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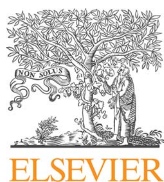

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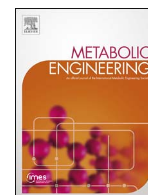

Original Research Article

Reaction kinetic analysis of the 3-hydroxypropionate/4-hydroxybutyrate CO_2 fixation cycle in extremely thermoacidophilic archaea

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A B S T R A C T

The 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle fixes CO_2 in extremely thermoacidophilic archaea and holds promise for metabolic engineering because of its thermostability and potentially rapid pathway kinetics. A reaction kinetics model was developed to examine the biological and biotechnological attributes of the 3HP/4HB cycle as it operates in *Metallosphaera sedula*, based on previous information as well as on kinetic parameters determined here for recombinant versions of five of the cycle enzymes (malonyl-CoA/succinyl-CoA reductase, 3-hydroxypropionyl-CoA synthetase, 3-hydroxypropionyl-CoA dehydratase, acryloyl-CoA reductase, and succinic semialdehyde reductase). The model correctly predicted previously observed features of the cycle: the 35–65% split of carbon flux through the acetyl-CoA and succinate branches, the high abundance and relative ratio of acetyl-CoA/propionyl-CoA carboxylase (ACC) and MCR, and the significance of ACC and hydroxybutyryl-CoA synthetase (HBCS) as regulated control points for the cycle. The model was then used to assess metabolic engineering strategies for incorporating CO_2 into chemical intermediates and products of biotechnological importance: acetyl-CoA, succinate, and 3-hydroxypropionate.

1. Introduction

Concerns over sustainability and global climate change have generated interest in developing biological systems for industrial production of fuels and chemicals, with particular interest in using inorganic carbon feed stocks, such as CO_2 (Conrado et al., 2013). To do so a CO_2 fixation pathway is needed, six of which are currently known: the 3-hydroxypropionate (3HP) bicycle, the dicarboxylate/4-hydroxybutyrate (DC/4HB) cycle, the reductive citric acid cycle, the reductive acetyl-CoA (Wood-Ljungdahl) pathway, the Calvin-Benson-Bassham cycle, and the 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle (Berg, 2011; Berg et al., 2010; Herter et al., 2002; Huber et al., 2008). There have been efforts to produce chemicals and fuels based on several of these pathways, including the 3HP bicycle, Calvin-Benson-Bassham cycle, reductive acetyl-CoA pathway, and, of interest here, the 3HP/4HB cycle (Keller et al., 2013; Li et al., 2012; Mattozzi et al., 2013; Muller et al., 2013; Ueki et al., 2014; Yuzawa et al., 2012) (see Table 1).

The 3HP/4HB cycle is a promising candidate for microbial production of chemicals from CO_2 for several reasons. First, it functions at high temperatures, allowing it to be used in an extremely thermophilic host with concomitant minimal risk of contamination and reduced cooling costs (Keller et al., 2015; Zeldes et al., 2015). Second, the 3HP/4HB cycle can function in either an aerobic or anaerobic host, unlike the DC/4HB and reductive acetyl-CoA pathways, which are found exclusively in anaerobic organisms (Fast and Papoutsakis, 2012). Third, it was shown that the 3HP/4HB cycle can drive rapid autotrophic growth with a doubling time of less than five hours (Hawkins

Abbreviations: 3HP/4HB, 3-hydroxypropionate/4-hydroxybutyrate cycle; ACC, acetyl-CoA/propionyl-CoA carboxylase; MCR, malonyl-CoA/succinyl-CoA reductase; MSR, Malonic semialdehyde reductase; HPCS, 3-hydroxypropionyl-CoA synthetase; HBCS, 4-hydroxybutyryl-CoA synthetase; HPCD, 3-hydroxypropionyl-CoA dehydratase; ACR, acryloyl-CoA reductase; MCE, methylmalonyl-CoA epimerase; MCM, methylmalonyl-CoA mutase; SSR, succinic semialdehyde reductase; HBCD, 4-hydroxybutyryl-CoA dehydratase; CCH/HBCD, bifunctional crotonoyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase; AACT, acetoacetyl-CoA β-ketothiolase; SSADH, succinic semialdehyde dehydrogenase; IPPASE, inorganic pyrophosphatase

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Table 1
Enzymes of the 3-hydroxypropionate/4-hydroxybutyrate cycle in *M. sedula*.

Enzyme name	Label	ORF (s)	Reaction (s) Catalyzed	[a]Reference
Acetyl-CoA/Propionyl-CoA carboxylase	ACC	Msed_0147 Msed_0148 Msed_1375	a) Acetyl-CoA+HCO_3^-+ATP↔Malonyl-CoA+ADP+P_i b) Propionyl-CoA+HCO_3^-+ATP↔(S)-Methylmalonyl-CoA+ADP+P_i	NP (Hugler et al., 2003)
Malonyl-CoA/Succinyl-CoA reductase (NADPH)	MCR	Msed_0709	a) Malonyl-CoA+NADPH↔Malonic semialdehyde +NADP+CoA b) Succinyl-CoA+NADPH↔Succinic semialdehyde +NADP+CoA	NCE (Kockelkorn and Fuchs, 2009) R (this work) R (*S. tokodaii*) (Alber et al., 2006)
Malonic semialdehyde reductase (NADPH)	MSR	Msed_1993	Malonic semialdehyde+NADPH↔3-Hydroxypropionate+NADP	R (Kockelkorn and Fuchs, 2009)
3-Hydroxypropionyl-CoA synthetase (AMP-forming)	HPCS	Msed_1456	3-Hydroxypropionate+ATP+CoA↔3-Hydroxypropionyl-CoA+AMP+PP_i	NP, R (*S. tokodaii*) (Alber et al., 2008) R (this work)
3-Hydroxypropionyl-CoA dehydratase	HPCD	Msed_2001	a) 3-Hydroxypropionyl-CoA↔Acryloyl-CoA+H_2O b) Crotonyl-CoA+H_2O↔(S)-3-hydroxybutyryl-CoA	NP (Teufel et al., 2009), R (this work)
Acryloyl-CoA reductase (NADPH)	ACR	Msed_1426	Acryloyl-CoA+NADPH↔Propionyl-CoA+NADP	R (*S. tokodaii*) (Teufel et al., 2009) R (this work)
Methylmalonyl-CoA epimerase	MCE	Msed_0639	(S)-Methylmalonyl-CoA↔(*R*)-Methylmalonyl-CoA	R (Marcheschi et al., 2012)
Methylmalonyl-CoA mutase	MCM	Msed_0638 Msed_2055	(*R*)-Methylmalonyl-CoA↔Succinyl-CoA	R (Marcheschi et al., 2012)
Succinic semialdehyde reductase (NADPH)	SSR	Msed_1424	Succinic semialdehyde+NADPH↔4-Hydroxybutyrate +NADP	R (Kockelkorn and Fuchs, 2009, this work)
4-Hydroxybutyryl-CoA synthetase (AMP-forming)	HBCS	Msed_0406	a) 4-Hydroxybutyrate+ATP+CoA↔4-Hydroxybutyryl-CoA+AMP+PP_i b) 3-Hydroxypropionate+ATP+CoA↔3-Hydroxypropionyl-CoA+AMP +PP_i	R (Hawkins et al., 2013)
4-Hydroxybutyryl-CoA dehydratase	HBCD	Msed_1321	4-Hydroxybutyryl-CoA↔Crotonyl-CoA+H_2O	R (Hawkins et al., 2014)
Crotonyl-CoA hydratase/(S)-3-Hydroxybutyryl-CoA dehydrogenase (NADH)	CCH/ HBCD	Msed_0399	a) Crotonyl-CoA+H_2O↔(S)-3-Hydroxybutyryl-CoA b) (S)-3-Hydroxybutyryl-CoA+NAD↔Acetoacetyl-CoA+NADH	NP (Ramos-Vera et al., 2011), R (Hawkins et al., 2014)
Acetoacetyl-CoA β-ketothiolase	AACT	Msed_0656	Acetoacetyl-CoA+CoA↔2 Acetyl-CoA	NP (Ramos-Vera et al., 2011), R (Hawkins et al., 2014)

[a] NCE=native cell extract; NP=native purified; R=recombinant purified.

et al., 2013), suggesting the potential for fast pathway kinetics. Components of the 3HP/4HB cycle can be identified in genomes within the crenarchaeal order Sulfolobales (Kockelkorn and Fuchs, 2009), and has been studied most intensively in the extremely thermoacidophilic archaeon *Metallosphaera sedula* (T_{opt}=73 °C; pH_{opt}=2.0) (Auernik and Kelly, 2010; Berg et al., 2010; Hawkins et al., 2014). The cycle can be divided into three sub-pathways to track the reduction of CO_2 into acetyl-CoA. In the first sub-pathway, acetyl-CoA is carboxylated by acetyl-CoA/propionyl-CoA carboxylase (ACC) and subsequently reduced to the stable intermediate 3HP (Fig. 1, reactions 1–3). In the second sub-pathway, 3HP is ligated to coenzyme A (CoA), reduced to propionyl-CoA, carboxylated by ACC, converted to succinyl-CoA, which is further reduced to the second stable intermediate 4HB (Fig. 1, reactions 4–12). In the third sub-pathway, 4HB is ligated to CoA and cleaved to regenerate the starting substrate and produce an additional molecule of acetyl-CoA (Fig. 1, reactions 13–17). Cellular intermediates used for biomass generation are drawn from the cycle through the intermediate acetyl-CoA, as well as through succinic semialdehyde via oxidation to succinate by succinic semialdehyde dehydrogenase (Fig. 1, reactions 18–22) (Estelmann et al., 2011). Isotopic labeling studies in *M. sedula* have shown that 65% of cellular intermediates are formed via succinate, while the remaining 35% of carbon enters cellular metabolism via acetyl-CoA (Estelmann et al., 2011). Putative cycle enzymes have been previously characterized to various extents, although some remain to be verified and characterized in purified form (Alber et al., 2006, 2008; Han et al., 2012; Hawkins et al., 2013, 2014; Hugler et al., 2003; Kockelkorn and Fuchs, 2009; Ramos-Vera et al., 2011; Teufel et al., 2009).

Outside of its natural context, there are many opportunities to use 3HP/4HB cycle as a route to renewable production of chemicals. The complete cycle for metabolic engineering could enable the production of chemicals directly from CO_2, while an alternative is to use portions of the complete cycle for production of chemicals from sugars via the intermediate acetyl-CoA. Prior to introducing all or parts of the 3HP/4HB cycle into a metabolically engineered host organism, it is useful to identify and address potential bottlenecks. To this end, genome-scale flux balance modeling has been used extensively to assist metabolic engineering of model microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae* (Kerkhoven et al., 2014; McCloskey et al., 2013). However, enzyme kinetics-based models can account for features such that the interplay of biochemical reaction pathways can be considered, making them valuable for testing metabolic engineering strategies (Kerkhoven et al., 2014; Loder et al., 2015). Here, we describe the development of such a model to explore aspects of the 3H/4HB cycle first as it operates in its native form in the model extreme thermoacidophile *Metallosphaera sedula* and then as a basis for production of bio-based fuels and chemicals, focusing on the cycle intermediates acetyl-CoA, 3HP, and succinate.

2. Materials and methods

2.1. Materials

Growth conditions for *M. sedula* (DSM 5348) and genomic DNA purification were conducted, as reported previously (Auernik and Kelly, 2010). Strains and vectors used for cloning included the pET-46b EK/LIC cloning Kit, pRSF-2 Ek/LIC Vector Kit, pCDF-2 Ek/LIC Vector Kit, Novablue GigaSingles™ *E. coli* competent cells (Novagen, San Diego, CA), and Rosetta™ (DE3) *E. coli* competent cells (Stratagene, La Jolla, CA). The reagents and devices used include: *n*-propionyl-Coenzyme A lithium salt, succinyl-CoA sodium salt, succinic semialdehyde solution, NADPH, ATP, and sodium acrylate (Sigma Chemical Co., St. Louis, MO); GelCode Blue Stain Reagent (Thermo Fisher Scientific Inc., Rockford, IL); Bio-Rad Protein Assay Dye Reagent (Hercules, CA), 3-hydroxypropionic acid (TCI America, http://www.tciamerica.net/); Quickload DNA Ladder (100 bp) (New England Biolabs, Ipswich,

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