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8

Biochemistry and Metabolism of *Toxoplasma gondii*

T. Asai and S. Tomavo

8.1 INTRODUCTION

That *Toxoplasma gondii* and all other parasites of the phylum Apicomplexa reside and replicate exclusively within eukaryotic cells suggests that these parasites depend on the metabolism of their hosts and that they have evolved metabolic pathways reflecting their intracellular lifestyle. In addition, these parasites may display specific or alternate metabolism as documented by the recent discovery of novel metabolic pathways in the remnant and vestigial plastid (apicoplast) found in many apicomplexan parasites. Evidence for unique parasite metabolic pathways has direct implications for understanding the parasite's requirements for intracellular growth. Further, these pathways may provide unique targets for compounds designed to inhibit and eradicate infection caused by these important human and animal pathogens.

While targeting unique parasite pathways is an attractive strategy, in practice the procurement of enough pure parasites for biochemical study or purification of parasite components such as enzymes is extremely difficult. Biochemical and metabolic studies of *T. gondii* have also been difficult to execute because of continuous problems with contamination of parasite preparations with host-cell components. Accordingly, only a few studies of biochemistry and metabolism of *T. gondii* have been reported.

Recent advances in gene engineering technology and molecular biology, including the genome database projects, are clearing many obstacles. For instance, it is now easy to obtain a predicted amino-acid sequence of an enzyme of *T. gondii* from the genome database (http://www.ToxoDB.org/). These data can be used to clone or synthesize the gene, and to produce a recombinant enzyme to characterize the enzyme. Bioinformatic schemes

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for several metabolic pathways of *T. gondii* have been compiled and released to the public at the genome database.

This chapter discusses aspects of metabolism, including carbohydrate metabolism, glucose metabolism during tachyzoite–bradyzoite differentiation, glycolipid anchors, nucleotide biosynthetic pathways, and NTPase, a peculiar enzyme unique to *T. gondii* and the closely related parasite *Neospora caninum*. It will also focus on new insights into distinct metabolic pathways, their evolutionary roots, and their contribution to *Toxoplasma gondii* survival during intracellular development and differentiation. In addition to this chapter, metabolism of the apicoplast and mitochondrion is discussed in Chapter 9. Chapter 19 encompasses a comprehensive discussion of amino-acid and nucleotide pathways with comparison to other Apicomplexa.

8.2 CARBOHYDRATE METABOLISM

8.2.1 Developmentally regulated expression of amylopectin in *T. gondii*

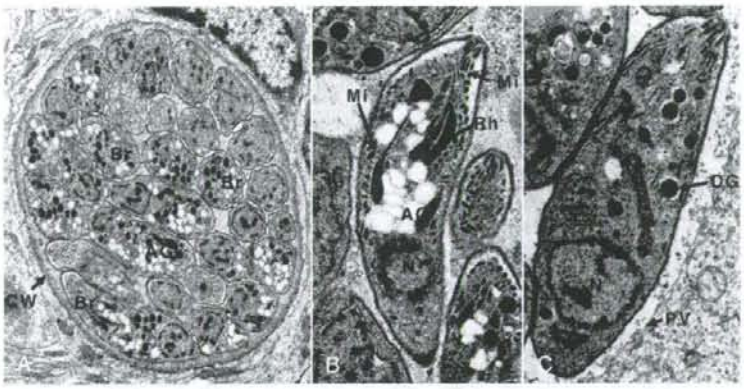
One evolutionary hallmark of the close relationship between the photosynthetic micro-organisms and apicomplexan parasites is defined by the existence of a storage polysaccharide named amylopectin in the cytoplasm of some apicomplexans. Another reflection of this relationship is the presence of a vestigial plastid 'apicoplast', discussed in Chapter 9. In contrast to plants, which contain starch defined as a branched amylopectin and amylose in the chloroplasts, the encysted bradyzoite and sporozoite forms of apicomplexans such as *T. gondii* (Coppin *et al.*, 2005), *Eimeria* (Karkhanis *et al.*, 1993), and *Cryptosporidium* (Harris *et al.*, 2004) accumulate amylopectin, a polymer of linear glucose that is not present in *Plasmodia* and *Babesia* species. It has been speculated that the disappearance of amylopectin in *Eimeria* sporozoites resulted in the inability of the parasite to establish successful infection in mice (Augustine, 1980; Nakai and Ogimoto, 1983). In *T. gondii*, the bradyzoites accumulate abundant amylopectin granules and numerous micronemes. In contrast, the tachyzoites lack amylopectin, and fewer micronemes are present (Figure 8.1). Amylopectin is probably consumed when the encysted and dormant bradyzoites switch into the rapidly replicating tachyzoites. It is postulated that amylopectin provides an energy source, as its degradation to glucose can provide metabolic intermediates or substrates for glycolysis or mitochondrial oxidative phosphorylation.

8.2.2 *T. gondii* displays a uniquely simple pathway for amylopectin synthesis

Using bioinformatic searches, several gene candidates encoding enzymes that are probably involved in amylopectin biosynthesis were identified (Coppin *et al.*, 2005). These putative enzymes can be grouped in two classes:

1. Enzymes that are involved in amylopectin synthesis, such as amylopectin synthase, branching enzymes, UDP-glucose pyrophosphorylase, isoamylase, indirect debranching enzyme, α-1, 4-glucanotransferase, and glycogenin
2. Enzymes for amylopectin degradation, like α-amylase, dikinase or R1 protein, phosphorylase, and α-glucosidase. Based on the presence of these enzymes, metabolic pathways and enzymes involved in amylopectin synthesis in *T. gondii* are probably similar to those of starch synthesis in the unicellular green algae *Chlamydomonas reinhardtii* (Figure 8.2).

Surprisingly, all of these genes are present in *Toxoplasma* genome as a unique copy, suggesting that redundant genes are not required for the synthesis of a genuine crystalline amylopectin in this protozoan parasite (Ball and Morell, 2003; Coppin *et al.*, 2005). This is in violation of the current dogma that suggests that redundancy of genes is required to build a crystalline starch in plants. Even in the simplest unicellular picophytoplanktonic green algae, *Ostreococcus tauri*, there is multiplicity



T. gondii Cyst Bradyzoite Tachyzoite

FIGURE 8.1 Transmission electron micrographs of bradyzoites (Br) within a tissue cyst (A). Note the presence of the cyst wall (CW) and numerous amylopectin granules (AG) in the cytoplasm of the bradyzoites. Panels B and C shows a higher magnification of ultrastructural morphology of bradyzoite and tachyzoite which lacks amylopectin granules. Rh, rhoptry; DG, dense granules; Mi, micronemes and M, mitochondrion; N, nucleus; CW, cyst wall; PV, parasitophorus vacuole.
This figure is reproduced in color in the color plate section.

of genes and redundancy of isoenzymes involved in starch synthesis (Ral *et al.*, 2004).

Only UDP-glucose pyrophosphorylase and UDP-glucose utilizing amylopectin synthase are found in *T. gondii*. Comparative genomic analyses involving the unicellular red alga *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004), the unicellular green alga *Chlamydomonas reinhardtii*, the yeast *Saccharomyces cerevisae*, and the bacteria *Escherichia coli* revealed that both *C. merolae* and *T. gondii* contain a UDP-glucose utilizing glycogen (starch) synthase-like sequences and glycogenins. These enzymes are specific for the eukaryote UDP-glucose based pathway. In addition, UDP-glucose utilizing glycogen synthase activity has been detected in the crude extract from *T. gondii* while only ADP-glucose dependent activity is present in *Chlamydomonas* lysates (Coppin *et al.*, 2005). *T. gondii* also contains an indirect debranching enzyme, a bifunctional enzyme that carries both α-1,4-glucanotransferase and amylo-1,6-glucosidase activities in fungi and animals (Figure 8.2).

However, the characteristic most typifying the amylopectin biosynthetic pathway in *T. gondii* is the presence of genes that are of plant origin. Among the genes that distinguish plant starch metabolism from those of the animal, fungal, and bacterial glycogen pathways are the isoamylase and R1 (glucan water dikinase activity)-like sequences in *T. gondii*. This suggests that both plant- and animal-like amylopectin biosynthetic pathways are required for the synthesis of crystalline amylopectin in the parasite (Figure 8.2).

8.2.3. Evolutionary origins of enzymes of amylopectin biosynthesis

Both plant- and animal-like metabolisms are probably involved in amylopectin biosynthesis in *T. gondii*. This is likely a signature of the evolutionary origin of apicomplexan parasites. These parasites contain a vestigial plastid 'apicoplast' that is derived from a secondary endosymbiosis with the engulfment of a unicellular algae (McFadden *et al.*,

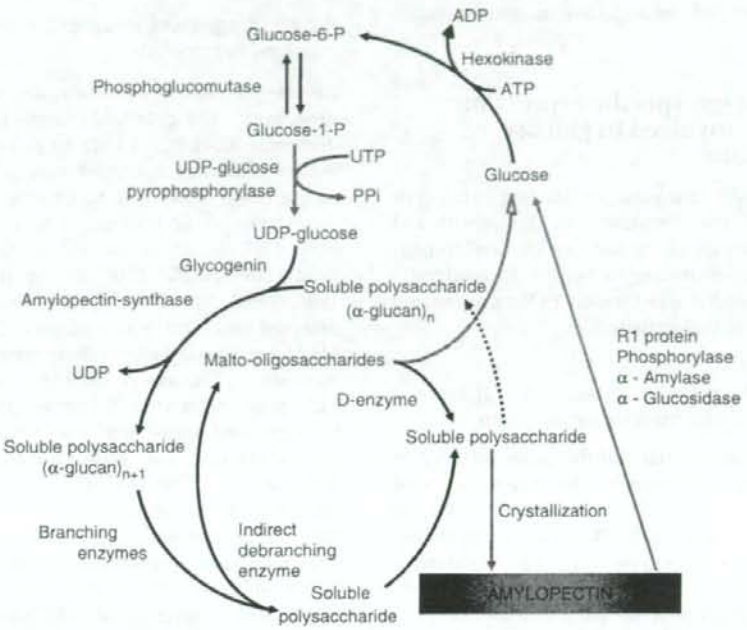


FIGURE 8.2 Proposed metabolic pathways involved in the biosynthesis of semi-crystalline polysaccharide storage, amylopectin of Toxoplasma gondii. The biosynthetic pathway and enzymes involved therein (glycogenin, UDP-glucose pyrophosphorylase, amylopectin synthase, branching enzymes, indirect debranching enzymes) are shown in black and red arrows, respectively (see color plate). The enzymes (α-amylase, R1 protein, phosphorylase and α-glucosidase) involved in the degradation pathway (green arrow) are indicated in brown. The putative genes encoding these enzymes have been identified in the genome sequence of T. gondii (http://www.ToxoDB.org/). The presence of active amylopectin synthase using UDP-glucose as a substrate has been assayed in tachyzoite and bradyzoite crude extracts.
This figure is reproduced in color in the color plate section.

1996; Köhler *et al.*, 1997; Cai, *et al.*, 2003; Waller *et al.*, 2003). Phylogenetic analyses performed with two key enzymes (amylopectin synthase and the R1 protein) demonstrate that the *T. gondii* amylopectin synthesis pathway has evolved from the red algal starch synthesizing machinery through a secondary endosymbiotic event (Coppin *et al.*, 2005). These phylogenetic data, together with the presence of the enzymes and the enzymatic activities described above, establish that apicomplexans and red algae such as *C. merolae* use a UDP-glucose pathway to build water-insoluble amylopectin. This is the pathway used by all floridean starch-accumulating organisms (Nyvall *et al.*, 1999).

It is equally apparent that apicomplexans also contain plant-like genes that are not found in yeast and mammals. These consist of genes that are required in plants and green algae and encode enzymes (water dikinase or R1 protein, α-1, 4-glucanotransferase or D-enzyme and iso-amylase) that are involved in the breakdown and synthesis of starch. The presence of these plant-like enzymes could be useful for the discovery of inhibitors that can interfere with the synthesis or

degradation of amylopectin in apicomplexan parasites.

8.2.4. Stage-specific expression of genes involved in glucose catabolism

It is noteworthy that some genes coding for enzymes involved in the biosynthesis of amylopectin and others in the glycolytic pathway are developmentally regulated during tachyzoite to bradyzoite stage conversion (see Chapter 13 for a discussion of bradyzoite differentiation).

8.2.4.1 Stage-specific expression of genes involved in amylopectin degradation

The expression pattern of the genes involved in amylopectin biosynthesis in tachyzoites and bradyzoites isolated from mouse brain cysts has been investigated by RT-PCR. Transcripts coding for enzymes known to be involved in the catabolic functions, such as the R1 protein, α-glucan phosphorylase, α-glucosidase and α-amylase, are preferentially expressed in bradyzoites (Coppin *et al.*, 2005). In constrast, transcripts coding for enzymes known to be involved in amylopectin synthesis (glycogenin, amylopectin synthase, branching enzyme) are preferentially expressed in tachyzoites but can also be detected at lower expression levels in bradyzoites.

This pattern is consistent with the production of amylopectin during differentiation of tachyzoites into bradyzoites, and with the mobilization of the glucose stores during bradyzoite to tachyzoite interconversion (Tomavo, 2001). Even though there is evidence for transcription of these genes, it remains to be determined whether the transcripts detected are translated into functional proteins and enzymes. Therefore, specific antibodies or enzymatic activities need to be tested in order to demonstrate protein synthesis. Even if the enzymatic activity of amylopectin synthase has been demonstrated in both tachyzoites and bradyzoites (Coppin *et al.*, 2005), post-transcriptional regulation may also occur on some of the stage-specific transcripts detected.

8.2.4.2 Stage-specific expression of genes involved in glycolysis

Glucose 6-phosphate isomerases and lactate dehydrogenases The glycolytic enzyme lactate dehydrogenase (LDH, EC 1.1.1.27) is a glycolytic enzyme that catalyses the interconversion of pyruvate to lactate using NAD+ as a co-enzyme (Figure 8.3). Two stage-specific LDH genes have been identified; the tachyzoite-specific *LDH1*, and the bradyzoite-specific *LDH2* (Yang and Parmley, 1995, 1997). The transcript of LDH2 was only detected in the bradyzoite stage, while mRNA of LDH1 was found in both bradyzoite and tachyzoite stages. The absence of LDH2 mRNA in the tachyzoite suggests that the transcription of LDH2 is suppressed during transition from the bradyzoite to the tachyzoite stage. Conversely, the data indicate that LDH1 is the only isoenzyme produced by the tachyzoites. The level of LDH2 mRNA increased markedly *in vitro* during bradyzoite induction, suggesting that transcription activation and/or mRNA stability may explain the stage-specific expression of LDH2 gene in *T. gondii* (Yang and Parmley, 1997). Because the predicted isoelectric points of the two LDHs are different, two-dimensional electrophoresis has been used to demonstrate that only one LDH protein is expressed in each developmental parasitic stage.

It has also been demonstrated that LDH1 and LDH2 share a unique structural feature with LDH from the malarial parasite *Plasmodium falciparum* (pLDH), namely a five-amino acid insertion into the substrate specificity loop. This insertion has only been observed in pLDH, LDH 1 and LDH2 (Bzik *et al.*, 1993; Yang and Parmley, 1997). All other LDH enzymes described so far do not contain this insertion. The insertion in LDH2 is identical to the insertion in pLDH (KSDKE), but differs slightly from the insertion in LDH1 (KPDSE).

Comparative studies on the kinetic properties of *T. gondii* LDH1 and LDH2 and *P. falciparum* LDH showed that LDH1 and LDH2 exhibit broader substrate specificity than pLDH. For both LDH1 and LDH2, 3-phenylpyruvate is an excellent substrate – even better than pyruvate when LDH2 was tested

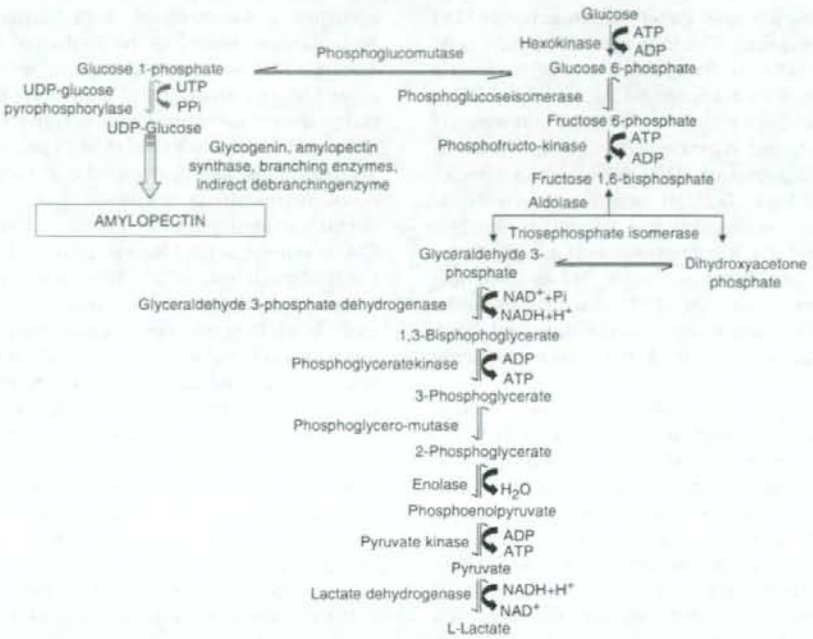


FIGURE 8.3 Schematic representation of the link between glycolysis and amylopectin biosynthesis. The classical pathway of glycolysis is shown in the right panel. Phosphoglucose isomerase, enolase and lactate dehydrogenase (shown in red; see color plate) are found as two iso-enzymes, each enzyme is stage-specifically expressed in the tachyzoites or in the bradyzoites (see more details in the text).
This figure is reproduced in color in the color plate section.

with both substrates. In contrast, pLDH does not utilize 3-phenylpyruvate (Dando *et al.*, 2001). In addition, both LDH1 and LDH2 can utilize the NAD analog 3-acetylpyridine adenine dinucleotide (APAD) efficiently, similarly to pLDH. A range of inhibitors including gossypol and derivatives inhibit LDH1, LDH2, and pLDH, but in general LDH2 is more sensitive than LDH1. LDH1 also shows substrate inhibition despite of the substitution in both LDH1 and LDH2 of a methionine for serine163, a residue that is thought to be critical for production of substrate inhibition (Dando *et al.*, 2001). Most importantly, gossypol and gossylic iminolactone have been shown to display inhibition of *T. gondii* tachyzoite growth in fibroblast cultures.

The differences in sensitivities to inhibitors between LDH1 and LDH2 further illustrate how these enzymes may have evolved to serve separate roles during stage development. It should be noted that these studies have been performed with recombinant enzymes. These observations remain to be confirmed with native purified enzymes, since other co-factors may also be involved in the modulation of enzymatic properties. Nevertheless, it can be speculated that the apparent greater sensitivity of recombinant LDH2 to gossypol and derivatives may be leads for the design of inhibitors that could be exploited as chemotherapeutic agents to eliminate cysts from chronically infected hosts.

For this purpose, the crystal structure of LDH may be required. Kavanagh *et al.* (2004) have crystallized LDH1 in its apoform and in its ternary complexes containing NAD+ or the NAD+-analogue 3-acetylpyridine adenine dinucleotide (APAD(+)) and sulphate or the inhibitor oxalate. Superimposition of LDH1 with human muscle- and heart-specific LDH isoforms reveals differences in residues that line the active site. This increases LDH1's hydrophobicity. It was concluded that these differences would aid in designing inhibitors specific for LDH1 that may be useful in treating toxoplasmic encephalitis and other complications that arise in immunocompromised patients.

Another glycolytic enzyme that is stage-specific is glucose 6-phosphate isomerase (G6-PI, EC 5.3.1.9), which catalyses the interconversion of glucose 6-phosphate to fructose 6-phosphate (Figure 8.3). A cDNA fragment encoding G6-PI was isolated from a bradyzoite-specific subtractive library and the full-length cDNA was used to complement an *E. coli* mutant lacking G6-PI (Dzierszinski *et al.*, 1999; Yahiaoui *et al.*, 1999). RT-PCR data have demonstrated that the transcript coding G6-PI is preferentially present in bradyzoites, while a minute amount can be detected in tachyzoites. Western blot analysis performed with specific polyclonal antibodies revealed G6-PI only in encysted bradyzoites, demonstrating the stage-specific expression of G6-PI in *T. gondii*. It remains to be determined, however, whether the other putative G6-PI coding genes presently described in the *Toxoplasma* genome correspond to the tachyzoite-specific G6-PI. Here, only the *T. gondii* cDNA coding G6-PI has been tested in *E. coli* complementation; its enzymatic activity with the purified enzyme has not been directly assayed.

Enolases Within the glycolytic pathway, enolase or ENO (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) catalyzes the conversion of 2-phosphoglycerate to phosphoenol pyruvate (Figure 8.3). As for LDH, two stage-specific enolase-coding genes have been described (Dzierszinski *et al.*, 1999). The two genes are located on the same chromosome and separated only by an intergenic sequence of 1.6 kilobases. Both transcript and protein corresponding to the product of the gene named ENO1 are only detected in bradyzoite,s while ENO2's transcript and protein are found in tachyzoites. The amino-acid identity between ENO1 and ENO2 was found to be 73 percent.

Interestingly, when compared with human and other mammalian's enolases, both enolases contain a pentapeptide insertion: EWGYC in ENO2, and the almost identical EWGWS motif in ENO1 (Dzierszinski *et al.*, 1999) and enolase of *Plasmodium falciparum* (Read *et al.*, 1994), respectively. In addition, another dipeptide EK/DK insertion was also found in ENO1 and ENO2 of *T. gondii* and in *P. falciparum* enolase. Superimposition of the model tridimensional structure of ENO1 or ENO2 with that of human enolase revealed a perfect match between their 3D-models except for the presence of two extra loops corresponding to the pentapeptide EWGWC and the dipeptide EK insertion, respectively. The presence of these two loops was also evident in *P. falciaprum* and, surprisingly, in plant enolases (Dzierszinski *et al.*, 1999).

The functions of these two loops were investigated by site-directed mutagenesis of the pentapeptide, the dipeptide, and both loops in the ENO1 recombinant enzymes (Dzierszinski *et al.*, 2001). The enzymatic properties of these mutated enzymes and of the wild-type enzyme demonstrated that the deletion of a single EK loop does not affect the K_m of the enzyme, but the deletion of both loops causes a 13-fold increase of the enzyme K_m. Deletion of the pentapeptide EWGWC gave a five-fold increase of the K_m compared to the values of the wild-type enzyme.

In addition, the K_m, V_{max}, and temperature stability of pure recombinant ENO1 and ENO2 enzymes were also compared. While the K_m values are identical, ENO1 and ENO2 display distinct V_{max} with a value three-fold higher for ENO2 than that for ENO1, suggesting that the two isoenzymes have the same affinity for the substrate 2-PGA but exhibit different rates of substrate consumption. The denaturation temperature of ENO1 was also found to be higher than that of ENO2, indicating that the tachyzoite ENO2 is more thermolabile than the bradyzoite ENO1.

The enzymatic properties of the two stage-specific enolases seem to be in good agreement with the metabolic and physiological adaptation required during *T. gondii* differentiation and encystation. It can be postulated that these enzymes play discrete biological functions that most probably involve profound carbohydrate metabolism modifications such as the biosynthesis or degradation of amylopectin that occurs during the stage conversion of *T. gondii*.

8.2.5 Parasite glycolytic enzymes involved in other biological functions

In eukaryotic cells, many kinds of multifunctional regulatory proteins have been identified that perform distinct biochemical functions in the nucleus, the cytoplasm, or both. Recent studies establish that metabolic enzymes display biological roles distinct from their cognate functions. Perhaps the best-studied examples are enzymes that double as eye-lens proteins essential for normal vision: lactate dehydrogenase (crystalline in ducks and crocodiles), a-enolase (crystalline in lamprey and turtles), and argininosuccinate lyase (crystalline in birds and reptiles) (Piatigorsky, 2003).

Another example of a multifunctional protein is glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which interacts with a wide variety of RNAs, including ribosomal RNA, tRNA, hammerhead ribozymes, and the 3′ untranslated region of many mRNAs. Proposed functions for GAPDH include regulation of helicase activity, tRNA and mRNA export, RNA unfolding, translational regulation, regulation of viral gene expression, and regulation of mRNA stability (Sirover, 2005). In *Plasmodium falciparum*, it has been shown that the N-terminal domain of GAPDH mediates GTPase Rab2-dependent recruitment to membranes, suggesting that GAPDH exerts non-glycolytic function(s) in the parasite, possibly including a role in vesicular transport and biogenesis of apical organelles (Daubenberger *et al.*, 2003).

In *T. gondii*, a number of studies have described several glycolytic enzymes with functions distinct from their primary functions. Using pull-down assays, Jewett and Sibley (2003) reported that the parasite aldolase is the molecular link between micronemal thrombospondin-anonymous related proteins (TRAP family) and the acto-myosin motor involved in apicomplexan parasite gliding and host-cell invasion. PRP1 (parafusin-related protein 1) is an orthologue of phosphoglucomutase that is associated with micronemes (Matheissen *et al.*, 2001, 2003).

To investigate the significance of *T. gondii* lactate dehydrogenases, LDH1 and LDH2, in the control of a metabolic flux during parasite differentiation, the expression of these two isoenzymes was knocked down in a stage-specific manner (Al-Anouti *et al.*, 2004). These LDH knockdown parasites exhibited variable growth rates in either the tachyzoite or the bradyzoite stage when compared with the wild-type parasites. Their differentiation processes were impaired *in vitro*, and they were unable to form tissue cysts in a murine model system. In addition, all mice infected with the knockdown of LDH1 and LDH2 expression gave rise to virulence-attenuated parasites, and survived a subsequent challenge with parental parasites at a dose that usually causes 100 percent mortality. It has been concluded that LDH expression is important for the cell cycle and differentiation of *T. gondii*. However, the precise mechanisms by which LDH knockdown impairs parasite growth and differentiation remain to be elucidated.

8.2.5.1 T.gondii *enolases are involved in nuclear functions*

Extensive studies performed on the stage-specific expression of enolases of *T. gondii* using the polyclonal antibodies specific to ENO1 and ENO2 revealed that both enolase isoenzymes can be detected in the nucleus of the parasite (Ferguson *et al.*, 2002). The accumulation of enolase signal in the nucleus is observed in both tachyzoites and bradyzoites, but only in dividing zoites. The nuclear signal of ENO1 in the brain of 12-day-infected mice is detected early in tachyzoites that are differentiating into bradyzoites. The expression of ENO1 in these intermediate zoites appears earlier than that of the classical bradyzoite surface

marker, P36 or SAG4, and these ENO1-expressing intermediate zoites are still expressing the tachyzoite SAG1 surface marker. In addition, it has been found that only the tachyzoite-specific ENO2 is expressed in the dividing sexual forms of *T. gondii* examined in infected cells derived from the cat. Taken together, these data suggest that this novel subcellular localization can be ascribed to novel nuclear activity displayed by ENO1 and ENO2.

It should be noted that the nuclear localization of enolase was first described in human cells where the enzyme binds to the c-Myc promoter and acts as transcriptional repressor in cancer cells (Feo *et al.*, 2000). The binding to DNA target and the domain in enolase that is involved in the transcriptional regulation has been identified (Subramanian and Miller, 2000). Interestingly, the factor isolated from cold-resistant mutants of *Arabidopsis thaliana* using genetic approaches was identified as *A. thaliana* enolase, which binds to a DNA motif similar to that described in human cells (Lee *et al.*, 2002). Thus, it could be postulated that *T. gondii* enolases might display similar transcriptional or other nuclear functions involved in the intracellular growth of the parasite. However, further experimental support needs to be provided for the precise nuclear activity of enolases in *T. gondii*.

8.3 GLYCOLIPID ANCHORS

8.3.1 Structure of *Toxoplasma* glycosylphosphatidylinositol and its role in membrane anchoring

Extensive studies have demonstrated that many proteins are attached to the eukaryote cell membranes via inositol-containing glycophospholipids (GPIs). In *T. gondii*, the major surface proteins are anchored to the parasite surface by a GPI-membrane anchor (Nagel and Boothroyd, 1989; Tomavo *et al.*, 1989). This type of anchor seems to be more frequently used in *Toxoplasma* and other protozoa than in higher eukaryotes.

When it became clear that numerous surface antigens of *T. gondii* are GPI-anchored, several studies established the structure and biosynthesis of these membrane anchors, and in particular of their putative precursors. The structure of GPI anchors of these proteins has been determined by combining metabolic labeling (tritiated glucosamine, mannose, galactose, palmitic and myristic acids, and inositol) and their sensitivity to a lipase named phosphatidylinositol phospholipase C (PI-PLC) (Tomavo *et al.*, 1989). The release of these proteins from the surface of live parasites, which causes a cross-reacting determinant (CRD) of the soluble forms to be accessible to anti-CRD serum of trypanosomes, also confirms that the major surface proteins (SAGs) of *Toxoplasma* possess GPI anchors.

Further investigations led to the determination of the *Toxoplasma* GPI anchor structure that is composed of the evolutionarily conserved linear GPI core, ethanolamine-PO_4-6Mana1-2Mana1-6Man1-4GlcNAca1-6-inositol on SAG1**61 (P30) and P23 (Figure 8.3, Tomavo *et al.*, 1992a, 1993). Candidate glycolipid precursors that are probably transferred *in bloc* to the nascent membrane proteins of *T. gondii* have been identified and isolated either in living parasites or in parasite extracts.

Four mature glycolipids and other intermediate forms have been characterized (Tomavo *et al.*, 1992b). These four major glycolipids have the same GPI core structure and can serve as preassembled precursors of GPI anchors linked to these proteins. In addition, a lipophilic 'low molecular weight' antigen (4.6 kDa), identified using human patient sera and monoclonal antibodies (Sharma *et al.*, 1983; Tomavo *et al.*, 1994), was shown to be glycophosphoinositols that are not linked to proteins (free GPIs) and localize in the plasma membrane of the parasites. These free-GPIs or low molecular weight antigens were shown to elicit an early immunoglobulin M response in humans.

The detailed structures of these free GPIs have been determined using metabolic labeling, enzymatic digestion, followed by classical chromatographic analysis, nuclear magnetic resonance, and fast-atom bombardment-mass spectroscopy (Striepen *et al.*, 1997). The following two GPI-structures were elucidated: the classical structure

(ethanolamine-PO4)-Mana1-2Mana1-6(GalNacβ1-4) Mana1-4GlcNa-inositol-PO4-lipid and a novel structure (ethanolamine-PO4)-Mana-2Mana1-6(Glca1-4GalNacβ1-4)Mana1-4GlcNa-inositol-PO4-lipid both with and without terminal ethanolamine phosphate. Only *T. gondii* GPIs bearing the unique glucose-N-acetylgalactosamine side branch are immunogenic in humans (Striepen *et al.*, 1997).

8.3.2 Role of GPIs in cell signaling and host immune response

Except for their role in membrane insertion of surface proteins in *T. gondii*, the biological functions of GPIs are presently unknown. In other eukaryotic systems, GPIs can display functions involved in signal transduction. One possible function of the GPI anchor might be to allow a closer association of the proteins with themselves and other surface proteins in the membrane (Tomavo, 1996). Consistent with this idea, genetically engineered transmembrane-anchored SAG1 does not show the usual observed association of GPI-anchored SAG1 with itself and/or other proteins (Seeber *et al.*, 1998).

As stated, *Toxoplasma* free-GPIs elucidate strong and early immunogenic responses during host infection. Data from other protozoa suggest that other functions of GPIs in host immune response are possible. In *Plasmodium falciparum*, the GPI moiety, free or associated with protein, induces tumor necrosis factor and interleukin 1 production by macrophages, and regulates metabolism in adipocytes (Schofield and Hackett, 1993). Deacylation with specific phospholipases abolishes cytokine induction. When administered to mice *in vivo* the malaria parasite GPI induces cytokine release, a transient pyrexia and hypoglycemia, and profound and lethal cachexia, in the presence of sensitizing agents. The data suggest that the GPI of *Plasmodium* is a potent glycolipid toxin that may be responsible for a novel pathogenic process. It has been further demonstrated that *Plasmodium* GPI directly and specifically increases cell adhesion molecule expression in HUVECs, and parasite cytoadherence (Schofield *et al.*, 1996). These parasites' GPIs induce rapid activation of a tyrosine kinase in macrophages.

The minimal structure requirement for tyrosine kinase activation is the evolutionarily conserved core glycan sequence Mana1,2Mana1,6Mana1-4GlcN1-6myo-inositol. The GPI alone appears sufficient to mimic the activities of malaria parasite extracts in the signaling pathway leading to TNF expression (Tachado *et al.*, 1997).

Thus, GPIs of intraerythrocytic *Plasmodium falciparum* induce pro-inflammatory cytokine responses. It was also reported that adults who have resistance to clinical malaria contain high levels of pertinent anti-GPI antibodies, whereas susceptible children lack or have low levels of short-lived antibody response. Individuals who were not exposed to *P. falciparum* completely lack anti-GPI antibodies. Absence of a pertinent anti-GPI antibody response correlated with malaria-specific anemia and fever, suggesting that anti-GPI antibodies provide protection against clinical malaria (Naik *et al.*, 2000). These results could be evaluated in studies aimed at the defining the activity of chemically defined structures for toxicity, and results would have implications for the development of GPI-based therapies or vaccines.

The *P. falciparum* GPI glycan consisting of the sequence NH(2)-CH(2)-CH(2)-PO(4)-(Mana1-2) 6Mana1-2Mana1-6Mana-4GlcNH(2)a1-6myo-inositol-1, 2-cyclic-phosphate was chemically synthesized, conjugated to carriers, and used to immunize mice infected with *P. berghei*, a rodent model of severe malaria. The recipients were substantially protected against malarial acidosis, pulmonary edema, cerebral syndrome, and fatality (Schofield *et al.*, 2002). Altogether, the above data suggest that GPI is a significant pro-inflammatory endotoxin of parasitic origin and it may contribute to pathogenesis and fatalities in humans. In addition, GPI may also be used as a prototype carbohydrate anti-toxin vaccine against malaria. It remains to be seen whether GPI has a similar role in clinical toxoplasmosis.

8.4 NUCLEOTIDE BIOSYNTHESIS

The most extensively studied metabolic pathways in *T. gondii* are those of pyrimidine and purine

nucleotide biosynthesis. These pathways provide the substrates for DNA/RNA biosynthesis, and are commonly targeted for chemotherapy. They are logical areas to study in the rapidly multiplying tachyzoite form. Due to limited material, nearly all studies have been performed exclusively in tachyzoites. Illustrations of the present overall knowledge of nucleotide biosynthesis in the tachyzoite form of *T. gondii* are shown in Figures 8.4–8.7. Comparative analyses of genome sequences for several Apicomplexa have revealed surprising differences among the Apicomplexa in nucleotide metabolism, which are discussed further in Chapter 19.

Early works determined that *T. gondii* has both *de novo* (Hill *et al.*, 1981; O'Sullivan *et al.*, 1981; Schwartzman and Pfefferkorn, 1981; Asai *et al.*, 1983a) and salvage (Pfefferkorn and Pfefferkorn, 1977, 1978; Pfefferkorn, 1978; O'Sullivan *et al.*, 1981; Iltzsch, 1993) pyrimidine nucleotide biosynthetic pathways. The *de novo* pyrimidine biosynthetic pathway is more important than the salvage pathway, and is essential for *T. gondii* growth and virulence. Pyrimidine auxotrophic mutants of *T. gondii* with disruption of the single copy carbamyl phosphate synthetase II gene (CPSII, E.C. 6.3.5.5) are avirulent in mice (Fox and Bzik, 2002). CPSII catalyzes the first step of the de novo pyrimidine biosynthetic pathway. Furthermore, pyrimidine starvation is one of the conditions that cause stage conversion from the tachyzoite to the bradyzoite (Bohne and Roos, 1997). The *de novo* pyrimidine biosynthetic pathway is shown in Figure 8.4.

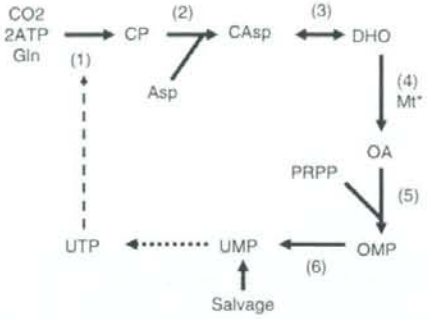


FIGURE 8.4 *T. gondii* pyrimidine de novo biosynthetic pathways. Solid lines represent activities that were detected in *T. gondii*, short dashed lines represent activities of uridylate kinase and nucleoside diphosphate kinase that are not considered to be *de novo* enzymes, and a long dashed line represents an inhibitory effect on CPSII by UTP. Abbreviations used are as follows: CP, carbamyl phosphate; CAsp, carbamyl asparatate; DHO, dihydroorotate; OA, orotic acid; OMP, orotidine 5′-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; Mt, mitochondrion. Enzyme activities are numbered as follows: (1) carbamyl phosphate synthetase II (CPSII), (2) asparatate carbamyltransferase (ATCase), (3) dihydroorotase (DHOase), (4) dihydroorotate dehydrogenase (DHO-DHase), (5) orotate phosphribosyltransferase (OPRTase), (6) orotidylate decarboxylase (ODCase). *DHO-DHase activity is present in membranous fraction and may link to the respiratory chain of mitochondrion.

8.4.1 Pyrimidine *de novo* biosynthetic pathway

The preliminary characterization of all six enzymes of the *de novo* pyrimidine biosynthetic pathway (Asai *et al.*, 1983a) indicated some distinctions between *T. gondii*-enzyme and host-enzyme activities. The mammalian host CPSII is part of a large multifunctional protein (CAD) composed of three enzymes: CPSII, aspartate carbamyltransferase (ATCase, E.C. 2.1.3.2), and dihydroorotase (DHOase, E.C. 3.5.2.3) (Mori and Tatibana, 1978; Davidson *et al.*, 1993). These three enzymes comprise the first three enzymes in the pathway. In contrast to mammalian CAD, *T. gondii* CPSII is a cytosolic protein that is an independent enzyme with approximate molecular mass of 540 kDa (Asai *et al.*, 1983a). *T. gondii* has a single CPSII gene which is interrupted by 36 introns. The predicted protein encoded by the 37 CPSII exons is a 1687 amino-acid polypeptide with approximate molecular mass of 186 kDa (Fox and Bzik, 2003). Consequently, the native *T. gondii* CPSII appears to be trimer of identical subunits.

This large CPSII is a common characteristic of other apicomplexan parasites (Flores *et al.*, 1994;

Chansiri and Bagnara, 1995). Bacteria and plants also possess the independent CPS proteins (Jones, 1980; Zhou *et al.*, 2000), however, the structural organization of these enzymes is different from those of mammalian and parasitic protozoan CPSII, including *T. gondii* CPSII.

The enzyme reaction of glutamine-dependent CPSII consists of two reactions; the reaction of glutamine amidotransferase (GATase, E.C. 2.4.2.14), and the reaction of CPS. *T. gondii* and other parasitic protozoa, including *Trypanosoma*, *Babesia*, and *Plasmodium*, express a bifunctional glutamine-dependent CPSII composed of an N-terminal GATase domain fused with C-terminal CPS domains (Aoki *et al.*, 1994; Flores *et al.*, 1994; Chansiri and Bagnara, 1995; Nara *et al.*, 1998; Gao *et al.*, 1999; Fox and Bzik, 2003). Bacteria and plants express a monofunctional GATase as well as a monofunctional CPS (Jones, 1980; Zhou *et al.*, 2000).

The mammalian CPSII is characteristically an allosterically controlled enzyme. Forward activation is by 5-phosphoribosyl-1-pyrophosphate (PRPP), and backward inhibition is by UTP (Jones, 1980). PRPP is a substrate of nucleotide biosynthesis, and provides the sugar phosphate moiety of nucleotides. The *T. gondii* CPSII activity is inhibited by UTP, but no activation by PRPP is reported (Asai *et al.*, 1983a). The regulatory domain for the allosteric control of mammalian CPSII is the C-terminal ~150 amino-acid domain (Liu *et al.*, 1994). The *T. gondii* CPSII contains significant amino-acid insertions in this expected regulatory domain (Fox and Bzik, 2003). Enlarged C-terminal regulatory domains are also reported for other apicomplexan parasites (Flores *et al.*, 1994; Chansiri and Bagnara, 1995). The lack of activation of *T. gondii* CPSII by PRPP may be due to this enlarged C-terminal domain.

The *T. gondii* ATCase is a cytosolic monofunctional enzyme protein with approximate molecular mass of 140 kDa (Asai *et al.*, 1983a). The open reading frame of cloned *T. gondii* ATCase cDNA encodes a putative 423 amino-acid polypeptide with a predicted molecular mass of 46.8 kDa. Recombinant *T. gondii* ATCase with catalytic activity exhibits a molecular mass of 144 kDa (Mejias-Torres and Zimmermann, 2002). Native *T. gondii* ATCase is likely to be trimer of identical subunits. Plant ATCase (Khan *et al.*, 1999) is inhibited by UMP, and *E. coli* ATCase (Wales *et al.*, 1999) is inhibited by CTP and UTP (*E. coli*). However, no significant effect by any nucleotide has been reported on the activity of *T. gondii* ATCase (Asai *et al.*, 1983a; Mejias-Torres and Zimmermann, 2002).

The *T. gondii* DHOase is a cytosolic monofunctional enzyme with approximate molecular mass of 70 kDa whose activity is not regulated by nucleotides (Asai *et al.*, 1983a). No characterization of the *T. gondii* DHOase gene has been reported.

Dihydroorotate dehydrogenase (DHO-DHase, E.C. 1.3.3.1) is the fourth enzyme of *de novo* pyrimidine biosynthetic pathway. DHO-DHases of living organisms are classified into two families. Found in some bacteria and some lower eukaryotes, family-1 enzymes are cytosolic proteins. On the other hand, family-2 enzymes are membrane-associated and link to the respiratory chain in mitochondria for their catalytic redox force (Bjornberg *et al.*, 1997). The *T. gondii* DHO-DHase is exclusively recovered in the particulate fraction of tachyzoite extract and inhibited by respiratory chain inhibitors (Asai *et al.*, 1983a). The predicted amino-acid sequence, with approximate molecular mass of 65 kDa from cloned cDNA, is most similar to family-2 DHO-DHases (Sierra-Pagan and Zimmermann, 2003).

Orotate phosphribosyltransferase (OPRTase, E.C. 2.4.2.10) is the fifth enzyme of the pathway, and orotidylate decarboxylase (ODCase, E.C. 4.1.1.23) is the sixth enzyme of the pathway. These are cytosolic enzymes, and co-sediment by sucrose gradient centrifugation at a position corresponding to a molecular mass of approximately 70 kDa (Asai *et al.*, 1983a). In some higher eukaryotes, these two enzymes are on the same polypeptide (Jones, 1980). In the apicomplexan parasite *Plasmodium falciparum*, the two enzymes exist as a multienzyme complex containing two subunits each of 33-kDa OPRTase and 38-kDa ODCase (Krungkrai *et al.*, 2005). It is not clear, but *T. gondii* OPRTase and ODCase are probably similar to the *P. falciparum* type.

8.4.2. Pyrimidine salvage biosynthetic pathway

The *T. gondii* tachyzoite pyrimidine salvage pathway is shown in Figure 8.5. *T. gondii* has five enzyme activities that are involved in the salvage of pyrimidine nucleosides and nucleobases: cytidine deaminase (E.C. 3.5.4.5), deoxycytidine deaminase (E.C. 3.5.4.14), uridine phosphorylase (E.C. 2.4.2.3), deoxyuridine phosphorylase (E.C. 2.4.2.23), and uracil phosphoribosyltransferase (UPRTase, E.C. 2.4.2.19) (Iltzsch, 1993). No detailed studies for the properties of pyrimidine nucleoside deaminases have been reported. Uridine phosphorylase has been characterized, and utilizes thymidine poorly (Chaudhary, Ting, Roos and Kim, personal communication).

It appears that all pyrimidine salvage in *T. gondii* proceeds through uracil, which is salvaged to the nucleotide level by UPRTase. However, the salvage pathway is not essential for tachyzoite viability and growth, since UPRTase-deficient mutants exhibit the same growth rate as normal tachyzoites (Donald and Roos, 1995). Although UPRTase may not be important for tachyzoite growth, UPRTase is thought to be a possible therapeutic target and is one of the most extensively studied enzymes in *T. gondii* at molecular level, including crystal structure analysis (Carter *et al.*, 1997; Schumacher *et al.*, 1998; Schumacher *et al.*, 2002). The *T. gondii* UPRTase recognizes uracil only, and no other naturally occurring pyrimidine and purine bases (Carter *et al.*, 1997). Without substrates or its activator GTP, recombinant *T. gondii* UPRTase behaves as a homodimer composed of two identical subunits (27 kDa). The physiologically active UPRTase is tetramer, and this tetrameric structure is stabilized by binding of GTP leading to a more active state (Schumacher *et al.*, 2002). The other native protozoan UPRTases from *Crithidia luciliae* (Asai *et al.*, 1990) and *Giardia intestinalis* (Dai *et al.*, 1995) also behave as homodimers.

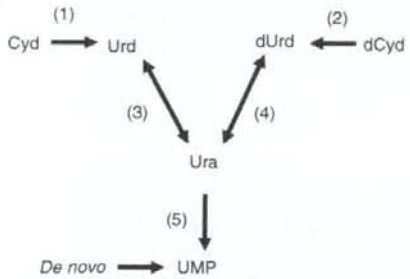


FIGURE 8.5 *T. gondii* pyrimidine salvage biosynthetic pathways. Solid lines represent activities that were detected in *T. gondii*. Abbreviations used are as follows: Cyd, cytidine; dCyd, deoxycytidine; Urd, uridine; dUrd, deoxyuridine; Ura, uracil. Enzyme activities are numbered as follows: (1) cytidine deaminase, (2) deoxycytidine deaminase, (3) uridine phosphorylase (4) deoxyuridine phosphorylase, (5) uracil phosphoribosyltransferase (UPRTase). It may be highly possible that deaminase activities are catalyzed by one enzyme and that phosphorylase activities are also catalyzed by one enzyme.

8.4.3 Purine salvage biosynthetic pathway

Like all parasitic protozoa and many intracellular pathogens, *T. gondii* is incapable of *de novo* purine nucleotide biosynthesis. It relies on salvage pathways for purine nucleotides that are essential for parasite growth and survival (Perrotto *et al.*, 1971; Schwartzman and Pfefferkorn, 1982; Krug *et al.*, 1989; Ullman and Carter, 1995). An illustration of the *T. gondii* tachyzoite purine-salvage pathway is shown in Figure 8.6. *T. gondii* is thought to have six enzyme activities that are involved in the salvage of purine nucleosides and nucleobases: adenine deaminase (E.C. 3.5.4.2), adenosine deaminase (E.C. 3.5.4.4), inosine phosphorylase (E.C. 2.4.2.1), guanosine phosphorylase (E.C. 2.4.2.15), adenosine kinase (AK, E.C. 2.7.1.20), and hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRTase, E.C. 2.4.2.8) (Chaudhary *et al.*, 2004). No detailed studies for the properties of purine nucleoside deaminase, purine base deaminase have been reported. A gene for adenine deaminase but not for adenosine deaminase has been identified in the *T. gondii* genome (Chaudhary *et al.*, 2004). Purine nucleoside phosphorylase has been characterized as having activity against inosine and guanosine, but not adenosine (Chaudhary *et al.*, 2006).

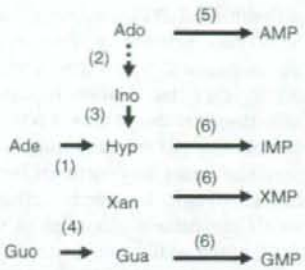


FIGURE 8.6 *T. gondii* purine salvage biosynthetic pathways. Solid lines represent activities or enzyme genomes that were detected in *T. gondii*, short dashed line represents activity of adenosine deaminase, the existence of which is highly suspected. Abbreviations used are as follows: Ade, adenine; Ado, adenosine; Ino, inosine; Guo, guanosine; Gua, guanine; Hyp, hypoxanthine; Xan, xanthine. Enzyme activities are numbered as follows; (1) adenine deaminase, (2) adenosine deaminase, (3) inosine phosphorylase, (4) guanosine phosphorylase, (5) adenosine kinase (AK), (6) hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRTase).

AK and HXGPRTase have been extensively studied at molecular level, including crystal structure analyses (Donald *et al.*, 1996; Schumacher *et al.*, 1996, 2000; Darling *et al.*, 1999; Heroux *et al.*, 1999a, 1999b; White *et al.*, 2000). Neither AK nor HXGPRTase is essential for *T. gondii* survival, since tachyzoites can survive elimination of either activity alone (Donald *et al.*, 1996; Sullivan *et al.*, 1999; Chaudhary *et al.*, 2004). The generation of a double knockout mutant of both AK and HXGPRTase is impossible, suggesting that *T. gondii* accommodates only these two routes of purine salvage (Chaudhary *et al.*, 2004). This situation is not common to all apicomplexan parasites, as discussed in Chapter 19. For instance, *Cryptosporidium* has AK but lacks HXGPRTase (Striepen *et al.*, 2004), and *Plasmodium* has HXGPRTase (Vasanthakumar *et al.*, 1990) but lacks AK (Chaudhary *et al.*, 2004).

The *T. gondii* AK is a monomeric protein with molecular mass of 39.3 kDa, and shares less than 30 percent sequence identity with the AKs of other organisms (Sullivan *et al.*, 1999). The *T. gondii* AK shows a strict specificity for adenosine among naturally occurring nucleosides, such as inosine and guanosine (Darling *et al.*, 1999).

Two isozymes of *T. gondii* HXGPRTase have been identified as the predicted translation products of differentially spliced mRNAs transcribed from a single gene (Donald *et al.*, 1996). The crystal structure of isozyme-I is tetramer, composed of four identical subunits (26.4 kDa) (Schumacher *et al.*, 1996; Heroux *et al.*, 1999a). The subunit of isozyme II possesses extra 49 amino acids that are inserted 7 amino acids downstream of the N-terminus, and the other sequence of isozyme II is identical to that of isozyme I (Donald *et al.*, 1996). The isozyme I is predominantly cytosolic, whereas the isozyme II localizes to the tachyzoite inner membrane complex, and the extra 49 amino-acids sequence of isozyme II contains a membrane-targeting signal (Chaudhary *et al.*, 2005). The two isozymes form heterotetramers when co-expressed in *E. coli* (White *et al.*, 2000) or in tachyzoite *in vivo* (Chaudhary *et al.*, 2005). For the membrane association of enzyme, at least two isozyme-II subunits in the tetramer are necessary (Chaudhary *et al.*, 2005). There is no significant difference in kinetic properties between isozymes, and it is not understood why *T. gondii* possesses two HXGPRTase isozymes.

The adenosine transporter in *T. gondii* has been identified, and a presence of additional permeation pathways for other purine nucleosides, purine bases of *T. gondii* has been suggested (Schwab *et al.*, 1995; De Kong *et al.*, 2003). The gene of *T. gondii* adenosine transporter is cloned and expressed in *Xenopus laevis* oocytes (Chiang *et al.*, 1999). Adenosine uptake by the expressed adenosine transporter is inhibited by various nucleosides, nucleoside analogs, hypoxanthine, guanine, and dipyridamole (Chiang *et al.*, 1999), suggesting that one transporter may play a role in all the transportations of nucleosides and nucleobases into the tachyzoite. Recent studies using nitrobenzylthioinosine (an inhibitor of nucleoside transporter in mammalian cells) and various non-physiological beta-L-enantiomers of purine nucleosides demonstrate that the *T. gondii* adenosine/purine nucleoside transporter(s) lacks stereospecificity and

substrate specificity in the transportation of purine nucleosides (Al Safarialani *et al.*, 2003). There is still no consensus regarding how many transport systems for purine nucleosides/nucleobases are present in the tachyzoite.

8.5 NUCLEOSIDE TRIPHOSPHATE HYDROLASE (NTPase)

8.5.1 Distinctive features and uniqueness

The tachyzoite form of *T. gondii* has a novel nucleoside triphosphate hydrolase (NTPase; EC 3.6.1.3), described for the first time in 1983 (Asai *et al.*, 1983b). The properties of the *T. gondii* NTPase, such as substrate specificity and divalent cation requirements, are most similar to those of E(ecto)-type ATPases (Plesner, 1995). E-type ATPases are ubiquitous in eukaryotic cells, and a number of parasitic protozoan E-type ATPases has been reported (see reviews in Plesner, 1995; Meyer-Fernandes, 2002). In the past half-century, there have been several suggestions regarding the function of E-type ATPase.

The physiological function of *T. gondii* NTPase is undoubtedly different from those of ubiquitous common E-type ATPases, for the reasons discussed below. Although its function is not yet understood, *T. gondii* NTPase must play an extraordinary and unique role for tachyzoite replication and survival.

The most striking feature of *T. gondii* NTPase is its great abundance in the tachyzoite cell. The *T. gondii* NTPase is one of the main proteins, and the calculated amount of NTPase protein is about 2–8 percent of the total tachyzoite proteins (Asai *et al.*, 1983b, 1987; Nakaar *et al.*, 1998). In contrast, the common E-type ATPases are in extremely low abundance. Even the most highly purified E-type ATPases ever reported would not be visible as a band in electrophoretic gels (Plesner, 1995).

The next striking feature of *T. gondii* NTPase is that the enzyme seems to be a dormant enzyme under ordinary conditions. Dithiol compounds, such as dithiothreitol (DTT), are essential for activation of NTPase activity *in vitro* (Asai *et al.*, 1983b). No common E-type ATPases that require activation by DTT have been reported. This feature, with the great abundance of NTPase in the tachyzoite cell, leads to the surprising conclusion that *T. gondii* conceals an enormous potency for ATP hydrolysis. In fact, the specific activity of ATP hydrolysis (U/mg protein), assayed in the presence of DTT, in the whole tachyzoite cell is about 5000 times higher than that of mouse spleen cell (Asai *et al.*, 1986).

The presence of DTT-activated NTPase in the closely related apicomplexan parasite *Neospora caninum* has also been reported (Asai *et al.*, 1998). However, DTT-activated NTPase enzymes have not been identified in other protozoa, including the apicomplexan parasites *Plasmodium berghei* (Asai *et al.*, 1986) and *Eimeria tenella* (Asai *et al.*, 1998). DTT-activated NTPase enzymes are thought to be restricted to *T. gondii* and *N. caninum*.

Another critical difference between the *T. gondii* NTPase and ubiquitous common E-type ATPases is that *T. gondii* NTPase seems to fulfill its function from outside of the tachyzoite cell. Tachyzoite replication occurs in the host cell within the parasitophorous vacuole. During formation of the parasitophorous vacuole, the tachyzoite secretes NTPase into the vacuole from the dense-granule – one of the secretory organelles of the tachyzoite (Bermudes *et al.*, 1994; Sibley *et al.*, 1994). The secreted soluble NTPase gradually associates with the intravacuolar network which is constructed within the vacuole, and it changes to a membrane-associated insoluble enzyme (Carruthers and Sibley, 1997). The ubiquitous common E-type ATPases, including other protozoan E-type enzymes, are not secreted from the cells (Plesner, 1995; Meyer-Fernandes, 2002). An illustration of NTPase in the tachyzoite-infected cell is shown in Figure 8.7.

8.5.2 NTPase isoforms and their molecular properties

The type I strains of *T. gondii*, which are acutely virulent in mice (Sibley and Boothroyd, 1992), contain two isoforms of NTPase (Bermudes *et al.*,

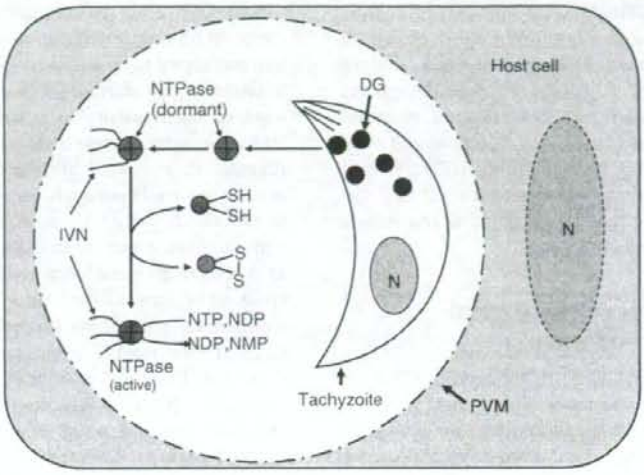


FIGURE 8.7 Behavior of NTPase in the tachyzoite-infected cell. NTPase is secreted from the dense granule of tachyzoite into the parasitophorous vacuolar space and associates to the intravacuolar network. Then, NTPase activity may be regulated by oxido-reduction change in its molecule affected by dithiol compound or unknown dithiol-disulfide oxidoreductase within parasitophorous vacuole. Abbreviations used are as follows: DG, dense granule; PVM, parasitophorous vacuole membrane; IVN, intravacuolar network; N, nucleous.
This figure is reproduced in color in the color plate section.

1994; Asai *et al.*, 1995). One of the isoforms (NTPase-I in Asai *et al.*, 1995; NTP3 in Bermudes *et al.*, 1994) preferentially hydrolyzes triphophate nucleosides, while another isoform (NTPase-II in Asai *et al.*, 1995; NTP1 in Bermudes *et al.*, 1994) hydrolyzes tri- and diphosphate nucleosides at approximately equal rates. The NTPase-I isoform appears to be present only in the type I virulent strains, while NTPase-II is universally present in all *T. gondii* strains (Asai *et al.*, 1995). Although the presence of NTPase-I seems to be one of the primary factors of virulence in mice, no direct evidence for a role in virulence has been proven. *Neospora caninum*, which is avirulent in mice, has only NTPase-I type enzyme, and no NTPase-II type enzyme has been detected (Asai *et al.*, 1998).

The complete cDNAs for NTPase-I and NTPase-II encode predicted open reading frames of the identical size that differ in 16 of 628 amino acids between the two isoforms (Asai *et al.*, 1995). The molecular mass of native NTPases are approximately 260 kDa, composed of four identical subunits with predicted molecular mass of 67 kDa (Asai *et al.*, 1995). Both isoforms of the NTPase contain an N-terminal hydrophobic signal peptide (25 amino acids) that is absent in native NTPase purified from the tachyzoites (Asai *et al.*, 1995). It is supposed that this signal peptide serves as a signal for transport of NTPase to the dense granule. Other dense-granule proteins have similar N-terminal hydrophobic signal peptides (Cesbron-Delauw *et al.*, 1996).

As mentioned above, the primary difference between these NTPase isoforms lies in their ability to hydrolyze nucleoside triphosphate versus diphosphate substrates. While NTPase-II hydrolyzes ATP to ADP and ADP to AMP at almost the same rate, both native and recombinant NTPase-Is hydrolyze ADP to AMP at a much slower rate – less than 1 percent of the rate for ATP (Asai *et al.*, 1995, 1998). This suggests that *T. gondii* NTPase should

be classified as NTP diphosphohydrolase (apyrase; EC 3.6.1.5), a new gene family of E-type ATPases. *T. gondii* NTPase has homology to apyrase, and an antibody against *T. gondii* NTPase recognizes apyrases of potato and *Schistosoma mansoni* (Vasconcelos *et al.*, 1996), and *Trypanosoma cruzi* (Fietto *et al.*, 2004). The abundant DTT-activated NTPase in *N. caninum* has no apyrase activity, and its substrate specificity is restricted to nucleoside triphosphate (Asai *et al.*, 1998).

8.5.3 Possible physiological function for NTPase

Protozoan parasites are purine auxotrophs (Berens *et al.*, 1995). It has been speculated that the NTPases of *T. gondii* are involved in the salvage of purine nucleosides from the host cell (Bermudes *et al.*, 1994). The *T. gondii* NTPase is secreted from the tachyzoite into the parasitophorous vacuole, therefore it contains a higher potency of hydrolyzing ATP to ADP and AMP (Figure 8.7). As mentioned earlier, purines are salvaged through adenosine kinase or HXGPRTase. However, the tachyzoite lacks an ecto-5′-nucleotidase activity that would dephosphorylate AMP to adenosine, the substrate of adenosine kinase (Ngo *et al.*, 2000). Furthermore, it is clear that the primary role of the NTPase of *N. caninum* tachyzoites is not likely to involve purine acquisition, since that parasite lacks enzymatic activities for progressively cleaving nucleotides to their monophosphate form (Asai *et al.*, 1998). It is therefore doubtful whether the *T. gondii* NTPases are involved in purine salvage.

Exposure of the tachyzoites to DTT can activate egress of previously non-motile intravacuolar tachyzoites within 60 seconds with a concurrent Ca^{2+} flux (Stommel *et al.*, 1997). Furthermore, *T. gondii* NTPase is found largely in an inactive oxidized form in the parasitophorous vacuole, and is readily activated by DTT. This leads to subsequent rapid depletion of host ATP and egress of tachyzoites (Silverman *et al.*, 1998). Whether or not the *T. gondii* NTPase is involved in naturally occurring tachyzoite egress from host cells, these phenomena are quite suggestive for the physiological function of *T. gondii* NTPase.

Since DTT is not present in nature, thioredoxin, the most abundant cellular-reducing dithiol catalyst, was tested as an activator. The reduced form of thioredoxin can activate NTPase, and the oxidized form of thioredoxin has a reversible effect on NTPase activity *in vitro* (Asai and Kim, 1987). This suggests that thioredoxin may regulate NTPase activity *in vivo*. However, host-cell thioredoxin may be excluded from the parasitophorous vacuole by the parasitophorous vacuolar membrane (Schwab *et al.*, 1994). Although glutathione itself has no activation effect on NTPase *in vitro* (Asai *et al.*, 1983b), it is reported that glutathione promoters activate a Ca^{2+} flux and decrease ATP levels in tachyzoite-infected fibroblasts (Stommel *et al.*, 2001). These investigators further postulate that the NTPase is activated by glutaredoxin, a small protein with an active center disulphide. Glutaredoxin may be reduced by glutathione, leading ultimately to tachyzoite egress. Finally, the tachyzoites control their egress by secreting glutaredoxin from their bodies into the parasitophorous vacuole for activation of NTPase (Stommel *et al.*, 2001). This model requires further testing, but it is possible that NTPase may be activated by unknown dithiol compound derived from the host cell or tachyzoites. Alternatively, NTPase activity may be regulated by an unknown protein disulfide oxidoreductase within the vacuole.

There is still no consensus for the physiological function of *T. gondii* NTPase. Despite this, it is thought that the *T. gondii* NTPase plays an extremely important function in tachyzoite replication and survival, since the enzyme is expressed mainly in tachyzoite form (Ferguson *et al.*, 1999). Attempts to disrupt the genes have failed, and antisense depletion studies suggest that NTPase is essential for parasite replication (Nakaar *et al.*, 1999).

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