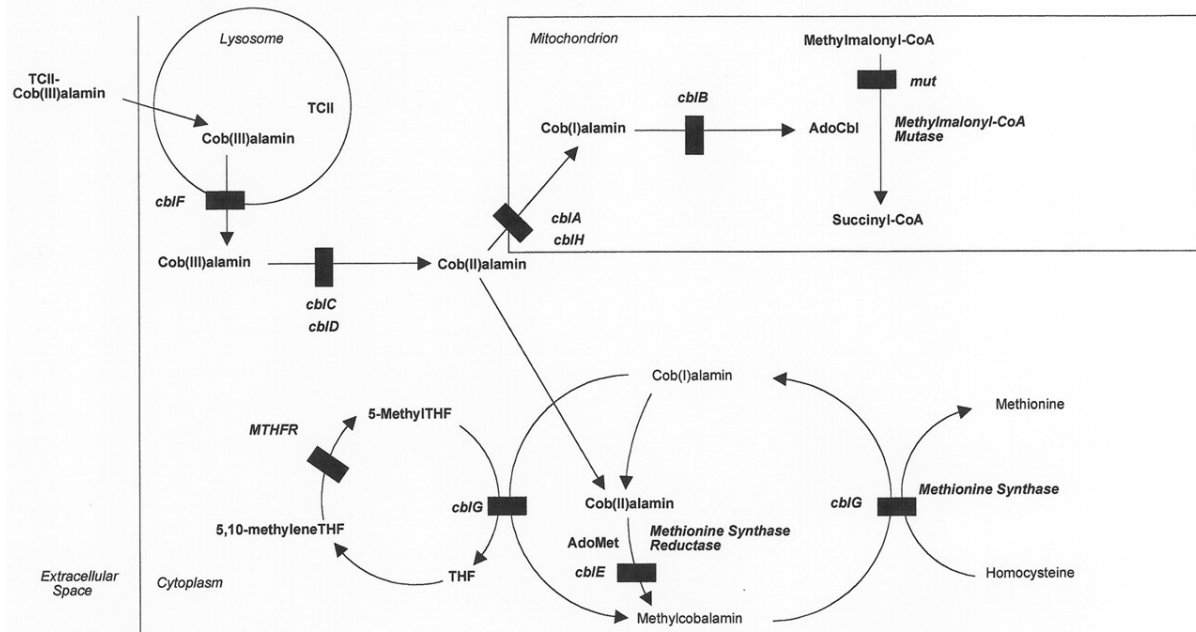


Figure 1.12 Cobalamin metabolism in mammalian cells (33). The pathways by which vitamin B₁₂ (cobalamin) is taken up by cells and converted to its active coenzyme derivatives are shown. The steps affected by inborn errors of cobalamin metabolism (*cbIA-cbIH*, *mut*), as well as *MTHFR* deficiency are shown. The methionine synthase reaction (*cbIG*) is shown twice in order to separate folate and homocysteine metabolism. Abbreviations: AdoCbl, Adenosylcobalamin; AdoMet, S-adenosylmethionine; 5-Methyl-THF, methyltetrahydrofolate; *MTHFR*, methylenetetrahydrofolate reductase; TCII, transcobalamin II.

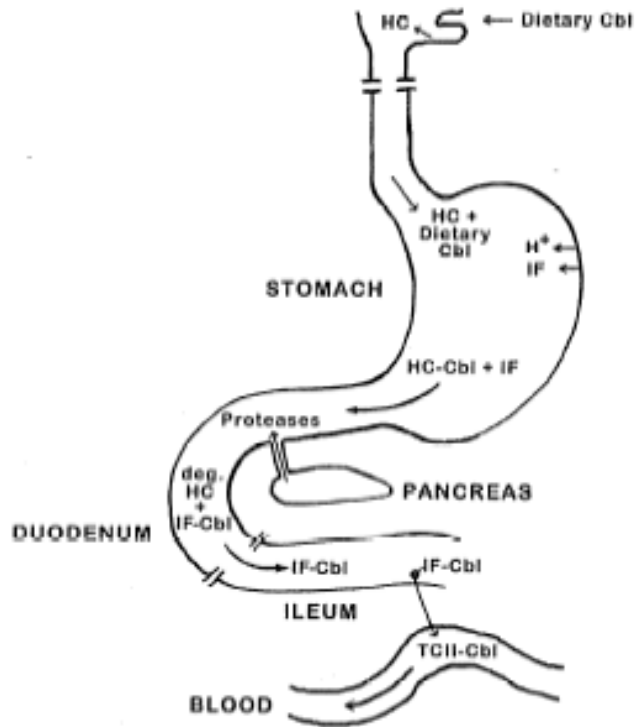


Figure 1.13 The gastrointestinal pathway for cobalamin absorption from dietary sources of cobalamin (73). Cbl: cobalamin; HC: haptocorrin (transcobalamin I/III); IF: intrinsic factor; TCII: transcobalamin II.

CHAPTER 2 FUNCTIONAL CHARACTERIZATION AND MUTATION ANALYSIS OF HUMAN ATP:COB(I)ALAMIN ADENOSYLTRANSFERASE

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Abstract

ATP:cob(I)alamin adenosyltransferase catalyzes the final step in the conversion of vitamin B₁₂ into the active coenzyme, adenosylcobalamin. Inherited defects in the gene for the human adenosyltransferase (hATR) result in methylmalonyl aciduria (MMA), a rare but life-threatening illness. In this study, we conducted a random mutagenesis of the hATR coding sequence. An ATR-deficient strain of *Salmonella* was used as a surrogate host to screen for mutations that impaired hATR activity *in vivo*. Fifty-seven missense mutations were isolated. These mapped to 30 positions of the hATR, 25 of which had not previously been shown to impair enzyme activity. Kinetic analysis and *in vivo* tests for enzyme activity were performed on the hATR variants, and mutations were mapped onto a hATR structural model. These studies functionally defined the hATR active site and tentatively implicated three amino acid residues in facilitating the reduction of cob(II)alamin to cob(I)alamin which is a prerequisite to adenosylation.

Introduction

In higher animals, two cobalamin (B₁₂)-dependent enzymes are known. Methylcobalamin-dependent methionine synthase is needed for recycling homocysteine to methionine (1, 2), and adenosylcobalamin-dependent methylmalonyl-CoA mutase (MCM) is essential for the conversion of propionyl-CoA to the TCA cycle intermediate, succinyl-CoA

(3). In higher animals, propionyl-CoA is produced from the breakdown of branched-chain amino acids as well as thymine, cholesterol, and odd-chain fatty acids. Hence, MCM is essential for the complete catabolism of these compounds (3).

Humans are incapable of synthesizing AdoCbl and MeCbl *de novo* and depend on assimilation of exogenous complex precursors such as vitamin B₁₂ (cyanocobalamin, CN-Cbl) and hydroxycobalamin (OH-Cbl). For AdoCbl synthesis, CN-Cbl is converted to GS-Cbl, successively reduced to cob(II)alamin and cob(I)alamin, and finally adenosylated to AdoCbl (4-6). For MeCbl synthesis, cob(II)alamin is reductively methylated while bound to MS (2, 7, 8). In humans, inherited defects in the genes for MCM (*mut*), MS (*MTR*), or the genes for cobalamin metabolism lead to methylmalonyl aciduria (MMA), homocystinuria, or combined disease (9, 10). Patients typically present within the first year of life with lethargy, failure to thrive, recurrent vomiting, dehydration, respiratory distress, and hypotonia and are prone to life-threatening acidosis (11).

The final step in the conversion of vitamin B₁₂ to AdoCbl is catalyzed by ATP:cob(I)alamin adenosyltransferase (ATR) (5, 12). Three families of ATRs that are unrelated in amino acid sequence have been identified: CobA type, PduO type and EutT type (13-16). The human adenosyltransferase (hATR) is a PduO type enzyme (17). The gene for hATR is *MMAB*, and defects in this gene underlie the *cblB* complementation group of isolated MMA (18). The crystal structures of the hATR and two related bacterial enzymes from *Lactobacillus reuteri* and *Thermoplasma acidophilum* were recently determined (19-21). These enzymes are trimers, where the monomer is a five-helix bundle and the active site is at the subunit interface. The structures of the human and *L. reuteri* enzymes were determined with MgATP bound which identified a unique ATP-binding motif (19, 20). These

studies also showed that binding of MgATP increased the order of ~20 N-terminal amino acids, creating a novel MgATP binding site that is part of a larger cleft proposed to include the cobalamin binding site. Thus far, a structure of a PduO-type ATR with cobalamin bound has not been reported, although a structure for a CobA-type ATR with cobalamin bound is available (22).

Recent spectroscopic studies indicate that in the presence of MgATP, hATR binds cob(II)alamin in a four-coordinate form in which dimethylbenzimidazole (DMB) is not coordinated to the cobalt in contrast to cob(II)alamin in solution at physiological pH where DMB is the lower axial ligand to cobalt (23). Dissociation of DMB changes the redox potential for the cob(II)alamin/cob(I)alamin couple from -610 mV to approximately -500 mV (24), and this increase in potential is proposed to facilitate cob(II)alamin reduction *in vivo* (25). In addition to preferentially binding cob(II)alamin in a DMB-off form, the hATR also binds AdoCbl DMB-off which may facilitate coenzyme transfer to MCM which binds cobalamin with H626 replacing DMB as the lower ligand to cobalt (25).

Studies with the hATR and the CobA and PduO ATRs from *Salmonella* indicate that these enzymes closely interact with partner reductases and that cob(I)alamin is not produced free in solution but rather exists as an enzyme-bound species that is rapidly adenosylated to AdoCbl (26-28). It has been proposed that this is essential to prevent byreaction of cob(I)alamin which is one of the strongest nucleophiles known in biology (29). Mechanistic studies of the PduO enzyme of *Salmonella* indicate a ternary complex mechanism rather than a substituted enzyme mechanism (30). Cumulatively, the studies mentioned above lead to a general model for ATR function in which MgATP binds first followed by cob(II)alamin and

cobalamin reductase. Subsequently, cob(II)alamin is reduced to cob(I)alamin which is adenosylated to AdoCbl.

To date, relatively little work has been reported that describes how amino acid changes affect the activity of PduO-type ATRs such as the human enzyme. The effects of four amino acid changes (R186W, R190H, R191W, and E193K) on hATR activity were determined, and three of the four corresponding amino acid residues were also studied in the *Thermoplasma* enzyme (21, 31). No general study on the effects of mutations on hATR activity and function has been reported. Therefore, we conducted a random mutagenesis of the hATR using *Salmonella* as a surrogate host to screen for changes in enzyme activity *in vivo*. 57 missense mutations at 30 positions were found to impair hATR activity *in vivo* in the surrogate host. These included mutations at 25 new sites that eliminate or reduce the hATR activity and hence could result in MMA if found in humans. In addition, we performed kinetic analysis and *in vivo* tests for enzyme activity on the hATR variants and mapped the mutations isolated onto a hATR structural model. These studies functionally defined the hATR active site and tentatively indicate a role for three amino acid residues in facilitating cob(II)alamin reduction by mediating the DMB-off transition.

Materials and Methods

Chemicals and Reagents. Dithiothreitol (DTT), OH-Cbl, and AdoCbl were purchased from Sigma (St. Louis, MO). Titanium(III) citrate was prepared as described previously (32). Isopropyl β -D-thiogalactopyranoside (IPTG) was from Diagnostic Chemical Ltd. (Charelottetown, PE). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA). Pefabloc SC PLUS was purchased from ICN Biomedicals, Inc. (Aurora, OH). Other chemicals were from Fisher Scientific (Norcross, GA).

Bacterial Strains, Media and Growth Conditions. The bacterial strains used in this study are listed in Table 2.1. The minimal medium used was NCE supplemented with 0.4% 1,2-propanediol (1,2-PD), 1 mM MgSO₄, 200 ng/mL OH-B₁₂, and 3 mM (each) valine, isoleucine, leucine, and threonine. LB (Luria-Bertani) medium was the rich medium used. MacConkey–1,2-PD–OH-Cbl indicator plates were composed of MacConkey agar base (Difco, Detroit, MI) supplemented with 1% 1,2-PD and 200 ng/mL OH-Cbl.

General Molecular and Protein Methods. Bacterial transformation, PCR, restriction enzyme digests, and other standard molecular and protein methods were performed as described previously (13, 33).

Cloning of hATR Coding Sequence for High Level Expression and Complementation of ATR-Deficient Salmonella. PCR was used to amplify hATR coding sequence. Primers were chosen that remove the predicted 36 amino acid N-terminal mitochondrial targeting sequence (MTS). Plasmid pNL166 provided the template DNA (17). The primers used for amplification were 5'-GCC GCC AGA TCT TAT GCC TCA GGG CGT GGA AGA CGG G-3' (forward) and 5'-GCC GCC AAG CTT ATC AGA GTC CCT CAG ACT CGG CCG-3' (reverse) for native hATR. Primer 5'-GCC GCC AGA TCT TAT GCA CCA TCA CCA TCA TCA CCA CCA TCC TCA GGG CGT GGA GAC GGG-3' was used to introduce an N-terminal His₈ tag, and primer 5'-GCC GCC AAG CTT ATC AAT GGT GGT GAT GAT GGT GAT GGT GGA GTC CCT CAG ACT CGG CCG-3' was used to introduce a C-terminal His₈ tag. These primers also introduced *Bgl*III and *Hind*III restriction sites into the PCR products which were used for cloning into pTA925 restricted with *Bgl*III and *Hind*III. Ligation mixtures were used to transform *E. coli* DH5α by electroporation, and transformants were selected on LB medium containing 25 ug/mL

kanamycin. Plasmid DNA was purified from these strains, and the DNA sequence of each clone was determined. Clones with the expected DNA sequence were restricted with *Sph*I and *Xba*I and ligated with a linker having the sequence 5'-CAA TTA ATA CGA CTC ACT ATA GAC CCC AGG CTT GAC ACT TTA TGC TTC CGG CTC TAT AAT GTG TGG AAT TGT GAG CGG T-3' and 5'-CTA GAC CGC TCA CAA TTC CAC ACA TTA TAG AGC CGG AAG CAT AAA GTG TCA AGC CTG GGG TCT ATA GTG AGT CGT ATT AAT TGC ATG-3'. Ligation mixtures were used to transform *E. coli* DH5 α by electroporation. Transformants were selected on LB medium with 25 ug/mL kanamycin. Plasmid DNA was purified from these strains, and the DNA sequence of the linker region was verified. The linker introduced both *E. coli* and T7 promoters which allowed high-level protein production in BL21DE3 RIL as well as complementation of the ATR-deficient *Salmonella* strain used for identification of hATR mutations.

Growth of hATR Expression Strains and Preparation of Cell Extract. The *E. coli* strains used for expression of hATR were grown on 400 mL LB supplemented with 25 ug/mL kanamycin at 16 °C in a New Brunswick Scientific Innova 4230 refrigerated shaker incubator with a shaking speed at 275 rpm. Cells were grown to an absorbance of 0.6–0.8 at 600 nm, and protein expression was induced by the addition of 0.5 mM IPTG. Cells were incubated at 16 °C with shaking at 275 rpm for an additional 18 h. Cells were removed from the incubator, held on ice for 10 min, and then harvested by centrifugation at 8000 rpm for 5 min using a Beckman JA-10 rotor. The cells were resuspended in 10 mL of 50 mM Tris and 300 mM NaCl (pH 7.5), and broken using a French pressure cell (Thermo Electron Corp.) at 10000 psi. Pefabloc SC PLUS was added to the cell extract to a concentration of 100 ug/mL to inhibit proteases. The crude cell extract was centrifuged at 16000 rpm for 30 min using a

Beckman JA-17 rotor to separate the soluble and insoluble fractions. The supernatant was the soluble fraction used for enzyme purification.

Western Blots. The soluble fractions of cell extracts were fractionated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was incubated at room temperature with gentle shaking in 50 mM Tris (pH 7.4), 200 mM NaCl, 0.1% Tween (TTBS), and 5% Milk blocking buffer for 30 minutes. The primary antibody used was the affinity-purified rabbit IgG anti-hATR diluted 1:1000. The secondary antibody was the goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (Bio-Rad) at a 1:3000 dilution. The membrane was prepared for detection using the alkaline phosphatase conjugate substrate kit (Bio-Rad).

ATP:Cob(I)alamin Adenosyltransferase Assays. ATR assays were performed as previously reported with some modifications (30). Reaction mixtures contained 200 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 10 mM KCl, 0.05 mM OH-Cbl, 0.5 mM ATP, and 1 mM titanium(III) citrate in a total volume of 2 mL. Reaction components (except for hATR) were dispensed into cuvettes inside an anaerobic chamber (Coy Laboratories, Grass Lake, MI). The cuvettes were sealed, removed from the chamber, and incubated at 37 °C for 5 min. Reactions were initiated by addition of purified recombinant hATR, and AdoCbl formation was measured by following the decrease in absorbance at 388 nm ($\epsilon_{388} = 24.9 \text{ cm}^{-1} \text{ mM}^{-1}$).

Random Mutagenesis and Screening of Candidate Human ATR Mutants. Random mutagenesis of the hATR gene was carried out using the *E. coli* mutator strain XL1-Red (Stratagene). pCF13 (His₈-hATR) and pCF15 (hATR-His₈) were transformed into XL1-Red by chemical transformation selecting for kanamycin resistance on LB medium. Approximately 3000 transformants were pooled and subcultured 5-10 times in 100 mL of LB

medium inoculated with 0.5 mL of an LB culture. pCF13 and pCF15 DNA was purified and used to transform the ATR-deficient bacterial mutant BE620 via electroporation. Transformants were screened on MacConkey-1,2-PD–OH-Cbl indicator plates supplemented with kanamycin at 25 µg/mL.

Growth Tests. AdoCbl-dependent growth on 1,2-PD minimal medium was performed as previously described in 48-well microtiter plates (13, 17).

UV-Visible Spectroscopy for Examination of the Lower Axial Ligand of AdoCbl.

Spectra were obtained using a Cary 50 Bio spectrophotometer (Varian). 1 mL soluble cell extract containing 10 mg of protein was added to 1 mL plastic cuvette, and the mixture was scanned from 300 to 600 nm as the baseline. Then, 2 µL of 5 mM AdoCbl (finally 5 µM) was added to the sample and gently mixed. After incubation at 37 °C for 2 minutes, the cuvette was scanned a second time from 300 to 600 nm.

EPR Spectroscopy for Examination of the Lower Axial Ligand of Cob(II)alamin.

Spectra were obtained using Bruker ER-200 EPR system. Cob(II)alamin was generated by photolysis of AdoCbl under anaerobic conditions in 10 mM Tris-HCl (pH 8.0). Purified hATR (2 mM) was incubated with 2 mM ATP anaerobically for 20 min at 4 °C. Then, cob(II)alamin was added to a final concentration of 100 µM. The mixture was equilibrated for 5 min at 37 °C and then injected into 4mm quartz EPR tubes.

DNA Sequencing. DNA sequencing was carried out by Iowa State University, at the DNA facility of the Iowa State University Office of Biotechnology.

Results

An *in vivo* Screen for hATR Mutants. Wild-type *Salmonella* produces red colonies on MacConkey-1,2-PD-B₁₂ indicator media as a result of AdoCbl-dependent 1,2-PD degradation

(34). *Salmonella* strain BE620 produces white colonies on similar media due to ATR deficiency (13). The phenotype of BE620 was complemented by pCF13 (His₈-hATR) or pCF15 (hATR-His₈), but not by a plasmid without an insert. Hence, pCF13 or pCF15 with a hATR inactivated by mutation was expected to produce white or pink colonies in BE620 rather than wild-type red, providing a convenient *in vivo* screen for hATR mutants.

E. coli mutator strain XL1-Red was used for random mutagenesis of pCF13 (His₈-hATR) and pCF15 (hATR-His₈). Mutagenized plasmid DNA was transformed into the ATR-deficient bacterial mutant *Salmonella* BE620, and resultant colonies were screened for color formation on MacConkey-1,2-PD-B₁₂ indicator media. Approximately 6000 transformants were screened, 3000 derived from each mutagenized plasmid. Of these, 185 produced colonies that were white or pink in color, suggesting that they carried a mutant hATR coding sequence. These included 57 single-base change missense mutations at 30 positions of the hATR gene with K78 and G97 each having two different mutations.

Effect of B₁₂ Concentration on the Activity of hATR Variants *in vivo*. We also used indicator plates with different amounts of OH-Cbl to view the sensitivity of the mutants to B₁₂ concentration. Eleven mutants, G63E, D64G, R76G, K78R, G97R, G97E, S126L, H183Y, R215K, D218N, and R225K, had an increased level of color formation (ATR activity) at 1500 nM compared to 150 nM OH-Cbl. Of these amino acid residues, G63 and K78 directly contact MgATP in the structural model, and residues D64, R76, S126, R215, D218, and R225 are at the surface of the proposed active site pocket in position to interact with MgATP or cobalamin. Thus, these results indicate that residues G63, D64, R76, K78, S126, R215, D218, and R225 are important to binding of MgATP/cobalamin by the hATR *in vivo*.

Results also showed that most of the mutants that were partly corrected by the increased B₁₂ concentration had an elevated K_m for MgATP, cobalamin, or both substrates. This finding is consistent with the proposal that MgATP and cobalamin binding are interrelated which was indicated by structural studies that showed MgATP binding leads to ordering of the N-terminal portion of the hATR with formation of a proposed cobalamin-binding site (19).

We also found that two hATR mutations that are partly corrected by increased B₁₂ levels *in vivo* (R215K and R225K) lack measurable activity *in vitro*, and two other B₁₂-sensitive mutations (G97R and H183Y) are at the inner part of the protein distant from the active site. Apparently, the R215K and R225K mutants are stabilized somewhat by the *in vivo* milieu, and G97R and H183Y affect the shape of the active site at a distance.

***In vivo* Stability of hATR Mutants.** The stability of the mutant hATR proteins in *Salmonella* BE620 was examined by Western blotting (Figure 2.1). Each lane contains 10 µg of protein. From these gels, it can be seen that most of the mutant hATRs were expressed at a level within ~2-fold of the control. However, mutant proteins C119Y, R186W, C189Y, R190C, and R191W were produced at much lower levels than the control, indicating that these mutations impair proper protein folding leading to degradation by cellular proteases. Most of the unstable mutants are located in a highly conserved region of the hATR, and three of five have been found in MMA patients (18).

B₁₂-Dependent Growth of hATR Mutants in a Surrogate Host. Ectopic expression of His₈-hATR, hATR-His₈, or native hATR in ATR-deficient *Salmonella* restored AdoCbl-dependent growth on 1,2-PD which was previously shown with native hATR (13) (Figure 2.2). Although the growth rate obtained via ectopic expression of His₈-ATR was approximately half of that with native hATR or hATR-His₈, it was still much higher than the

control; hence, we were able to further evaluate the *in vivo* activity of all hATR mutants with growth studies. With one exception (T161I), none of the hATR missense mutants enhanced the growth of ATR-deficient *Salmonella* BE620 on 1,2-PD. The T161I mutant was capable of slow growth. These results indicate that the 32 hATR missense mutants isolated here had little to no ATR activity *in vivo* when *Salmonella* was used a surrogate host.

Kinetic Values for Human ATR Mutants. Using purified enzymes (Figure 2.3), kinetic constants were determined for the wild-type and mutant hATRs. K_m and k_{cat} values were determined by nonlinear regression with Grafit (Table 2.2). When the K_m values for ATP were determined, saturating levels of cob(I)alamin (50 μ M) were added to assay mixtures and the ATP concentration was varied from 0 to 200 μ M. Similarly, saturating levels of ATP (500 μ M) and varying levels of cob(I)alamin from 0 to 50 μ M were used to determine the K_m values for cob(I)alamin.

Controls showed that hATR-His₈ and native hATR had similar activity. The N-terminal His₈ tag enzyme had a lower k_{cat} value (58%), showing that the N-terminal tag affected enzyme activity somewhat. Kinetic analysis of the hATR missense mutants indicated four distinct groups.

Group I consisted of 19 mutants (K78Q, E84K, G87R, D90N, E91K, L92S, S94L, R186W, C189Y, R190C, R191W, E193K, R194G, F212S, R215K, S217R, L220P, L223P, and R225K) that had no activity even with a 10-fold excess of ATP and cob(I)alamin compared to the standard assay (13). Most of these had mutations that changed conserved amino acid residues (17). Among these, R186W, R190C, R191W, and E193K have been found in methylmalonic aciduria patients (18). Note that although R186W, C189Y, R190C, and R191W were unstable when expressed in ATR-deficient *Salmonella* BE620 at moderate

levels (Figure 2.1), it was still possible to purify these proteins following high-level expression in *E. coli* BL21DE3 RIL.

Group II consisted of three mutants that exhibited relatively large changes in the K_m values for both ATP and cob(I)alamin: R76G, F83S, and S126L. Their K_m values for ATP were $45.89 \pm 8.17 \mu\text{M}$, $215 \pm 37 \mu\text{M}$, and $87.70 \pm 40.98 \mu\text{M}$, respectively, while the wild-type was $6.90 \pm 0.36 \mu\text{M}$. K_m values for cob(I)alamin were $9.09 \pm 1.89 \mu\text{M}$, $30.2 \pm 12.5 \mu\text{M}$, and $14.25 \pm 4.49 \mu\text{M}$, respectively, while the wild-type was $1.60 \pm 0.55 \mu\text{M}$. Residue F83 has direct contact with MgATP in the crystal structure, and R76 and S126 map to the surface of the active site pocket. Hence, these residues are likely critical for proper binding of MgATP and/or cobalamin.

Group III mutants (G63E, D64G, S68F, K78R, G97R, and D218N) showed modest changes or wild-type K_m values for ATP and cob(I)alamin, but a substantially reduced V_{max} . Three of these residues interact directly with MgATP (G63, S68, and K78), and the remainder of the residues (D64, G97, and D218) map to the surface of the proposed active site pocket. Hence, these amino acid residues likely define the enzyme active site.

Group IV mutants (G97E, C119Y, T161I, and H183Y) had kinetic constants in the wild-type range. This raises the question of why these mutants were inactive in *Salmonella in vivo*. Western blots showed that hATR-C119Y was produced only at a low level *in vivo* (Figure 2.1); hence, it may have been inactive *in vivo* due to instability. However, the G97E, T161I, and H183Y hATR variants were all produced at near wild-type levels *in vivo*. We speculate that these mutations interfere with the reduction of cob(II)alamin to cob(I)alamin *in vivo* and thereby impair hATR activity. For the *in vitro* assay, cob(I)alamin is provided by

use of a strong chemical reductant Ti(III); hence, a defect that impairs the reduction of cob(II)alamin to cob(I)alamin would not be detected by this test.

Effect of Mutations on AdoCbl Binding. Prior studies showed that binding of AdoCbl to native hATR results in a large blue shift in its UV-visible spectrum from 540 to 460 nm because AdoCbl is bound base-off (without DMB as the lower axial ligand to cobalt) (25). It was proposed that this mode of binding allows efficient transfer of AdoCbl to MCM which binds AdoCbl in the base-off mode with H626 replacing DMB as the lower axial ligand of cobalt (25). Therefore, we examined the effects of hATR missense mutants on the DMB-off transition. Controls showed that binding of AdoCbl to native hATR and hATR-His₈ resulted in the anticipated blue shift in the UV-Visible spectrum (Figure 2.4A). Unexpectedly, however, binding of AdoCbl to His₈-hATR resulted in no obvious changes in the UV-Visible spectrum (Figure 2.4B). This indicated that the N-terminal His₈ tag interfered with the ability of the hATR to mediate the base-off transition of AdoCbl. Consequently, further studies of this transition focused on variants of the hATR with the C-terminal His₈ tag.

Among the hATR missense mutants with C-terminal His₈ tag (S68F, K78Q, K78R, R186W, and R190C) mediated blue shifts similar to that of the wild-type hATR. However, the majority of the hATR missense mutants, including D64G, F83S, G87R, D90N, E91K, L92S, S94L, C189Y, R191W, E193K, R194G, F212S, S217R, L220P, and L223P, lacked a measurable blue shift. The fact that most of the hATR mutants were unable to mediate the base-off transition of AdoCbl suggests that this conversion is a key aspect of the enzyme mechanism and that most of these mutations affect cobalamin binding.

Effect of Mutations on Cob(II)alamin Binding. Previous studies showed that in the presence of ATP, hATR binds cob(II)alamin in the base-off form (23, 25). Base-off binding

is proposed to raise the $\text{Co}^{2+/1+}$ reduction potential from -610 to -500mV, thus activating the cob(II)alamin for reduction (24). Prior studies distinguished base-on and base-off cob(II)alamin by EPR spectroscopy (23). Base-on cob(II)alamin exhibits an EPR spectrum in which the octet of hyperfine lines is split into triplets (Figure 2.5A). In contrast, base-off cob(II)alamin has high-field lines that appear as singlets (Figure 2.5B). We examined cob(II)alamin binding by hATR and variants using EPR spectroscopy. Controls showed that His₈-hATR bound cob(II)alamin base-on (Figure 2.5C) and that hATR-His₈ bound cob(II)alamin base-off (Figure 2.5D). This indicated that the N-terminal His₈ tag interfered with the ability of the hATR to mediate the base-off transition of cob(II)alamin which was also seen in the case of AdoCbl binding (see above). Among the hATR missense mutants with the C-terminal His₈ tag, only S68F, K78Q, K78R, R186W, and R190C exhibited EPR spectra indicating base-off binding of cob(II)alamin. This result was consistent with that of AdoCbl base-off transition experiments described above, suggesting that base-off binding of AdoCbl and cob(II)alamin is mechanistically related.

Discussion

In this study, the hATR was subjected to random mutagenesis and an ATR-deficient *Salmonella* strain was used as a surrogate host to facilitate isolation and characterization of mutations that impaired hATR activity. This approach allowed isolation of a number of single-base change mutations that impaired hATR activity. Single-base changes are much more likely to arise in natural populations compared to two or three simultaneous base changes which are often used in site-directed mutagenesis studies. Thus, the mutations studied here are more likely to have relevance to MMA than multibase changes created by site-directed mutagenesis. In total, 57 missense mutations were found at 30 different sites of

the hATR, five of which were previously shown to be altered in MMA patients and 25 of which were at new sites.

In addition to identifying mutations that have potential relevance to MMA, the studies reported here also help to functionally define the hATR active site. Analysis of hATR variants indicated that two small regions are particularly important for enzyme activity, E84–S94 and R186–R194. In the E84–S94 region, mutations in seven of 12 positions abolished enzyme activity, while in the R186–R194 region changes in six of nine residues eliminated activity. This suggests that these two short conserved regions are part of the hATR active site which is consistent with the crystal structure determined by Schubert and Hill (19).

To realize a more detailed understanding of how the mutations isolated here affected hATR activity, we mapped them onto a hATR structural model with MgATP bound. The crystal shows that MgATP binding involves the formation of numerous hydrogen bonds and van der Waals interactions. ATP binding also increased the order of ~20 N-terminal amino acids, creating a novel ATP-binding site that was part of a larger cleft proposed to include the cobalamin binding site (19). Thirteen missense mutations isolated in this study affected residues directly involved in MgATP binding (G63E, D64G, S68F, K78R, K78Q, F83S, G87R, R190C, E193K, R194G, R215K, S217R, and D218N) (Figure 2.6A), and two missense mutations changed residues proposed to be involved in cobalamin binding (D90N and R186W). Hence, these mutations functionally define the hATR active site.

We also examined hATR mutations that resulted in an abnormal K_m value for cob(I)alamin or sensitivity to hydroxycobalamin concentration *in vivo*. In addition to residues D90, F170, R186, and F221 which were previously suggested to aid cobalamin binding (19), we found that S94 sits at the inner surface of the proposed cobalamin binding

site and that mutation S94L resulted in an enzyme that was inactive *in vitro* and *in vivo*, suggesting a role for S94 in cobalamin binding not apparent in the crystal structure (Figure 2.6B).

The crystal structure of the hATR indicates a trimer structure is required for enzyme activity since the active site is at the monomer interfaces. The trimer interface contains two charged rings (E84/R195 and E91/R191) and two phenylalanine rings that form a solid core (19). Among these positions, three missense mutants were obtained (E84K, E91K, and R191W). Each lacked detectable hATR activity *in vivo* and *in vitro*, indicating that these positions are likely important for trimer formation. We did not obtain mutations that alter the phenylalanine residues at the core of the trimer. However, mutant L92S (Figure 2.6C), which is located at the trimer interface, also lacked measurable activity *in vitro* and *in vivo*. L92 may make contact with V188 at the interface of the neighbor subunit. In addition, two mutants were obtained (L220P and L223P) that are expected to produce kinks in helix $\alpha 5$ which is involved in active site formation. These two mutants also lacked measurable activity *in vitro* and *in vivo*.

Recent spectroscopic studies indicate that the hATR binds cobalamin as a four-coordinate species in which DMB is no longer bonded to the central cobalt atom (23). This mode of binding (DMB-off) is proposed to raise the $\text{Co}^{2+}/1^{+}$ midpoint potential 110 mV (from -610 to -500 mV) bringing it into the physiological range (24). We found three hATR variants that had near normal activity *in vitro* but were stable and inactive *in vivo* (G97E, T161I, and H183Y). We speculate that these mutant enzymes might be unable to mediate the DMB-off transition *in vivo* and hence be inactive since formation of cob(I)alamin is a prerequisite for adenosylation. Such mutants would be expected to be inactive *in vivo* but active *in vitro*.

because cob(I)alamin is provided through use of a strong chemical reductant. Alternatively, mutations G97E, T161I, and H183Y might impair interaction of the hATR with a partner reductase. Studies in several systems indicate that ATRs directly interact with cognate reductases to protect the highly reactive cob(I)alamin intermediate from side reactions (26-28). Impairment of this interaction would be expected to produce ATR that is active *in vitro* but not *in vivo* which was found for variants G97E, T161I, and H183Y. However, G97, T161, and H183 map to the interior of the hATR (Figure 2.6D); hence, it seems unlikely they would be able to interact with a partner protein. Therefore, we favor a possible role for G97, T161, and H183 in stabilizing four-coordinate cob(II)alamin. Each of these residues maps near a hydrophobic loop between $\alpha 3$ and $\alpha 4$ which we tentatively suggested is involved in DMB binding or otherwise facilitating cob(II)alamin reduction.

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Table 2.1 Bacterial Strains Used in This Study

strain	genotype
<i>Escherichia coli</i>	
DH5 α	F ⁻ λ^- <i>endA1 hsdR17 relA1 supE44 thi-1 recA1 gyrA96 relA1</i> $\Delta(lacZYA-argF)U169$ ($\phi 80dlacZ\Delta M15$)
BL21DE3 RIL.....	F ⁻ <i>ompT hsdS</i> ($r_B^- m_B^-$) <i>dcm</i> ⁺ Tet ^r <i>gal</i> λ (DE3) <i>endA</i> Hte [<i>argU ileY leuW</i> Cam ^r]
XL-1 Red.....	<i>endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutt</i> Tn10 (tet ^r) ^a (bought from Stratagene)
BE119.....	BL21DE3 RIL/pTA925 (T7 expression vector without insert)
BE260.....	DH5 α /pNL166 (pLac22- hATR)
BE702.....	BL21DE3 RIL/pCF13 (His ₈ -hATR)
BE704.....	BL21DE3 RIL/pCF15 (hATR-His ₈)
BE706.....	BL21DE3 RIL/pCF19 (native hATR)
<i>Salmonella enterica</i> serovar Typhimurium LT2	
BE121.....	$\Delta pduO651 cobA366::Tn10dCam$
BE620.....	$\Delta pduO651cobA366::dCam \Delta 1955$ (<i>zfa3646</i> *Tn10* <i>zfa3648</i>)
BE701.....	BE620/pCF13 (His ₈ -hATR)
BE703.....	BE620/pCF15 (hATR-His ₈)
BE705.....	BE620/pCF19 (native hATR)
BE707.....	BE620/pCF (modified pTA925)

Table 2.2 Kinetics Constants for hATR Variants ^a

	K_{mATP} (μM)	$K_{\text{mcoB(I)}}$ (μM)	k_{cat} (min^{-1})	$k_{\text{cat}}/K_{\text{mATP}}$ ($\mu\text{M}^{-1} \text{min}^{-1}$)	$k_{\text{cat}}/K_{\text{mcoB(I)}}$ ($\mu\text{M}^{-1} \text{min}^{-1}$)
His ₈ -hATR	7.19 ± 0.10	1.58 ± 0.65	2.77 ± 0.27	0.39	1.75
hATR-His ₈	7.35 ± 0.67	1.60 ± 0.44	4.96 ± 0.26	0.67	3.10
native hATR	6.90 ± 0.36	1.60 ± 0.55	4.75 ± 0.23	0.69	2.97
G63E	12.4 ± 1.2	2.24 ± 0.60	0.49 ± 0.03	0.039	0.22
D64G	20.5 ± 2.8	1.94 ± 0.64	0.53 ± 0.04	0.026	0.27
S68F	17.2 ± 2.8	1.86 ± 0.55	0.78 ± 0.05	0.045	0.42
R76G	45.9 ± 8.2	9.09 ± 1.89	2.58 ± 1.20	0.056	0.28
K78R	8.26 ± 0.68	2.25 ± 0.35	0.32 ± 0.05	0.039	0.14
F83S	215 ± 37	30.2 ± 12.5	2.07 ± 1.58	0.010	0.07
G97R	20.3 ± 8.4	3.03 ± 0.62	0.80 ± 0.06	0.039	0.26
G97E	5.23 ± 1.25	2.52 ± 0.79	2.38 ± 0.13	0.46	0.94
C119Y	14.1 ± 1.7	5.13 ± 1.82	4.06 ± 0.30	0.29	0.79
S126L	87.7 ± 41	14.3 ± 4.5	0.58 ± 0.08	0.007	0.04
T161I	5.00 ± 0.91	2.71 ± 0.25	2.29 ± 0.05	0.46	0.85
H183Y	9.97 ± 2.01	7.13 ± 1.06	2.83 ± 0.43	0.28	0.40
D218N	2.87 ± 0.40	1.72 ± 0.43	0.73 ± 0.02	0.25	0.42

^a: The values shown are based on three replicates. Average values were used to calculate $k_{\text{cat}}/K_{\text{m}}$. Mutants without measurable ability are not shown in this table. K_{m} and k_{cat} values were determined by nonlinear regression with Grafit[®].

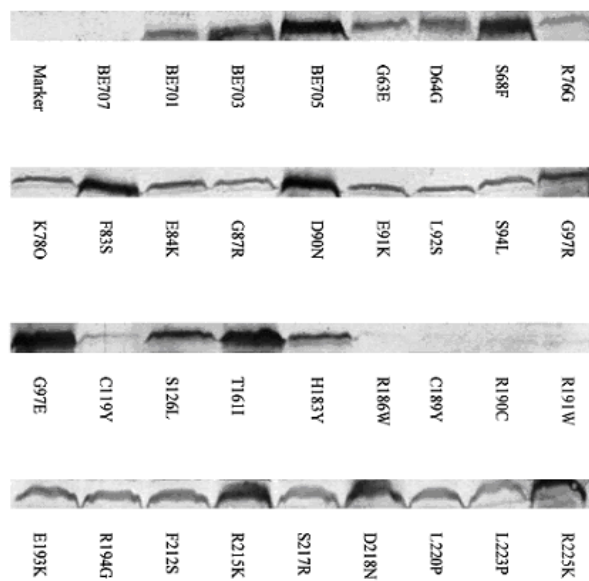


Figure 2.1 Western blotting analysis of hATR mutants. All lanes were loaded with 10 μ g of soluble cell extract. The following strains were used: strain BE707 (no insert control), strain BE701 (His₈-hATR), strain BE703 (hATR-His₈), and strain BE705 (native hATR).

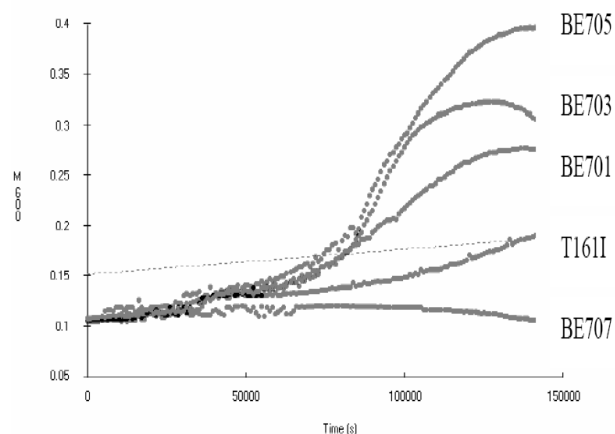


Figure 2.2 Growth tests with hATR mutants using *Salmonella* as a surrogate host. The following strains were used: BE701 (His₈-hATR), BE703 (hATR-His₈), BE705 (native hATR), and BE707 (no insert control). The mutants were grown on minimal medium with 1,2-PD as the only carbon source at 37 °C with shaking for 40 h. Cell growth was assessed by measuring OD₆₀₀. The control strain containing plasmid without insert (BE707) was unable to grow on 1,2-PD-B₁₂ minimal medium due to a lack of ATR activity.

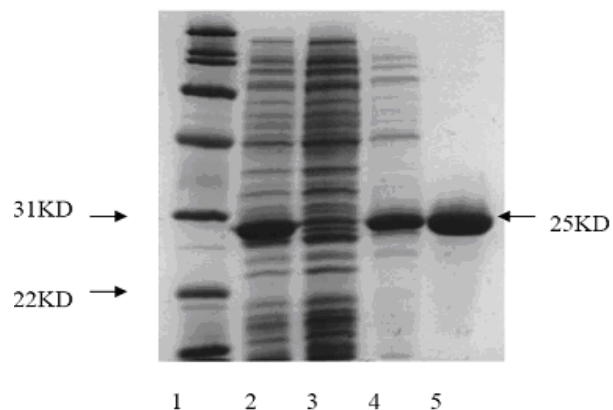


Figure 2.3 SDS-PAGE analysis of hATR-His₈ purification: Lane 1, molecular mass marker; lane 2, 10 µg of soluble extract of strain BE704 (hATR-His₈); lane 3, 10 µg of flow-through fraction; lane 4, 5 µg of 80 mM imidazole wash fraction; and lane 5, 5 µg of 250 mM imidazole elution fraction. The molecular mass of hATR-His₈ is about 25 kDa. The gel contained 12% acrylamide.

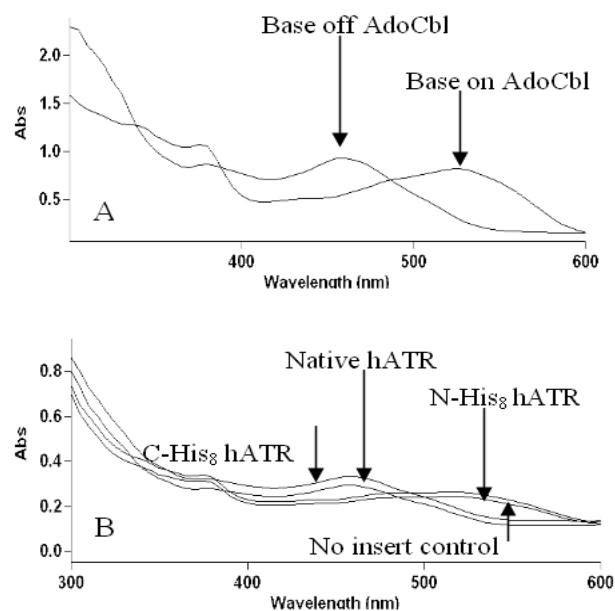


Figure 2.4 Binding of AdoCbl by hATR. UV-visible spectra of (A) AdoCbl in the base-on and base-off conformations and (B) AdoCbl bound to recombinant hATRs. 1 mL soluble cell extract containing 10 mg of protein was scanned as the baseline. Then, AdoCbl was added to the sample and gently mixed. After incubation at 37 °C for 2 min, the cuvette was scanned a second time (shown). The strains used for hATR production were BE702 (His₈-hATR), BE704 (hATR-His₈), BE706 (native hATR), and BE119 (no insert control).

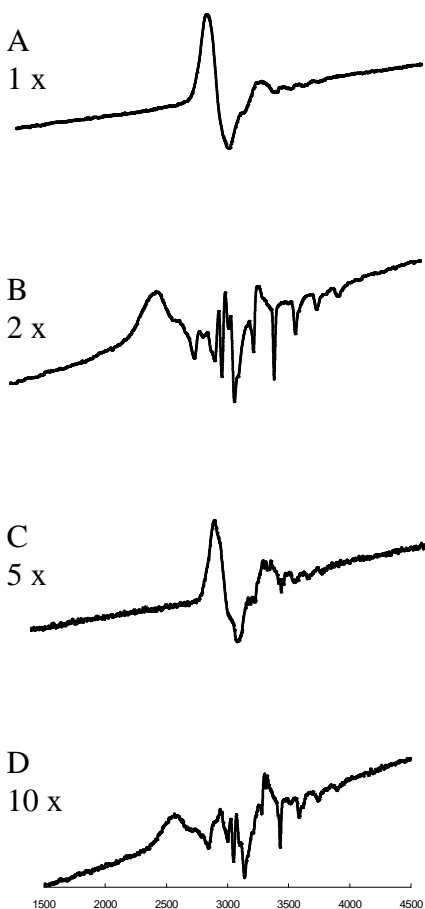


Figure 2.5 X-Band EPR spectra of (A) base-on cob(II)alamin [1mM cob(II)alamin in 10 mM Tris-HCl buffer (pH 8.0)], (B) base-off cob(II)alamin [1mM cob(II)alamin in 1% H₃PO₄], (C) 100 μ M cob(II)alamin in the presence of 2 mM purified His₈-hATR and 2 mM ATP, and (D) 100 μ M cob(II)alamin in the presence of 2 mM purified hATR-His₈ and 2 mM ATP. Spectra were collected at 100K with a modulation amplitude of 4G, a modulation frequency of 100 KHz, a time constant of 100 ms and a microwave power of 20 mW.

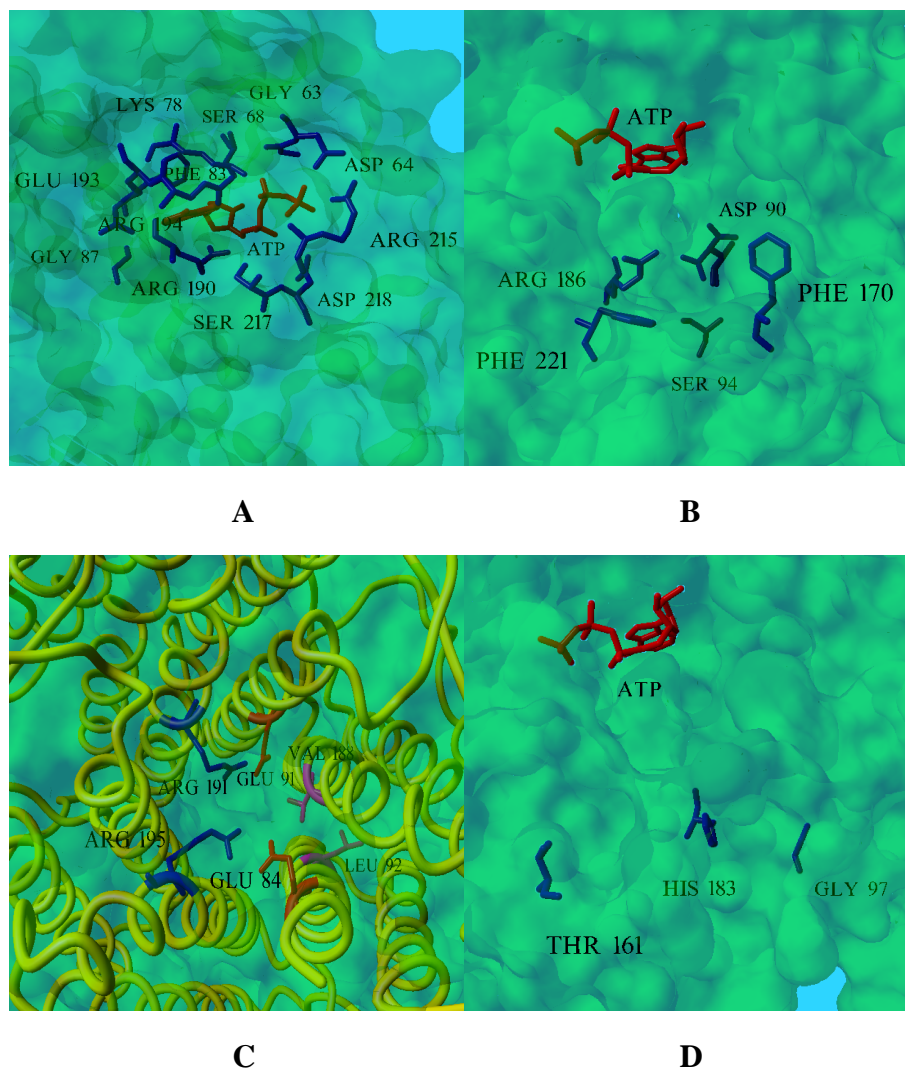


Figure 2.6 Mapping of mutations on the hATR structural model. (A) Side view of mutations involved in MgATP binding. (B) Side view of mutations at the surface of the proposed cobalamin binding site. (C) Top view of mutations at the interface of the hATR trimer. (D) Side view of mutations proposed to facilitate the base-off transition. This figure was made using Yasara[®].

CHAPTER 3 THE PDUX ENZYME OF *SALMONELLA ENTERICA* IS AN L-THREONINE KINASE USED FOR COENZYME B₁₂ SYNTHESIS

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Abstract

Here, the PduX enzyme of *Salmonella enterica* is shown to be an L-threonine kinase used for the *de novo* synthesis of coenzyme B₁₂ and the assimilation of cobyrinic acid (Cby). PduX with a C-terminal His tag (PduX-His₆) was produced at high levels in *Escherichia coli*, purified by nickel-affinity chromatography and partially characterized. ³¹P NMR spectroscopy established that purified PduX-His₆ catalyzed the conversion of L-threonine and ATP to L-threonine-*O*-3-phosphate and ADP. Enzyme assays showed that ATP was the preferred substrate compared with GTP, CTP or UTP. PduX displayed Michaelis-Menten kinetics with respect to both ATP and L-threonine and nonlinear regression was used to determine the following kinetic constants: $V_{\max} = 62.1 \pm 3.6 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$; $K_{\text{mATP}} = 54.7 \pm 5.7 \text{ }\mu\text{M}$ and $K_{\text{mThr}} = 146.1 \pm 8.4 \text{ }\mu\text{M}$. Growth studies showed that *pduX* mutants were impaired for the synthesis of coenzyme B₁₂ *de novo* and from Cby, but not from cobinamide which was the expected phenotype for an L-threonine kinase mutant. The defect in Cby assimilation was corrected by ectopic expression of *pduX* or by supplementation of growth medium with L-threonine-*O*-3-phosphate, providing further support that PduX is an L-threonine kinase. In addition, a bioassay showed that a *pduX* mutant was impaired for the *de novo* synthesis of coenzyme B₁₂ as expected. Collectively, the genetic and biochemical studies presented here show that PduX is an L-threonine kinase used for AdoCbl synthesis.

To our knowledge, PduX is the first enzyme shown to phosphorylate free L-threonine and first L-threonine kinase shown to function in coenzyme B₁₂ synthesis.

Introduction

The B₁₂ coenzymes (adenosylcobalamin, AdoCbl, and methylcobalamin, MeCbl) are required cofactors for at least 15 different enzymes that are widely distributed in nature and are essential for human health (1, 2). AdoCbl and MeCbl are synthesized *de novo* by certain prokaryotes and from corrinoid precursors by a broader range of organisms (1, 2). The synthesis of B₁₂ has been studied extensively in *Salmonella enterica* (3, 4). This organism carries out *de novo* synthesis under anaerobic conditions and assimilates corrinoids such as vitamin B₁₂, cobinamide (Cbi) and cobyrinic acid (Cby) under both aerobic and anaerobic conditions (3, 5). Corrinoid assimilation begins with transport into the cytoplasm by the B₁₂ uptake system (*btu*) followed by synthetic steps specific to a particular corrinoid (Figure 3.1) (6-8). In the case of Cbi, an adenosyl-group is added to the central cobalt atom to form Adenosyl-Cbi which is phosphorylated to Adenosyl-Cbi-phosphate (9, 10). Subsequently, the nucleotide loop is assembled in three steps to form AdoCbl (3). Lastly, a portion of the AdoCbl is converted to MeCbl (11, 12).

Many of the steps used for Cbi assimilation are also used for the assimilation of Cby as well as the *de novo* synthesis of AdoCbl and MeCbl (Figure 3.1). A key difference is that Cby assimilation and *de novo* synthesis require (R)-1-amino-2-propanol-O-2-phosphate (AP-P) whereas the conversion of Cbi to the B₁₂ coenzymes does not (3) (Figure 3.1). Prior studies showed that AP-P was produced by decarboxylation of L-threonine-O-3-phosphate (L-Thr-P) which was catalyzed by the CobD enzyme (13). These findings predicted that an L-threonine (L-Thr) kinase would be required for the *de novo* synthesis of AdoCbl and

MeCbl, and the assimilation of Cby. However, this enzyme has not been identified in any system.

In *S. enterica*, AdoCbl and MeCbl are required cofactors for three enzymes (5). MeCbl-dependent methionine synthase (MetH) is used to convert homocysteine to methionine (14); AdoCbl-dependent diol dehydratase and ethanolamine ammonia lyase are required for growth on 1,2-propanediol (1,2-PD) and ethanolamine, respectively (15, 16). The genes specific for 1,2-PD utilization (*pdu*) are found in a large contiguous cluster (15, 17). Bioinformatic studies tentatively suggest that the last gene of the *pdu* operon (*pduX*) encodes an L-Thr kinase (18). The PduX protein has homology to GHMP (galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase) family of kinases and a number of PduX homologues are encoded by genes located proximal to genes for AdoCbl biosynthesis. However, no experimental studies of PduX homologues have been conducted in any system.

Here, we present genetic and biochemical studies that show PduX is an L-Thr kinase used for the *de novo* synthesis of AdoCbl and the assimilation of Cby. To our knowledge, PduX is the first enzyme shown to phosphorylate free L-Thr.

Experimental Procedures

Chemicals and Reagents. Antibiotics, Cbi, L-Thr-P, (R)-1-Amino-2-propanol (AP), nucleoside triphosphates and nucleoside diphosphates were purchased from Sigma-Aldrich (St. Louis, MO). Isopropyl- β -D-thiogalactopyranoside (IPTG) was from Diagnostic Chemical Ltd., Charelottetown, Prince Edward Island, Canada. Restriction enzymes and T4 DNA ligase were from New England Biolabs, Beverly, MA. Pefabloc SC PLUS was purchased from ICN Biomedicals, Inc. Aurora, OH. Other chemicals were from Fisher

Scientific, Norcross, GA. Cby was kindly provided by Kathy Krasny from the laboratory of J. Escalante-Semerena.

Bacterial Strains, Media and Growth Conditions. The bacterial strains used in this study are listed in Table 3.1. The minimal medium used was NCE supplemented with 0.4% 1,2-PD, 1 mM MgSO₄, 50 µM Ferric citrate, 1 µM 5,6-dimethylbenzimidazole (DMB) and 3 mM (each) valine, isoleucine, leucine, and threonine. LB (Luria-Bertani) medium was the rich medium used.

General Molecular Methods. Agarose gel electrophoresis was performed as described previously (19). Plasmid DNA was purified by QIAGEN (Chatsworth, CA) products according to the manufacturer's instructions. Following restriction digestion and PCR amplification, DNA was purified by Promega Wizard PCR Preps (Madison, WI). Restriction digests were carried out using standard protocols (19). For ligation of DNA fragments, T4 DNA ligase was used according to the manufacturer's instructions. Electroporation was carried out as previously described (17).

Protein Methods. Polyacrylamide gel electrophoresis (PAGE) was performed by using Bio-Rad Ready gels and Bio-Rad Mini-Protean II electrophoresis cells according to the manufacturer's instructions. Following gel electrophoresis, Coomassie Brilliant Blue R-250 was used to stain proteins. The protein concentration of solutions was determined by using Bio-Rad protein assay reagent.

Construction of Plasmids for Production of PduX and PduX-His₆. PCR was used to amplify the *pduX* coding sequence from pEM55 (17). The primers used for amplification were 5'-GCC GCC AGA TCT ATG CGC GCA CAC TAT TCG TAC CT-3' and 5'-GCC GCC AAG CTT ATC ACT GCA GTT TGA CCC CGC CA-3'. The reverse primer used for

fusing six histidine residues to the C-terminus of PduX was 5'-GCC GCC AAG CTT ATC AAT GAT GAT GAT GAT GAT GCT GCA GTT TGA CCC CGC CA-3'. These PCR primers introduced *Bgl*III and *Hind*III restriction sites that were used for cloning into vector pTA925 or pLac22 (20). Following ligation, clones were introduced into *Escherichia coli* DH5 α by electroporation, and transformants were selected by plating on LB agar supplemented with 25 $\mu\text{g ml}^{-1}$ kanamycin (pTA925) or 100 $\mu\text{g ml}^{-1}$ ampicillin (pLac22). Pure cultures were prepared from selected transformants. The presence of insert DNA was verified by restriction analysis or PCR, and the DNA sequence of selected *pduX* clones was determined. Clones with the expected DNA sequence were used for further study.

DNA Sequencing. DNA sequencing was carried out by Iowa State University, DNA facility of Iowa State University Office of Biotechnology using automated sequencing equipment from Applied Biosystems Inc.

Growth Curves. Growth media are described in the figure legends. To prepare the inoculum, 2 ml LB cultures were incubated overnight at 37°C, and then cells were collected by centrifugation and suspended in growth curve medium. Media were inoculated to a density of 0.15 absorbance units, and the cell growth was monitored by measuring the optical density at 600 nm using a BioTek Synergy microplate reader and 48-well flat-bottom plates (Falcon). Each well was inoculated with 0.3 ml of culture and incubated at 37°C as described (21).

Corrinoid Extraction and Quantification. Selected strains of *S. enterica* were grown aerobically on 5 ml LB medium supplemented with 1% 1,2-PD, 1 μM DMB and 5 μM CoCl₂ overnight at 37°C in 17 \times 100 mm test tubes. Cells were collected by centrifugation and suspended in 1 ml, 50 mM Tris pH 7.0. The cell suspension was transferred into 3 ml serum

vials. The vials were sealed and flushed with helium for 3 minutes. The suspension was autoclaved for 10 minutes at 121°C, then placed on ice for 10 minutes and centrifuged at $25,000 \times g$ for 1 hour at 4°C. The corrinoids present in the supernatant were quantified using a bioassay based on the AdoCbl-dependent growth of *S. enterica* strain BE86 on the ethanolamine minimal medium. Growth measurements were carried out using a microplate reader as described above for “growth curves”. Quantitation was based on a standard curve of vitamin B₁₂ concentration versus maximum cell density of BE86. The assay was linear from 0 to 20 nM vitamin B₁₂ with an r^2 value of 0.9843.

Growth of PduX Expression Strains. The *E. coli* strains used for expression of *pduX* were grown on 400 ml LB supplemented with 25 $\mu\text{g ml}^{-1}$ kanamycin at 16°C in a New Brunswick Scientific, Innova 4230, refrigerated shaker- incubator with shaking speed at 275 rpm. Cells were grown to an absorbance of 0.6–0.8 at 600 nm, and protein expression was induced by the addition of 0.5 mM IPTG. Cells were incubated at 16 °C with shaking at 275 rpm for an additional 18 hours and harvested by centrifugation at $5000 \times g$ for 10 minutes at 4°C using a Beckman JA-10 rotor.

Preparation of Cell Extract of *Salmonella enterica*. About 2.0 g of cell paste was suspended in 8 ml of 50 mM Tris (pH 7.5), 300 mM NaCl, 20 mM imidazole and broken by French pressure (Thermo Electron Corp.) at 20,000 psi. Pefabloc SC PLUS was added to the cell extract to a concentration of 100 $\mu\text{g ml}^{-1}$ to inhibit proteases. The crude cell extract was centrifuged at $35000 \times g$ for 30 minutes at 4°C using a Beckman JA-17 rotor to separate the soluble and insoluble fractions. The supernatant was the soluble fraction used for enzyme purification.

Purification of PduX-His₆. Eight ml soluble cell extract from an *E. coli* expression strain was loaded onto a column containing 5 ml Ni-NTA Superflow resin (QIAGEN) previously equilibrated with 50 mM Tris (pH 7.5), 300 mM NaCl, 20 mM imidazole. The column was washed with 5 ml of 50 mM Tris (pH 7.5), 300 mM NaCl, 80 mM imidazole, then the enzyme bound to the column was eluted with 2.5 ml 50 mM Tris (pH 7.5), 300 mM NaCl, 300 mM imidazole. The purified protein was desalted by using PD-10 desalting column (GE Healthcare Life Sciences) and eluted with 3.5 ml 10 mM Tris (pH7.5) following the manufacturer's instructions.

PduX Enzyme Assay. The activity of PduX was measured by using an ADP Quest assay kit according to the manufacturer's instructions (DiscoverRx, Fremont, CA). The assay uses a coupled enzyme reaction system to generate hydrogen peroxide from ADP (ADP reacts with PEP to form pyruvate by pyruvate kinase, and then pyruvate oxidase generates hydrogen peroxide). Hydrogen peroxide when combined with Acetyl Dihydroxy Phenoxazine (ADHP) in the presence of peroxidase generates the fluorescently active resorufin dye. Standard assay mixtures contained 15 mM HEPES (pH 7.4), 20 mM NaCl, 10 mM MgCl₂ in a total volume of 0.1 mL. Activity was determined by monitoring the fluorescence intensity with a BioTek Synergy HT microplate reader. The excitation wavelength was 530 nm and emission wavelength was 590 nm. To minimize background fluorescence, 96-well black microplates were used (Greiner Bio-one, Frickenhausen, Germany). For kinetic studies, the concentrations of certain assay components varied as indicated in the text. Quantitation was based on standard curves made with ATP, GTP, CTP or UTP.

³¹P NMR Spectroscopy. The products of the PduX reaction were analyzed by ³¹P nuclear magnetic resonance (NMR) spectroscopy. Reactions were performed using conditions described for the PduX enzyme assay with 1 mg purified PduX-His₆ in a final volume of 1 ml. The reaction mixtures were incubated at 37°C. Protein was removed by a Vivaspin 500, 10K filtration device. Samples were transferred to 5 mm NMR tubes (WILMAD). 50 µL 100% D₂O was added (finally 5%). ³¹P NMR spectra of reference compounds and kinase reaction products were obtained with a 11.7-T magnet (Biomolecular Nuclear Magnetic Resonance Facility, Iowa State University, Ames) and a Bruker DRX 500 spectrometer at the following settings: frequency, 202.347 MHz; excitation pulse width, 8.1 µs; pulse repetition delay, 6 s; and spectral width, 12.136 kHz. Spectra were processed with TOPSPIN 1.3 software from Bruker. Chemical shifts were referenced to triphenyl phosphate, which was set to -18.0 ppm.

High Pressure Liquid Chromatography (HPLC). A Mono Q HR5/5 column (GE Healthcare, Piscataway, NJ) was used with a Varian ProStar system that included a model 230 solvent delivery module, a model 430 autosampler, and a model 325 UV-Vis detector (Varian, Palo Alto, CA). Buffers A and B contained 20 mM Tris (pH 8.0) and 50 mM and 1 M NaCl, respectively. The flow rate was 1 ml min⁻¹, and analytes were eluted with a linear gradient from 50 mM to 700 mM NaCl over 10 minutes. The compounds were monitored at 260 nm. The retention times for ATP, ADP, GTP, GDP, UTP, UDP, CTP and CDP were 4.46, 6.05, 5.18, 6.28, 4.37, 5.63, 4.09 and 5.47 min, respectively.

Results

PduX is Needed for *Salmonella enterica* Growth on 1,2-PD Minimal Medium Supplemented with Cby, but not on Similar Medium Supplemented with Cbi. L-Thr

kinase is predicted to be required for the synthesis of AdoCbl from Cby, but not from Cbi (Figure 3.1). Therefore, we measured aerobic growth of *S. enterica* and a *pduX* deletion mutant (BE892) on 1,2-PD minimal medium supplemented with Cby or Cbi. Under these conditions, growth requires the synthesis of AdoCbl from Cby or Cbi (15).

Results showed that a *pduX* mutant was significantly impaired for growth on 1,2-PD minimal medium supplemented with Cby, but grew well on similar medium supplemented with Cbi (Figure 3.2). In contrast, wild-type *S. enterica* grew well on 1,2-PD medium supplemented with either Cby or Cbi. The generation times of *S. enterica* with Cbi and Cby were 4.2 and 5.8 hours, while those of the *pduX* mutant were 4.2 and 17.8 hours, respectively. These are the expected phenotypes of an L-Thr kinase mutant. Normal growth with Cbi indicates that the 1,2-PD degradative pathway is intact, and that corrinoid uptake is unimpaired since a single system is used for uptake of Cbi, Cby and other corrinoids (6-8). It also shows that *pduX* mutants can carry out all the steps needed for the conversion of Cbi to AdoCbl (Figure 3.1). Thus, the inability of *pduX* mutants to use Cby can be attributed to a defect in the conversion of Cby to AdoCbi-phosphate (Figure 3.1). This process requires four enzymes: 1. an adenosyltransferase (CobA); 2. an enzyme that converts AdoCby to AdoCbi-phosphate (CbiB); 3. an L-Thr-P decarboxylase (CobD) and 4. an L-Thr kinase (9, 13, 22). The CobA, CbiB and CobD enzyme have been characterized in *S. enterica* (9, 13, 22). Hence, the above studies suggest that PduX is an L-Thr kinase used for the conversion of Cby to AdoCbl.

A PduX Deletion Mutation is Complemented by Ectopic Expression of PduX. A *pduX* expression plasmid (pLac22-*pduX*) was introduced into a *pduX* deletion mutant by electroporation. This plasmid fully corrected the growth defect of a *pduX* mutant on 1,2-PD

minimal medium supplemented with Cby (the generation time was 6.9 hours compared with 6.1 hours for wild-type) (Figure 3.3). In contrast, plasmid without insert had little effect on growth (the generation time is 17.4 hours). This showed that the observed phenotype (poor growth with Cby) resulted from the *pduX* mutation but not from polarity or an unknown mutation.

Supplementation of Growth Media with L-threonine-phosphate Substantially Corrects the Growth Defect of a PduX Mutant. In many cases, biosynthetic mutants are corrected by supplementation of growth medium with a downstream metabolite. Studies conducted here showed that 100 μ M L-Thr-P substantially corrected the growth defect of a *pduX* mutant on 1,2-PD minimum media containing Cby (Figure 3.4). With 100 μ M L-Thr-P the generation time for a *pduX* deletion mutant was 8.8 hours compared with a generation time of 8.3 hours for the wild-type strain. Both strains reached a maximum optical density at 600 nm of about 0.4. These results indicate that PduX has a role in the synthesis of L-Thr-P.

(R)-1-amino-2-propanol (AP) is Ineffective for Correction of a PduX Deletion Mutant. Prior studies showed that a *cobD* mutant was partly corrected for the synthesis of MeCbl from Cby by addition of AP to growth media (23). This and subsequent findings indicated that unknown kinase phosphorylated AP to AP-P bypassing the *cobD* defect (Figure 3.1) (13). Therefore, PduX was tested for AP kinase activity. However, results were negative. Both a *cobD* mutant and a *pduX cobD* double mutant grew similarly on minimal glucose medium supplemented with Cby and AP indicating that *S. enterica* converts AP to AP-P in the absence of PduX (Figure 3.9). Concentrations of AP between 1 mM and 20 mM were tested and in no case did the PduX⁺ strain grow better than the PduX⁻ strain. The studies described above were conducted in a *metE* background under conditions where the

conversion of Cby to MeCbl is required for growth in minimal medium (24). Tests of PduX for AP kinase activity using 1,2-PD minimal medium supplemented with Cby and enzyme assays with purified recombinant enzyme were also negative. Thus, results indicate that PduX does not phosphorylate AP.

PduX is Needed for *Salmonella enterica* Growth on Minimal Ethanolamine Medium Supplemented with Cby, but not on Similar Medium Supplemented with Cbi. The *pduX* gene is the last gene in the *pdu* operon (17). Measurable expression of this operon requires 1,2-PD (15, 25-27). This raised the question of whether PduX supports ethanolamine degradation. Results showed that a *pduX* mutant was unable to grow on ethanolamine minimal medium supplemented with Cby, but grew well on similar medium supplemented with Cbi (Figure 3.5). In contrast, wild-type *S. enterica* grew well on ethanolamine medium supplemented with either Cby or Cbi. The generation times of *S. enterica* with Cbi and Cby were 4.3 and 4.4 hours, while those of the *pduX* mutant were 4.3 and 33.1 hours, respectively. In addition, the growth defect of the *pduX* mutant on ethanolamine with Cby was fully corrected by ectopic expression of *pduX*, showing this defect resulted from the *pduX* mutation, but not from polarity or an unknown mutation. Hence, *pduX* is used for AdoCbl synthesis even in the absence of 1,2-PD which is required for transcription of the *pdu* operon (15, 17, 26, 27). This finding is surprising to us since it suggests that *pduX* has a different regulatory pattern than upstream genes presumed to be in the same operon.

Thr-P but not AP Supplementation Allows Growth of a PduX Mutant on Ethanolamine with Cby. The growth defect of a *pduX* mutant on minimal ethanolamine medium supplemented Cby was corrected by addition of Thr-P, but not by addition of AP-P.

This was similar to results obtained with 1,2-PD (above) and provided further evidence that PduX is an L-Thr kinase.

Production of MeCbl Sufficient to Support Methionine Biosynthesis is Independent of PduX. *Salmonella enterica metE* mutants require MeCbl for methionine biosynthesis; hence, L-Thr kinase should be required for growth of *metE* strains on minimal medium supplemented with Cby (Figure 3.1) (14). However, results showed that a *metE pduX* double mutant grew only slightly slower than wild-type on minimal glucose medium supplemented with Cby. Doubling times for the wild-type, *metE* mutant and *metE pduX* double mutant were 2.0, 2.0 and 3.8 hours, respectively. This suggested that PduX contributed to MeCbl synthesis, but *S. enterica* also produced a second L-Thr kinase which allowed substantial growth of a *metE* mutant as is further explained in the discussion.

PduX is Used for the *De Novo* Synthesis of B₁₂. L-Thr kinase is expected to be required for the *de novo* synthesis of AdoCbl (Figure 3.1) (12). Therefore, we used a bioassay to quantitate *de novo* synthesis of AdoCbl and MeCbl by *S. enterica*, a *pduX* mutant and a *cbiB* mutant (as negative control). These strains produced B₁₂ in the following amounts (pmole per gram wet cells): 2620 ± 112 , 280 ± 11 and undetectable, respectively. Thus, a *pduX* mutant produced about 11% as much B₁₂ as the wild-type strain. *S. enterica* BE86 was used for the bioassay. This strain grows on ethanolamine minimal medium supplemented with complete corrinoids such as AdoCbl and MeCbl, but not on similar medium supplemented with corrinoids such as AdoCby-P which might in a *pduX* mutant.

High-level Expression of the PduX-His₆ Protein. PduX with a 6× C-terminal His tag (PduX-His₆) was produced at high levels via an *E. coli* T7 expression system and purified by Nickel-affinity chromatography (Figure 3.6). This procedure allowed the isolation PduX-His₆

that appeared nearly homogeneous following Coomassie staining. The apparent molecular mass of PduX-His₆ by SDS-PAGE was approximately 35 kDa which was close to the expected value of 33.7 kDa.

ADP and L-threonine-O-3-phosphate are the Products of the PduX-His₆ Reaction.

³¹P NMR spectroscopy was used to identify the products of the PduX reaction. Figure 3.7A shows the ³¹P NMR spectrum of a kinase reaction mixture prior to addition of purified PduX-His₆ enzyme. The triplet centered at -22.5 ppm corresponds to the β-phosphate of ATP; the two doublets centered at -7.1 ppm and -11.6 ppm correspond to the γ- and α-phosphates of ATP, respectively. Figure 3.7B shows the ³¹P NMR spectrum of ADP standard. The two doublets centered at -7.2 ppm and -11.2 ppm correspond to the β- and α-phosphates of ADP, respectively. Figure 3.7D shows the complete reaction mixture after 2 hours of incubation at 37°C with 200 μg of purified PduX-His₆. The triplet corresponding to the β-phosphate of ATP (-22.5 ppm) is absent. The two doublets centered at -7.2 ppm and -11.2 ppm of the complete reaction mixture correlate well with the two doublets of ADP standard (Figure 3.7B). The singlet at 2.3 ppm corresponds to the phosphate group of L-Thr-P (Figure 3.7C). Figure 3.7E shows the intermediate reaction mixture after 30 minutes of incubation at 37°C with 200 μg of purified PduX-His₆. It shows clearly that the intermediate mixture contains L-Thr as well as both ATP and ADP (Figure 3.7F). Hence, these results indicate that ADP and L-Thr-P are the products of the PduX reaction.

***In Vitro* Activity of PduX-His₆.** To test whether purified PduX-His₆ had L-Thr kinase activity, we used a continuous fluorometric assay that measured the nucleoside diphosphate product (described in the experimental procedures section). With ATP as the substrate, the specific activity of purified PduX-His₆ was 56.3 nmol min⁻¹ mg protein⁻¹. In the absence of

ATP, no activity was detected. In the absence of PduX, L-Thr or both, activity decreased by 90%. The remaining 10% activity appears to have resulted from ATP-dependent background fluorescence. Thus, enzyme assays indicate that PduX has L-Thr kinase activity.

Linearity and Nucleoside Triphosphates Specificity of the PduX Reaction. The fluorometric assay described above as well as an HPLC-based assay were used to determine the substrate specificity of PduX. Both assay methods gave similar results. Setting ATP to 100%, the activity of PduX-His₆ with CTP, GTP, or UTP was 6, 11, and 3 %, respectively, by the fluorometric method and 6, 12 and 4%, respectively, by HPLC assay. Assays contained 3 μ M purified PduX-His₆ and 200 μ M nucleoside triphosphates. The effect of PduX concentration on enzyme activity was also determined. Results showed that the rate of reaction was proportional to PduX-His₆ concentration from 0.006 to 6 μ M when 200 μ M ATP and 500 μ M L-Thr were used as substrates. Linear regression yielded an r^2 value of 0.998.

K_m and V_{max} Values for PduX. Purified PduX-His₆ displayed Michaelis-Menten kinetics with respect to both ATP and L-Thr (Figure 3.8). Based on nonlinear regression, the K_m values for ATP and L-Thr were $54.7 \pm 5.7 \mu\text{M}$, and $146.1 \pm 8.4 \mu\text{M}$, respectively. The enzyme V_{max} was $62.8 \pm 3.6 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ when L-Thr was varied and $61.4 \pm 3.6 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ when ATP was varied. The average of these two values is $62.1 \pm 3.6 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$. Similar kinetic constants were derived from double-reciprocal plots: The K_m values for ATP and L-Thr were $60.9 \pm 9.7 \mu\text{M}$ and $127.0 \pm 11.3 \mu\text{M}$, respectively. V_{max} values were $58.6 \pm 6.2 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ when L-Thr was varied and $57.2 \pm 5.8 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ when ATP was varied. The average of these two values is $57.9 \pm 6.0 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$. When the K_m values for ATP were determined, saturating levels of

L-Thr (500 μ M) were added to assay mixtures while varying the concentration of ATP. Similarly, saturating levels of ATP (200 μ M) were added to assays when the K_m values for L-Thr were determined. Purified PduX-His₆ was used at a concentration of 3 μ M. The values used for kinetic calculations were the average of three measurements of the initial reaction rate.

Discussion

Work done in a number of laboratories has defined many steps in the *de novo* synthesis of AdoCbl and MeCbl (3, 5, 28-32). Of particular relevance to this work are the studies reported by Brushaber *et al.* which showed the CobD enzyme catalyzes the decarboxylation L-Thr-P to AP-P (13). This finding predicted that B₁₂ synthesis would require an L-Thr kinase. Subsequently, Rodionov *et al.* (18) proposed that PduX homologues might be L-Thr kinases involved in B₁₂ synthesis. This was based sequence similarity to GHMP family of kinases and the observation that *pduX* genes were proximal to B₁₂ biosynthetic genes in several instances. However, it is well-known that functional predictions based on sequence similarity and gene proximity are tentative. In addition, a number of PduX homologues are encoded by genes unlinked to B₁₂ biosynthetic genes raising the possibility of functional diversity among PduX homologues.

Here, we presented *in vitro* and *in vivo* evidence that the *pduX* gene of *S. enterica* encodes an L-Thr kinase involved in the *de novo* synthesis AdoCbl and the assimilation of Cby. ³¹P NMR spectroscopy indicated that purified PduX-His₆ catalyzes the conversion of ATP and L-Thr to ADP and L-Thr-P (Figure 3.7). Enzyme assays indicated the ATP was preferred substrate for PduX compared to GTP, CTP and UTP. The V_{max} of PduX (62 nmol min⁻¹ mg protein⁻¹) is within the range determined for other B₁₂ biosynthetic enzymes which

have relatively low activity in accord with the low levels of AdoCbl and MeCbl that are required to support B₁₂-dependent processes (5). The K_m value of PduX for ATP (55 μ M) is well below the ATP pool sizes in rapidly growing cells (1-3 mM) (33). The K_m value for L-Thr (146 μ M) is close to measured pool sizes (70-400 μ M) for *E. coli* which is a close relative of *S. enterica* (34). Thus, *in vitro* studies demonstrated that purified recombinant PduX has L-Thr kinase activity with kinetic parameters suitable to a role in AdoCbl synthesis.

In addition to the above *in vitro* studies, genetic tests demonstrated that PduX has a role in the synthesis of AdoCbl *in vivo*. A bioassay and growth tests showed that a *pduX* deletion mutant was impaired for the *de novo* synthesis AdoCbl and for the assimilation of Cby. In addition, the growth defects of a *pduX* mutant were corrected by addition of L-Thr-P to minimal media indicating that PduX is needed for L-Thr-P synthesis.

A point of note is that bioassays found B₁₂ in cultures grown under aerobic conditions. It is well-established that *S. enterica* only synthesizes B₁₂ *de novo* in the absence of oxygen (35). The bioassays conducted here and unpublished studies done in our laboratory indicate that *S. enterica* synthesizes B₁₂ under aerobic incubation conditions at high cell densities. Presumably, oxygen is depleted by cell respiration allowing B₁₂ synthesis. This helps explain the paradoxical observation that *S. enterica* synthesizes B₁₂ only in the absence of oxygen, but requires oxygen for degradation of ethanolamine and 1,2-PD as sole carbon and energy sources (5).

The genetic tests performed here also indicated that a *pduX* mutant synthesized enough MeCbl to support substantial growth of a *metE* mutant. This suggests that *S. enterica* expresses an L-Thr kinase in addition to PduX, but raises the question why is this second

kinase unable to support ethanolamine or 1,2-PD degradation (Figures 3.2 and 3.5). One explanation is that the second kinase is induced during growth on glucose. Alternatively, the activity of the second kinase might be insufficient for 1,2-PD and ethanolamine degradation. A *pduX* mutant grew slowly on 1,2-PD minimal medium supplemented with Cby (Figure 3.2) suggesting a second kinase with low activity. Prior studies showed that the MeCbl needed to support growth of a *metE* mutant (methionine biosynthesis) was about 100-fold less than the amount of AdoCbl needed to support 1,2-PD and ethanolamine degradation. Hence, a second L-Thr kinase that has low activity compared to PduX is indicated.

To our knowledge, PduX is the first enzyme reported to transfer a phosphoryl-group to free L-Thr. Sequence similarity indicates that PduX is a member of the GHMP kinase family (18). This family includes members that transfer the γ -phosphoryl-group of ATP to acceptors such as mevalonate, homoserine, galactose, and developmental protein Xol-1 (36). A feature of PduX we think is interesting is that it phosphorylates free L-Thr which is also required for protein synthesis. Presumably, PduX would need to be regulated to prevent L-Thr depletion or a futile cycle that needlessly consumes ATP. However, regulation of the activity of the PduX enzyme has not been studied.

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TABLE 3.1 Bacterial strains used in this study

Species and strain	Genotype
<i>Escherichia coli</i>	
DH5 α	F ⁻ λ^- <i>endA1 hsdR17 relA1 supE44 thi-1 recA1 gyrA96 relA1</i> $\Delta(lacZYA-argF)U169$ ($\phi 80dlacZ\Delta M15$)
BL21DE3 RIL.....	F ⁻ <i>ompT hsdS</i> ($r_B^- m_B^-$) <i>dcm</i> ⁺ Tet ^r <i>gal</i> λ (DE3) <i>endA</i> Hte [<i>argU ileY leuW</i> Cam ^r]
BE119.....	BL21DE3 RIL/pTA925
BE945.....	DH5 α /pTA925 - <i>pduX</i> -His ₆
BE963.....	BL21DE3 RIL/ pTA925- <i>pduX</i> -His ₆
<i>Salmonella enterica</i> serovar Typhimurium LT2	
BE86.....	DEL1715[(<i>cbiD24</i>)*MudJ*(zea-3666)]
BE200.....	Del <i>pduX668</i>
BE287.....	pLac22
BE438.....	<i>cbiB24::mudJ</i>
BE892.....	Del <i>pduX685::Kan</i>
BE933.....	Del <i>pduX685::kan</i> /pLac22
BE935.....	Del <i>pduX685::Kan</i> /pLac22- <i>pduX</i>
BE938.....	Del <i>pduX668 cobD498::mudJ</i>

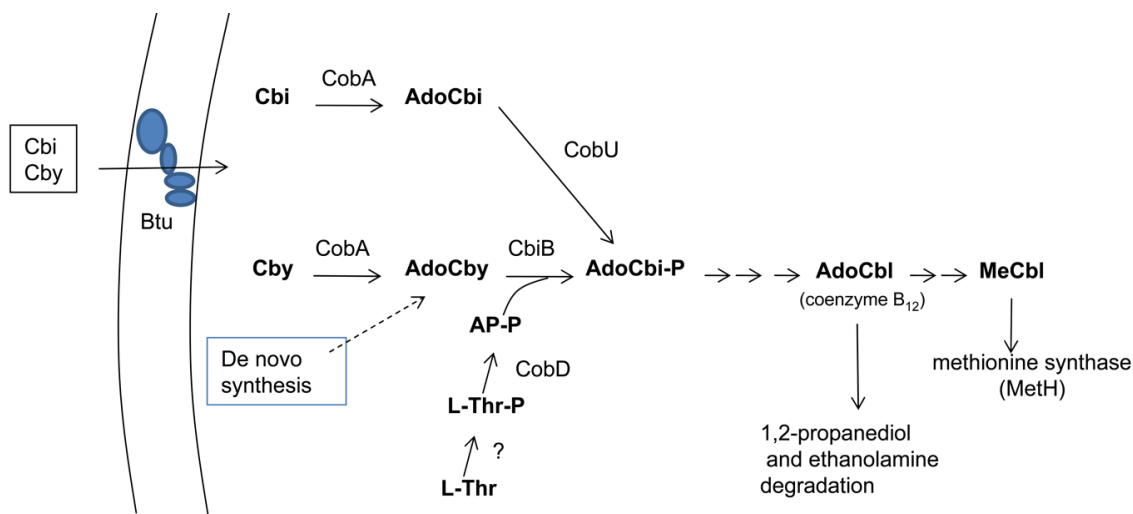


Figure 3.1 Pathways for assimilation of Cbi and Cby in *Salmonella enterica*. AdoCbl and MeCbl are the forms of B₁₂ that function as enzymatic cofactors. The assimilation of Cbi and Cby and the later steps of the de novo synthesis of the B₁₂ coenzymes share a number of enzymatic steps. A key difference is that the conversion of L-Threonine-O-3-phosphate to (R)-1-amino-2-propanol-O-2-phosphate is unnecessary for the assimilation of Cbi. Abbreviations: Btu, B₁₂ uptake system; Cbi, cobinamide; Cby, cobyrinic acid; AdoCbi, adenosylcobinamide; AdoCby, adenosylcobyrinic acid; AdoCbi-P, adenosylcobinamide phosphate; AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin; L-Thr, L-threonine; L-Thr-P, L-threonine-O-3-phosphate; AP-P, (R)-1-amino-2-propanol-O-2-phosphate.

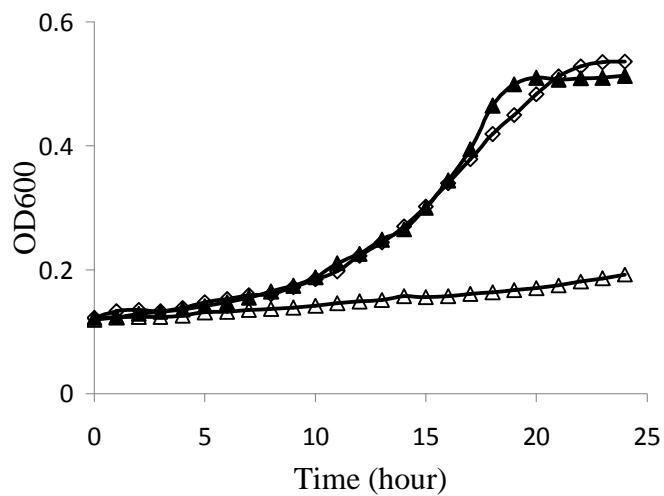


Figure 3.2 Effects of a *pduX* deletion on 1,2-propanediol degradation with Cbi or Cby. Cells were grown in minimal 1,2-propanediol medium supplemented with 50 nM Cbi or Cby. Solid triangle: BE892 (Del *pduX*) with Cbi; Open diamond: wild-type with Cby; Open triangle: BE892 (Del *pduX*) with Cby.

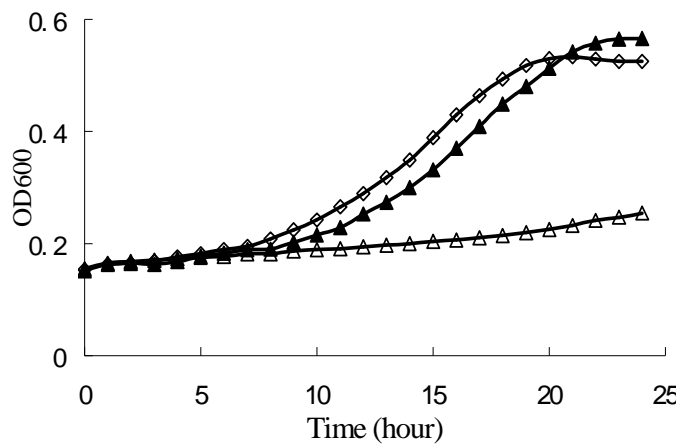


Figure 3.3 Complementation of the *pduX* deletion by ectopic expression of by *pduX*. Cells were grown in minimal 1,2-propanediol medium supplemented with 50 nM Cby. Open diamond: wild-type *S. enterica*; Solid triangle: BE935 (Del *pduX*/pLac22-*pduX*); Open triangle: BE933 (Del *pduX* /pLac22).

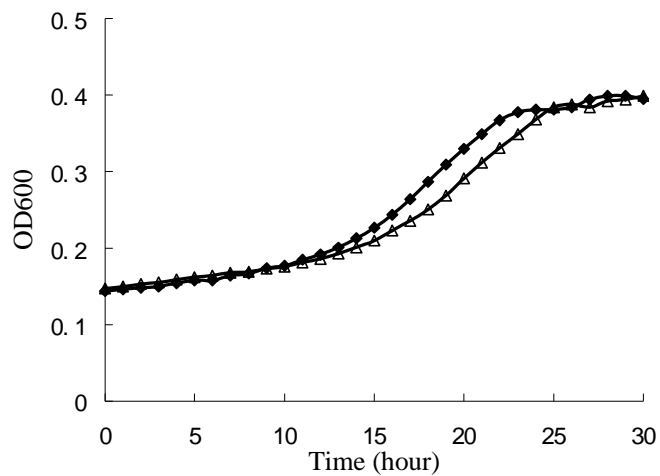


Figure 3.4 Effects of L-threonine phosphate on growth of a *pduX* deletion. Cells were grown in minimal 1,2-propanediol medium supplemented with 50 nM Cby and 100 μ M L-Thr-P. Solid diamond: wild-type *S. enterica*; Open triangle: BE892 (Del *pduX*).

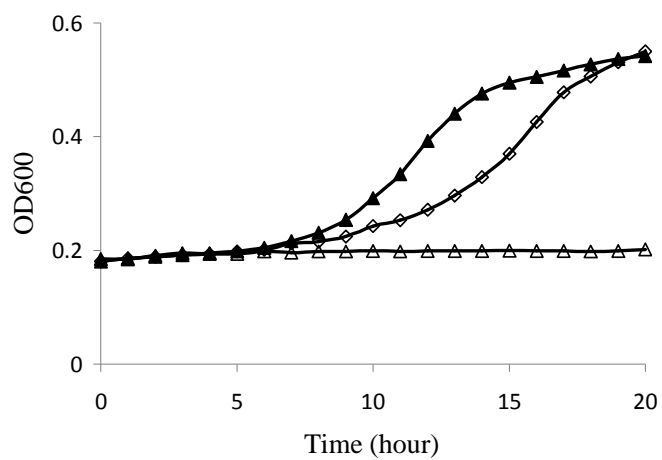


Figure 3.5 Effects of a *pduX* deletion on ethanolamine degradation with Cbi or Cby. Cells were grown in minimal ethanolamine medium supplemented with 50 nM Cbi or Cby. Solid triangle: BE892 (Del *pduX*) with Cbi; Open diamond: wild-type with Cby; Open triangle: BE892 (Del *pduX*) with Cby.

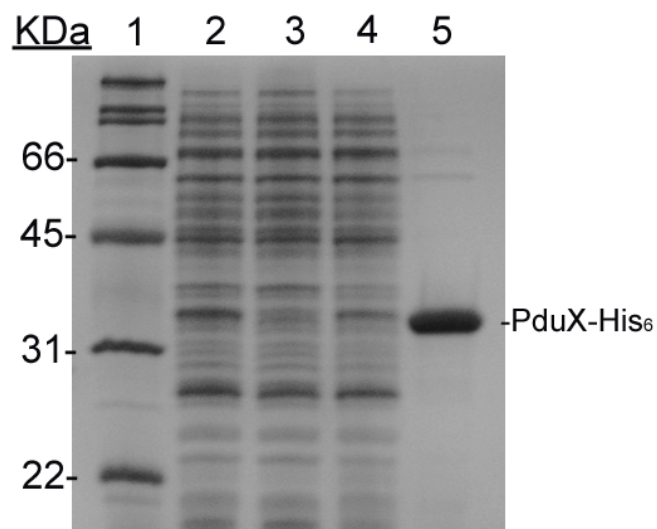


Figure 3.6 SDS-PAGE analysis of PduX-His₆ purification. Lane 1, molecular mass markers; Lane 2, 5 µg of soluble extract of strain BE963 (PduX-His₆); Lane 3, 5 µg of flow through fraction; Lane 4, 5 µg of 80 mM imidazole wash fraction; Lane 5, 5 µg of 300 mM imidazole elution fraction. The gel contained 12% acrylamide and was stained with Coomassie Brilliant Blue.

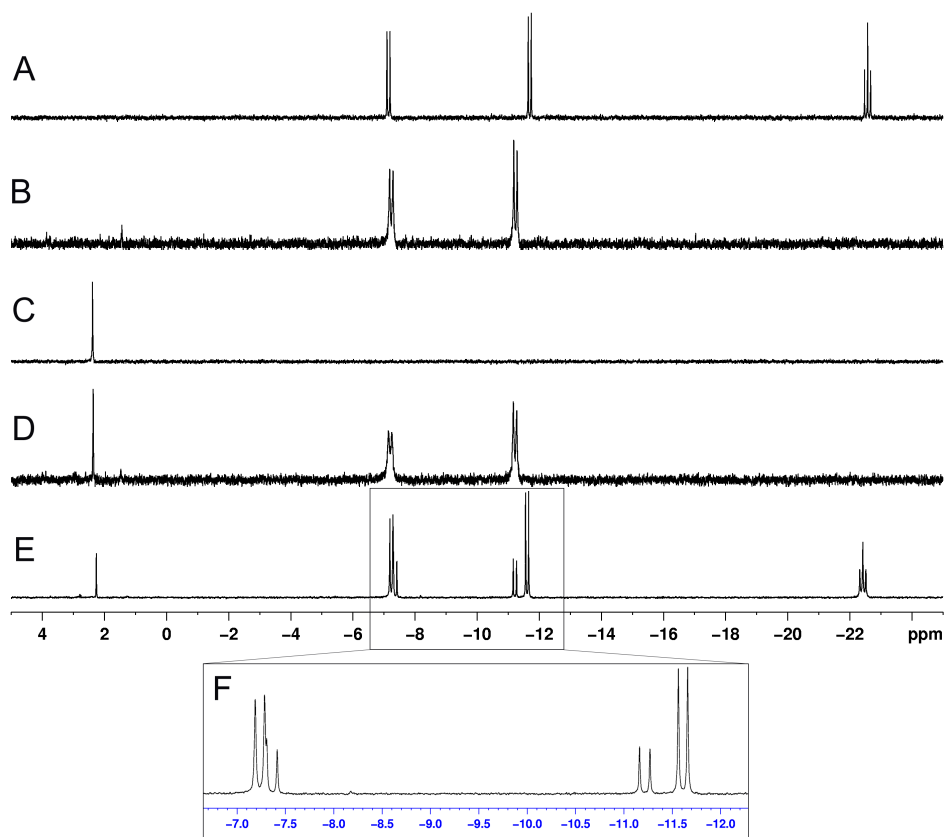


Figure 3.7 ^{31}P NMR spectra of a PduX reaction. (A) Reaction mixture containing 1 mM ATP and 1 mM L-threonine prior to addition of enzyme. (B) ADP standard (1 mM) (C) L-threonine-*O*-3-phosphate standard (1 mM). (D) Complete reaction after 2 hours of incubation with 200 μg of purified PduX-His₆. (E) Intermediate reaction after 30 minutes of incubation with 200 μg of purified PduX-His₆. (F) Expanded view of intermediate reaction from chemical shift -7.0 to -12.0 ppm. The chemical shifts observed were -7.1 ppm, -11.6 ppm, and -22.5 ppm for the γ -, α -, and β -phosphates of ATP, respectively, -7.2 ppm and -11.2 ppm for the β - and α -phosphates of ADP, respectively, and 2.3 ppm for L-threonine-*O*-3-phosphate. All chemical shifts are referenced to triphenyl phosphate, which was set to -18.0 ppm.

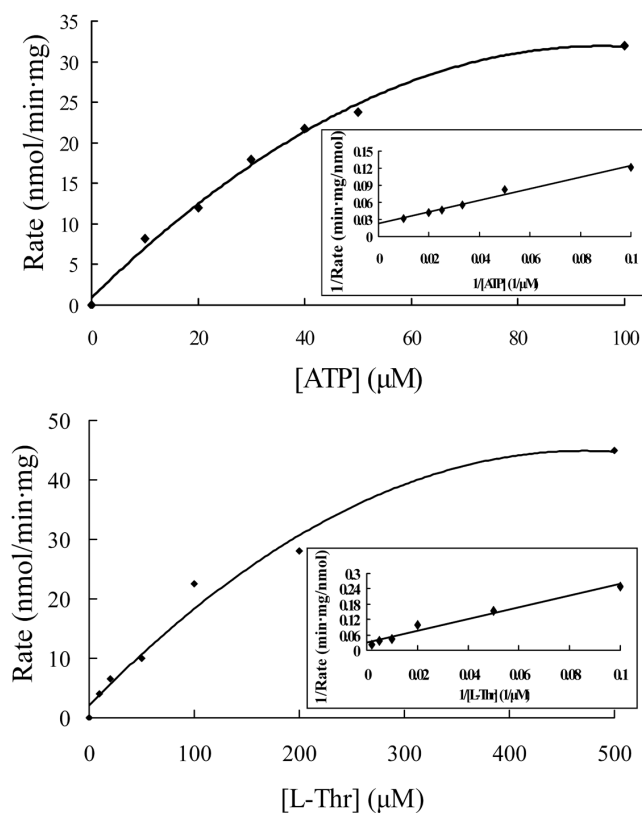


Figure 3.8 Kinetic analyses of PduX. Enzyme assays were performed with 3 μM purified PduX-His₆ protein. When the K_m value for ATP was determined, L-threonine was held at 500 μM and the concentration of ATP was varied. When the K_m value for L-threonine was determined ATP was held at 200 μM and the concentration of L-threonine was varied. The values shown are the average of three measurements of the initial reaction rate. The Insets are the double-reciprocal plots of the kinetic data.

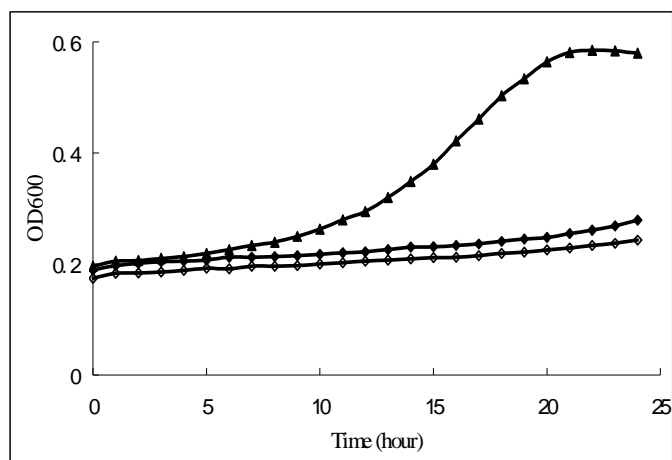


Figure 3.9 Effects of (R)-1-amino-2-propanol (AP) on growth of a *pduX* deletion. Cells were grown in minimal 1,2-propanediol medium supplemented with 50 nM Cby and 20 mM AP. Solid diamond: wild-type *S. enterica*; Open diamond: Del *pduX*; Closed diamond: Del *pduX cobD*.

CHAPTER 4 KINETIC AND FUNCTIONAL ANALYSIS OF L-THREONINE KINASE, THE PDUX ENZYME OF *SALMONELLA ENTERICA*

A paper in preparation

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Abstract

The PduX enzyme of *Salmonella enterica* is an L-threonine kinase used for the *de novo* synthesis of coenzyme B₁₂ and the assimilation of cobyrinic acid. PduX with an N-terminal histidines tag (His₈-PduX) was produced in *Escherichia coli* and purified. The recombinant enzyme was soluble and active. Kinetic analysis indicated an ordered ternary complex mechanism in which ATP is the first substrate to bind. The lack of obvious pH and isotope effects indicated that proton transfer is unlikely to be involved directly in the rate determining step of catalysis. Based on a multiple sequence alignment of PduX homologues and other GHMP family members, 14 PduX variants having changes at 10 conserved serine/threonine and aspartate/glutamate sites were constructed by site-directed mutagenesis. Each variant was produced in *E. coli* and purified. Comparison of the CD spectra and kinetic properties of the PduX variants to those of the wild-type enzyme indicated Glu-24 and Asp-135 are needed for proper folding, Ser-99 and Glu-132 are used for ATP binding, Ser-253 and Ser-255 are critical L-threonine binding while Ser-100 is essential to catalysis but its precise role is uncertain. To our knowledge, the studies reported here are the first to investigate the catalytic mechanism of L-threonine kinase from any organism.

Introduction

The B₁₂ coenzymes (adenosylcobalamin, AdoCbl, and methylcobalamin, MeCbl) are the largest cofactors known in biology. They are essential for the human health and have important metabolic roles in many microbes (1,2). The B₁₂ coenzymes are synthesized *de novo* by certain prokaryotes and from corrinoid precursors by a broader range of organisms (1,2). The synthesis of B₁₂ has been studied extensively in *Salmonella enterica* (3,4). This organism carries out *de novo* synthesis under anaerobic conditions and assimilates corrinoids such as cobinamide (Cbi) and cobyrinic acid (Cby) under both aerobic and anaerobic conditions (3,5). We recently showed that the PduX enzyme of *S. enterica* is a L-threonine kinase, which is required for the *de novo* synthesis of AdoCbl and MeCbl, and the assimilation of Cby (6). By sequence similarity, the PduX enzyme in *Salmonella enterica* belongs to GHMP (galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase) family (7), which is a novel family of kinases found in metabolic pathways of small molecules. Although there has been no three-dimensional structure of PduX or other L-threonine kinases reported to date, the crystal structures of several GHMP kinases have been solved (8-15) (16-19) (20,21) (22). These structures reveal a unique kinase fold and a novel nucleotide-binding mode that are conserved among members of the GHMP kinase family. All members contain three common motifs (23). Motif I is at the N-terminal region and its function is uncertain. Motif II is the most conserved one with a typical sequence Pro-X-X-X-Gly-Leu-X-Ser-Ser-Ala. This region is involved in nucleotide binding and distinguishes the GHMP family from other protein kinase families. Motif III is near the C-terminus containing a small glycine-rich loop that appears well-positioned to interact with substrate that is phosphorylated by ATP (24). However, there are highly divergent sequences

outside these three motifs and modes of oligomerization, substrate-binding and catalytic mechanism vary among different members (14).

Based on structural and biochemical data, members of GHMP family are subdivided into two groups depending on their catalytic mechanisms. In the first group, which includes rat mevalonate kinase, the crystal structure shows an aspartate residue in the active site positioned to act as a catalytic base that abstracts a proton from substrate hydroxyl group. In addition, there is a lysine residue that is thought to reduce the pK_a of the substrate hydroxyl group and to stabilize the penta-coordinated γ -phosphoryl group of ATP (18). In the second group, such as *Escherichia coli* homoserine kinase, there appears to be no suitable residue positioned to act as a catalytic base. Consequently, it has been hypothesized that homoserine kinase achieves catalysis through transition state stabilization alone (14).

Here, we investigate the catalytic mechanism of PduX by a series of kinetic studies and mutational analyses of 10 invariant serine/threonine and aspartate/glutamate residues. Results indicate an ordered ternary complex mechanism in which ATP binds first and a catalytic mechanism that involves primarily transition state stabilization, rather than acid-base catalysis. To our knowledge these are the first reported studies on the catalytic mechanism of L-threonine kinase from any organism.

Experimental Procedures

Chemicals and Reagents. Adenylyl imidodiphosphate (AMP-PNP), L-threonine phosphate, nucleoside triphosphates and nucleoside diphosphates were purchased from Sigma-Aldrich (St. Louis, MO). Restriction enzymes and T4 DNA ligase were from New England Biolabs, Beverly, MA. Pefabloc SC PLUS was purchased from ICN Biomedicals Inc. (Aurora, OH). Other chemicals were from Fisher Scientific (Norcross, GA).

Cloning Expression and Purification of His₈-PduX. PCR was used to amplify the *pduX* coding sequence from pEM55 (25). The forward primer for fusing eight histidines to the N-terminus was 5'-GCC GCC AGA TCT TAT GCA CCA TCA CCA TCA TCA CCA CCA TCG CGC ACA CTA TTC GTA CC-3'. The reverse primer was 5'-GCC GCC AAG CTT ATC ACT GCA GTT TGA CCC CGC C-3'. The cloning procedure and the protein production and purification procedure were carried out as previously described (6).

Kinase activity assay. The activity of PduX was measured by using an ADP Quest assay kit according to the manufacturer's instructions (DiscoverRx, Fremont, CA) as previously described (6). To minimize background fluorescence, 96-well black microplates were used (Greiner Bio-one, Frickenhausen, Germany). For kinetic studies, the concentrations of certain assay components were varied as indicated in the text. All data were analyzed by nonlinear curve fitting using the program GraFit (Erithacus Software Limited).

Site-Directed Mutagenesis of His₈-PduX. Mutations were introduced into the His₈-PduX-pTA925 (6) construct using the QuikChange II-E Site-Directed Mutagenesis Kit (Stratagene). Briefly, PCR was used to amplify the entire plasmid from two complementary primers which both contained the desired mutation. The template plasmid was then digested with *Dpn* I and transformed into *E. coli* XL-1 Blue. Pure cultures were prepared from selected transformants and the plasmids were purified. Clones with the expected DNA sequence were used for further study. All mutant PduX proteins were produced and purified by the same method as the wild-type.

Circular Dichroism (CD) spectra. CD spectra of the mutants used in this study were collected using a Jasco J-710 spectropolarimeter (cell length = 1 cm). The samples contained enzyme (4.0 μ M) and K₂HPO₄ (10 mM), pH 8.4. CD data were collected from 190 to 250 nm

with scanning speed 50 nm/min, resolution 0.2 nm, band width 1.0 nm, sensitivity 20 mdeg and response 8 seconds at room temperature. CD data were analyzed by program JFIT.

DNA sequencing. DNA sequencing was carried out by Iowa State University, DNA facility of Iowa State University Office of Biotechnology using automated sequencing equipment from Applied Biosystems Inc.

Results

The PduX L-threonine kinase reaction proceeds via an ordered, ternary complex mechanism. For a bi-substrate reaction, there are two classes of kinetic mechanisms: one involves a ternary complex of both substrates and the enzyme, and the other is the ‘Ping Pong’ mechanism (26). In the ‘Ping Pong’ model, one substrate binds to the enzyme, undergoes some forms of chemical change such as covalent modification of the enzyme, then the first product is released and the second substrate binds, often receiving the chemical group from the enzyme. A diagnostic feature of this mechanism is that when the concentration of one substrate is fixed and the other varied, a double reciprocal plot of rate versus concentration of the variable substrate will be parallel with similar plots at different fixed substrate concentrations (26,27). Kinetic analyses of His₈-PduX showed that such lines converged and were not parallel excluding the ‘ping-pong’ mechanism (Figure 4.1). Hence, the PduX reaction most likely proceeds via a ternary complex mechanism in which the enzyme binds to both substrates before a chemical reaction occurs. There are three possible ternary complex mechanisms, one random and two ordered. In a random mechanism either of the substrates may bind first, and the binding of one does not influence the binding of the other. In an ordered mechanism, one substrate must bind first and this binding is a pre-requisite for interaction with the second substrate (27).

Studies of product inhibition can provide information about the order of substrate binding (26,28). Therefore, we examined the effect of L-threonine-*O*-phosphate on the PduX reaction over a range of concentrations (Figure 4.2). L-threonine phosphate is noncompetitive *versus* both ATP and L-threonine. If the reaction proceeded by a random mechanism, L-threonine-*O*-phosphate would be a competitive inhibitor of both substrates. Hence, a random ternary complex mechanism is rejected and an ordered mechanism inferred. Competitive inhibition is also observed in an ordered mechanism between the final product released and the first substrate to bind since both interact with the same form of the enzyme (27). Since L-threonine-*O*-phosphate was a noncompetitive inhibitor with respect to both L-threonine and ATP as shown, we conclude that L-threonine-*O*-phosphate is the first product released. This means that ADP must be the second product to be released and symmetry suggests that ATP is the first substrate bound.

The effects of the other product of the PduX reaction (ADP) as an inhibitor could not be assessed using the coupled enzyme assay system since ADP is a substrate for the coupling enzyme. However, competitive dead-end inhibitors, which block binding of one substrate to the active site, are also useful for determining the binding order. If such an inhibitor is competitive with substrate A and uncompetitive with substrate B, then A is second to bind (26). Here, we used AMP-PNP as a competitive dead-end inhibitor *versus* ATP. Inhibition by AMP-PNP was determined over a range of AMP-PNP concentrations while varying the ATP concentration with L-threonine at saturating levels and vice versa (Figure 4.3). AMP-PNP is competitive *versus* ATP and noncompetitive *versus* L-threonine. We also used L-valine as a dead-end analog *versus* L-threonine (Figure 4.4). L-valine is an uncompetitive inhibitor *versus* ATP and a competitive inhibitor *versus* L-threonine. Thus, studies using AMP-PNP and

L-valine as dead-end inhibitors support the product inhibition studies using L-threonine-*O*-phosphate which also indicated that ATP is the first substrate to bind to PduX. Controls established that AMP-PNP inhibition of pyruvate kinase, which was the coupling enzyme used in these studies, was not significant and did not influence the rate measurements.

Effect of pH on PduX activity. To investigate a possible role for ionizable residues in the pduX reaction mechanism, we examined kinetic parameters as a function of pH (Figure 4.5). The reaction buffer system was composed of MES, HEPES, CHES or a mixture of buffers (total of 100 mM) with 100 mM KCl and 10 mM MgCl₂ with pH range from 5.5 to 9.0. This is the pH range in which the PduX is stable and soluble. The buffer components were examined separately and had no effect on the enzyme activity. The pH values of the buffers were determined before the addition of 20% (v/v) dimethylsulphoxide (DMSO), a water miscible aprotic solvent. The apparent k_{cat} profile has a maximum from pH 7 to 8.5, but no dramatic changes were observed on either side (The lowest value was only 14% less than the maximum value.). The apparent specificity constants also varied little with pH. The $\text{p}K_a$ of ionizable groups depends upon the dielectric constant of the solvent. Altering the polarity of the solvent can therefore alter the $\text{p}K_a$ values of groups in enzyme active sites. The pH profile for PduX was repeated in the presence of 20% DMSO. The apparent k_{cat} values showed the same trend but were about 10% lower compared to values obtained in the absence of DMSO. The apparent specificity constants showed little variation over the pH range examined (Figure 4.5). Hence, ionizable groups do not appear to participate directly in catalysis.

Isotope effects on PduX activity. Solvated deuterons have substantially different physical properties than protons. Substituting deuterium oxide for water in an enzyme reaction can thus provide information whether proton transfer is involved in the reaction. The effects of adding increasing amounts of deuterium oxide on the kinetic parameters of PduX were determined at pH 8.0 (Figure 4.6). There was a very small of linear decrease in apparent turnover numbers as the mole fraction of deuterium oxide increased. There was no obvious effect on the specificity constants for either substrate. Thus, proton transfer does not appear to be critical to the PduX catalytic mechanism.

Selection of Mutagenesis Targets. The choice of the appropriate amino acid targets for site-directed mutagenesis was based on sequence comparisons among related enzymes and previous mutation analysis on other members of GHMP family (29-32). Highly conserved residues in all members of GHMP family are likely to participate in the common steps of catalysis, while residues that are conserved within one family but are different in others are candidates for specific interactions with the substrates. The amino acid sequences of PduX homologues (that are annotated as functioning in B₁₂-dependent 1,2-propanediol degradation) from various species were aligned by using the ClustalX program. Four highly conserved motifs were identified (Figure 4.7). Motif I of PduX occurs in approximately the same place as the non-ATP substrate binding site in the other family members but the sequence is quite different. Therefore, motif I of PduX may form part of the L-threonine-binding site. Motif II is well-conserved throughout the GHMP family (equivalent to Motif II in GHMP family) and interacts with the phosphates of ATP. Motif III is also well-conserved across the GHMP family (equivalent to Motif III in GHMP family) and is close to the substrate binding sites.

Motif IV is unique to PduX. Since it is near the ATP-binding motif, it is possible that this motif forms part of the active site or ATP-binding site.

The catalytic function of carboxylate side chains in phosphotransferase reactions has been well documented for several GHMP family enzymes (29,32). In view of these results, we investigated two conserved glutamates (Glu-24 and Glu-132) and two invariant aspartates (Asp-103 and Asp-135) that are located in highly conserved motifs (Figure 4.7). Each residue was replaced with alanine and its isosteric substitution (glutamine replaces glutamate and asparagine replaces aspartate).

There is also precedent for participation of hydroxyl groups from serine or threonine in substrate binding and/or catalysis in GHMP family members (14,18,30,31). A proposed mechanistic role for such residues is H-bonding to oxygen atoms of the ATP phosphoryl chain (9,18,21). Elimination of side chains that function in phosphoryl chain orientation by hydrogen-bonding interactions can correlate with large diminutions in catalytic rate (30,31). From sequences alignment we found four invariant serine residues (Ser-99, Ser-100, Ser-253, and Ser-255) and two conserved threonines (Thr-101 and Thr-134) in highly conserved motifs (Figure 4.7). We replaced these residues with alanines.

Structural integrity of PduX variants. We used CD spectra to detect protein secondary structure changes caused by amino acid replacements. CD data were analyzed and fitted by JFIT program. The wild-type CD spectrum shows the PduX has 67.01% helical, 19.30% sheet and 13.69% coil structure (Figure 4.8). This result is consistent with the previously solved crystal structures of GHMP family kinases which contain large proportion of helical structures (9,17,18). The CD spectra of the PduX variants were compared to that of the wild-type enzyme (Table 4.1). Mutations E24A and D135A resulted in a large decrease in

helical structure (E24A drops to 54.27% and D135A decreases to 48.05%) indicating that these two conserved sites might be involved in proper folding of PduX enzyme. Other PduX variants showed no obvious changes in the CD spectra indicating that the mutations did not lead to major changes in folding.

Kinetic Characterization of PduX mutants. Using purified enzymes, kinetic constants were determined for the wild-type and mutant PduX enzymes. K_m and k_{cat} values were determined by nonlinear regression with Grafit (Table 4.2). When the K_m values for ATP were determined, saturating levels of L-threonine (5 mM) were added to assay mixtures and the ATP concentration was varied from 0 to 500 μ M. Similarly, saturating levels of ATP (2 mM) and varying levels of L-threonine from 0 to 500 μ M were used to determine the K_m values for L-threonine.

Three mutants (E24A, S100A and D135A) had no activity even with saturating levels of both substrates (ATP 2 mM, L-threonine 5 mM), so their kinetic parameters could not be determined. Two of these (E24A and D135A) had abnormal CD spectra indicating improper folding. Six mutants (E24Q, T101A, D103A, D103N, T134A and D135N) had kinetic parameters similar to those of wild-type PduX. The remaining five mutants had altered kinetic properties compared to wild-type PduX. Mutations S253A and S255A increased the K_m for L-threonine more than 30-fold indicating that these residues may be involved in binding of L-threonine. Mutations S99A, E132Q, and E132A, had K_m values for ATP more than 20-fold higher than wild-type indicating a possible role in ATP binding.

Discussion

Previous studies of GHMP family members have revealed three kinetic mechanisms, depending on the enzyme. The first group uses an ordered mechanism in which ATP was the

first substrate to bind including galactokinase from rat liver (33), yeast (34), and human (35), and homoserine kinases from *E. coli* (36), and *Methanococcus jannaschii* (37). The second group appears to have an ordered mechanism in which the non-ATP substrate binds first including broad bean galactokinase (38) and hog liver mevalonate kinase (39). The last group was shown to proceed by a random ternary complex mechanism such as *E. coli* galactokinase (40) and *Streptococcus pneumoniae* phosphomevalonate kinase (41). It is interesting that GHMP family members use different kinetic mechanisms despite the existence of highly similar primary structures. This suggests that this superfamily interacts with their substrates using similar chemical functions, but that their dynamics are quite different. The studies of PduX L-threonine kinase presented here are consistent with an ordered ternary complex mechanism in which ATP binds first and L-threonine second. The most likely explanation for an ordered mechanism is that the binding of the first substrate induces a conformational change in the enzyme resulting in the formation of a high-affinity binding site for the second substrate.

We also determined the effects of pH and deuterium oxide on the kinetic parameters of PduX. pH-dependent changes in the apparent $k_{\text{cat}}/K_{\text{m}}$ reveals information concerning changes in the state of ionization of essential functional groups on the enzyme or substrates that affect activity, while the apparent turnover numbers profile reflects changes in the state of ionization of groups on the central enzyme–substrate complexes. For PduX enzyme, a small, pH-dependent effect on the apparent turnover number was measured. However, this effect was much smaller than expected if a pH-sensitive group was involved directly in catalysis in which case at least one unit difference on log scale is expected (26,27). Similarly, only a small kinetic isotope effect on k_{cat} was observed with deuterium oxide. We conclude that this

was due to an indirect effect on catalysis rather than an effect on a critical proton transfer event that drives the reaction. The apparent specificity constants for ATP and L-threonine were essentially unaffected over the pH range used in our experiments. They were also unchanged in the presence of DMSO and no solvent kinetic isotope effect was observed for either k_{cat} or $k_{\text{cat}}/K_{\text{m}}$. This indicates that there are no critical ionizable residues involved in the interaction of the free enzyme with ATP or in the interaction between the enzyme–ATP complex with L-threonine.

Within the GHMP kinase family, different members use different catalytic mechanisms. Studies on mevalonate kinase and phosphomevalonate kinase have suggested a mechanism involving a conserved aspartic acid residue as a possible catalytic base whose role is to activate the acceptor hydroxyl group of the substrate. A conserved lysine residue near the acceptor hydroxyl group is proposed to lower its $\text{p}K_{\text{a}}$ facilitating proton abstraction (15,17,18,24). In contrast, in homoserine kinase, no strong nucleophile sits near the acceptor hydroxyl group. Phosphoryl transfer in homoserine kinase has been suggested to occur by direct nucleophilic attack on the γ -phosphate group of ATP by the δ -hydroxyl of homoserine (14). In this mechanism, homoserine is stabilized by the formation of a hydrogen bond to asp-141, which is not conserved in the superfamily. The side chain hydroxyl group of thr-183 interacts with the β -phosphate of the nucleotide and thus may help to stabilize the transition state (14). In another GHMP member, galactokinase, there is a conserved asp-83 that may serve to abstract the proton from the hydroxyl group of galactose for its subsequent attack on the γ -phosphorus of ATP. Additionally, there is an arg-36 residue that corresponds to the lysine in mevalonate kinase which is hypothesized to lower the $\text{p}K_{\text{a}}$ of the substrate hydroxyl group (8). However, kinetics studies on the galactokinase showed little variation in the

turnover number and specificity constants with respect to pH and negligible effects on addition of increasing mole fractions of deuterium oxide in place of water (34,35). Hence, there is a controversy as to whether the conserved aspartate in galactokinase functions as a catalytic base or rather is simply involved in substrate binding (10). Our studies on the effects of pH and deuterium oxide on the kinetics of PduX indicated that this enzyme is similar to homoserine kinase which lacks a catalytic base in the active site, and that it probably derives its catalytic power from its ability to stabilize the transition state.

Most of our understanding of the catalytic mechanisms of GHMP family members comes from the analysis of crystal structures with substrates or substrate analogs bound. Only limited mutagenesis data are available on the roles of key functional groups (29,42-44). Here, we changed ten highly conserved amino acids of PduX, which have carboxylate- or hydroxyl-containing side chains, and tested the effect of these mutations on structure and kinetic parameters. Our alignment results showed there are four highly conserved motifs in PduX homologues. In motif I, Glu-24 is the only invariant residue and it is also well conserved in the GHMP family. The replacement of Glu-24 with alanine resulted in large secondary structure changes (about 30 amino acids changed from a helical structure to random coil) and complete loss of activity. Since alanine has a small side chain without any charge or polarity, we made the isosteric substitution of glutamine for Glu-24. This mutant had almost the same secondary structure and similar kinetic behavior to wild-type, with only 2-fold decrease in specific activity and modest increase in apparent K_m values for both substrates (2-fold increase for ATP and 3.5-fold increase for L-threonine). These results indicated Glu-24 is probably involved in folding rather than having a direct role in catalysis.

Motif II is the most highly conserved motif in the GHMP family. It forms a distinct ATP binding loop not found in other kinase families (23). In this motif, we mutated four sites: Thr-101 and Asp-103 which are only highly conserved in PduX homologues and Ser-99 and Ser-100 which are conserved across the entire GHMP family. None of these mutations caused an obvious change in secondary structure based on CD analyses. T101A showed only a 15% decrease in apparent turnover number and approximately a 2-fold increase in K_m for both ATP and L-threonine. Replacement of Asp-103 with alanine or asparagine did not cause a significant change in kinetic parameters. It seems that Thr-101 and Asp-103 have no key function in substrate binding or catalysis even though they are conserved among PduX homologues. In contrast, mutant S99A had a 25-fold higher K_m for ATP, a 1.4-fold higher K_m for L-threonine and 1.2-fold decrease in k_{cat} indicating that Ser-99 is involved in ATP binding. Mutant S100A had no activity at all even with 2 mM ATP (40-fold $K_{m,ATP}$ for wild-type). Hence, we propose a critical role of the hydroxyl group of Ser-100 in catalysis of the PduX reaction. Ser-100 of PduX corresponds to Ser-145 in human and Ser-121 in yeast mevalonate diphosphate decarboxylase. Previous mutagenesis analysis of human mevalonate kinase proposed Ser-145 functions also as a hydrogen bond donor to a phosphoryl oxygen of bound substrate or product nucleotide (30). In yeast mevalonate diphosphate decarboxylase they proposed that Ser-121 functions in orienting the phosphoryl chain to optimize the angle of attack on the γ -phosphate of ATP (31). We propose that Ser-100 could interact with γ -phosphate of ATP to stabilize the transition state of the reaction corresponding to Thr-183 in homoserine kinase which has a similar catalytic mechanism to PduX (14). However, an understanding of exact function of Ser-100 in the PduX L-threonine kinase reaction will require further.

Mutagenesis was also used to examine the function of conserved motif III in PduX. S253A and S255A both showed large increases in apparent K_m values for L-threonine (37-fold and 29-fold individually). Since both substitutions had no major impact (less than 2-fold) on turnover rates or the specificity constants for ATP, the large increase in $K_{m,L-Thr}$ reflects a significant diminution in L-threonine binding efficiency.

Motif IV is conserved only in the PduX homologues but the specific sites within this motif that were changed for this study are also well-conserved across the GHMP family. Several crystal structures of GHMP members show a glutamate residue, corresponding to Glu-132 in PduX, coordinates with Mg^{2+} ion thus activates the γ -phosphate of ATP (14,15,18). When we changed glutamate 132 of PduX to alanine or glutamine, the K_m value for ATP increased 37-fold and 23-fold respectively, with only minor (less than 1.5-fold) change in the k_{cat} or the k_{cat}/K_m for L-threonine. The T134A mutation had little effect on the kinetics of PduX. Motif IV also contains conserved residue Asp-135. As mentioned above, there are two branches of GHMP kinase family, distinguished by the presence or absence of an aspartate residue that plays a role as a catalytic base. In homoserine kinase subfamily, this aspartate residues is absent (14). However, in mevalonate kinase and phosphomevalonate kinase subfamilies, both crystal structures and mutagenesis indicated that this aspartate functions as a catalytic base (15,17,18,24,29,32). In the case of PduX, replacement of Asp-135 with alanine caused loss of both structural integrity and enzyme activity, while substitution with asparagine had little effect (only 2-fold decrease in apparent specificity activity). Hence, in PduX, Asp-135 appears to have primarily a structural role.

In summary, the four conserved motifs in PduX subfamily play different roles in the phosphoryl-transfer process. Motifs II and IV are mainly for ATP binding while motif III is

for L-threonine binding. Glu-24 and Asp-135 are needed for proper folding. Collectively, the studies presented here help to define catalytic mechanism of L-threonine kinase, a key enzyme in the *de novo* synthesis of coenzyme B₁₂ and further expand our understanding of the catalytic properties of the GHMP superfamily.

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Table 4.1 Circular Dichroism (CD) Fitted Data for PduX Mutants

	Helix %	Sheet %	Coil %	R factor %
Wild-type	67 ± 5	19 ± 3	14 ± 3	9 ± 2
E24Q	64 ± 5	20 ± 3	15 ± 3	6 ± 2
E24A	54 ± 4	19 ± 3	27 ± 4	8 ± 2
S99A	66 ± 5	20 ± 3	14 ± 3	7 ± 2
S100A	65 ± 5	26 ± 3	9 ± 2	7 ± 2
T101A	68 ± 5	22 ± 3	9 ± 2	7 ± 2
D103N	63 ± 5	21 ± 3	16 ± 3	7 ± 2
D103A	65 ± 5	21 ± 3	14 ± 3	8 ± 2
E132Q	68 ± 5	22 ± 3	10 ± 2	9 ± 2
E132A	68 ± 5	16 ± 3	16 ± 3	9 ± 2
T134A	67 ± 5	24 ± 3	9 ± 2	7 ± 2
D135N	64 ± 5	20 ± 3	17 ± 3	9 ± 2
D135A	48 ± 4	22 ± 3	30 ± 3	8 ± 2
S253A	66 ± 5	24 ± 3	10 ± 2	8 ± 2
S255A	66 ± 5	18 ± 3	16 ± 3	8 ± 2

The values shown are based on three replicates.

Table 4.2 Kinetics Constants for PduX Mutants ^a

	$K_{m,ATP}$ (μ M)	$K_{m,L-Thr}$ (μ M)	$k_{cat,ATP}$ (min^{-1})	$k_{cat,L-Thr}$ (min^{-1})	$k_{cat}/K_{m,ATP}$ $10^{-4}(\mu\text{M}^{-1}\text{min}^{-1})$	$k_{cat}/K_{m,L-Thr}$ $10^{-4}(\mu\text{M}^{-1}\text{min}^{-1})$
Wild-type	55 ± 3.8	127 ± 3.9	2.56 ± 0.16	2.62 ± 0.17	466	206
E24Q	117 ± 8.0	463 ± 44	1.12 ± 0.07	1.13 ± 0.05	96	24
S99A	1374 ± 96	176 ± 17	2.03 ± 0.22	1.95 ± 0.18	15	111
T101A	98 ± 6.2	220 ± 14	2.13 ± 0.13	2.22 ± 0.17	218	101
D103N	141 ± 13	184 ± 12	2.03 ± 0.21	2.15 ± 0.13	144	117
D103A	163 ± 11	258 ± 18	1.88 ± 0.09	1.93 ± 0.12	116	75
E132Q	1264 ± 152	144 ± 25	1.63 ± 0.08	1.55 ± 0.07	13	108
E132A	1979 ± 213	198 ± 23	1.23 ± 0.10	1.16 ± 0.11	6	59
T134A	87 ± 8.4	142 ± 16	2.05 ± 0.15	2.12 ± 0.15	236	149
D135N	68 ± 5.6	273 ± 9.1	1.04 ± 0.11	0.96 ± 0.09	154	35
S253A	76 ± 5.6	4698 ± 232	1.56 ± 0.14	1.72 ± 0.09	205	4
S255A	74 ± 7.2	3708 ± 404	1.36 ± 0.17	1.43 ± 0.16	185	4

^a: $k_{cat,ATP}$ means apparent turnover numbers derived from variable ATP experiments. $k_{cat,L-Thr}$ means apparent turnover numbers derived from variable L-threonine experiments. Mean values of k_{cat} and K_m were used to calculate k_{cat}/K_m . Mutants without measurable ability (E24A, S100A and D135A) are not shown in this table. The values shown are based on three replicates.

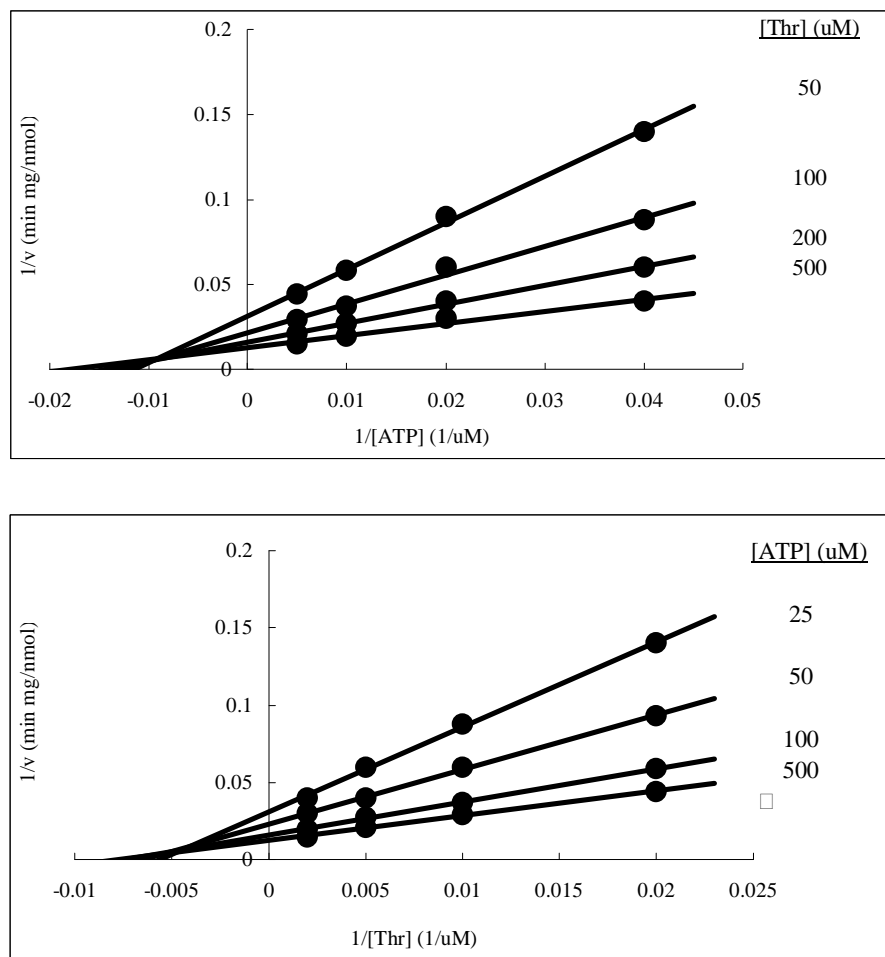
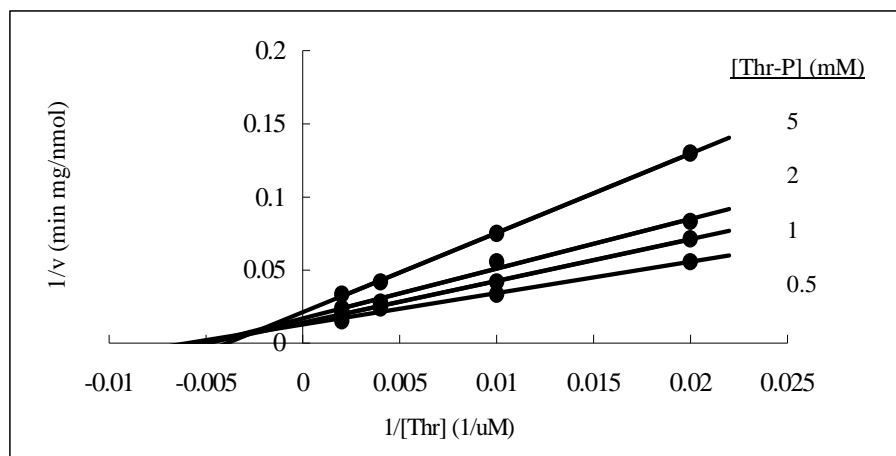
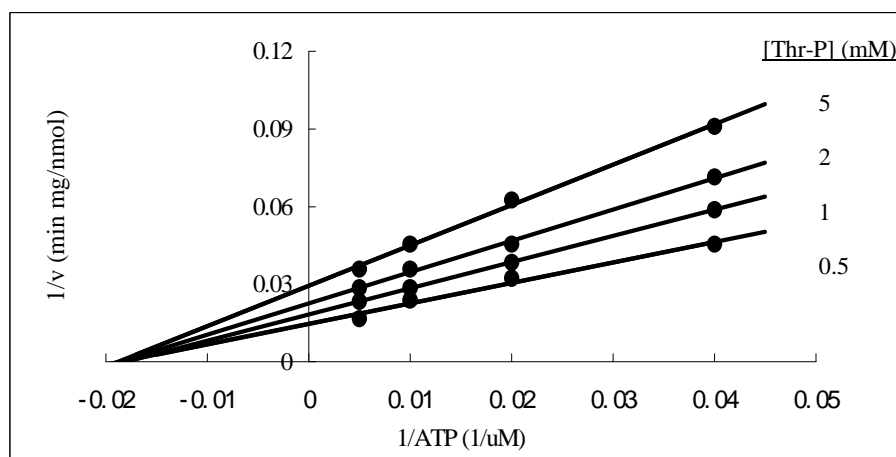


Figure 4.1 Initial-rate study of the PduX reaction. Double reciprocal plots of rate versus substrate concentration with one substrate varied and the other held at a saturating level (ATP at 2 mM and L-threonine at 5 mM). The reaction conditions were as follows: PduX enzyme (10 nM), ATP and L-threonine concentration as indicated. Standard assay mixtures contained 15 mM HEPES (pH 7.4), 20 mM NaCl, 10 mM $MgCl_2$ in a total volume of 0.1 mL. The values shown are based on three replicates.

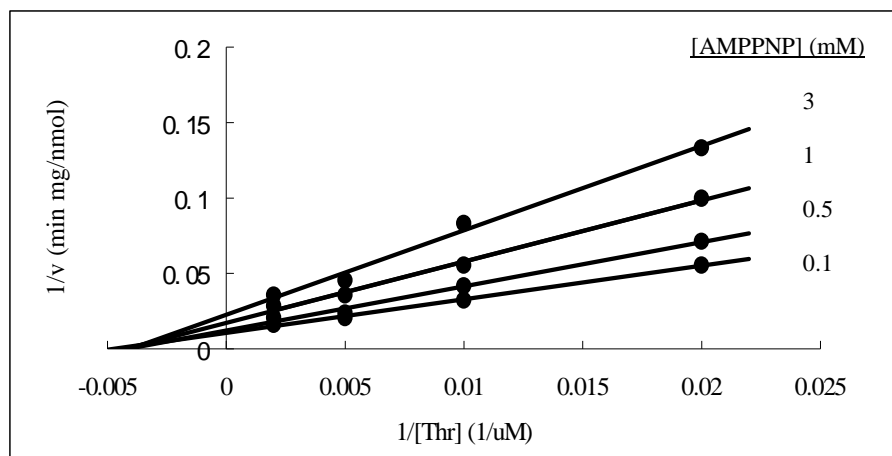


A

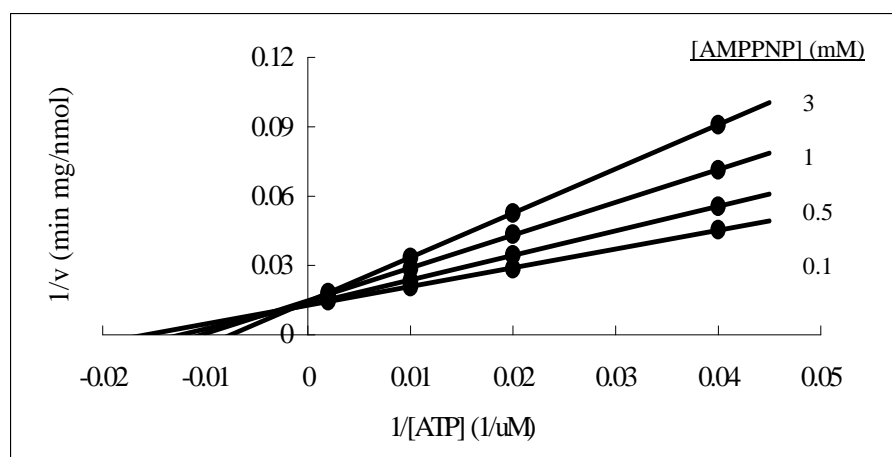


B

Figure 4.2 Kinetic analysis of product inhibition by L-Thr-P. A) L-Thr-P is a noncompetitive inhibitor of L-threonine in the PduX reaction. ATP concentration was held at 2 mM. B) L-Thr-P is a noncompetitive inhibitor of ATP in the PduX reaction. L-threonine concentration was held at 5 mM. The reaction conditions were the same as standard assay. The values shown are based on three replicates.

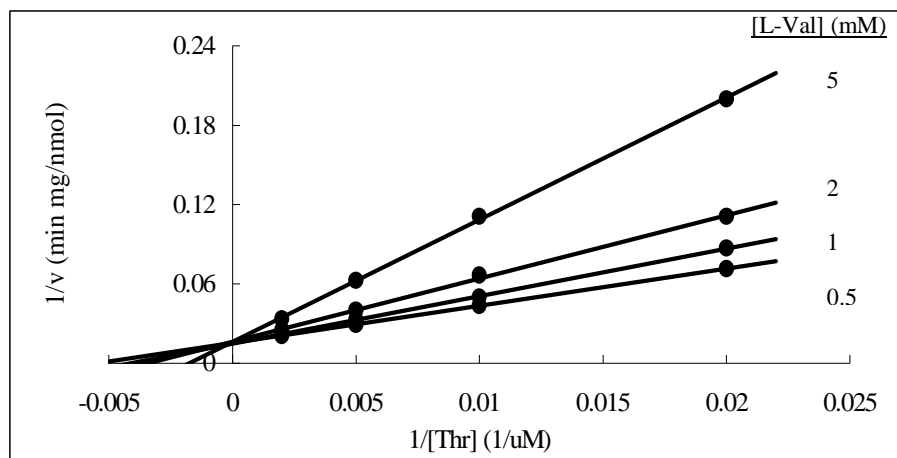


A

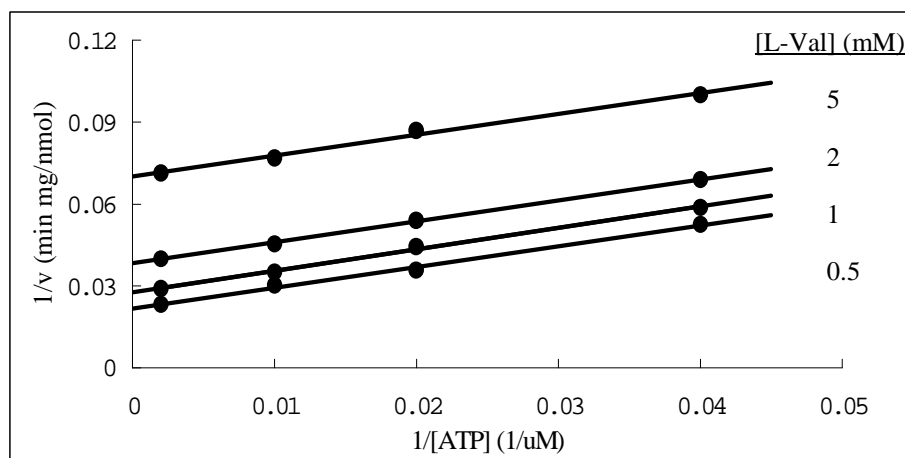


B

Figure 4.3 Kinetic analysis of dead-end inhibition by the ATP analog AMP-PNP. A) AMP-PNP is a noncompetitive inhibitor of L-threonine in the PduX reaction. ATP concentration was held at 2 mM. B) AMP-PNP is a competitive inhibitor of ATP in the PduX reaction. L-threonine concentration was held at 5 mM. The reaction conditions were the same as standard assay. The values shown are based on three replicates.



A



B

Figure 4.4 Kinetic analysis of dead-end inhibition by the L-threonine analog L-valine. A) L-Val is a competitive inhibitor of L-threonine in the PduX reaction. ATP concentration was held at 100 μ M. B) L-Val is an uncompetitive inhibitor of ATP in the PduX reaction. L-threonine concentration was held at 300 μ M. The reaction conditions were the same as standard assay. The values shown are based on three replicates.

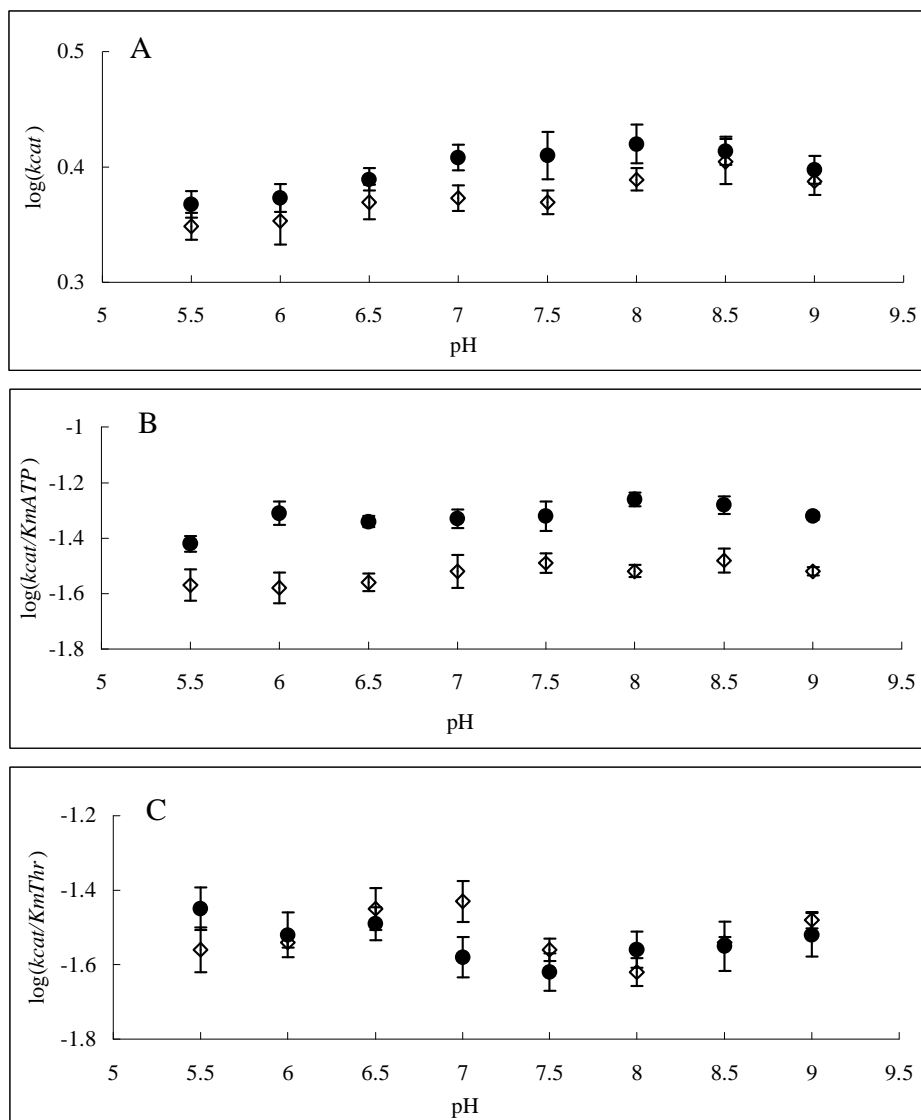


Figure 4.5 The effects of pH on the kinetic parameters of the PduX enzyme. Three kinetic constants, k_{cat} , $k_{cat}/K_{m,ATP}$, and $k_{cat}/K_{m,L-Thr}$, were determined over the pH range 5.5–9.0. Reactions were performed in the absence (solid circles) and presence (open diamond) of 20% (v/v) DMSO. (A) The pH effects on the apparent turnover number (k_{cat}). Values for k_{cat} were determined with variable L-threonine and variable ATP concentrations at each pH value with similar results. Average values are shown on the figure. (B) The pH effects on the apparent specificity constant of ATP ($k_{cat}/K_{m,ATP}$). (C) The pH effects on the apparent specificity constant of L-threonine ($k_{cat}/K_{m,L-Thr}$). The values shown are based on three replicates.

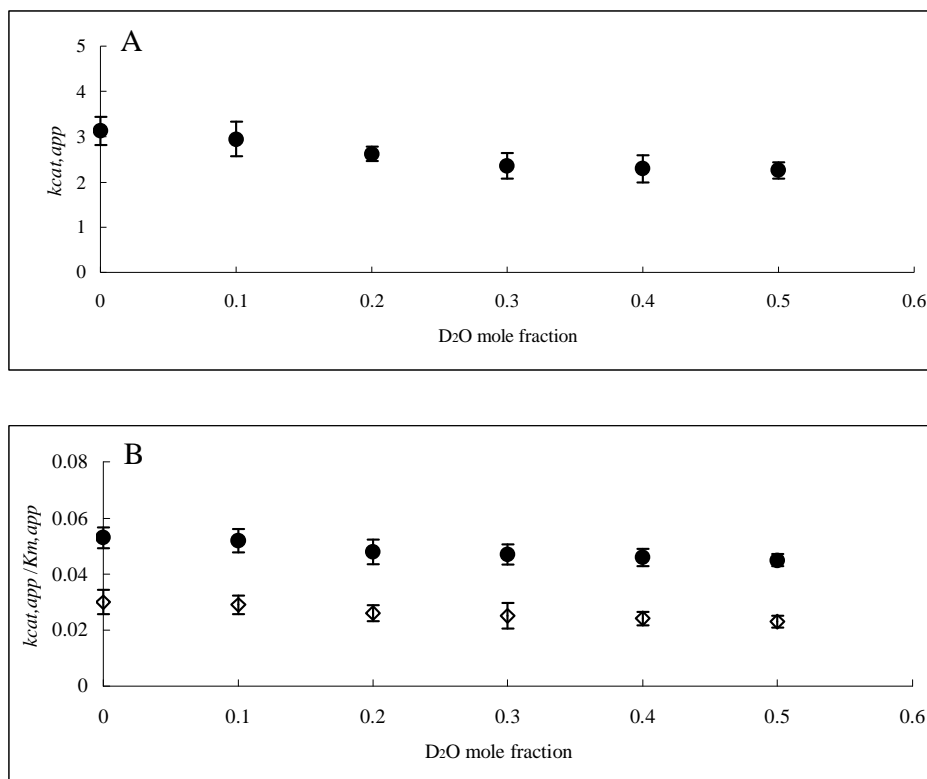


Figure 4.6 The effects of deuterium oxide on the kinetic parameters of the PduX enzyme. Three kinetic constants, k_{cat} , $k_{cat}/K_{m,ATP}$, and $k_{cat}/K_{m,L-Thr}$, were determined at pH 8.0. (A) The kinetic isotope effect on the apparent turnover number (k_{cat}). Values for k_{cat} were derived from both variable L-threonine and variable ATP experiments at each D₂O mole fraction and similar values were obtained. Average values are shown on the figure. (B) The isotope effects on the apparent specificity constant of ATP (solid circles), and the apparent specificity constant of L-threonine (open diamonds). The values shown are based on three replicates.

PduX Motif	I	II	IV	III	
GHMP Motif	I	II	none	III	
	24	99	132	253	
	*	*** *	* **	* *	GeneBank number
<i>S. enterica</i>	PASCGELIQG	PVAKGMASSTADI	IEPTDS	GLNVAHSGSVVG	AAD39022
<i>C. freundii</i>	PASCGELIQG	PIAKGMASSTADI	LEPTDS	GVNVAHSGSVVG	CAM57303
<i>K. pneumoniae</i>	PASCGELIQG	PVAKGMASSTADI	LEPTDS	GLNVAHSGSVVG	YP_001336851
<i>P. luminescens</i>	PASCGELIQG	PMAGKGLASSTADI	LEPTDS	GLNVAHSGSVVG	NP_930200
<i>Y. enterocolitica</i>	PASCGELLQG	PVAKGLASSTADI	LEPTDS	GLNVAHSGSVIG	YP_001008181
<i>L. welshimeri</i>	PASCGELLQG	PVAKGMASSTADI	LEPTDS	GINVSHSGTVVG	YP_849325
<i>A. metalliredigens</i>	PGSCGELIQG	PMGKGMASSTADL	IEPTDS	GVNVAHSGTVMG	YP_001317970
<i>H. modesticaldum</i>	PATCGELAQG	PSGKGLATSTADI	VEPSDG	GVNVAHSGTVLG	ABZ84953
<i>T. carboxydovorans</i>	PGTCGELVQG	PIGKGMASSTADI	IEPTDG	GVNVAHSGTVLG	ZP_01666223
<i>M. thermoacetica</i>	PGACGELVQG	PPGKGLASSTADV	VEPSDG	GVNTAHSGTVIG	YP_429960
<i>C. hydrogenoformans</i>	CGTAGELVQG	PEGKGMASSTADL	VEPTDG	GVNVAHSGSGIG	YP_359626
<i>N. thermophilus</i>	PLTCGEWVQG	PSAQGFGTSSADI	IEPSDT	GVIRAHSGTALG	ZP_02852581
<i>D. hafniense</i>	PGTCGEWVQG	LIGKGMASSTADM	IEPSDP	GVITAHSGTLLA	ZP_01372324
<i>B. pseudomallei</i>	YCSFGELLQG	PIGKGLSSSSADL	IEPTDG	GLVTAHSGTCIG	EDS87805
<i>A. friuliensis</i>	FGTFGELLQG	PEGKGLASSSADL	IEPTDG	GVVVGHS GTTLG	CAD32905
<i>S. sanguinis</i>	PGSCGELFQG	PISKGYSSSTADM	IEPTDS	GLNVAHSGTLVG	YP_001034508
<i>B. thuringiensis</i>	CGTCGELVQG	MEGKGMASSTADI	IEPSDG	GISVAHSGSILS	YP_001573827
<i>C. botulinum</i>	PGSFGELILQG	PRGKGLASSTADL	IEPTDS	GIIGCHSGDALG	YP_001253487

Figure 4.7 Motifs found in a multiple sequence alignment of PduX homologues. A multiple sequence alignment identified four conserved motifs in PduX. The corresponding motif in the GHMP family is indicated. Motif four is characteristic of PduX. Motif I is only weakly conserved. Deduced amino acid sequences of 18 PduX homologues were aligned using the program ClustalX. Residue numbering corresponds to the PduX enzyme of *Salmonella*. Invariant residues were marked by stars. The abbreviations for organisms in sequence are *Salmonella enterica*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Photobacterium luminescens*, *Yersinia enterocolitica*, *Listeria welshimeri*, *Alkaliphilus metalliredigens*, *Heliobacterium modesticaldum*, *Thermosinus carboxydovorans*, *Moorella thermoacetica*, *Carboxydotherrmus hydrogenoformans*, *Natranaerobius thermophilus*, *Desulfotobacterium hafniense*, *Burkholderia pseudomallei*, *Actinoplanes friuliensis*, *Streptococcus sanguinis*, *Bacillus thuringiensis*, *Clostridium botulinum*.

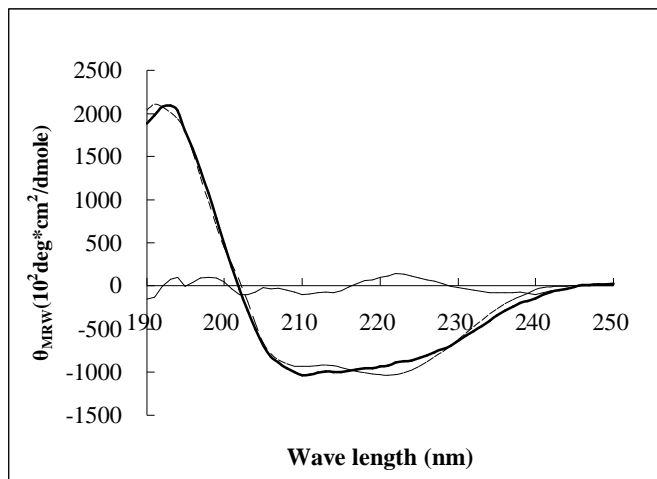


Figure 4.8 CD spectra of the PduX enzyme. CD spectra used in this study were collected using a Jasco J-710 spectropolarimeter (cell length = 1 cm). The sample contained PduX enzyme (4.0 μM), K_2HPO_4 (10 mM). CD data were collected from 190 to 250 nm with resolution 0.2 nm, band width 1.0 nm, sensitivity 20 mdeg and response 8 seconds at room temperature. CD data were analyzed by program JFIT. Solid line is experimental data, dot line is the fitted values and interval line indicates the error.

CHAPTER 5 SUMMARY AND GENERAL CONCLUSIONS

This dissertation focuses on the kinetic and mutational analysis of enzymes involved in biosynthesis of coenzyme B₁₂ especially for the upper and lower ligands.

Chapter 2 is about the upper ligand (adenosyl group) formation. ATP:cob(I)alamin adenosyltransferase catalyzes the transfer of adenosyl group from ATP. No general study on the effects of mutations on hATR activity and function has been reported before. Therefore, we conducted a random mutagenesis of the hATR using *Salmonella* as a surrogate host to screen for changes in enzyme activity *in vivo*.

- 1) Fifty-seven missense mutations at 30 positions were found to impair hATR activity *in vivo* in the surrogate host. These included mutations at 25 new sites that eliminate or reduce the hATR activity and hence could result in methylmalonyl aciduria if found in humans.
- 2) We also used indicator plates with different amounts of OH-Cbl to view the sensitivity of the mutants to B₁₂ concentration. Eleven mutants, G63E, D64G, R76G, K78R, G97R, G97E, S126L, H183Y, R215K, D218N, and R225K, had an increased level of color formation (ATR activity) at higher concentration of OH-Cbl indicating these residues are important to binding of MgATP/cobalamin by the hATR *in vivo*.
- 3) The stability of the mutant hATR proteins in *Salmonella* was examined by western blotting. Mutant proteins C119Y, R186W, C189Y, R190C, and R191W were produced at much lower levels than the wild-type, indicating that these mutations impair proper protein folding leading to degradation by cellular proteases.

- 4) Kinetic constants were determined for the wild-type and mutant hATRs. K_m and k_{cat} values were determined by nonlinear regression with Grafit. Group I consisted of 19 mutants (K78Q, E84K, G87R, D90N, E91K, L92S, S94L, R186W, C189Y, R190C, R191W, E193K, R194G, F212S, R215K, S217R, L220P, L223P, and R225K) that had no activity even with a 10-fold excess of ATP and cob(I)alamin compared to the standard assay. Group II consisted of three mutants (R76G, F83S, and S126L) that exhibited relatively large changes in the K_m values for both ATP and cob(I)alamin. Group III mutants (G63E, D64G, S68F, K78R, G97R, and D218N) showed modest changes or wild-type K_m values for ATP and cob(I)alamin, but a substantially reduced V_{max} . Group IV mutants (G97E, C119Y, T161I, and H183Y) had kinetic constants in the wild-type range.
- 5) We used UV-visible spectra to determine the effect of mutations on AdoCbl Binding. S68F, K78Q, K78R, R186W, and R190C mediated blue shifts similar to that of the wild-type hATR. However, the majority of the hATR missense mutants lacked a measurable blue shift.
- 6) We also used EPR spectra to determine the effect of mutation on cob(II)alamin binding. The results were consistent to those in UV-visible spectra study, only S68F, K78Q, K78R, R186W, and R190C exhibited EPR spectra indicating base-off binding of cob(II)alamin.

Chapter 3 and 4 are related to the lower ligand (DMB) formation. Previous work proposed that PduX homologues might be L-threonine kinases involved in B₁₂ synthesis. This was based sequence similarity to GHMP family of kinases and the observation that

pduX genes were proximal to B₁₂ biosynthetic genes in several instances. However, there was no direct evidence before. Here, we presented *in vitro* and *in vivo* evidence that the *pduX* gene of *Salmonella enterica* encodes an L-Thr kinase involved in the *de novo* synthesis AdoCbl and the assimilation of Cby.

- 1) PduX is needed for *Salmonella enterica* growth on 1,2-PD minimal medium supplemented with Cby, but not on similar medium supplemented with Cbi.
- 2) A Pdux deletion mutation is complemented by ectopic expression of Pdux.
- 3) Supplementation of growth media with L-threonine-phosphate substantially corrects the growth defect of a PduX mutant.
- 4) A bioassay and growth tests showed that a pduX deletion mutant was impaired for the *de novo* synthesis AdoCbl and for the assimilation of Cby.
- 5) ³¹P NMR spectroscopy indicated that purified PduX-His₆ catalyzes the conversion of ATP and L-Thr to ADP and L-Thr-P. And there are no other intermediate produced in the reaction.
- 6) Enzyme assays indicated the ATP was preferred substrate for PduX compared to GTP, CTP and UTP. Based on nonlinear regression, the K_m values for ATP and L-Thr were $54.7 \pm 5.7 \mu\text{M}$, and $146.1 \pm 8.4 \mu\text{M}$, respectively. The enzyme V_{\max} was $62.8 \pm 3.6 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$.
- 7) Previous studies of GHMP family members have revealed three kinetic mechanisms, depending on the enzyme. The studies of PduX L-threonine kinase presented here are consistent with an ordered ternary complex mechanism in which ATP binds first and L-threonine second.

- 8) We also determined the effects of pH and deuterium oxide on the kinetic parameters of PduX. A small pH-dependent effect on the apparent turnover number was measured. Similarly, only a small kinetic isotope effect on k_{cat} was observed with deuterium oxide. These indicate that there are no critical ionizable residues involved in the interaction of the free enzyme with ATP or in the interaction between the enzyme–ATP complex with L-threonine. It probably derives its catalytic power from its ability to stabilize the transition state.
- 9) We conducted site-directed mutagenesis at several conserved residues among PduX homologues and GHMP family members. We used CD spectra to detect protein secondary structure changes caused by amino acid replacements. Mutations E24A and D135A resulted in a large decrease in helical structure indicating that these two conserved sites might be involved in proper folding of PduX enzyme.
- 10) Kinetic constants were determined for the wild-type and mutant PduX enzymes. Three mutants (E24A, S100A and D135A) had no activity even with saturating levels of both substrates. Two of these (E24A and D135A) had abnormal CD spectra indicating improper folding. Six mutants (E24Q, T101A, D103A, D103N, T134A and D135N) had kinetic parameters similar to those of wild-type PduX. The remaining five mutants had altered kinetic properties compared to wild-type PduX. Mutations S253A and S255A increased the K_m for L-threonine more than 30-fold indicating that these residues may be involved in binding of L-threonine. Mutations S99A, E132Q, and E132A, had K_m values for ATP more than 20-fold higher than wild-type indicating a possible role in ATP binding.

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