Review

Structure and function of enzymes involved in the anaerobic degradation of L-threonine to propionate

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In *Escherichia coli* and *Salmonella typhimurium*, L-threonine is cleaved non-oxidatively to propionate via 2-ketobutyrate by biodegradative threonine deaminase, 2-ketobutyrate formate-lyase (or pyruvate formate-lyase), phosphotransacetylase and propionate kinase. In the anaerobic condition, L-threonine is converted to the energy-rich keto acid and this is subsequently catabolised to produce ATP via substrate-level phosphorylation, providing a source of energy to the cells. Most of the enzymes involved in the degradation of L-threonine to propionate are encoded by the anaerobically regulated *tdc* operon. In the recent past, extensive structural and biochemical studies have been carried out on these enzymes by various groups. Besides detailed structural and functional insights, these studies have also shown the similarities and differences between the other related enzymes present in the metabolic network. In this paper, we review the structural and biochemical studies carried out on these enzymes.

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1. Introduction

The sequencing of microbial genomes containing several thousand genes is the underlying driving force for studies aimed at understanding metabolic networks at a deeper level of complexity. Advances in molecular biology techniques and genomic data have jointly helped in identifying new metabolic pathways present in various microorganisms. Many of these metabolic pathways utilize a large number of enzymes with diverse catalytic mechanisms. With the availability of the sequence as well as the probable role of many such enzymes in various metabolic pathways, structure–function studies on a large number of enzymes have been carried out by various research groups in the recent past. In the past few years, our group has carried out structure–function studies on some of the enzymes involved in propionate metabolism from *Salmonella typhimurium*. Propionate, following acetate, is the second most abundant low molecular-mass carbon compound found in the soil. It is mainly formed during *β*-oxidation of odd-numbered carbon-chain fatty acids, fermentation of carbohydrates, oxidative degradation of the branched-chain amino acids valine and isoleucine, and from the carbon skeletons of threonine and methionine. Here we review structure–function studies on four different enzymes – biodegradative threonine deaminase (TdcB), 2-ketobutyrate formate-lyase (TdcE), phosphotransacetylase (Pta) and propionate kinase (TdcD) – involved in the anaerobic degradation of L-threonine to propionate.

During the formation of L-isoleucine from L-threonine, involvement of 2-ketobutyrate as a precursor for L-isoleucine synthesis has been well documented (Umbarger 1996) (figure 1a). While working with extracts derived

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Abbreviations used: Acetyl-P, acetyl phosphate; AMPPNP, adenylyl imidodiphosphate; Ap_4A, diadenosine 5′, 5‴-P^1, P^4-tetra-phosphate; ASKHA superfamily, acetate and sugar kinase, heat shock cognate 70 and actin superfamily; CoA, coenzyme A; CobB or CbiA, cobyrinic acid a,c-diamide synthase; CobQ or CbiP, cobyric acid synthase; DTBS, dethiobiotin synthetase; IlvA, biosynthetic threonine deaminase; MinD, ATPase; MtPta, *Methanosarcina thermophila* Pta; PFL, pyruvate formate-lyase; PLP, pyridoxal 5′-phosphate; Pta, phosphotransacetylase; TdcB, biodegradative threonine deaminase; TdcD, propionate kinase; TdcE, 2-ketobutyrate formate-lyase

from *Clostridium tetanomorphum*, Tokushige *et al* (1963) demonstrated *in vitro* that 2-ketobutyrate formed from L-threonine can be catabolised to propionate via a propionyl phosphate intermediate. However, the route of degradation of L-threonine to propionate remained enigmatic for a long time. In 1987, Van Dyk and LaRossa (1987) showed that in *S. typhimurium,* Pta and acetate kinase are involved in the degradation of 2-ketobutyrate via propionyl coenzyme A (CoA). Later on in 1998, Hesslinger *et al* showed that the *tdcE* and *tdcD* genes present in the *tdc* operon with TdcE and TdcD activity, respectively, along with Pta catalyses the conversion of 2-ketobutyrate to propionate in *Escherichia coli* and *S. typhimurium* (Hesslinger *et al* 1998) (figure 1b). Based on these findings, it was suggested that the extended *tdc* operon (*tdcABCDEFG*) (figure 2) encodes components of an anaerobically inducible, catabolite-repressible pathway, which generates one molecule of ATP from the degradation of L-threonine and L-serine (Sawers 1998) (figure 1b). Under aerobic conditions, threonine can also be converted to glycine by threonine dehydrogenase (Tdh) and 2-amino-3-ketobutyrate:CoA ligase (Kbl). However, in *E. coli* this pathway is not a major route of threonine degradation (Sawers 1998).

Except Pta, the other three enzymes involved in the anaerobic degradation of L-threonine to propionate are encoded by the multicistronic *tdcABCDEFG* operon (figure 2). Expression of the *tdc* operon is highly complex, being affected by at least five transcription factors. These include the cAMP-receptor protein (CRP), the DNA bending and binding protein integration host factor (IHF), histone-like protein (HU) and two transcription factors encoded by the *tdc* locus, TdcR and TdcA (Ganduri *et al* 1993; Schweizer and Datta 1989; Wu and Datta 1992; Wu *et al* 1992). Fumarate nitrate reduction regulator (FNR), a class II transcription factor which activates gene expression when *E. coli* grows anaerobically, has been shown to have an indirect effect on anaerobic induction of *tdc* expression, possibly by controlling levels of an important metabolite (Chattopadhyay *et al* 1997). The global transcription factor CRP provides the principal control of operon expression, with the LysR-like TdcA protein acting as an upstream regulator, possibly responding to L-threonine levels in the growth medium (Ganduri *et al* 1993). Induction of operon expression occurs anaerobically in the absence of catabolite-repressing sugars such as glucose and pyruvate (Sawers 2001; Wu *et al* 1992). Previous studies have revealed that this catabolite repression can be overcome by the activity of a small HU from *Clostridium pasteurianum* (Sawers *et al* 1998). This alleviation of catabolite repression may be related in some way to a protein-induced alteration in the topological status of the DNA (Sawers 2001).

Since induced expression of enzymes involved in the anaerobic breakdown of L-threonine takes place in the absence of glucose and oxygen in the medium, during which energy level inside the cell is low, formation of ATP and propionate from L-threonine provides a source of energy to the cells. In *E. coli* and *S. typhimurium*, propionate can be further catabolised to pyruvate and succinate by the 2-methylcitric acid cycle. In the recent past, the three-dimensional structures of enzymes (or their close homologues) involved in the anaerobic degradation of L-threonine to propionate have been determined by various groups. It is thus an appropriate time to present in this review recent advances in our understanding of the structure and function of these enzymes. The review focuses on the enzymatic properties available in the literature. These data are not available in the direction of ATP formation for all the four enzymes, which would have been biologically more appropriate.

2. Biodegradative threonine deaminase

Biodegradative TdcB catalyses the first reaction in the anaerobic breakdown of L-threonine to propionate (figure 1b). Two distinctly different pyridoxal 5′-phosphate (PLP)-containing threonine deaminases (EC 4.3.1.19), one biosynthetic and the other biodegradative, are present in *E. coli* and *S. typhimurium* (Luginbuhl *et al* 1974; Umbarger and Brown 1957). Both the enzymes catalyse the deamination of L-threonine to yield 2-ketobutyrate and ammonia. Biosynthetic threonine deaminase (IlvA) activity is allosterically inhibited by the end-product of the pathway, L-isoleucine, and activated by the product of a parallel pathway, L-valine (Eisenstein 1991). IlvA has been extensively studied as the model system for investigations on feedback inhibition and allosteric regulation (Monod *et al* 1965; Umbarger 1996). Biodegradative (catabolic) threonine deaminase, encoded by the gene *tdcB* in *E. coli* and *S. typhimurium,* has been shown to be synthesized when the organism is grown anaerobically in a medium containing high concentrations of amino acids and no glucose (Wood and Gunsalus 1949). TdcB shares 34% sequence identity with the N-terminal domain of IlvA of *E. coli* and does not contain the sequence corresponding to the C-terminal regulatory domain (Higgins *et al* 1994). TdcB has been shown to be inhibited by the reaction product 2-ketobutyrate, other α-keto acids and by certain intermediary metabolites of the tricarboxylic acid cycle (Bhadra and Datta 1978; Feldman and Datta 1975; Park and Datta 1979). There is no significant difference in the sensitivity of the biosynthetic and biodegradative (in the presence of AMP) enzyme with respect to inhibition by 2-ketobutyrate (Shizuta *et al* 1973). Unlike IlvA, TdcB is insensitive to L-isoleucine and L-valine, and is activated by adenosine monophosphate (AMP). In the presence of AMP, the enzymatic activity is enhanced due to a large decrease in K_M for L-threonine and an apparent increase in V_{max} (Bhadra

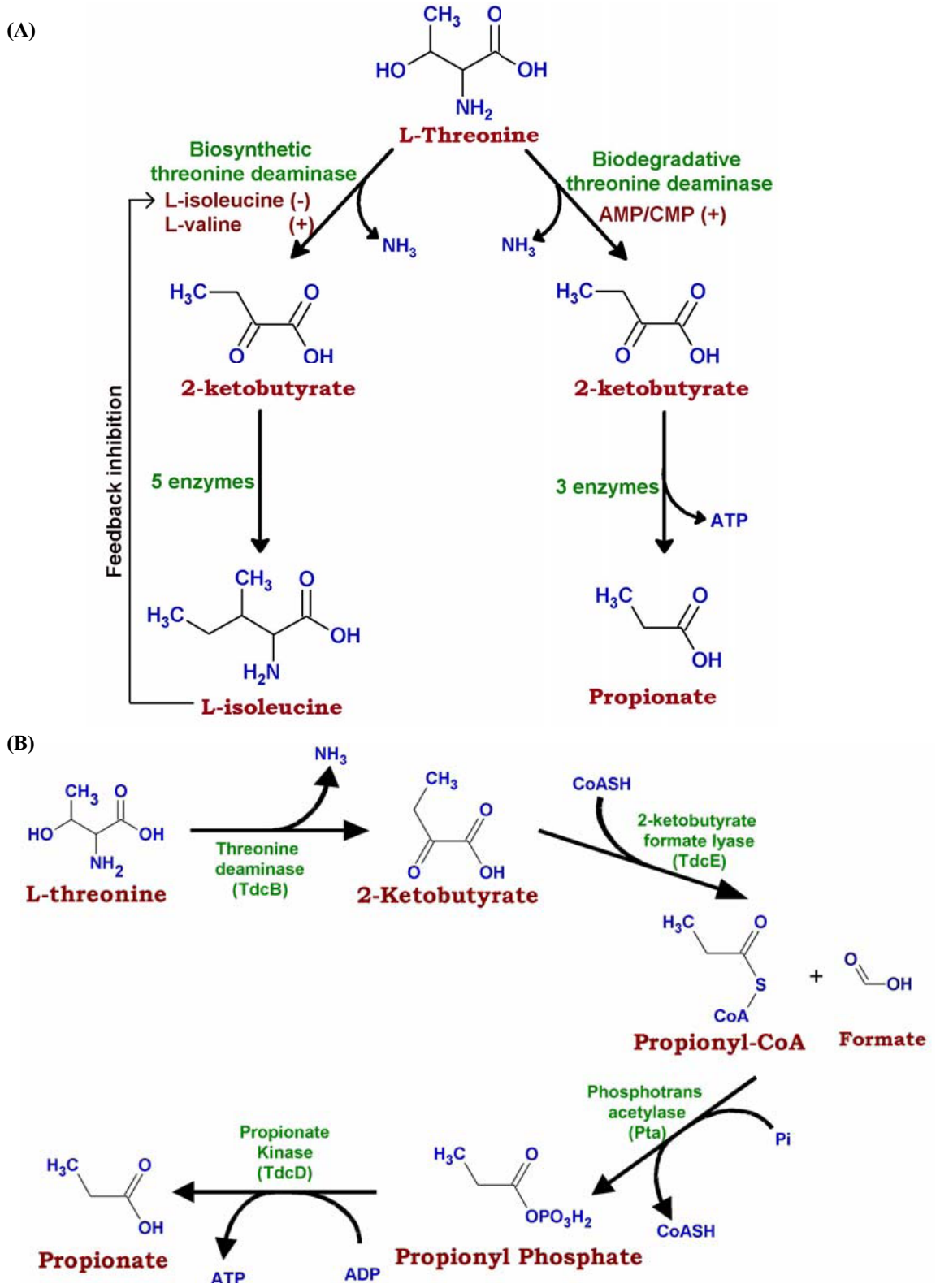

Figure 1. L-threonine metabolism. **(A)** During growth under aerobic conditions, L-threonine is used in the synthesis of L-isoleucine whereas under anaerobic and low energy level conditions, it is degraded to propionate with the generation of one molecule of ATP. **(B)** Metabolic pathway showing the anaerobic degradation of L-threonine to propionate via 2-ketobutyrate.

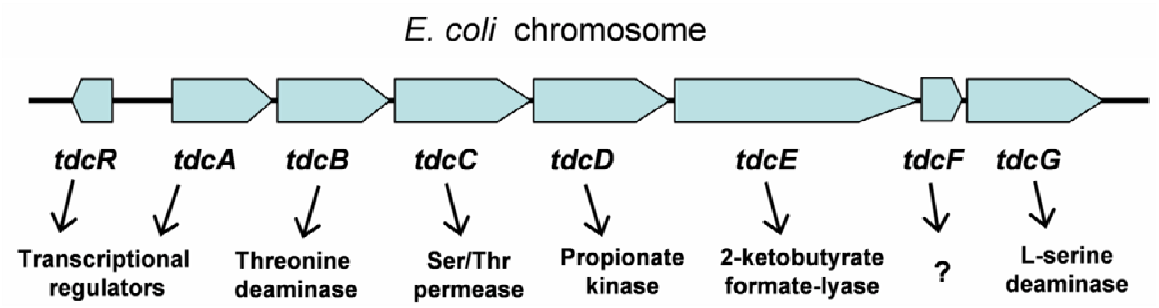

Figure 2. Genetic organization of the *tdc* operon in *E. coli*. The function of the gene product is written below the respective gene. The physiological function of TdcF is unknown.

and Datta 1978; Dunne and Wood 1975; Shizuta and Hayaishi 1976). Among other mononucleotide phosphates, cytidine monophosphate (CMP) showed significant enzyme activation compared with guanosine monophosphate (GMP), uridine monophosphate (UMP) and inosine monophosphate (IMP). Further, no enzymatic activation was observed in the presence of adenosine triphosphate (ATP) whereas adenosine diphosphate (ADP) showed a slight activation (Nakazawa *et al* 1967; Rabinowitz *et al* 1968). Unlike IlvA, which shows sigmoidal kinetics, TdcB exhibits Michaelis–Menten kinetics. The K_M for both biosynthetic (IlvA) and biodegradative (TdcB) threonine deaminase is in the mM range. It is 8 mM in the absence of L-isoleucine for IlvA and 16 mM for TdcB in the presence of AMP (Eisenstein 1991; Simanshu *et al* 2006). Using purified *S. typhimurium* TdcB, the K_M value for L-threonine was estimated as 123 mM (Simanshu *et al* 2006). In the presence of AMP and CMP, the K_M value of the enzyme for L-threonine was reduced by approximately 7.7- and 3.5-fold, respectively, whereas the V_{max} was increased by 9- and 3-fold, respectively. Studies by various groups have shown that in the absence of AMP, TdcB exists in monomer–dimer equilibrium at low concentration (Bhadra and Datta 1978; Gerlt *et al* 1973; Shizuta *et al* 1973; Simanshu *et al* 2006; Whanger *et al* 1968). This equilibrium shifts toward the tetrameric form as the concentration of TdcB is increased. However, even at low concentrations of TdcB, the presence of AMP/CMP induces oligomerization from monomer to tetramer.

Recently, we have reported the first crystal structures of TdcB and its complex with the activator molecule CMP (Simanshu *et al* 2006) (figure 3). In the native structure, TdcB is in a dimeric form whereas in complex with CMP, it forms a tetramer, which appears as a dimer of dimers. TdcB exhibits fold type II, characteristic of the β-family of pyridoxal 5′-phosphate (PLP)-dependent enzymes. The structure consists of a small and a large domain, both assuming an α/β structure with an open twisted β-sheet. Between the two domains, there is a large internal gap that provides space for the active site. The PLP cofactor is covalently bound as a Schiff base to the ε-amino group of Lys58. The pyridine ring of PLP forms a hydrogen bond to Ser311 and Asn85, whereas the phosphate moiety of PLP forms hydrogen bonds with Gly184, Gly185, Gly186, Gly187, Leu188 and Ile189. The substrate threonine is expected to bind near PLP in a cavity formed mainly by His86, Pro152, Tyr153, Val158 and Gln162.

The structure of the TdcB–CMP complex revealed the exact site of CMP binding and its role in the formation of higher oligomers as well as in enzyme activation (Simanshu *et al* 2006). Tetrameric TdcB binds to four molecules of CMP, two molecules at each of the dimer interfaces (figure 3b). CMP interacts with residues from two different subunits (Arg53, Thr54, Gln88, Asp119, Tyr120 and Asn314 from one subunit, and Asn34, Gln275 and Lys278 from the other subunit) and leads to the formation of a tight dimer, which in turn interacts with another dimer forming a tetramer. In the absence of CMP, TdcB forms a relatively loose dimer, which seems to explain the monomer–oligomer equilibrium observed in solution. Structural superposition of dimers of dimeric and tetrameric TdcB shows differences in the arrangement of the two subunits. Comparison of the dimer structure in the ligand-free and CMP-bound forms suggests that the changes induced by ligand binding at the dimer interface are essential for tetramerization. Most of the tertiary structural changes induced by CMP binding are associated with either the residues of the small domain lining the entry to the active site pocket or the residues at the dimer interface (Simanshu *et al* 2006). The differences observed at the dimer interface, and in the tertiary and quaternary structures of TdcB in the absence and presence of CMP, appear to account for the enzyme activation and increased binding affinity for L-threonine.

It is believed that when N1 of the PLP pyridine is protonated by an acidic amino acid residue, the pyridine ring of PLP can form a quinone-like resonance structure. The α-hydrogen of the PLP–amino acid aldimine is readily

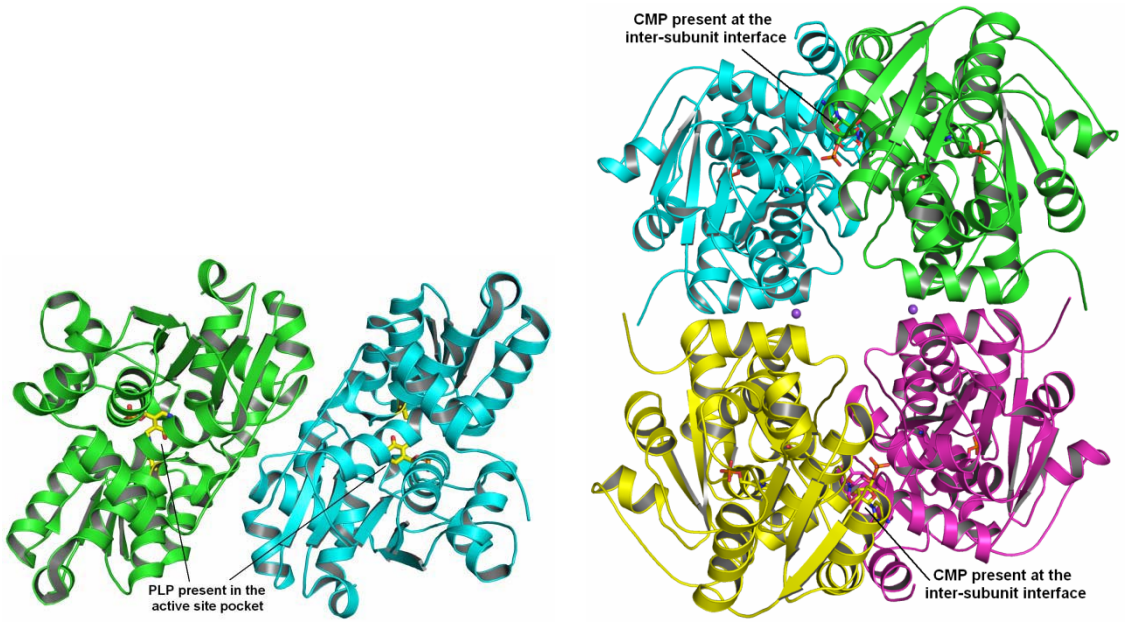

Figure 3. Crystal structures of **(A)** dimeric and **(B)** tetrameric forms of *Salmonella typhimurium* biodegradative threonine deaminase. The PLP cofactor covalently bound as a Schiff base to Lys58 (yielding an internal aldimine) and CMP present at the dimer interface are shown in the stick model. These figures were prepared with the program PyMOL (DeLano 2002) using the atomic coordinates from the Protein Data Bank (PDB codes 2GN1 and 2GN2).

abstracted by a basic residue (Schiff base Lys) because the resulting carbanion can form a resonance-stabilized structure. When N1 of the PLP pyridine is not protonated, as in serine and threonine deaminases, abstraction of α-hydrogen is not as easy as in cases with protonated pyridine because the resulting carbanion cannot form the resonance-stabilized structure (Yamada *et al* 2003). Due to the presence of a neutral amino acid (Ser311) as a hydrogen bond partner of the N1 atom of PLP and the presence of a carboxyl group of the substrate in a neutral environment in TdcB, it is difficult to abstract the C_α hydrogen from the substrate present in the external aldimine form (Simanshu *et al* 2006). Therefore, it has been proposed that in TdcB the reaction proceeds via the carbanion intermediate rather than the quinonoid intermediate.

3. 2-ketobutyrate formate-lyase

2-ketobutyrate formate-lyase (TdcE, EC 2.3.1.-) is a glycyl radical enzyme that catalyses the conversion of 2-ketobutyrate to propionyl-CoA and formate (figure 1b) (Hesslinger *et al* 1998). It has been shown that TdcE has a substrate spectrum similar to that of pyruvate formate-lyase (PFL, EC 2.3.1.54) and the two enzymes can substitute for each other in anaerobic catabolism (Hesslinger *et al* 1998; Sawers *et al* 1998). PFL catalyses the conversion of pyruvate to acetyl-CoA and formate, which has a central role in anaerobic glucose fermentation in *E. coli* and other bacteria. TdcE has a short 4-amino acid N-terminal extension compared with PFL and shares 79% identity (89% overall similarity) spanning the complete length of the amino acid sequence, suggesting a similar structure and catalytic properties. It has been shown that 2-ketobutyrate functions as a substrate for both TdcE and PFL (Hesslinger *et al* 1998). *In vitro* studies have shown that TdcE accepts both 2-ketobutyrate and pyruvate with equal efficiency whereas pyruvate is the preferred substrate for the PFL enzyme (Hesslinger *et al* 1998).

The active form of PFL contains a relatively stable glycyl radical (Gly734 in the case of *E. coli* PFL) (Knappe *et al* 1984; Wagner *et al* 1992). Activation of PFL to the radical-bearing species occurs only anaerobically and is catalysed by an iron–sulphur cluster containing PFL-activating enzyme (EC 1.97.1.4), which generates the glycyl radical using a 5′-deoxyadenosyl radical derived from S-adenosyl methionine (Frey *et al* 1994). In PFL, pyruvate or its analogue oxamate, is required for activation (Chase and Rabinowitz 1968; Knappe *et al* 1974). Like PFL, the enzymatic activity shown by TdcE is absolutely dependent on a glycyl radical-activating enzyme and on CoA, and is sensitive to oxygen (Hesslinger *et al* 1998). Induction of the protein-based radical

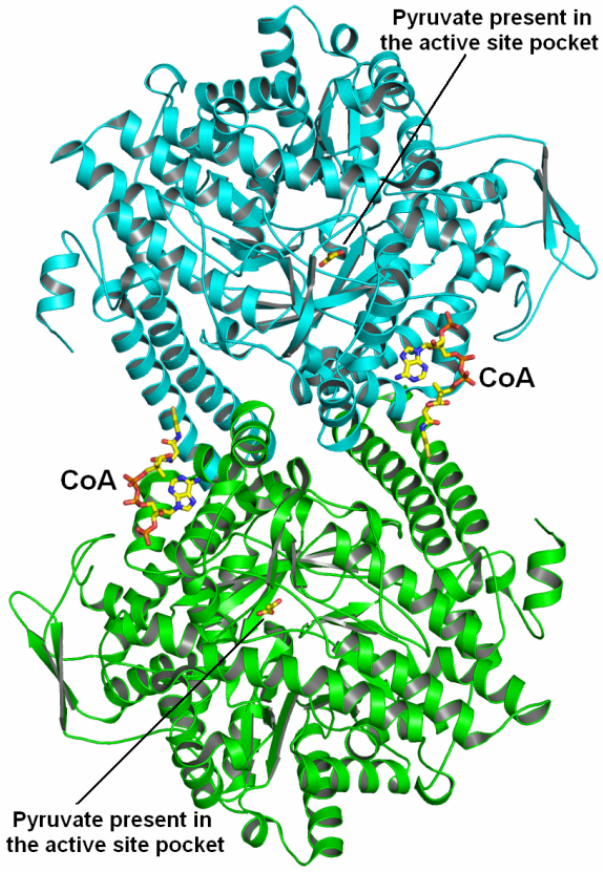

Figure 4. Quaternary structure of *Escherichia coli* pyruvate formate-lyase (non-radical form) in complex with pyruvate and CoA. This figure was prepared with the program PyMOL (DeLano 2002) using the atomic coordinates from the Protein Data Bank (PDB code 1H16).

in TdcE is also catalysed by PFL-activating enzyme (Sawers *et al* 1998). PFL-activating enzyme is the first example of an activase which is able to convert two distinct polypeptides to the catalytically active, radical-bearing species (Hesslinger *et al* 1998). Glycyl radical-bearing enzymes such as PFL display half-of-the-sites reactivity, so that only one of the glycine residues of the dimer is in the radical state at any time (Unkrig *et al* 1989). It has been suggested that the conformational change during activation of one PFL monomer makes the glycine in the other monomer inaccessible to the activating enzyme. Because of the high sequence similarity, substrate structure and the reaction catalyzed, we expect similar catalytic mechanisms in PFL and TdcE. So, the reaction catalysed by TdcE is expected to occur as two half-reactions involving a propionyl enzyme intermediate (E + 2-ketobutyrate = propionyl-E + formate; propionyl-E + CoA = E + propionyl-CoA).

PFL remains the first and best studied example of an enzyme with a catalytically important main-chain radical. The crystal structure of *E. coli* PFL has been determined with various ligands (Becker *et al* 1999; Becker and Kabsch 2002; Lehtio *et al* 2002) (figure 4). The tertiary structure of the enzyme shows a ten-stranded α/β-barrel consisting of two sets of five parallel α/β units assembled in an antiparallel manner. These features also relate PFL enzymes to another class of radical enzymes, the class III ribonucleotide reductases. The active site residues Cys418, Cys419 and Gly734 are present at the tips of short loops, each connecting two-stranded anti-parallel β-sheets. These loops protrude from the top and bottom surfaces into the centre of the α/β-barrel, where the Cα atoms of cysteine and glycine residues approach within a distance of 1.8 Å. These three catalytic residues involved in the reaction mechanism of PFL were also found to be fully conserved in TdcE from *E. coli* and *S. typhimurium* (Cys423, Cys424 and Gly739). Replacement of an alanine residue present near the methyl group of pyruvate in the PFL–pyruvate complex structure (Becker and Kabsch 2002) by a glycine residue in TdcE provides a plausible structural rationale for the different substrate specificity in these two enzymes. In *E. coli*, PFL has been shown to use a radical mechanism to reversibly cleave the C1–C2 bond of pyruvate using the Gly734 radical and two cysteine residues (Cys418, Cys419) (Becker *et al* 1999). The glycyl radical (Gly734) is thought to generate a thiyl radical at Cys418/419 needed for homolytic substrate cleavage. In the structure of *E. coli* PFL (non-radical form) in complex with pyruvate and CoA (Becker and Kabsch 2002), CoA is present in a *syn* conformation awaiting pyruvate cleavage. After cleavage of pyruvate, CoA is expected to change to an *anti* conformation, without affecting the adenine-binding mode of CoA, and then thiol of CoA could pick up the acetyl group generated from pyruvate cleavage.

4. Phosphotransacetylase

During anaerobic degradation of L-threonine to propionate, conversion of propionyl-CoA to propionyl phosphate is catalysed by an enzyme called Pta or phosphate acetyltransferase (Pta; EC 2.3.1.8) (figure 1b) (Van Dyk and LaRossa 1987; Sawers 1998; Sawers *et al* 1998). Pta also plays a key role in acetate metabolism by catalysing the interconversion of acetyl-CoA and acetyl-phosphate, a low molecular mass phosphate donor to several response regulators *in vitro* (McCleary and Stock 1994; McCleary *et al* 1993; Wanner and Wilmes-Riesenberg 1992).

Native as well as liganded Pta from various sources has been shown to exist as dimers in solution as well as in crystal structures (Shimizu *et al* 1969; Pelroy and Whiteley 1972; Iyer *et al* 2004; Xu *et al* 2004; Xu *et al* 2005; Lawerence and Ferry 2006) (figure 5). The first crystal structure of native Pta was reported from the archaeon *Methanosarcina thermophila* (MtPta) (Iyer *et al* 2004). The MtPta monomer

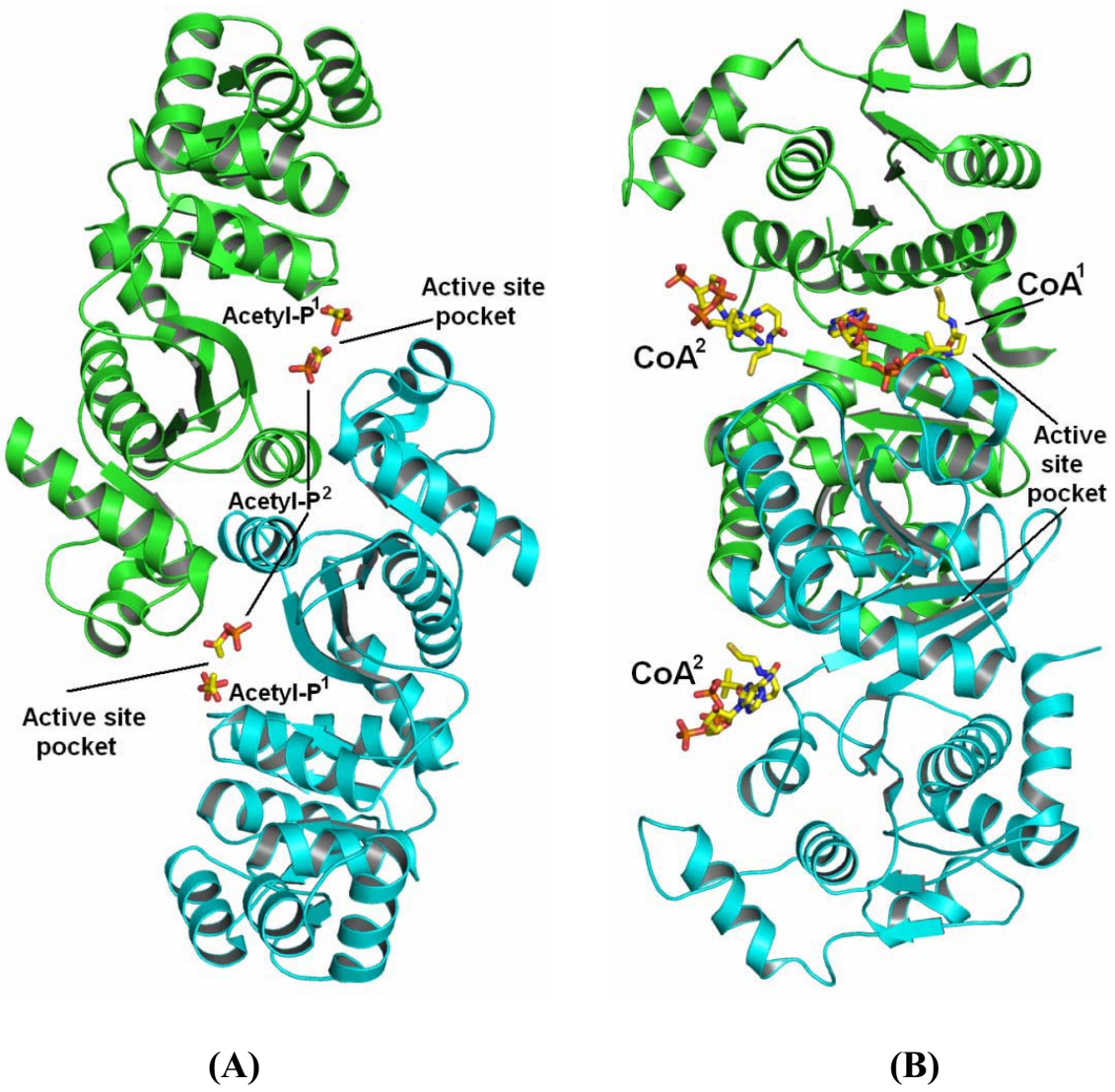

Figure 5. Quaternary structure of phosphotransacetylase **(A)** from *Bacillus subtilis* in complex with acetyl phosphate and **(B)** from *Methanosarcina thermophila* in complex with coenzyme A. These figures have been drawn in different orientations for better clarity. Figures were prepared with the program PyMOL (DeLano 2002) using the atomic coordinates from the Protein Data Bank (PDB codes 1XCO and 2AF3).

is an extended molecule composed of two α/β domains (I and II). Domain I contains a parallel five-stranded β-sheet sandwiched by α-helices on each side, and domain II, involved in dimerization, contains a mixed six-stranded β-sheet surrounded by three and two helices on opposite sides. These two domains are separated by a distinct interdomain cleft proposed as the substrate binding site. More recently, Pta structure has also been determined from two more bacterial sources, *Streptococcus pyogenes* (Xu *et al* 2004) and *Bacillus subtilis* (Xu *et al* 2005), and found to have similar overall architecture and oligomeric status, with similar dimerization mode, as observed in MtPta.

Extensive biochemical and structural studies on Pta have provided detailed information regarding the active site architecture and reaction mechanism (Iyer *et al* 2004; Xu *et al* 2005; Lawrence and Ferry 2006; Lawrence *et al* 2006). The crystal structure of *B. subtilis* Pta (BsPta) in complex with acetyl-P showed four molecules of acetyl-P bound per monomer (Xu *et al* 2005). Two of them (acetyl-P^1 and acetyl-P^2) bind in the interdomain cleft but in reverse orientations and interact with highly conserved residues; these are likely to be the potential substrate molecules capable of undergoing catalysis (figure 5a). The other two molecules (acetyl~P^3 and acetyl~P^4) were found near the lower entrance of the cleft

(Xu *et al* 2005), probably representing non-specific binding. However, an isothermal titration calorimetric experiment on MtPta suggested only one binding site for acetyl-P per monomer (Lawrence *et al* 2006). Perhaps only one binding site is of high affinity. The other three low-affinity binding sites reveal bound ligand in the crystal structure, probably due to the high concentration of the ligand (50 mM) present in the mother liquor used for soaking the native crystal. Crystal structures of MtPta in complex with CoA revealed one CoA^1 bound to the proposed active site cleft with its reactive sulphydryl group proximal to the residue implicated in catalysis (Lawrence *et al* 2006) (figure 5b). The additional CoA^2 molecule bound at the periphery of the cleft, which is exposed to solvents, is speculated to have either a regulatory role or act as the loading site to preorient CoA (Lawrence *et al* 2006) during catalysis. Based on a close inspection of the CoA^1 in the MtPta active site pocket, Ser309, Asp316 and Arg310 were identified as potential catalytic residues. The catalytic mechanism as proposed for MtPta proceeds through abstraction of the sulphydryl proton by Asp316 from CoA^1 followed by attack of thiolate anion on the carbonyl carbon of acetyl-P (Lawrence *et al* 2006). Arg310 may facilitate catalysis by optimizing the position of acetyl-P by interacting with its phosphate group. The negatively charged transition state formed during the reaction mechanism is proposed to be stabilized by the catalytic Ser309 (Lawrence *et al* 2006). Formation of acetyl-CoA is followed by the abstraction of a proton from Asp316 by the resulting PO_4^{3-} ion. This neutralizes one of the negative charges of the phosphate and regenerates deprotonated Asp316 for another round of catalysis (Lawrence *et al* 2006). Although combined X-ray and kinetic analyses of Pta from different sources were informative, several key questions remain unanswered. These include the functional relevance of the CoA^2 binding site found in MtPta, determination of the specific acetyl-P molecule (acetyl~P^1 or acetyl~P^2) undergoing catalysis, and the role of acetyl~P^3 and acetyl~P^4, if any.

The Pta polypeptides of *E. coli* and *S. typhimurium* consist of 714 amino acid residues and share 96% amino acid sequence identity between them. Sequences belonging to the other well characterized Ptas align only to the C-terminal part and do not possess the sequence corresponding to the N-terminal region present in *E. coli* and *S. typhimurium* enzymes (N-Pta) (Higgins *et al* 1994). The N-Pta domain has been shown to be homologous to dethiobiotin synthetase (DTBS), cobyric acid synthase (CobQ or CbiP), cobyrinic acid a,c-diamide synthase (CobB or CbiA) and ATPase (MinD) (Galperin and Grishin 2000). In N-Pta, this region, referred to as the DTBS-like region, showed general conservation of residues involved in ATP binding, as found in homologous proteins and thus N-Pta might bind ATP in a similar manner (Galperin and Grishin 2000). This domain is suggested to regulate the Pta activity in response to ATP (Galperin and Grishin 2000). These observations were in agreement with earlier studies which show that $NADH+H^+$, ATP and its analogues inhibit *E. coli* Pta (Suzuki 1969; Suzuki *et al* 1969) but do not affect enzymes lacking the N-Pta domain (Rado and Hoch 1973; Suzuki *et al* 1969). The C-terminal region of the N-Pta domain shows homology with the DRTGG domain of unknown function, named after some of the most conserved residues corresponding to Asp281–Arg282 and Thr305–Gly307 of *S. typhimurium* Pta.

5. Propionate kinase

Propionate kinase (TdcD; EC 2.7.2.15) catalyses the last step of this metabolic process by enabling the conversion of propionyl phosphate and ADP to propionate and ATP (figure 1b) (Hesslinger *et al* 1998). TdcD exhibits 38–42% amino acid sequence identity with acetate kinase. Acetate, an analogue of propionate, has also been reported to be a phosphate acceptor in propionate kinases. The K_M value for ATP using the purified enzyme was 112 μM whereas the K_M values for propionate and acetate were 2.3 mM and 26.9 mM, respectively, showing that TdcD has about ten times higher affinity for propionate than for acetate (Simanshu *et al* 2005). In 2005, the structure of propionate kinase from *S. typhimurium* in complex with ADP and the non-hydrolysable ATP analogue AMPPNP was first determined by our group (Simanshu *et al* 2005). Propionate kinase contains a fold with the topology $\beta\beta\beta\alpha\beta\alpha\beta\alpha$, similar to that of acetate and sugar kinase, heat shock cognate 70 (Hsc70) and actin, the ASKHA superfamily of phosphotransferases (Buss *et al* 2001). A ribbon representation of the dimeric enzyme is shown in figure 6. Each subunit comprises two domains of unequal size. The propionate- and nucleotide-binding sites are present in a cleft between the two domains. Both the domains contain a core secondary structure $\beta\beta\beta\alpha\beta\alpha\beta\alpha$, which is similar to that of acetate kinase/glycerol kinase/hexokinase/Hsc70/actin. These proteins differ from each other in the subdomains inserted between particular secondary structural elements. The α-helices present at the C-terminal ends of both the domains extend and form part of the other domain. The structure of TdcD-AMPPNP approximates the optimal position of the γ-phosphate group poised for catalysis (figure 6) (Simanshu *et al* 2005). The cryoprotectant ethylene glycol observed in the proposed propionate-binding site in the AMPPNP-bound TdcD complex structure probably reflects the mode of propionate binding. Aliphatic carbon atoms of ethylene glycol are present in a hydrophobic pocket formed by Ala88, Leu117, His118, Phe174 and Pro227. A less bulky residue, glycine, is present in all the butyrate kinase sequences, and a more bulky residue, valine, is found in all the acetate kinases at positions equivalent to Ala88, which is present at the

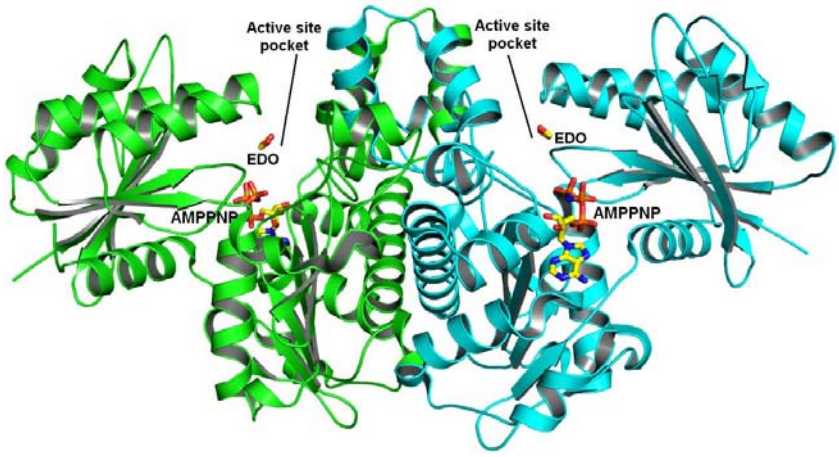

Figure 6. Quaternary structure of *Salmonella typhimurium* propionate kinase bound with AMPPNP and ethylene glycol (EDO). Ethylene glycol binds near the proposed binding site of propionate. This figure was prepared with the program PyMOL (DeLano 2002) using the atomic coordinates from the Protein Data Bank (PDB code 1X3N).

bottom of the hydrophobic pocket in propionate kinase. These observations suggest that the size of the hydrophobic pocket is a determinant of substrate specificity in acetate, propionate and butyrate kinases (Ingram-Smith *et al* 2005; Simanshu *et al* 2005). Comparison of the TdcD complex structures with members of the ASKHA superfamily, along with results obtained from various site-directed mutagenesis experiments with acetate kinase (Buss *et al* 2001; Miles *et al* 2001) and hexokinase (Zeng and Fromm 1995), has permitted identification of the essential residues involved in substrate binding and catalysis. The active site residues expected to play an important role during catalysis in propionate kinase are Asn11, Arg86, Asp143, His175, Arg236 and Glu381. Structural details obtained from the TdcD complex structures indicate that once the substrate binds in the cleft, the proposed substrate-induced domain movement occurs, which positions the reactants, brings the active site residues present in both the domains close to the reactants, and shields the active site pocket from the surrounding solvent.

Studies on acetate kinase, a homologue of propionate kinase with significant amino acid sequence identity, have shown an inversion of configuration during phosphoryl transfer (Blattler and Knowles 1979), suggesting either a direct in-line transfer or a covalent triple displacement mechanism (Spector 1980) involving two phosphoenzyme intermediates. The direct in-line transfer mechanism was supported by steady-state kinetics as well as stereochemical evidence (Miles *et al* 2002), whereas the triple displacement mechanism was proposed when a phosphoenzyme was isolated after incubating *E. coli* acetate kinase with radiolabelled ATP or acetyl-P, and the isolated phosphoenzyme was able to transfer the phosphoryl group to ADP or acetate (Anthony and Spector 1970). Recent structural and biochemical studies carried out on acetate and propionate kinases have provided additional support for the direct in-line transfer of the phosphoryl group from ATP to acetate/propionate (Gorrell *et al* 2005; Simanshu *et al* 2005).

In the well-characterized members of the ASKHA superfamily, ATP phosphoryl transfer or hydrolysis is coupled to a large conformational change in which the two domains close around the active site cleft. The significant amino acid sequence similarity between TdcD and acetate kinase facilitated the study of domain movement, which indicated large domain motion in these two enzymes (Simanshu *et al* 2005). These studies also indicated that the conformation assumed by the two domains in the nucleotide-bound structure of TdcD represent an intermediate point in the pathway of domain closure.

Our studies on propionate kinase from *S. typhimurium* provided evidence for a novel diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) synthetic activity (Simanshu *et al* 2007). Crystals of TdcD obtained in the presence of ATP clearly showed Ap_4A bound in the active site pocket of the enzyme. In these structures, Ap_4A is present in an extended conformation with one adenosine moiety present in the nucleotide-binding site and other in the proposed propionate-binding site. Further, mass spectroscopic studies and co-crystallization trials of TdcD with commercially available Ap_4A confirmed its formation and binding to the enzyme. The formation of Ap_4A by TdcD in the presence of ATP is similar to the Ap_4A synthetic activity of Ap_4A phosphorylase. In the case of TdcD, initially ATP might undergo hydrolysis to yield ADP, which could in turn react with excess ATP present in the crystallization mixture

leading to the formation of Ap_4A in the presence of Mg^{2+}. It has been proposed that these dinucleotides such as Ap_4A may act as alarmones, alerting the cells to the onset of oxidative stress (Varshavsky 1983).

6. Summary

Even though the conversion of L-threonine to propionate via 2-ketobutyrate was recognized well over 40 years ago, it took almost three decades to identify the exact route of reaction and the enzymes involved in it. Dehydration of L-threonine to 2-ketobutyrate is the first committed step in isoleucine biosynthesis as well as in propionate formation. It is likely that during periods in which the level of energy in the cells is low, the higher concentration of AMP provides a signal for the activation and conversion of L-threonine to propionate and ATP. In the absence of the C-terminal domain of IlvA, which plays an important role in feedback inhibition by L-isoleucine, AMP seems to regulate the enzymatic activity of TdcB. The other three enzymes TdcE, Pta and TdcD convert the 2-ketobutyrate formed from L-threonine into propionate with the generation of an energy-rich ATP molecule. Extensive biochemical, kinetic and structural studies carried out on these enzymes have shown interesting results, which have helped in understanding the structure–function relationship in these enzymes.

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