ABSTRACT

Acetate and propionate are low molecular mass carbon compounds found abundantly in the soil. Although these compounds have been extensively used as food preservatives because of their ability to inhibit microbial growth, surprisingly, bacteria such as *Escherichia coli* and *Salmonella typhimurium* are able to grow on propionate as their sole carbon and energy source. Only in the presence of glucose, acetate and other short chain fatty acids inhibit microbial growth.

Propionate is produced during the β-oxidation of odd-numbered carbon-chain fatty acids, fermentation of carbohydrates, oxidative degradation of branched-chain amino acids such as valine and isoleucine. They are also produced during the catabolism of threonine, methionine, thymine and cholesterol. In *Escherichia coli* and *Salmonella typhimurium*, enzymes involved in the degradation of L-serine and L-threonine to acetate and propionate, respectively, are encoded by the anaerobically regulated tdc operon. L-threonine is anaerobically degraded to propionate in four consecutive reaction steps catalyzed by biodegradative threonine deaminase (TdcB), 2-ketobutyrate formate lyase (TdcE), phosphotransacetylase (Pta) and propionate kinase (TdcD). Detailed studies on the structure and function of two of these enzymes have earlier been carried out in our laboratory. However, these studies did not reveal the precise substrate binding site in *Salmonella typhimurium* TdcD (*St*TdcD). It was also not possible to provide a satisfactory explanation of the structural basis of substrate specificity. The present studies were therefore aimed at locating the substrate binding site, elucidating the structural basis of substrate specificity and mechanism of catalysis of *St*TdcD.

Oxalic acid is toxic to almost all organisms and its excessive occurrence leads to a variety of pathological conditions. In humans and other vertebrates, secretion of oxalic acid leads to formation of low soluble calcium oxalate, which precipitates as kidney stones. Formation of kidney stones is aggravated by lack of enzymes that catabolize oxalate. Oxalate oxidase, oxalate decarboxylase and oxalyl-CoA decarboxylase constitute three distinct categories of oxalate degrading enzymes. *Photorhabdus luminescens* is a Gram-negative, symbiotic bacterium associated with the entomopathogenic nematodes of the family *Heterorhabditidae*. Novel insecticidal genes from these symbiotic bacteria are now being examined for their potential in generating pest resistant transgenic plants. As part of this project, the three-dimensional X-ray crystal structure of an oxalate oxidase (OXDC) enzyme from *Photorhabdus luminescens* (*Pl*OXDC) was determined.

The introductory chapter (**Chapter I**) of the thesis presents the earlier investigations carried out in the laboratory on the structure and function of *StTdcD*. It also provides a summary of the earlier literature pertaining to propionate metabolism in *S. typhimurium*. The crystal structure of *St*TdcD in the apo form as well as in complex with ADP and the non-hydrolysable nucleotide analog AMPPNP were determined by earlier Dr. Simanshu (Simanshu *et al*., 2005 , 2008). Subsequently, Dr Chittori determined the structures of the enzyme in complex with various other nucleotides (Chittori *et al*., 2013). These studies along with enzyme assays performed by Chittori revealed that *St*TdcD possesses broad specificity and it could be activated by various nucleotides and metal ions and catalyzes phosphorylation of both propionate and acetate (Chittori *et al*., 2013). In spite of these extensive studies, the precise mode of binding of the substrate propionate to *St*TdcD could not be elucidated. The chapter also presents a summary of the literature on oxalate, its toxic effects and enzymes that degrade oxalate. The importance of structural and functional studies on oxalate degrading enzymes and other enzymes encoded by *Photorhabdus luminescens* is also briefly discussed.

All the experimental protocols and computational methods applicable for most of the investigations reported in Chapters 4, 5 and 6 are presented in **Chapter II**. The experimental procedures described include cloning, overexpression, purification, enzymatic assays, crystallization and X-ray diffraction data collection. Computational methods covered include summary of crystallographic theory and details of various programs used during data processing, structure solution, refinement, model building, validation and analysis. The databases that were used in the course of these investigations are also cited.

The experience gained during attempts to determine the structure of *St*TdcD by single wavelength anomalous dispersion (SAD) method is described in **Chapter III**. The impetus for this work was the urge to examine the power of SAD technique making use of a newly acquired rotating anode X-ray generator equipped with a chromium anode. As expected, the structure determined by SAD was very close to the earlier determined structure of *St*TdcD. The structure contained a citrate, which was part of the crystallization cocktail at the active site. This is in contrast with acetate kinase, where it was found that citrate binds at the dimeric interface. The present studies demonstrated that the identification of a plausible regulatory site at the interface of dimeric structure in acetokinases based on the structure of acetate kinase (Chittori *et al*., 2013) is not valid for propionate kinase.

Extensive efforts carried out to obtain structures of *St*TdcD and its mutants *St*TdcD A88V and *St*TdcD G207A complexed with either the substrate or substrate analogues provided several crystal structures. In most of these structures, the ligand was bound at a position distinct from the substrate binding site. These structures and their analysis are described **Chapter IV**. Asn206 was transformed from a disallowed region to an allowed region of the Ramachandran map in these structures whenever an anion was bound at the position corresponding to the γ- phosphate of the nucleotide substrate. This structural transformation might enhance the affinity of the enzyme for the substrate. In the structure of *St*TdcD A88V in complex with AMPPNP, AMPPNP was found to be cleaved to AMP and PNP either due to catalytic activity of the enzyme or due to radiation damage. The released PNP probably reacted with propionate forming propionyl-pyrophosphate. These structures also demonstrate that the nucleotide site readily accommodates the substrate or substrate analogues in the absence of a bound nucleotide.

*St*TdcD catalyzes the Mg2+ ion dependent inter-conversion of propionate and ATP to propionyl phosphate and ADP. Two distinct catalytic mechanisms have been proposed for the phosphoryl transfer reaction catalyzed by acetokinase family enzymes: 1) direct-in-line transfer mechanism and 2) triple displacement mechanism (Anthony and Spector, 1972; Matte *et al*., 1998). In both, the configuration of the transferred phosphate undergoes an inversion, which has been experimentally demonstrated. Structural studies carried out with the view of elucidating the catalytic mechanism of *St*TdcD is described in **Chapter V**. Fortunately, it was possible to obtain the crystal structures of *St*TdcD and its mutants with propionate and AMPPNP bound at the active site. The structure supported an associative SN2 type direct in-line transfer mechanism of catalysis. The studies also revealed that Arg236 and His175 are catalytically important residues. As suggested earlier, Ala88 has a major role in specificity determination. However, Ala88 is not the sole determinant of specificity. Active site volume determining residues, Arg86, His118, Asp143 and the segment Pro116-Leu117-His118 are also important for substrate specificity. The catalytic mechanism proposed in this chapter may also be applicable to other acetokinase family members.

The final **Chapter VI** describes three different crystal structures of *Pl*OXDC. As expected from sequence similarity with *B. subtilis* and *T. maritima* OXDCs, *Pl*OXDC polypeptide was found to possess a bicupin structure. However, the functional unit was a trimer in contrast to *Bs*OXDC which functions as a hexamer. The difference is shown to be due to the disorder in the amino terminal segment of *Pl*OXDC. The polypeptide was truncated during purification by a non-specific cleavage at residue Lys26 either by thrombin used for

cleaving the covalently attached GST tag or by some other protease. However, in the crystal structure, the amino terminal 90 residues were disordered. The observed trimeric form of *Pl*OXDC may represent its inherent nature or a result of the missing N-terminal residues. There is some controversy in the literature on whether both or only one cupin domain of the protomer is catalytically active. The structures presented in this chapter provided significant information on the mode of ligand binding to *Pl*OXDC. In one of the structures, EDO was bound to both the cupin domains and was involved in similar interactions with protein atoms. This may imply that the substrate binds at both the sites and both cupin domains may have catalytic function.

The thesis ends with a short note on future perspectives. It is clear that substantial work has been carried out on acetokinases. These studies have provided significant understanding of their structure and function. In the future, appropriate site-specific mutations of the substrate specificity determining residues may be made and their effect on enzyme specificity could be studied. Similarly, mutagenesis experiments could be performed to inter-convert acetate, propionate and butyrate kinases. These studies will provide deeper insights on intricacies of enzyme function. In contrast to the work on short chain fatty acid kinases, work on OXDC should be considered preliminary and further biochemical and structural studies are needed to illustrate the catalytic mechanism and examine if the protein is a suitable candidate for generating transgenic crops resistant to insect pests.

The following manuscripts have been published or will be communicated for publication based on the results presented in the thesis.