Chapter 8
Cryptosporidium Metabolism

Guan Zhu and Fengguang Guo

Abstract Rather than the presence of unique metabolic pathways, it is the absence of many pathways that characterizes the metabolism of *Cryptosporidium*. In fact, this genus of parasites has lost its ability of synthesizing de novo virtually all nutrients such as amino acids, nucleotides and fatty acids, thus relying on a large number of transporters to scavenge nutrients from the host. Members of this genus lack an apicoplast and associated pathways that are present in other apicomplexans. They lack cytochrome-based respiration, and rely mainly on glycolysis for energy production. Core metabolic pathways are highly streamlined, and redundancy is rare. These features make *Cryptosporidium* different from other apicomplexans. This chapter summarizes these features based on the analysis of genome sequences and published biochemical data in the context of drug targets and drug development.

8.1 Introduction

The genus *Cryptosporidium* comprises many species with different host specificities. *C. parvum* and *C. hominis* are the major human pathogens. The genomes of *C. parvum* and *C. hominis* have been reported (Abrahamsen et al. 2004; Xu et al. 2004), that of *C. muris* has been sequenced but remains to be published, and many more are being sequenced by an NIH-funded "Comparative Genomics of *Cryptosporidium* Species" project (http://www.genome.gov/26525388). The three sequenced genomes are accessible at GenBank and EuPathDB (http://www.EuPathDB.org). The availability of these genome sequences has greatly contributed to our understanding of parasite biology, and provided new opportunities to study *Cryptosporidium* metabolism. Currently, our

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knowledge on *Cryptosporidium* metabolism is mainly derived from genome annotations and analyses. A growing but limited number of enzymes have been investigated for their functions, biochemical features and potentials as therapeutic targets. However, systematic studies at the pathway level are still limited. Two reviews on *Cryptosporidium* biochemistry and metabolism were published as a book chapter in 2008 and a journal article in 2010 (Rider and Zhu 2010; Zhu 2008), These publications were mainly based on the *C. parvum* and *C. hominis* genomes and biochemical data available then. In this chapter, we will highlight the major metabolic features in *Cryptosporidium* with the addition of the newly sequenced *C. muris* genome and up-to-date biochemical data.

8.2 General Features of *Cryptosporidium* Metabolism

Phylogenetic and phylogenomic analyses have consistently placed *Cryptosporidium* at the base of the Apicomplexa with a closer relationship to gregarines than coccidia and haematozoa (Barta and Thompson 2006; Templeton et al. 2010; Zhu et al. 2000a). This view differs from the conventional taxonomy that considers *Cryptosporidium* as a sister group to the intestinal coccidia. The divergence of cryptosporidia from coccidia is also supported at the genomic and metabolic level. *Cryptosporidium* species have highly compact genomes (i.e., ~10 Mb), which are 3–5 times smaller than those of other apicomplexans and feature short intergenic sequences and very few introns (Abrahamsen et al. 2004; Xu et al. 2004). More importantly, this genus of parasites has lost the ability to synthesize virtually any nutrients de novo, such as amino acids, nucleotides and fatty acids. *Cryptosporidium* also lacks an apicoplast and mitochondrial genomes and associated metabolic pathways that are present in coccidia and haematozoa (Abrahamsen et al. 2004; Xu et al. 2004; Zhu et al. 2000b). Members of the genus possess a mitochondrial remnant that lacks the cytochrome-based respiratory chain, and retains only limited functions such as the assembly of ion-sulfur clusters (Lei et al. 2010; Kang et al. 2008; Keithly et al. 2005; Slapeta and Keithly 2004; Roberts et al. 2004; Riordan et al. 2003). Therefore, *Cryptosporidium* metabolism is extremely simplified. This feature differentiates these species from the evolutionarily more closely related gregarines that are able to synthesize many nutrients (e.g., Templeton et al. 2010).

Because *Cryptosporidium* cannot synthesize most nutrients de novo, it relies on a large family of transporters to scavenge nutrients such as amino acids (~11 transporters), sugars (~9), and nucleotides (at least one) (Abrahamsen et al. 2004; Xu et al. 2004). About 24 putative ATP-binding cassette (ABC) transporters can be identified in the *C. parvum* genome, but their substrates remain to be determined (Benitez et al. 2007; Li and Mun 2005; Bonafonte et al. 2004; Zapata et al. 2002; Perkins et al. 1999). ABC transporters are a large family of proteins with various substrate preferences including ions, sugars, amino acids, peptides, lipids, sterols and drugs. Therefore, many of them could be utilized by the parasite to scavenge

nutrients. The parasite also possesses at least seven P-type ATPases (P-ATPases) that are mostly involved in cation transport. Among them, a putative Ca^{2+}-ATPase and a heavy metal ATPase with binding specificity for reduced copper [Cu(I)] have been reported (LaGier et al. 2002, 2001; Zhu and Keithly 1997). One of the CpATPases belongs to the phospholipid transporter family and may be involved in lipid transport or membrane remodeling.

8.3 Carbohydrate and Energy Metabolisms

Carbohydrates are a source of energy and serve as building blocks for various biomolecules. *Cryptosporidium* is able to synthesize amylopectin as an energy storage polysaccharide. This biosynthetic capability is supported by earlier biochemical analyses and the presence of two glycogen branching enzymes (Harris et al. 2004; Zhang et al. 2012). The parasite can use polysaccharides, disaccharides or hexoses (e.g., glucose) to produce pyruvate and acetyl-CoA via the glycolytic pathway (Fig. 8.1). However, it employs two pyrophosphate-dependent phosphofructokinase (PPi-PFK) isoforms, rather than an ATP-PFK, to minimize the consumption of ATP. It also uses a unique pyruvate: $NADP^+$ oxidoreductase comprised of pyruvate:ferredoxin oxidoreductase (PFO) and P450 reductase domains, rather than a pyruvate dehydrogenase complex to convert pyruvate into acetyl-CoA (Abrahamsen et al. 2004; Ctrnacta et al. 2006; Rotte et al. 2001). It has been speculated that $NADP^+$ oxidoreductase is associated with the anti-cryptosporidial action of nitazoxanide (NTZ) that is currently the only drug approved by the FDA to treat cryptosporidial infections in immune-competent patients (Coombs and Muller 2002). Unlike classic inhibitor-enzyme interactions, NTZ may not act on $NADP^+$ oxidoreductase. Rather, it is converted to a biotoxic free radical molecule by the PFO domain, similar to the reductive activation of 5-nitroimidazole metronidazole by PFO in the anaerobic protists *Trichomonas* and *Giardia* (Leitsch et al. 2011; Crossnoe et al. 2002; Yarlett et al. 1986). To better understand the enzymes in the carbohydrate pathway, protein crystals have been obtained for *C. parvum* LDH, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), and pyruvate kinase (Nguyen et al. 2011; Cook et al. 2009; Senkovich et al. 2005). The crystal structures of pyruvate kinase and triosephosphate isomerase are resolved, and the structure of the active site of pyruvate kinase displays no obvious difference compared to the human homologue (Nguyen et al. 2011; Cook et al. 2009).

There are at least two types of cryptosporidial mitochondrial remnants: the "intestinal-type" *C. parvum* and *C. hominis* lack both the tricarboxylic acid (TCA) cycle and the cytochrome-based respiratory chain (Abrahamsen et al. 2004; Xu et al. 2004); whereas the "gastric-type" *C. muris* similarly lacks the respiratory chain but, based on the *C. muris* genome annotation, retains a complete set of enzymes for the TCA cycle and a type II NADH dehydrogenase (unpublished). The absence of an electron transport chain in all three species indicates that their mitochondria are unlikely to be a major source of energy,

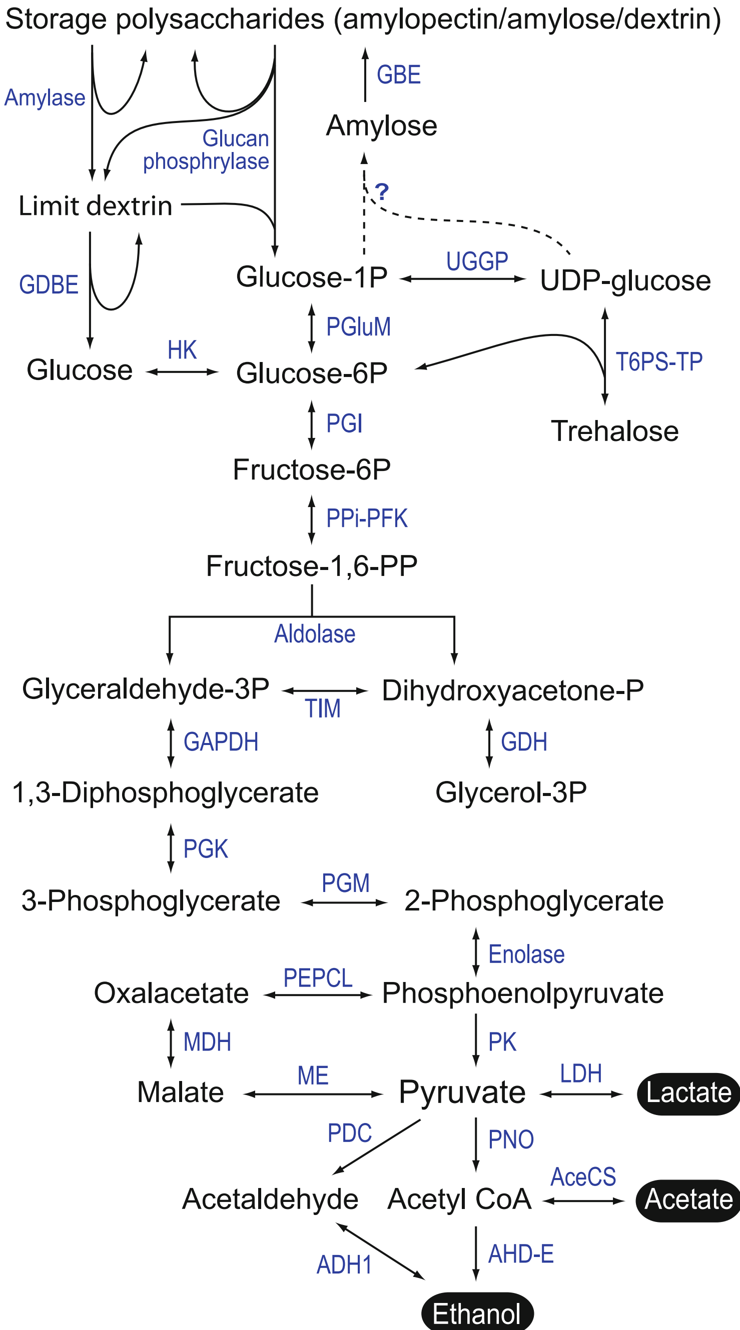

Fig. 8.1 Illustration of the carbohydrate metabolic pathway in *Cryptosporidium*, in which the core components are glycolytic and fermentative enzymes. Abbreviations: *AceCS* acetyl-CoA synthetase (also known as acetate-CoA ligase), *ADH1* alcohol dehydrogenase 1 (monofunctional), *ADH-E* type E alcohol dehydrogenase (bifunctional), *GDH* glycerol phosphate dehydrogenase, *GAPDH* glyceraldehyde phosphate dehydrogenase, *GBE* glycogen branching enzyme, *GDBE* glycogen

although it is possible that a certain level of electron potential may be generated via the type II NADH dehydrogenase in *C. muris*. Without respiration, the TCA cycle is possibly used by *C. muris* to supply intermediate metabolites. Additionally, all three genomes encode a plant-type alternative oxidase, which may be involved in detoxification of oxygen and/or generating certain electron potential.

Cryptosporidium does not carry a mitochondrial genome and the machinery for the replication, transcription and translation of organellar genomes. In addition to the enzymes discussed above, their nuclear genomes also encode a number of other proteins with mitochondrial-targeting signals, which include several translocases of outer and inner membranes, heat-shock proteins (HSPs), solute carriers, nucleotide anti-porters, ferredoxin and ferredoxin reductase and a small set of enzymes involved in ion-sulfur [Fe-S] cluster assembly (Abrahamsen et al. 2004; Lei et al. 2010; Kang et al. 2008; Mogi and Kita 2010; LaGier et al. 2003). It is believed that the [Fe-S] cluster assembly is one of the core functions retained in the mitochondrial remnant.

Collectively, we may conclude that *Cryptosporidium* relies mainly on glycolysis to produce energy. This notion is also supported by the presence of fermentative enzymes for producing three organic end products to avoid the accumulation of pyruvate and acetyl-CoA at the end of glycolysis (Fig. 8.1). These include lactate produced by lactate dehydrogenase (LDH), acetic acid by acetyl-CoA synthetase (AceCS; also known as acetate-CoA ligase, AceCL) and alcohol by a type-E bifunctional alcohol dehydrogenase (ADH-E) from acetyl-CoA or monofunctional ADH coupled with pyruvate decarboxylase from pyruvate (Zhang et al. 2012). Among them, LDH and ADH are bacterial-type enzymes. *Cryptosporidium* LDH originated from malate dehydrogenase (MDH) by a relatively recent gene duplication event which occurred after this genus separated from other apicomplexans. In fact, all apicomplexan MDH and LDH are bacterial-type, derived from an α-proteobacterial MDH (Zhu and Keithly 2002; Madern et al. 2004). Interestingly, a recent microarray-based transcriptome analysis also revealed that LDH has the highest level of expression among all genes in *C. parvum* oocysts, suggesting that the parasite mainly depends on LDH to keep the glycolytic pathway unobstructed in the external environment (Zhang et al. 2012).

Cryptosporidium possesses a plant-type pathway for synthesizing trehalose, which is accomplished by UDP-glucose/galactose pyrophosphorylase (UGGP) and a bifunctional enzyme fusion containing trehalose-6P synthase and trehalose phosphatase (T6PS-TP) (Yu et al. 2010). The presence of trehalose in *C. parvum* oocysts has been confirmed biochemically (Yu et al. 2010). However, the genome

Fig. 8.1 (continued) debranching enzyme, *HK* hexokinase, *LDH* lactate dehydrogenase, *MDH* malate dehydrogenase, *ME* malic-enzyme, *PDC* pyruvate decarboxylase, *PEPCL* phosphoenolpyruvate carboxylase, *PGI* phosphoglucose isomerase, *PGluM* phosphoglucose mutase, *PGK* phosphoglycerate kinase, *PGM* phosphoglycerate mutase, *PK* pyruvate kinase, *PNO* pyruvate: $NADP^+$ oxidoreductase, *PPi-PFK* pyrophosphate-dependent phosphofructokinase, *T6PS-TP* trehalose-6-phosphate synthase-trehalose phosphatase, *TIM* triosephosphate isomerase, *UGGP* UDP-galactose/glucose pyrophosphorylase

lacks trehalase, suggesting that the parasite is unlikely to reuse trehalose as a carbon source, unless using reversed reactions by UGGP and T6PS-TP. This differs from the intestinal coccidian *Eimeria* that has a trehalase, but lacks UGGP and T6PS-TP. Instead, *Eimeria* possesses a mannitol cycle that is absent in most other apicomplexans, including *Cryptosporidium* (Coombs and Muller 2002; Schmatz 1989). Trehalose and mannitol are known to function as anti-desiccants, antioxidants or protein-stabilizing agents in microorganisms, plants and some invertebrates, thus likely playing an important role in protecting the parasite against environmental stress.

The glycolytic pathway also provides GDP-mannose derived from fructose-6P or mannose-6P for N-glycan biosynthesis. *Cryptosporidium* appears to have a complete set of enzymes for synthesizing N-glycans in the lumen of the endoplasmic reticulum (ER) (e.g., various asparagine-linked glycosylation [ALG] transferases and an oligosaccharidyl-lipid flippase RFT1). N-glycan synthesis is also connected to the GPI (glycosyl-phosphatidyl-inositol) anchor synthesis. Like other apicomplexans and protists, these parasites lack enzymes to make more complex N-glycans in the Golgi apparatus that are common in fungi and plants. The *C. parvum* genome encodes ~30 mucin-like proteins, many of which are (or are predicted to be) membrane or secretory proteins based on the presence of signal peptides (e.g., Cevallos et al. 2000a, b; Barnes et al. 1998; Chatterjee et al. 2010). Most of the mucins contain both N- and O-glycosylation sites, and at least four enzymes involved in mucin-type O-glycosylation have been identified in the *C. parvum* genome, including UDP-N-acetyl-D-galactosamine-polypetide N-acetyl-galactosaminyl transferases (Wanyiri and Ward 2006). The involvement of mucins in parasite attachment to, and invasion of, host cells is being actively investigated. Binding of some of the mucin-like proteins by antibodies can block or reduce infection in vitro and/or in vivo, suggesting that mucins may be targets for developing immunotherapeutics (Wanyiri and Ward 2006).

8.4 Amino Acid Metabolism

Cryptosporidium cannot synthesize any amino acids de novo, which differs from other apicomplexans that possess complete pathways to make at least some amino acids. Instead, it retains only enzymes to interconvert certain amino acids coupled with other metabolic pathways. These include: glutamine synthetase for recycling glutamate produced by GMP synthetase back to glutamine; serine hydroxymethyl transferase for converting glycine to serine within the folate cycle; asparagine synthetase for producing asparagine from aspartate (which may be required to recycle ammonia released by AMP-deaminase); S-adenosylmethionine (SAM or AdoMet) synthetase to catalyze the formation of SAM to serve as an important methyl donor for transmethylation; and S-adenosylhomocysteine (SAH) synthase (SAHS; also known as SAH hydrolase, SAHH) for converting SAH derived from SAM after transmethylation to homocysteine and adenosine.

Among these enzymes, the general molecular and biochemical features of *C. parvum* SAHH (CpSAHH) has been characterized. Its inhibitors D-eritadenine and 9-(S)-(2, 3-dihydroxypropyl)adenine [(S)-DHPA] display efficacy at low micromolar levels against growth of *C. parvum* in vitro (Ctrnacta et al. 2007, 2010).

8.5 Nucleotide Metabolism

Most apicomplexans scavenge purines, but are capable of synthesizing pyrimidines de novo. However, *Cryptosporidium* lacks synthetic pathways for both purines and pyrimidines. The highly simplified purine salvage pathway starts with the uptake of adenosine by a nucleoside transporter. Adenosine is converted to AMP by adenosine kinase, IMP by AMP deaminase, XMP by IMP dehydrogenase (IMPDH), and GMP by GMP synthase (Fig. 8.2) (Striepen and Kissinger 2004). IMPDH genes in *C. parvum* and *C. hominis* were acquired from ε-proteobacteria, and differ from the eukaryotic type IMPDHs found in humans, animals and other apicomplexans (Striepen et al. 2002). However, an IMPDH gene has yet to be identified in the current version of the *C. muris* genome. Based on the essential role of this enzyme, *C. muris* needs IMPDH unless it directly scavenges GMP from the host. The *C. parvum* and *C. hominis* IMPDH genes are located at the end of chromosome 6. As this region is not represented in the sequenced *C. muris* genome, it is likely that a *C. muris* IMPDH gene is located in an unsequenced region.

Because *Cryptosporidium* lacks hypoxanthine-xanthine-guanine phosphoribosyl transferase to serve as an alternative purine salvaging pathway, blocking this AMP-GMP pathway can effectively kill the parasite. The bacterial-type IMPDH that is highly divergent from humans and animals has been considered an attractive drug target. Its protein structure has been determined, and a number of potent inhibitors have been identified and are being evaluated for drug development (Johnson et al. 2013; Gorla et al. 2012; Sharling et al. 2010; Umejiego et al. 2004, 2008).

For pyrimidine salvaging, *Cryptosporidium* may utilize uracil, uridine and cystidine by converting them to UMP and CMP by uridine kinase (UK) and a bifunctional enzyme with UK fused to uracil phosphoribosyltransferase (UK-UPRT) (Fig. 8.2). Thymidine can also be used and converted to dTMP by a bacterial-type thymidine kinase (TK), which is subsequently converted to dUMP by thymidylate synthase (TS) coupled with the folate cycle, and to dCMP by dCMP deaminase. dCMP, CMP and UMP can be further converted to dCDP/dCTP, CDP/CTP and UDP/UTP by a multi-functional UMP kinase and UDP kinase. The three pyrimidine nucleotide pathways are interconnected by a ribonucleoside-diphosphate reductase enzyme. Therefore, inhibition of a single pathway may be insufficient to block the pyrimidine synthesis, unless interconvertion is restricted by the presence of rate-limiting enzymes and/or if the supply of any single source of precursors is limited. Although the pyrimidine salvaging pathways appear to be redundant, a recent study has shown that

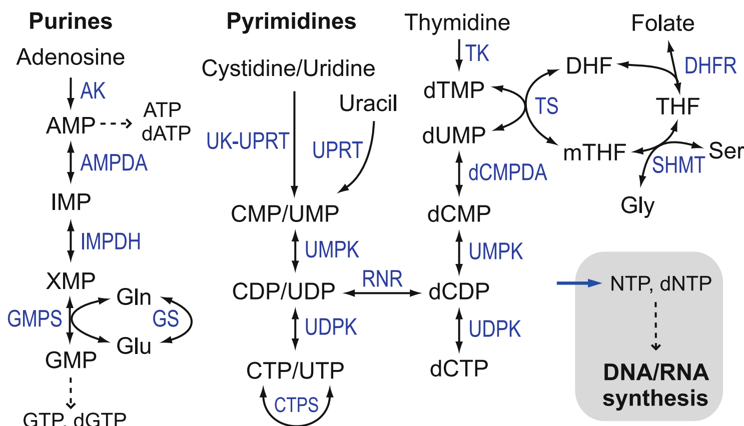

Fig. 8.2 Streamlined purine and pyrimidine salvaging pathways and folate cycle in *Cryptosporidium*. Abbreviations: *AK* adenosine kinase, *AMPDA* AMP deaminase, *CTPS* CTP synthase, *dCMPDA* dCMP deaminase, *DHFR* dihydrofolate reductase, *GMPS* GMP synthase (glutamine-hydrolyzing), *IMPDH* IMP dehydrogenase, *RNR* ribonucleoside-diphosphate reductase, *SHMT* serine hydroxymethyl transferase, *TK* thymidine kinase, *TS* thymidylate synthase, *UDPK* UDP/CDP kinase, *UK* uridine kinase, *UMPK* UMP/CMP kinase, *UPRT* uracil phosphoribosyltransferase

TK-mediated pro-drug activation may be utilized as an effective strategy for treating cryptosporidiosis (Sun et al. 2010).

In the folate cycle, dihydrofolate reductase and thymidylate synthase (DHFR-TS) are fused into a bifunctional enzyme in apicomplexans and some other protists (Vasquez et al. 1996). The linker between the DHFR and TS domains in *Cryptosporidium* is unique as it contains an 11-residue α-helix with extensive interactions with the opposite DHFR-TS monomer of the homodimeric enzyme (O'Neil et al. 2003). The active site of *C. parvum* DHFR contains unique residues that are analogous to the point mutations associated with antifolate resistance in other DHFRs, suggesting CpDHFR may be intrinsically resistant to some antifolate inhibitors (Vasquez et al. 1996). However, several novel CpDHFR inhibitors have been identified using a yeast complementation system and structure-based virtual screens, but their anti-cryptosporidial activity in vitro or in vivo remains to be determined (Senkovich et al. 2009; Martucci et al. 2009; Bolstad et al. 2008; Popov et al. 2006; Anderson 2005; Lau et al. 2001; Brophy et al. 2000).

8.6 Lipid Metabolism

Fatty acids are a source of energy in many organisms and major components of all biomembranes. However, *Cryptosporidium* is unable to use fatty acids as an energy source due to the absence of the β-oxidation pathway. It also lacks an apicoplast and

its associated pathways such as isoprenoid synthesis and the Type II fatty acid synthase (FAS) system. Therefore, the parasite cannot synthesize fatty acids (Zhu 2004). However, *Cryptosporidium* possesses a 25-kb intronless Type I FAS gene that resembles bacterial polyketide synthase (PKS) and predicts a ~900 kDa megasynthase comprised of at least 21 enzymatic domains (Zhu et al. 2000c). The basic biochemical features have been studied using recombinant proteins. Its N-terminal loading unit containing an acyl-ligase (AL) and an acyl-carrier protein (ACP) has a substrate preference towards long chain fatty acids (LCFAs), indicating that CpFAS1 functions as a fatty acid "elongase" rather than synthesizing fatty acids (Zhu et al. 2004). This notion is further supported by functional analysis of its C-terminal reductase domain that is only active with very long chain (VLC) fatty acyl-CoAs (i.e., >C20:0) (Zhu et al. 2010). The reductase domain-catalyzed reductive reaction may release final products as fatty acyl aldehydes or fatty acyl alcohol, which differs from classic Type I FAS (in humans and animals) and Type II FAS (in prokaryotes, plants and plastid-containing apicomplexans) that use thioesterase to release acyl chains as fatty acids by hydrolysis (Zhu 2004). Between the loading unit and the reductase domain are three internal acyl elongation modules, each consisting of a complete set of 6 enzymes: (1) ACP for carrying acyl chains; (2) acyl-transferase (AT) for loading malonyl-CoA and transferring an acyl-chain from the previous module to ACP; (3) ketoacyl-ACP synthase (KS) for condensing a two-carbon (C2) unit from malonyl-CoA into the acyl-chain by a carboxylation reaction; (4) ketoacyl-ACP reductase for the reduction of a keto group; (5) hydroxyacyl-ACP dehydrase for the dehydration of a hydroxyl group; and (6) enoyl-ACP reductase for the reduction of double bonds (Zhu 2004; Zhu et al. 2000c, 2004, 2010). Therefore, at least three C2 units can be added into the acyl precursors (e.g., C16:0 palmitic acid) to form very long fatty acyl chains (e.g., C22:0) that are released as fatty acyl aldehydes or alcohol. Because aldehydes are biotoxic, fatty acyl alcohols are likely the final products that can be produced by two series of reductive reactions.

In parallel to Type I FAS, the *Cryptosporidium* genome also encodes a giant 45-kb intronless PKS, which represents the first PKS discovered in a protist (Zhu et al. 2002). Molecular and biochemical analysis reveals that CpPKS1 is similarly structured as CpFAS1, but contains seven internal acyl elongation modules that lack one or more of the five enzymatic domains. Therefore, elongated acyl chains will contain keto groups, hydroxyl groups and/or double bonds, which are characteristic of polyketides. The CpPKS1 loading unit also displays substrate preference towards LCFAs, suggesting the final product(s) may contain 30 or more carbons (Fritzler and Zhu 2007).

The ACP domains in all types of FAS and PKS systems require a post-translational modification by phosphopantetheinyl transferase (PPT) to add a prosthetic phosphopantetheine to a serine residue to become a functional holo-ACP. There are two types of PPT with different substrate preferences: SFP-type for activating Type I ACPs and ACPS-type for Type II ACPs. In fact, the types of PPT present in various apicomplexans match well with the types of FAS systems. For example, *Cryptosporidium* possesses only Type I FAS and SFP-PPT,

Plasmodium has only Type II FAS and ACPS-PPT, while *Toxoplasma* contains both types I and II FAS and both SFP-PPT and ACPS-PPT. The activation of CpFAS1-ACP domains by SFP-PPT has been biochemically characterized and demonstrated (Cai et al. 2005).

In addition to type I FAS and PKS, *Cryptosporidium* possesses another set of enzymes capable of elongating fatty acyl chains, in which the substrates are fatty acyl-CoA thioesters, rather than acyl-ACP. The hallmark enzyme is a long chain acyl elongase (LCE). The biochemical features of CpLCE1 have been characterized using recombinant protein expressed in HEK-293 T cells as its expression in bacterial systems was found to be difficult (Fritzler et al. 2007). CpLCE1 is a membrane protein localized on the surface of sporozoites and the parasitophorous vacuole membrane (PVM). Localization on the surface contrasts with CpFAS1 and CpPKS1 that are mainly cytosolic. CpLCE1 is able to add a single C2 unit to LCFAs with substrate preference towards C14:0 myristoyl-CoA and C16:0 palmitoyl-CoA (Fritzler et al. 2007).

Long chain fatty acyl-CoA synthetase (ACS; aka fatty acid-CoA ligase, ACL) is another family of important enzymes in lipid metabolism. It catalyzes the first reaction in all fatty acid metabolisms by activating free fatty acids to form fatty acyl-CoA thioesters except for the Type I and II FAS/PKS systems. In fact, AL domains in Type I FAS/PKS systems share similar molecular and biochemical properties with ACS, and can also catalyze the formation of fatty acyl-CoA (Fritzler and Zhu 2007). *Cryptosporidium* possesses three ACS genes, which are under investigation in our laboratory. Our data have shown that CpACS enzymes prefer LCFAs as substrates and their inhibitors could inhibit the growth of *C. parvum*, suggesting that ACS can be explored as a novel therapeutic target in the parasite (unpublished observation).

Cryptosporidium also possesses a long-type fatty acyl-CoA binding protein (ACBP) that is responsible for restraining the movement of fatty acyl-CoA and/or forming an acyl-CoA pool in cells. This function is important, as free fatty acyl-CoA may be harmful to cellular membranes due to its “detergent effect” if it is not restrained or immediately routed into other metabolic pathways. CpACBP1 is also a membrane protein localized to PVM. It prefers binding to LC and VLC fatty acyl-CoA thioesters (Zeng et al. 2006). More recently, a fluorescence assay was developed for CpACBP1 and used to screen 1,040 known drugs, from which 28 drugs displayed inhibitory effects on CpACBP1 at sub-micromolar concentrations. Among them, four drugs (i.e., broxyquinoline, cloxyquin, cloxacillin sodium and sodium dehydrocholate) displayed efficacies against the growth of *C. parvum* with ID_{50} values at low micromolar levels. This observation raises hopes for potential repurposing of known drugs to treat cryptosporidiosis (Fritzler and Zhu 2012).

Two distinct oxysterol binding protein (OSBP)-related proteins (ORPs) have also been identified in *C. parvum*, designated as CpORP1 and CpORP2 (Zeng and Zhu 2006). The short-type CpOPR1 contains only a ligand binding domain, while the long-type CpORP2 contains Pleckstrin homology and ligand-binding domains. Lipid–protein overlay assays have revealed that CpORP1 and CpORP2 could specifically bind to phosphatidic acid, various phosphatidylinositol phosphates

(PIPs), and sulfatide, but not to other types of lipids with simple heads. However, cholesterol was not a ligand for these two proteins. Like CpLCE1 and ACBP, CpORP1 also localized to the PVM, while CpORP2 localized only in intracellular merozoites (Zeng and Zhu 2006).

Due to the incapability of synthesizing fatty acids de novo, *Cryptosporidium* needs to scavenge fatty acids/lipids from the host. However, it is unclear how the parasite scavenges lipids as no specific fatty acid or lipid transporters have been identified or experimentally validated. A recent study has provided strong evidence that *C. parvum* is able to scavenge cholesterol from host cells and from the intestinal lumen (Ehrenman et al. 2013). Among lipoproteins, LDL is an important source of cholesterol, and *C. parvum* can obtain cholesterol that is incorporated into micelles and internalized into enterocytes by the NPC1L1 transporter. Pharmacological blockage of NPC1L1 function by ezetimibe or moderate down-regulation of NPC1L1 expression decreases parasite infectivity (Ehrenman et al. 2013).

The presence of acyl-CoA binding protein, long-chain acyl elongase, acyl-CoA synthetase and OSBP-related protein in PVM indicates that this unique membrane structure is involved in lipid metabolism including transport, activation and/or elongation of fatty acids in *Cryptosporidium*. Some ACS proteins in bacteria and yeast are also known to function as fatty acid transporters (Black and DiRusso 2007; DiRusso and Black 1999). Based on the most recent data, we have formulated a working hypothesis that fatty acids may be directly transported into the parasite via an undefined pathway(s) as free fatty acids, or by a PVM-specific ACS coupled with the formation of fatty acyl-CoA (Fig. 8.3). Free fatty acids may be elongated by the Type I FAS or PKS, or activated by ACS within the parasite to form fatty acyl-CoA. All fatty acyl-CoA thioesters may be immediately routed into subsequent metabolic pathways (such synthesis of complex lipids and biomembranes, or protein palmitoylation), undergo chain elongation by LCE, or bound to ACBP to form an acyl-CoA pool before entering subsequent pathways. *Cryptosporidium* may also scavenge phospholipids as implied by the presence of a type IV P-ATPase (cgd7_1760) with predicted substrate affinity towards phospholipids. However, it remains to be determined if this transporter is truly involved in lipid scavenging or is simply responsible for intracellular trafficking of phospholipids in parasite cells.

Cryptosporidium has a small set of enzymes involved in synthesis of complex lipids. In glycerolipid synthesis, it only retains enzymes for conversions between 1, 2-diacyl-sn-glycerol-3-phosphate, 1,2-diacyl-sn-glycerol and triacylglycerol by phosphatidate phosphatase, diacylglycerol kinase, and diacylglycerol acyltransferase 1, indicating that the parasite may store fatty acids in the form of triacylglycerol. Phosphatidyl-ethanolamine may be synthesized from diacyl-sn-glycerol and CDP-ethanolamine (produced from phospho-ethanolamine) by ethanolamine-phosphotransferase (ETHPT), or via the diacyl-sn-glycerol-3-phosphate to CDP-diacyl-glycerol to phosphatidyl-L-serine to phosphatidyl-entholamine pathway by CDP-diacylglycerol synthase (CDS), phosphatidylserine synthase and phosphatidylserine decarboxylase (PSDC). However, a phosphatidylserine synthase gene has not be identified in the *Cryptosporidium* genomes.

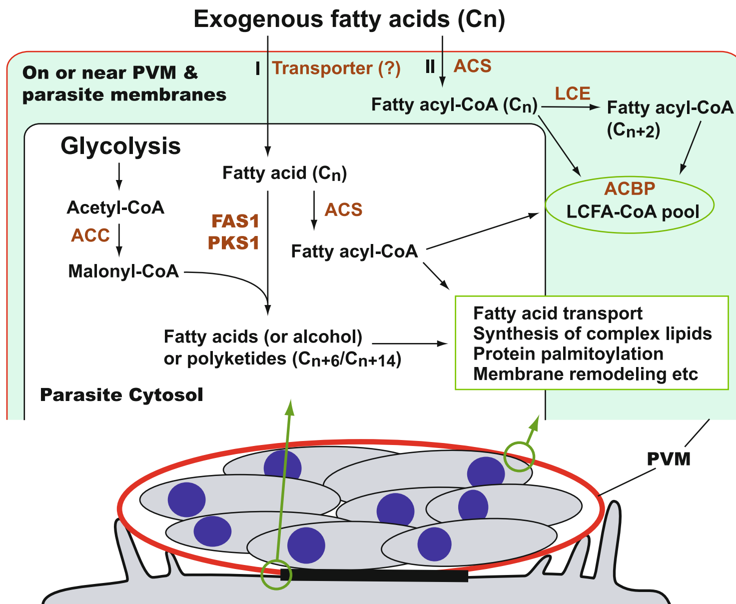

Fig. 8.3 Working hypothesis on the fatty acid metabolism associated with the parasitophorous vacuole membrane (PVM) in *Cryptosporidium*. Abbreviations: *ACBP* fatty acyl-CoA binding protein, *ACC* acetyl-CoA carboxylase, *ACS* Fatty acyl-CoA synthetase, *FAS1* Type I FAS, *LCE* long chain fatty acyl-elongase, *LCFA-CoA* long chain fatty acyl-CoA, *PKS1* Type I PKS

Additionally, enzymes involved in synthesizing lipoproteins and glycolipids are present in the *Cryptosporidium* genomes, which include up to nine DHHC family palmitoyl transferases for post-translational S-palmitoylation of proteins, and PIG-A, PIG-C, PIG-H, PIG-P, PIG-M and PIG-U involved in GPI anchor synthesis.

8.7 Stress-Related Pathways

Cryptosporidium must face various external and internal environmental stresses, including UV irradiation, temperature changes and dehydration in the natural environment, as well as hazardous chemicals, free radical molecules, drugs and host immune responses at various life cycle stages. The parasite genome encodes several classes of proteins that are important in handling these stresses, including heat shock proteins (HSPs) and various anti-oxidant molecules. HSPs are well known stress proteins that are generally up-regulated as part of the stress responses by participating in protein folding, maintaining proper protein conformation, and monitoring cellular proteins. The expression of the *CpHSP70* gene has been found to be highly up-regulated in response to chlorine-based oxidants and heat treatment (Bajszar and Dekonenko 2010).

Cryptosporidium possesses a number of anti-oxidant molecules, including superoxide dismutase (SOD), glutathione S-transferase, glutathione peroxidase, thioredoxin reductase, and a number of thioredoxin related proteins (e.g., Kang et al. 2008; Zhang et al. 2012; Yoon et al. 2012). In *C. parvum* oocysts, all three glutaredoxin-associated genes and five out of 13 thioredoxin-associated genes are expressed at various levels. Upon UV treatment, three putative t-complex protein 1 (TCP-1) subunits and several thioredoxin-associated genes are up-regulated in oocysts, whereas various HSP/DNAj family members, SOD and glutaredoxin-related genes are not up-regulated or are even down-regulated, suggesting that different stress proteins play different roles in responses to different stresses (Zhang et al. 2012).

DNA damage may occur more frequently in the natural environment than during DNA replication in the host cell due to the exposure of oocysts to UV irradiation. *Cryptosporidium* possesses a machinery for DNA excision repair. Several genes encoding excision repair enzymes were found to be up-regulated upon UV treatment, including the replication protein large subunit 1B (CpRPA1B) (Zhang et al. 2012; Rider and Zhu 2008; Rochelle et al. 2005). There are two types of RPA1 proteins in *Cryptosporidium* (i.e., RPA1A and RPA1B) which are involved in DNA replication, repair and recombination (Rider and Zhu 2008; Zhu et al. 1999; Millership and Zhu 2002). Several studies have indicated that RPA1A is mainly responsible for general DNA replication in the parasite, whereas RPA1B may play a role in DNA recombination and repair (Rider and Zhu 2008; Rider et al. 2005).

Trehalose synthesis is another important anti-stress pathway as described in Sect. 8.3 (Yu et al. 2010). Despite *Cryptosporidium* lacking amino acid synthetic pathways, a single standalone bacterial-type tryptophan synthase β-subunit gene is present in the *C. parvum* and *C. hominis* genomes. It is known that tryptophan starvation is one of the innate immunity's strategies in humans and animals to kill cells infected with certain pathogens including *Toxoplasma* and probably *Cryptosporidium* by activating the tryptophan degradation pathway (MacKenzie et al. 2007; Habara-Ohkubo et al. 1993). It is possible that *Cryptosporidium* may use tryptophan synthase β-subunit to synthesize tryptophan from indole present in the gut to evade tryptophan depletion.

8.8 Conclusions

Cryptosporidium is extremely well adapted to a parasitic life style. These parasites rely on the host to supply virtually all nutrients for their highly streamlined metabolic pathways. The insensitivity of *Cryptosporidium* to many anti-apicomplexan drugs is explained by the absence of the drug targets which are common in other apicomplexans. Examples of such pathways are de novo biosynthetic pathways and the cytochrome-based respiratory chain. Evolutionary divergence from other apicomplexans (e.g., bacterial type IMPDH and DHFR-TS with unusual amino acids at the active site) was also observed and may be relevant to

drug design. The availability of whole genome sequences provides opportunities not only to better understand the unique metabolic features of these parasites, but also to identify key enzymes and study their biochemical features of interest to drug development. Indeed, a number of potential drug targets have been proposed and/or are currently pursued by various laboratories, including protein kinases and enzymes in the carbohydrate, energy, nucleotide, and fatty acid metabolic pathways.

Currently, research on *Cryptosporidium* is still hampered by the lack of genetic tools and by technical difficulties in manipulating of the parasite in the laboratory. Therefore, recombinant protein-based biochemical analysis is still the best approach to study the metabolism and characterize potential drug targets. On the other hand, although gene knockout or knockdown tools are not available to validate drug targets, we can still effectively predict potential drug targets in the streamlined core metabolic pathways that are essential to the parasite. Drug development against cryptosporidial infection has been progressing slowly, but promising new data are being reported (see also Chap. 11). Research will be accelerated by new knowledge generated by biochemical analysis, target-based high-throughput screening of drugs, and structure-based analysis of protein-inhibitor interactions.

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