Biochimica et Biophysica Acta 1834 (2013) 2036–2044

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Mechanistic features of *Salmonella typhimurium* propionate kinase (TdcD): Insights from kinetic and crystallographic studies

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ARTICLE INFO

Article history:
Received 24 December 2012
Received in revised form 5 May 2013
Accepted 20 May 2013
Available online 5 June 2013

Keywords:
Salmonella typhimurium
Short-chain fatty acid metabolism
Enzyme assay
X-ray crystallography
Protein dynamics

ABSTRACT

Short-chain fatty acids (SCFAs) play a major role in carbon cycle and can be utilized as a source of carbon and energy by bacteria. *Salmonella typhimurium* propionate kinase (*St*TdcD) catalyzes reversible transfer of the γ-phosphate of ATP to propionate during L-threonine degradation to propionate. Kinetic analysis revealed that *St*TdcD possesses broad ligand specificity and could be activated by various SCFAs (propionate > acetate ≈ butyrate), nucleotides (ATP ≈ GTP > CTP ≈ TTP; dATP > dGTP > dCTP) and metal ions ($Mg^{2+} \approx Mn^{2+} > Co^{2+}$). Inhibition of *St*TdcD by tricarboxylic acid (TCA) cycle intermediates such as citrate, succinate, α-ketoglutarate and malate suggests that the enzyme could be under plausible feedback regulation. Crystal structures of *St*TdcD bound to PO_4 (phosphate), AMP, ATP, Ap4 (adenosine tetraphosphate), GMP, GDP, GTP, CMP and CTP revealed that binding of nucleotide mainly involves hydrophobic interactions with the base moiety and could account for the broad biochemical specificity observed between the enzyme and nucleotides. Modeling and site-directed mutagenesis studies suggest Ala88 to be an important residue involved in determining the rate of catalysis with SCFA substrates. Molecular dynamics simulations on monomeric and dimeric forms of *St*TdcD revealed plausible open and closed states, and also suggested role for dimerization in stabilizing segment 235–290 involved in interfacial interactions and ligand binding. Observation of an ethylene glycol molecule bound sufficiently close to the γ-phosphate in *St*TdcD complexes with triphosphate nucleotides supports direct in-line phosphoryl transfer.

1. Introduction

Short-chain fatty acids (SCFAs; aliphatic chains of up to 6 carbons) such as acetate, propionate and butyrate play a major role in carbon cycle and, in the absence of a preferred nutrient, could serve as carbon and energy source for bacteria [1,2]. Advances in molecular biology techniques and availability of genomic data on *Escherichia coli* [3] and *Salmonella typhimurium* [4] have helped in identifying metabolic pathways that are responsible for the degradation of SCFAs in bacteria. Further, biochemical and genetic studies of these pathways in *E. coli* and *S. typhimurium* revealed the importance of an anaerobically regulated *tdc* operon (*tdcABCDEFG*) consisting of genes coding for enzymes essential for the degradation of serine and threonine to energy-rich keto acids that could be catabolized to acetate and propionate [5–8].

Propionate kinase (TdcD) catalyzes reversible transfer of phosphate from propionyl-phosphate to ADP during L-threonine degradation to propionate resulting in the formation of energy-rich ATP [5]. TdcD is homologous to relatively well characterized acetate kinase (AckA) [9–14]. These enzymes belong to the acetokinase family of enzymes (Pfam: PF00871; SCOP: 53080) that also includes butyrate kinases. We have earlier carried out biochemical characterization of *S. typhimurium* TdcD (*St*TdcD) and determined its crystal structures in the unliganded state as well as in complex with ADP, a non-hydrolyzable ATP analog AMPPNP, and Ap4A (diadenosine tetraphosphate) [15,16]. *St*TdcD is a homodimeric enzyme with each monomer consisting of two domains with an active site pocket formed between the inter-domain cleft. Both the domains contain a core of secondary structure ββββαβαβα, which is similar to the fold of proteins belonging to acetate and sugar kinase/heat shock cognate (Hsc) 70/actin (ASKHA) superfamily [17,18]. Among the members of ASKHA superfamily, phosphoryl transfer is coupled to a conformational change in which the two domains close around the active site pocket [12,15,17–19]. The domain movement is required to bring the active site residues from the moving domain (domain-I) into close proximity of the substrate, anchor the reactants and shield the reaction intermediates from the surrounding solvent during catalysis.

In the present study we carried out in-depth kinetic characterization of *St*TdcD, which revealed broad specificity and relatively low affinity

Abbreviations: SCFA, Short-chain fatty acid; TdcD, propionate kinase; AckA, acetate kinase; ASKHA, Acetate and Sugar Kinase/Heat shock cognate (Hsc) 70/Actin

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http://dx.doi.org/10.1016/j.bbapap.2013.05.020

Table 1
Kinetic constants determined for *St*TdcD.

	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
SCFA				
Acetate[a]	25.3 ± 2.4	155 ± 24	120 ± 17	4.7 ± 1.2
Propionate[a]	2.5 ± 0.2	140 ± 18	108 ± 14	43.2 ± 4.8
Butyrate	35.2 ± 4.0	78 ± 10	60 ± 22	1.7 ± 1.0
Nucleotide				
ATP[a]	126 ± 15	155 ± 24	120 ± 17	1.0 ± 0.2
GTP	115 ± 23	150 ± 13	113 ± 25	1.0 ± 0.3
UTP	214 ± 17	108 ± 15	81 ± 20	0.26 ± 0.11
CTP	226 ± 20	95 ± 20	73 ± 15	0.31 ± 0.08

Values reported are mean ± standard deviation from three independent experiments.
[a] A similar value of kinetic constant for these ligands has been earlier reported from our lab [15].

for its ligands and, inhibition by metabolites of TCA cycle. In our earlier study, ethylene glycol (EDO) used as the cryoprotectant was observed in the proposed propionate binding site (hereafter referred to as SCFA-I site) and was suggested to reflect the mode of propionate binding [15]. Analysis of *St*TdcD-nucleotide complexes determined in the present studies suggested a distinct site of propionate binding (hereafter referred to as SCFA-II site) closer to the γ-phosphate of ATP, supporting a direct in-line phosphoryl transfer mechanism of catalysis. Molecular dynamics simulations of monomer and dimeric forms of *St*TdcD revealed plausible open and closed states consistent with inter-domain motion during catalysis.

2. Materials and methods

2.1. Enzyme kinetics

Biochemical activity of *St*TdcD was examined by coupling ADP produced by the enzyme to the oxidation of NADH using pyruvate kinase (PK) and lactate dehydrogenase (LDH) as coupling enzymes [15]. The standard assay mixture of 0.5 ml contained 50 mM HEPES–NaOH pH 7.5, 25 mM propionate, 1 mM ATP, 1.5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 0.25 mM NADH, 15 units of PK and 20 units of LDH. The reaction was initiated by the addition of the enzyme and monitored at 340 nm for 10 min. Kinetic constants were determined by fitting the initial velocity versus substrate concentration (0.1–10 K_m) to the Michaelis–Menten equation (Table 1). Enzymatic activity of the enzyme was also examined for alternative SCFA and nucleotides by replacing propionate or ATP, respectively, in the assay mixture (Table 1 and Supplementary Table S1). Metal ion dependence of *St*TdcD was examined following the method described by Rose et al. [20] using acetate as the SCFA substrate. Briefly, the reaction of the product acetyl-phosphate with neutral hydroxylamine leading to the formation of acetyl-hydroxamate was followed by estimating the colored complex (ferric-hydroxamate, $\lambda_{max} = 540$ nm) formed in the presence of ferric ions. Inhibition of *St*TdcD activity by various metabolites and their analogs was examined by incubation (15 min at 25 °C) of the enzyme with the corresponding compound prior to kinetic measurements.

Table 2
Crystal parameters and refinement statistics for *St*TdcD-nucleotide complexes.

Parameter	ATP	GTP	CTP
Crystal parameters			
Crystallization condition	0.1 M Bis-Tris pH 6.5, 35% pentaerythritol ethoxylate	0.1 M Bis-Tris pH 6.5, 30% pentaerythritol ethoxylate	0.1 M Bis-Tris pH 6.5, 30% pentaerythritol ethoxylate
Complexation method	Soaking	Soaking	Soaking
Resolution range (Å)	50.0–2.95 (3.06–2.95)	50.0–2.65 (2.74–2.65)	50.0–2.70 (2.80–2.70)
Space group	$P3_121$	$P3_121$	$P3_121$
Unit cell parameters	a = b = 111.4, c = 66.7	a = b = 110.3, c = 66.5	a = b = 110.7, c = 66.5
Observed reflections	82575	157877	84256
Unique reflections	10303	13746	12988
Multiplicity	8.0	11.5	6.5
Completeness (%)	99.2 (97.7)	99.2 (97.2)	97.3 (98.6)
Mean [<I>/σ(<I>)] [a]	15.7 (3.2)	18.3 (4.2)	6.6 (6.2)
R_{merge} (%) [b]	6.4 (51.9)	9.7 (49.5)	11.6 (34.7)
Matthews coefficient ($Å^3$ Da^{-1})	2.64	2.58	2.60
Solvent content (%)	53.44	52.31	52.64
Refinement statistics			
R-factors (%)[c]			
$R_{work\ +\ free}/R_{work}/R_{free}$	23.7/23.4/29.1	22.2/21.9/27.4	24.0/23.8/28.4
No. of atoms			
Protein/water/EDO/Lig[d]	2897/28/16 (4)/31 (1)	2948/85/8 (2)/32 (1)	2940/74/16 (4)/29 (1)
Average B-factor ($Å^2$)			
Protein/water/EDO/Lig[d]	63.3/53.1/79.1/99.6	45.1/42.4/65.2/81.4	48.8/37.9/57.2/60.4
RMS deviations			
Bond length (Å)	0.006	0.006	0.005
Bond angle (°)	0.987	0.917	0.895
Dihedral angle (°)	4.735	4.587	4.364
Chiral-center restraints ($Å^3$)	0.065	0.058	0.059
General planes (Å)	0.003	0.003	0.003
Ramachandran map (%/number)			
Most favored region	90.8/314	91.6/317	91.0/315
Allowed region	9.0/31	8.4/29	9.0/31
Generously allowed region	0.3/1	0/0	0/0
Disallowed region	0/0	0/0	0/0

Values in parentheses refer to the highest resolution shell.
[a] I is the integrated intensity and σ(I) is the estimated standard deviation of that intensity.
[b] $R_{merge} = \sum_{hkl} \sum_i | I_i(hkl) - <I(hkl)> | / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of the intensity of reflection hkl and <I(hkl)> is the mean intensity.
[c] $R_{work} = \Sigma(|F_{obs}|-|F_{calc}|)/\Sigma|F_{obs}|$; R_{free} was calculated similarly using 5% of the reflection that were excluded from the refinement.
[d] Lig refers to the nucleotide ligand bound to the respective *St*TdcD-complex; number in parentheses indicates the number of molecules.