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How Is Encystment Regulated in Giardia Intestinalis

Iryna G. Tsarukyanova
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HOW IS ENCYSTMENT REGULATED IN *GIARDIA INTESTINALIS*

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To those who mean the world to me. To those who supported, encouraged, and inspired me. To those who make me who I am: my father, my mother, my husband, and my son.
ACKNOWLEDGMENT

I would like to thank my adviser Dr. Harry van Keulen for his guidance, support, and his friendship. I would also like to thank my Dissertation committee members: Dr. Donald Lindmark, Dr. Edward Jarroll, Dr. Girish Shukla, Dr. Paul Doerder, and Dr. Dennis Stuehr for their valuable advises and attention to my work. I would like to give a special thanks to Dr. Edward Jarroll and Dr. Donald Lindmark for their help with the supplies, support and encouragement. It is a great pleasure to know you and to work with you. I would also like to thank my peers and colleagues, those who shared this journey with me, who supported me on the daily basis and made this more fun. This work was also partially supported by Doctoral Dissertation Research Expense Award and Fellowship Program to IGT.
HOW IS ENCYSTMENT REGULATED IN GIARDIA INTESTINALIS

IRYNA G. TSARUKYANOVA

ABSTRACT

*Giardia intestinalis* is a protozoan parasite causing water-borne infection: giardiasis. The infective stage of the parasite is a cyst, protected from the environment by a cyst wall of protein and a complex polysaccharide called giardan.

The initiation and the key for cyst wall biosynthesis is amination and isomerization of fructose 6-phosphate by glucosamine 6-phosphate deaminase (GNP). Fructose 6-phosphate is also an intermediate in glycolysis, the main, but not the only energy producing pathway in *Giardia*. The alternative energy source is arginine which is metabolized through the arginine dihydrolase pathway. The latter pathway is up-regulated upon encystment. However, the triggering mechanism is still unknown. The hypothesis developed and discussed in this dissertation is that the switch between glycolysis and cyst wall synthesis is a key point for encystment regulation and is controlled, at least in part by nitric oxide (NO).

The discovery that *Giardia* is able to synthesize NO leads to the conclusion that it may use protein nitrosylation to regulate cellular differentiation in a similar way as it takes place in higher eukaryotes. This study aims to show that key proteins for glycolysis and the encystment pathway are posttranslationally modified by S-nitrosylation. The latter modification regulates protein activity and function. In some cases it is achieved via the change of protein cellular localization. This study demonstrates that *Giardia* produces NO and nitrosylates proteins. Various techniques were used: fluorescent staining of cells,
the Griess assay, and the biotin-switch method. GNP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were identified as key nitrosylated proteins. Incubation of recombinant GNP with an NO donor affects the catalytic activity of the enzyme, suggesting that the enzyme’s affinity for the substrate increases upon nitrosylation. In vitro mutational studies identified two cysteines (position 156 and 230) within GNP as targeted residues. GAPDH’s activity, on the other hand, is significantly decreased during encystment in comparison with GAPDH from non-encysting trophozoites. Incubation of trophozoite lysates with NO donors also significantly decreased the enzyme activity in a time-dependent manner, showing that nitrosylation might be acting as an allosteric effector on enzymes and changing their catalytic properties and in that way, the metabolic flow of carbons during *Giardia* encystment.
# TABLE OF CONTENTS

ABSTRACT..................................................................................................................VI

LIST OF TABLES...........................................................................................................X

LIST OF FIGURES.........................................................................................................XI

CHAPTER I — INTRODUCTION

Introduction..................................................................................................................1
Giadiasis.....................................................................................................................1
Biology of *Giardia*...................................................................................................2
Metabolism................................................................................................................7
Encystment.................................................................................................................9
Molecular biology......................................................................................................11
Post-translational protein modification and its effect on their activity..............12
Nitrosylation..............................................................................................................13
Ubiquination..............................................................................................................15

CHAPTER II- MATERIALS AND METHODS

Materials and methods............................................................................................22

CHAPTER III – POST-TRANSLATIONAL MODIFICATION OF GNP AND ITS EFFECT ON THE ENZYME’S CATALYTIC PROPERTIES

Introduction..............................................................................................................26
Materials and methods..........................................................................................33
Results.......................................................................................................................39
Discussion................................................................................................................53

CHAPTER IV – ANALYSIS OF NO-SYNTHESIZING ACTIVITY IN *GIARDIA*

Introduction..............................................................................................................67
Materials and methods..........................................................................................71
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Comparison of the <em>Giardia</em> genome with the human genome</td>
<td>12</td>
</tr>
<tr>
<td>Table 2</td>
<td>Comparative properties of native and recombinant gGNPs</td>
<td>30</td>
</tr>
<tr>
<td>Table 3</td>
<td>Primers to introduce mutations by PCR in recombinant glucosamine 6-phosphate deaminase</td>
<td>36</td>
</tr>
<tr>
<td>Table 4</td>
<td>Comparison of kinetic parameters of recombinant and mutant GN</td>
<td>52</td>
</tr>
<tr>
<td>Table 5</td>
<td>Catalytic parameters of wild type GNP</td>
<td>53</td>
</tr>
<tr>
<td>Table 6</td>
<td>giNOS- and GDH-specific primers</td>
<td>75</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1  *Giardia intestinalis*: scanning electron micrographs and diagrams of microtubular cytoskeleton.................................3

Figure 2  Diagram of *Giardia* life cycle......................................................5

Figure 3  Arginine dihydrolase pathway.........................................................8

Figure 4  Encystment specific vesicles (ESV) in the encystsing *Giardia*..............10

Figure 5  The pathway of giardan synthesis....................................................27

Figure 6  Alignments of GNP from *Giardia* (*Gia*GNP), *B. subtilis* (*Bsu*GNP), and *E. coli* (*Eco*GNP).........................................................29

Figure 7  Western blot analysis of GNP in trophozoites and 24 h-encysting cells..........................................................39

Figure 8  Western blot and enzymatic analysis of GNP........................................41

Figure 9  Western blot analysis of GNP using trophozoites incubated with different concentrations of NO-donor..................42

Figure 10  Nitrosylated proteins captured with avidin-agarose...............................44

Figure 11  Sequence of GNP with mutated sites indicated......................................45

Figure 12  Comparative biotin-switch analysis of wild type and mutated GNP.....46

Figure 13  Effect of nitrosylation on catalytic parameters of recombinant GNP ........................................................47

Figure 14  Effect of nitrosylation on catalytic parameters of recombinant mutant GNP..........................................................48

Figure 15  Effect of nitrosylation on catalytic parameters of recombinant mutant C219 GNP..........................................................50
Figure 32  Specific activity of *G. intestinalis* GAPDH during encystment........107

Figure 33  Alignments of human, bacterial, and *Giardia* GAPDH..................108

Figure 34  Inhibition of *Giardia* GAPDH activity by different concentrations of cysteine-NO.................................................................109

Figure 35  Effect of time on the inhibition of *Giardia* activity by fixed concentration of cysteine-NO.........................................................109

Figure 36  Western Blot of *Giardia* proteins screened with anti-GADPH serum ...........................................................................................................110
CHAPTER I
INTRODUCTION

*Giardia intestinalis* is a protozoan parasite that colonizes the upper small intestine and causes one of the most widely spread water-borne infections, giardiasis. The World Health Organization estimates that approximately 200 million people are carriers may play a significant role in the spread of infection, especially among children. Outbreaks of giardiasis in the USA often start in daycare centers. According to the Environmental Protection Agency the percentage of infected children ranges from 7 to 54 among different populations and regions. Giardiasis causes nutrient malabsorption and severe dehydration, which is especially dangerous in children under 5 years old. Some studies indicate severe defects in the growth and development of the infected infants (Adam, 2001; Bope et al., 2012; Savioli et al., 2006).

GIARDIASIS

Giardiasis is initiated by ingestion of cysts through contaminated food or water (e.g. sewage discharge, run-off from livestock) (Fig. 2). However, a direct fecal-oral route often occurs among children in childcare facilities and also among homosexuals.
Introduction of as few as 10 cysts is enough to establish an infection (Adam, 1991). After exposure to the acidic environment of the stomach cysts undergo excystation, and the released trophozoites colonize the lumen of the small intestine, mostly the duodenum and jejunum. As parasites travel down the intestine they undergo encystment, and fully formed cysts are released into the environment (Fig. 2). The majority of infected individuals stay asymptomatic, while others develop clinical symptoms in 1-3 weeks (Gardner and Hill, 2001).

**BIOLOGY OF GIARDIA**

*Giardia* is a binucleated, amitochondriate flagellated protozoan parasite. Although its exact phylogenetic position is uncertain, the small subunit RNA gene sequence analysis supports its early divergence in the phylogenetic tree along with *Trichomonas*. *Giardia* belongs to the kingdom Excavata, phylum Fornicata, order Diplomonads (Simpson et al., 2002). According to morphological characteristics the genus *Giardia* is divided into three species: *G. agilis* (amphibian parasite), *G. muris* (parasite of rodents, birds, and reptiles), and *G. intestinalis*, which was originally identified as a human parasite (Adam, 1991). The precise analysis of small subunit (16S) rRNA in various isolates showed that *G. intestinalis*, also known as *G. duodenalis* and *G. lamblia*, could be found in many wild and domestic mammals, excluding muskrats, voles, and some mice (van Keulen et al., 2002). Therefore, it is more accurate to say that *Giardia intestinalis* exists in eight different genetic “assemblages”, ranging from A to H. Different assemblages carry genetically determined host specificity. For instance assemblages A and B are capable of causing infection in humans. Some of these assemblages may be transmitted from animals to humans and vice versa (H. van Keulen et al., 2002; Cotton et al., 2011).
*Giardia* has a simple life cycle, consisting of two forms: a trophozoite (trophic stage), responsible for the symptoms (Fig. 1, A-B); and an infective cyst (Fig. 1, C).

![Giardia images](A,C)

Figure 1. *Giardia intestinalis*: scanning electron micrographs (panels A-C) and diagram of microtubular cytoskeleton (panel D).

(A) *Giardia* trophozoite; (B) *Giardia* trophozoites colonizing intestinal villi, (C) in vivo derived cyst at CSU. Taken from Erlandsen et al. (1996).

(D) Diagram of a *Giardia* trophozoite’s: microtubular skeleton of the trophozoite is characterized by four pairs of flagella (anterior (afl), caudal (c), posterior (pfl), ventral (vfl)), the ventral disc (vd), the funis (fn) and the median body (mb). Nuclei are labeled (N). The spatial position is demonstrated by the following planes: left-right (L-R), dorsal-ventral (D-V), anterior-posterior (A-P). Taken from Sagolla et al. (2006).
The trophozoite is a pear-shaped motile form of approximately 6 by 10 µm with four pairs of flagella and two nuclei (both are transcriptionally active). The cytoskeleton is extensive and includes a body, the median body, and a ventral disk on the anterior two-thirds of the ventral surface (Fig. 1, D). *Giardia* trophozoites persist in the host small intestine attached to the epithelium (Fig. 1, B) using suction power of the ventral disk (Adam, 1991; Lujan et al., 1997). The median body serves as the major morphological distinguishing structure among *Giardia* species due to high variability in its shape, number and position, however, its exact function is not known (Piva and Benchimol, 2004). There are no mitochondria or Golgi apparatus in trophozoites (Adam, 1991).

The cyst is resistant to the external environment and gastric acid and is approximately 5 by 8 µm (Adam, 1991). It is protected by the cyst wall, which consists of two layers: an outer filamentous layer (7 - 15 nm in width) consisting of galactosamine, most likely in the form of N-acetylgalactosamine, and polypeptides, and an inner double membrane layer (Jarroll et al., 1989; Jarroll et al., 1995, Manning et al., 1992; Gerwig et al., 2002). Although *Giardia* lacks true mitochondria, both trophozoites and cysts contain mitosomes: mitochondria highly reduced in structure and function, found in some unicellular eukaryotes, and lysosomes or lysosome-like vacuoles, enriched in cysteine proteases (Lindmark, 1988; Adam, 1991; Dolezal et al., 2005).

The goal of the current project is to investigate the regulation of the encystment mechanism with focus on the primary enzymes initiating cyst wall formation. Understanding encystment regulation will allow us to identify the major control points of this process and identify new and powerful therapeutic targets. The working hypothesis is that this regulation is through protein modification.
Clinical signs of infection may include but are not limited to, nausea, vomiting, weight loss, bloating, abdominal pain, and diarrhea (Langford et al., 2002; Cotton et al., 2011). Increasing evidence has also implicated Giardia pathogenesis in the development of chronic gastrointestinal disorders including Irritable Bowel Syndrome (IBS) (Cotton et al., 2011). Tight attachment between Giardia trophozoites and intestinal epithelial cells triggers a series of events that culminate in the production of diarrhea (Cotton et al., 2011). Giardia induces a caspase-dependant apoptosis of intestinal epithelium, a
reduction in total intestinal absorptive surface area, and a shortening of epithelial brush border microvilli. The shortening of the microvilli results in nutrient and electrolyte malabsorption, anion hypersecretion and increased intestinal secretion. The osmotic gradient, created due to the above mentioned conditions, draws water into the lumen of the small intestines (Cotton et al., 2011). Although giardiasis is a non-invasive infection, the intestinal permeability is increased as a result of apoptosis (Langford et al., 2002; Cotton et al., 2011).

B lymphocytes and IgA play a central role in clearance of the infection, but most probably not by direct killing but rather by detachment of the parasite (Langford et al., 2002). At the same time the exposure to the host epithelial cells results in the induced expression of certain Giardia genes involved in the evasion of the host’s humoral immune response. The main proteins that are involved in this mechanism are variant surface proteins (VSP), only one of which is expressed at a time, surface lectins, and cysteine proteases that cleave human IgA (Cotton et al., 2011). The other mechanism is by the consumption of exogenous arginine, a key metabolite required for production of epithelial nitric oxide which is giardiastatic (Cotton et al., 2011). Giardia also releases an “enterotoxin” that causes excessive ion secretion and intestinal fluid accumulation and other unknown products that modulate the behavior of dendritic cells (Cotton et al., 2011).

Giardiasis is diagnosed using IFA, ELISA, and PCR, however stool sample microscopy is still a “golden standard” (Gardner and Hill, 2001; Savioli, Smith and Thompson, 2006).
Among the drugs used to treat giardiasis are nitroimidazoles, quinacrine, furazolidone, benzimidazoles, and paromomycin (Gardner and Hill, 2001). Metronidazole and tinidazole are the only nitroimidazole drugs available for treatment in the USA, and the most commonly used worldwide (Gardner and Hill, 2001). After entering the trophozoite the drug is activated and covalently binds DNA causing its damage (Gardner and Hill, 2001). Metronidazole completely inhibits the trophozoite’s oxygen uptake and motility resulting in cell death; however, it has no effect on the cyst’s oxygen uptake or their ability to excyst in patients (Jarroll and Lindmark, 1990). This leads to the excretion of high numbers of viable cysts by those treated individuals. Metronizazole also has side effects such as headache, vertigo, nausea, and metallic taste in the mouth (Gardner and Hill, 2001). The drug can be carcinogenic in mice and rats at high doses over a long period of administration (Gardner and Hill, 2001). Tinidazole, on the other hand, is effective at a single dose and is well tolerated (Adam, 1991). Quinacrine, despite its effectiveness against giardiasis, causes severe side effects, such as toxic psychosis (Adam, 1991; Gardner and Hill, 2001). A number of other drugs such as paromomycin, azithromycin, and rifampin were successfully applied in vitro; however, they have not been tested extensively in clinical settings (Adam, 1991; Gardner and Hill, 2001).

**METABOLISM**

*Giardia* is classified as an aerotolerant anaerobe. The parasite catabolizes glucose through the Embden-Meyerhof-Parnas and pentose phosphate pathways. The pyruvate produced in glycolysis is incompletely oxidized to CO$_2$, acetate, ethanol, and alanine. Depending on oxygen availability the ratio of acetate/ethanol varies. Acetate dominates
under aerobic conditions, while ethanol is the main anaerobically produced product (Jarroll and Lindmark, 1990). The energy is generated by substrate level phosphorylation. ATP can also be produced from arginine via the **arginine dihydrolase pathway (ADHP)** (Schofield et al., 1990). *Giardia* obtains arginine from the host in exchange of ornithine and converts it to citrulline, which is converted to ornithine and carbamoyl-P (Knodler et al., 1995). Carbamoyl-P is subsequently converted to CO$_2$ and NH$_4^+$ releasing ATP (Fig. 3).

![Figure 3. Arginine dihydrolase pathway.](image)

The enzymes are labeled as follows: 1. Arginine deiminase (ADI); 2. Ornithine transcarbamoylase (OTC), 3. Carbamate kinase (CK). Taken from Adam (2001).

In the presence of oxygen trophozoites respire by a flavin-iron sulfur protein-mediated electron transport system. In 1989, Paget et al. demonstrated that *G. muris* cysts respire oxygen at approximately 10 - 20% of the trophozoite’s rate. *Giardia* relies on salvage rather than de novo synthesis of lipids, purines, and pyrimidines (Aldritt et al., 1985; Jarroll et al., 1981; Jarroll and Lindmark, 1990; Kulda and Nohýnková 1995; Wang and Aldritt, 1983). All the lipids, including sterols, sterol esters, mono-, di-,
triacylglycerides, and phospholipids Giardia seems to obtain from serum in in vitro experiments and bile from its host (Jarroll and Lindmark, 1990; Luján et al., 1996). However, Gillin et al. (1986) showed that G. intestinalis can grow in serum-free medium, containing cholesterol and six bile salts instead.

**ENCYSTMENT**

The conditions that trigger the encystment process in trophozoites remain unknown. Some authors believe that it is bile, or more precisely bile salts and a shift in pH that causes encystment (Gillin et al., 1987; Gillin et al., 1989; Schupp et al., 1988; Kane et al., 1991). However, Sterling et al. (1988) demonstrated that trophozoites undergo encystment even under bile starvation conditions. In 1996 Luján et al. demonstrated that the encystment process is controlled by cholesterol availability. Analyzing different regions of infected human and several animal intestines Luján et al. (1997) showed that nonencysting parasites concentrate in the jejunum where lipid absorption takes place. This, along with the parasite’s dependence on host lipids, pointed to cholesterol starvation as the main encystment triggering factor. Moreover, it was shown that trace amounts of salts solubilize lipids making them more available for the cell, while a high concentration inhibits cholesterol uptake (Farthing et al., 1985). The mechanism of sensing low cholesterol levels and passing the signal to the parasite nucleus remains unclear.

During encystment, trophozoites express cyst antigens after 6 h and develop Golgi-like components or encystment specific vesicles (ESV) (Fig. 4) after 6 - 18 h (Reiner,
Among cyst-specific protein are three proteins rich in leucine which are delivered to the cyst wall via ESVs (cyst wall protein; CWP 1 - 3) (Luján et al., 1995; Mowatt et al., 1995; Sun et al., 2003). *Giardia*’s ability to use exogenous glucose is lost 10 h post-induction (Jarroll et al., 2001). Approximately at the same time the initiation of cyst wall fibril assembly was shown by Chavez-Munglia et al. (2007) using ruthenium red staining. The cyst wall is assembled from separate fibrils organized in bundles which grow and overlap forming a mesh like structure. The highest growth rate is observed between 30 - 50 h of encystment and ceases by 65 h (Erlandsen et al., 1989; Luján et al., 1995; Arguello-Garcia et al., 2002).

Experiments by Su et al. (2007) showed that neomycin and puromycin selectively affected *Giardia* gene expression. Among genes that were affected are enzymes involved...
in glycolysis and ADHP (GAPDH, ornithine transcarbamoylase, carbamate kinase), as well as CWP 1 - 3. These observations point to the existence of a common signaling pathway that connects the encystment, glycolysis and ADHP. The other possible candidates for participation in the signaling pathway are protein kinase C (PKC) and PKA. PKC inhibitors block encystment in a dose-dependent manner, and PKA was stimulated by intracellular cAMP during encystment (Bazan-Tejeda et al., 2007).

**MOLECULAR BIOLOGY**

*Giardia* is a polyploid organism. Its ploidy and the chromosome numbers vary, depending on the strain from 4N to 12N and from 5 to 8 (Adam, 2001). Trophozoites divide by binary cell fission, the molecular mechanism of which is not known (Adam, 1991). *Giardia* has a very compact genome with genes arranged in a single transcription unit; introns are rare (so far reported in only three genes) (Vanacova et al., 2003; Jeffares, 2006; Roy and Gilbert, 2006). The promoter elements of *Giardia* are short, AT-rich and located within 70 - 150 bp upstream of the transcription start site. Beside the core promoter element *Giardia* also has multiple initiator sequences that are able to drive transcription independently, producing high numbers of sterile transcripts (Vanacova et al., 2003). *Giardia* mRNA possesses unusually short 5’ and 3’ untranslated regions. Differential gene expression was recorded during encystment and excystation. The *Giardia* genome encodes a Myb-like transcription factor, and Myb-binding sequences were identified in at least 5 genes induced upon encystment: cyst wall proteins 1 - 3, glucosamine 6-phosphate deaminase (GNP), and the Myb-like gene itself (Sun et al., 2002).
When the *Giardia* genome is compared to the human genome, it is especially striking that *Giardia* has a high gene density and that the distance between genes is very small (Manning et al., 2011; Venter et al., 2001). Some other differences are shown in Table 1.

**Table 1. Comparison of the *Giardia* genome with the human genome.** Based on data presented by Manning et al. (2011) and Venter et al. (2001).

<table>
<thead>
<tr>
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<th>Giardia</th>
<th>Human</th>
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<tr>
<td>Genome size in basepairs</td>
<td>11.7 x 10⁶</td>
<td>2.66 x 10⁹</td>
</tr>
<tr>
<td>G + C content</td>
<td>49 %</td>
<td>38 %</td>
</tr>
<tr>
<td>Annotated genes</td>
<td>6,470</td>
<td>26,383</td>
</tr>
<tr>
<td>Average gene size (bp)</td>
<td>1,283</td>
<td>27,000</td>
</tr>
<tr>
<td>Hypothetical genes</td>
<td>ND*</td>
<td>12,731</td>
</tr>
<tr>
<td>Intergenic distance (bp)</td>
<td>372</td>
<td>ND*</td>
</tr>
<tr>
<td>Gene density</td>
<td>0.58/kbp</td>
<td>0.005 - 0.023/kbp</td>
</tr>
<tr>
<td>Number of protein kinases</td>
<td>80</td>
<td>868</td>
</tr>
</tbody>
</table>

*ND - not determined

**POST-TRANSLATIONAL PROTEIN MODIFICATION AND ITS EFFECT ON THEIR ACTIVITY**

Reversible post-translational modifications are widely used to regulate protein activity (Gill, 2004). They provide a cell with a fast and effective mechanism of accommodation to changing environmental conditions. The use of multiprotein complexes and reversible protein binding and modification appears to be a universal process in cells. It allows for quick changes in biological regulation using fast on and off switching of processes, and adds a whole new dimension to the control of gene expression. However, the variety of modifications is extremely large and complex. Proteins can be modified by attachment of small chemical groups, such as methylation,
acetylation, glutathionylation, nitrosylation, phosphorylation, to mention just a few, or attachment of larger molecules such as sugars, lipids, or other polypeptides, for instance, ubiquitin.

**NITROSYLATION**

Nitric oxide (NO) is a small highly diffusible molecule that is widely distributed in nature. Intracellularly it is produced by an enzyme nitric oxide synthase (NOS), which converts the amino acid arginine into citrulline with the release of NO. Depending on the cell’s nature there are three general types of NOS: neuronal, epithelial, and inducible (specific for macrophages). NO was originally discovered in mammals as one of the endothelial-derived blood vessels’ relaxing factors and a macrophage-derived molecule that mediates killing of tumor and bacterial cells (Gaston et al., 2003; Hara and Snyder, 2006; Ischiropoulos, 2005). Since then, many other functions have been attributed to NO. Some of them are inhibition of platelet aggregation and leukocyte adherence, ion-channel activity and mitochondrial function, immune response modulation and killing of microbial agents, apoptosis and tissue-damaging in autoimmune diseases (Hernández-Campos et al., 2003). NO is also produced by some invertebrates (insects and mollusks), nematodes (*Ascaris suum, Dirofilaria immitis*, and *Brugia sp.*), plants, prokaryotes, and protozoa (Hernández-Campos et al., 2003). In prokaryotes, NO serves as an endogenous regulator of gene expression and also is an intermediate of denitrification processes (Cutruzzola, 1999; Spiro, 2006; Zumft, 2002). NO-producing ability of protozoa as well as the function of the molecule is not well studied.
NO reacts with cysteine residues in proteins, causing S-nitrosothiol formation. This post-translational modification plays a major role in intra- and inter-cellular signaling (Gaston et al., 2003). The chemical term used for this process is nitrosation; however, in the literature this protein modification is commonly called nitrosylation. Some authors have used the consensus motif [G/S/T/C/Y/N/Q] [K/R/H/D/E] C [D, E] to predict protein nitrosylation. The amino acids arginine (R) and aspartate (D) that flank cysteine in the motif make NO more electrophilic, serving as the partial electron acceptor (Stamler et al., 1997; Gaston et al., 2003). At the same time others suggest that nitrosylation is rather commanded by the cysteine environment, provided by a protein’s tertiary structure (Marino and Gladyshev, 2009). There are two well-recognized nitrosylation targets: soluble guanylate cyclase and the cytochrome oxidase of complex 4 in the mitochondrial electron transport chain (Morton and Vermehen, 2007). In many biological systems transferring of NO$^+$ to a sulfhydryl group affects protein localization and function or affects gene expression (Mohr et al., 1999). Here are just a few presently known targets of so called S-nitrosylation: bovine serum albumin, tissue-type plasminogen activator, GAPDH, oncogenic p21$^{ras}$, and some transcription activators (Mohr et al., 1999). Nitrosylation could inactivate as well as activate protein catalytic activities. A good example of this is glutathione S-transferase. The microsomal enzyme is two-fold upregulated following nitrosylation, while the cytosolic one is downregulated (Ji et al., 2002). Recently NO production has been detected in *Trichomonas vaginalis* and *G. intestinalis* (Harris et al., 2006); NOS activity was measured using radioactively labeled arginine. Confocal microscopy using the fluorescent indicator specific for oxidized NO showed population heterogeneity, suggesting a cell cycle dependence of NO.
database (McArthur et al., 2000) search resulted in finding a gene annotated as inducible NOS (iNOS). Typical NOS is composed of two domains: an oxygenase and a reductase. The former serves for binding the substrate and biopterin (Mitchell et al., 2007). The *Giardia* enzyme clearly misses the oxygenase domain, however, it has a reductase-like domain. Therefore, it cannot be a typical NOS.

**UBIQUINATION**

Ubiquination is a reversible post-translational modification of cellular proteins. It is present in eukaryotes and archaeabacteria, but absent from eubacteria (Krebber et al., 1994). Originally discovered as a mediator for protein proteasomal or lysosomal degradation, later involved into the regulation of the cell cycle, protein trafficking, DNA repair, apoptosis, and signal transduction (Kimura and Tanaka, 2010; Claugue and Urbé, 2006). Ubiquitin (Ub) is a highly conserved 76 amino acid protein that can form an iso-peptide bond between its carboxy-terminal glycine and the ε-amino group of lysine residues in a target protein (Kimura and Tanaka, 2010). The mammalian genome has four Ub genes: two code for poly-Ub, while the other two code for fusions with ribosomal proteins. Yeasts have a single poly-Ub gene and three ribosome fusion genes. Knocking out Ub-encoding genes results in vulnerability to stresses in yeasts as well as infertility, neurodegeneration and embryonic lethality in mice (Kimura and Tanaka, 2010).

Protein ubiquination is catalyzed by the well-coordinated action of three enzymes: an Ub-activating enzyme (E1), an Ub-conjugating enzyme (E2), and an Ub ligase (E3). Each one is highly specific for its substrate (Kimura and Tanaka, 2010). Ub itself possesses seven internal lysines making formation of a poly-Ub chain possible. As a result,
cytosolic Ub can be found as a monomer, substrate-conjugated mono- or poly-Ub, or an unanchored Ub chain (Kimura and Tanaka, 2010). The common sites for Ub polymerization are lysine 48 and 63 (Gill, 2004). In order to interact with a proteasome, the Ub chain has to be at least four subunits long and is usually attached through lysine 48, however, mono-ubiquination is sufficient for degradation of one of the transcription factors from the nuclear factor-κB family (Ciechanover, 1998; Claugue and Urbé, 2006; Gill, 2004; Kravtsova-Ivantsiv et al., 2009). Targeting to the lysosome on the other hand, as was shown in yeast, requires at least two Ub molecules linked through lysine 63, however, there are exceptions (Claugue and Urbé, 2006). Protein modifications via Ub attachment are usually predetermined by either phosphorylation or by internal small N-terminal motifs, such as a D-box, or a KEN box. The D-box is a consensus sequence of R-A/T-A-L-G-X-I/V-G/T-N, while the KEN box is K-E-N-X-X-X-N, where X stands for any amino acid (Jadhav and Wooten, 2009). Ubiquination could be reversed by Ub-specific proteases called deubiquitinating enzymes (Dubs). These enzymes are also involved in the processing of Ub precursor’s terminal domain (carrying an R-G-G sequence) to reveal C-terminal glycine (Ciechanover, 1998). It is estimated that mammalian cells possess approximately 600 E3s and 100 Dubs (Kimura and Tanaka, 2010). The pool of cellular Ub is maintained at a certain level through transcriptional regulation, and a coordinated work of E3 ligases, 26S proteasome, and Dubs (Ciechanover, 1998; Kimura and Tanaka, 2010).

While polyubiquination in general targets proteins for degradation, single Ub attachment regulates membrane protein trafficking, transcription factor activity, subcellular localization, protein-protein interactions, and endocytosis (Claugue and Urbé,
Mono-ubiquination serves as a sufficient signal for cell surface protein internalization and a sorting signal for transmembrane and biosynthetic proteins in yeasts and mammals (Schnell and Hicke, 2003). Histones H2B, H2A, H1 and H3 were modified by a single Ub or a short chain. In yeast, H2B ubiquination is required for meiosis and influences silencing at the telomere. H2B ubiquination is also required for methylation of H3 at multiple sites (Gill, 2004; Schnell and Hicke, 2003). Several Ub-binding domains (UBD) interacting with two hydrophobic patches on the Ub surface have been characterized (Gill, 2004; Schnell and Hicke, 2003). Among them are Ub-associated domain, Ub-interacting motif, and Ub E2 variant (Gill, 2004). A number of cellular proteins, such as the above mentioned proteins are involved in endocytosis or sorting and the budding machinery carry one of the UBDs. These proteins can bind mono- or poly-Ub, otherwise recruit E2 and E3 and promote mono-ubiquination (Schnell and Hicke, 2003). Schnell and Hicke (2003) suggested that Ub-binding proteins protect a mono-ubiquitinated partner from poly-Ub chain formation.

A number of Ub-related proteins have been identified in different organisms. Among them are yeast RUB 1, Parkin, and a group of small Ub-related modifier (SUMO) in mammals. Parkin is implicated in the pathogenesis of certain forms of Parkinson, while SUMO (exactly SUMO1) is primarily known for its targeting of RanGAP1 (nuclear transport protein) to the nuclear pore (Ciechanover, 1998). SUMO has about 20% overall similarity with Ub, however, it has a different distribution of the charged residues and a 15 amino acids N-terminal extension (Gill, 2004; Jadhav and Wooten, 2009). Only one SUMO protein was reported in yeasts (Wilkinson and Henley, 2010). Mammals have
four different SUMO isoforms (SUMO 1-4), however, SUMO 1 is the most commonly found one (Jadhav and Wooten, 2009). Unlike ubiquitin SUMO in general functions as a monomer (Gill, 2004). The process of SUMOylation is similar, though much simpler compared to ubiquination (Gill, 2004; Yeh, 2009). It usually targets lysine in the consensus sequence Ψ-K-X-E (where Ψ represents large hydrophobic amino acids, while X stands for any amino acid) (Jadhav and Wooten, 2009). In some cases, SUMOylation depends on previous phosphorylation of a target protein (similar to Ub), an example of that is heat shock protein 1 (Andreou and Tavernakaris, 2009). In order to be linked to a lysine SUMO has to be activated by proteolytic cleavage. The cleavage reveals two terminal glycine residues and is performed by sentrin/SUMO-specific proteases (SENPs). The same SENP (two identified in yeast and six in humans) are responsible for deconjugation of SUMO. The rest of the SUMOylation machinery engages a single conjugating enzyme, and a limited number of ligases (Wilkinson and Henley, 2010; Yeh, 2009). Coordination of SUMO attachment and deconjugation is achieved through transcriptional regulation of SENP as well as their import/export between nucleus and cytosol (Yeh, 2009). SUMOylation usually results in alteration of target protein localization, activity, or interaction with binding partners. It is rarely associated with protein degradation (Andreou and Tavernakaris, 2009). SUMOylation could also protect proteins from degradation through the ubiquitin-proteasomal pathway (Andreou and Tavernakaris, 2009). Unlike ubiquination SUMOylation is hard to detect as only about 1% of total cellular proteins are modified (Wilkinson and Henley, 2010). Most of the known SUMO-modified targets are nuclear proteins as SUMOylation is frequently associated with transcriptional regulation (Andreou and Tavernakaris, 2009; Gill, 2004).
If ubiquination of transcriptional factors and histones in general results in increased gene expression, SUMOylation is associated with decreased gene expression. It is achieved through either binding to transcriptional (co)repressors or promotion of interactions with proteins that repress transcription (Gill, 2004). At the same time SUMOylated proteins are involved in genome integrity, DNA repair, apoptosis, signal transduction and mitochondrial fission (Andreou and Tavernakaris, 2009; Gill, 2004; Scorrano and Liu, 2009). It seems like modifications via attachment of SUMO and Ub are antagonistic, as they often target the same lysine residue, however, they do not compete directly (Gill, 2004; Wilkinson and Henley, 2010). SUMO also competes with acetylation of the same lysine (Wilkinson and Henley, 2010). Among proteins targeted by SUMOylation are: nuclear pore complexes RanGAP1, chromatin structure regulating histone H4, transcriptional co-activators HDAC1 and 4, transcriptional factors involved in cytokine activity, tumor growth and other signal transduction pathways (p53, cIRF-1, Myb, Smad4) (Gill, 2004). An example of regulation of subcellular localization is RanGAP. Non-modified protein is typically found in the cytosol, whereas the SUMOylated one is associated with a nuclear pore (Gill, 2004). A number of proteins are associated with SUMO and a conserved SUMO-interacting motif (SIM) has been identified (Andreou and Tavernakaris, 2009; Gill, 2004; Wilkinson and Henley, 2010). Among the proteins associated are histone deacetylases 2 and 6 (HDAC2 and HDAC6) (Andreou and Tavernakaris, 2009). A cellular SUMOylation system is also exploited by several bacterial and viral pathogens to establish an infection. It was shown that Yersinia pestis (bacteria causing the black plaque), encodes SUMO protease, whose activity is important for suppression of the host immune system (Gill, 2004).
The ubiquitin protein as well as ubiquination and deubiquination are conserved across eukaryotes (Ponder and Bogyo, 2007). Both the number of Ub molecules and location of the modification are responsible for the following protein destiny. Considering their complex life cycle, various mechanisms of host immune system evasion, and establishment of an infection, parasites should possess a well regulated system of protein expression–degradation. However, protein modification by means of ubiquination is not very well studied in protozoa. Ub-like modifiers, including SUMO and their deconjugating enzymes were identified in *Plasmodium, Toxoplasma, Leishmania, Trypanosoma, Entamoeba, Giardia, Cryptosporidium, and Theileria spp.* (Ponder and Bogyo, 2007).

The presence of a single Ub gene in *Giardia* was reported first by Krebber et al. in 1994. Since then the entire ubiquination-dependent protein degradation machinery, including two E1, six E2, three E3, four deubiquitinating enzymes, as well as a 20S proteasome were identified in *Giardia* (Emmerlich et al., 1999; Gallego et al., 2007). In addition a Ub-ribosomal fusion gene was described (Gallego et al., 2007). Further analysis showed that one out of two E1 enzymes was likely a SUMO protein activating enzyme (Gallego et al., 2007). Isolated *Giardia* 20S proteasome complexes resembled the higher eukaryote proteasome; however, it was not active possibly due to damage during the process of purification (Emmerlich et al., 1999; Emmerlich et al., 2001; Paugam et al., 2003). Transcriptional studies (Gallego et al., 2007) did not detect a difference in Ub level between trophozoites, encysting cells and those undergoing excystation. At the same time this group registered a significant increase in SUMO-activating E1 and a significant decrease in E3 ligase transcript level after 6 h of encystment. A different
group, however, showed an increased expression of the Ub gene (Gen Bank # 70050) along with GNP1 and cyst wall protein 2 (CWP2) during encystment (Eligio-Garcia et al., 2011). However, close scrutiny of this manuscript revealed that GNP2 not GNP1 was analyzed and that not ubiquitin itself but an ubiquitin carboxyterminal hydrolase (EC 3.1.2.15, ORF 102710 in the Giardia Database) was upregulated at the transcription level. Therefore nothing is yet known with certainty about transcriptional control of Ub in Giardia.

The processes of encystment and excystation involve differential gene expression and quick and specific protein turnover, which may be indicated by the presence of the Ub/proteasome system. Some researchers reported persistent Ub expression during the parasite’s differentiation processes, whereas, variation in transcription was observed in activating (E1), conjugating (E2), and ligating (E3) enzymes (Touz et al., 2002).

In summary there is experimental evidence that F6P serves as a substrate for the energy production and cyst wall synthesis. There is also experimental evidence that G. intestinalis produces NO, the molecule that operates as a cell cycle regulator in many systems. The primary questions addressed in this dissertation are:

1. Is there nitrosylation in G. intestinalis?
2. What enzymes involved in the energy production and cyst wall synthesis are nitrosylated? What is the effect of enzyme nitrosylation?
3. What enzyme is responsible for the NO production?
CHAPTER II

MATERIALS AND METHODS

Chemicals that were used in the experiments were supplied by Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburg, PA) unless otherwise indicated.

Cell culture and encystment – *G. intestinalis* trophozoites (Assemblage A, strain MR4) were grown axenically at 37 °C in Diamond’s TYI-S-33 medium, containing 1 mg/ml bovine bile, with the pH adjusted to 7.0 (Lopez et al., 2003). For encystment, the growth medium was replaced with TYI-S-33 medium with 10 mg/ml bovine bile, adjusted to pH 7.6. Encystment is asynchronous and normally 70–80% of the trophozoites completely transform into cysts within 48 – 72 h. The process of encystment was monitored using light microscopy and cysts were identified on the basis of shape and water resistance. Encystment was determined from the number of cysts formed and is presented as a percentage of the total cell number.
Protein concentration — Protein concentration was measured using the Bio-Rad (Bio-Rad, Hercules, CA) method with Bovine Serum Albumin (BSA) as standard or using Bradford reagent (Amresco, Solon, OH).

SDS-Polyacrylamide gel (SDS-PAG) electrophoresis — PAG electrophoresis was done according to the method of Laemmli (Laemmli, 1970) with modifications. Protein samples were loaded onto 15.8% SDS-PAGs and electrophoresed in buffer (25 mM Tris-HCL, 250 mM glycine, 0.1% SDS, pH 8.3). Between 10 - 15 µg of total protein were usually loaded per well. In Western Blot analysis of GNP from trophozoites and cells induced to encyst for various lengths of time, protein samples were separated using Novex® 4-20 % Tris-Glycine Midi gels (Life technologies, Grand Island, NY).

Western Blot analysis — SDS-PAGs were transferred routinely to Immobilon Polyvinylidene Fluoride (PVDF) membranes (Fisher Scientific) for 1 h at 1 mA/cm² using a semi-dry transblotter according to the manufacturer’s specifications (Fisher Scientific). After blotting, the membranes were blocked with 5 % non-fat dry milk dissolved in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl (TBS) plus 0.1 % Triton X-100 (TBS-T) overnight at 4˚C. Blots were routinely incubated with primary antibodies at appropriate dilution for 2 h at room temperature with agitation. Afterwards the blots were washed thoroughly with TBS with 0.05% Triton X-100 for 1 h at room temperature with agitation. Washes were changed every 15 min. Secondary goat anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:10,000 dilution. The blots were incubated with secondary antibodies for 1 h at room temperature with agitation. The blots were washed as described previously.
The final wash was performed with TBS only. Blots were developed using Pierce ECL Western Blotting Substrate (Fisher Scientific).

Detection of Nitric oxide – NO production by encysting and non-encysting Giardia trophozoites was estimated spectrophotometrically as formed nitrite (NO$_2^-$) using the Griess Reagent System (1% sulfanilamide in 0.1 M HCl and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED) (Promega Corporation). Briefly, 50 µl of cellular lysates were incubated with 50 µl of sulfanilamide solution for 5 min protected from light. Following that 50 µl of NED were added to the mixture and incubated for another 5 min. The absorbance of the formed azocompound was measured with a Perkin Elmer 1420 Multilabel Counter microtiter plate reader with a 570 nm filter for 1 sec (see Chapter III for the details).

Nitric Oxide Donor – Nitrosocysteine (cys-NO) was synthesized using the protocol of Martínez-Ruiz and Lamas (2007). The concentration was measured using a Beckman DU-64 spectrophotometer using the molar absorption coefficient $\varepsilon_{338} = 900 \text{ M}^{-1} \text{ cm}^{-1}$ and diluted as indicated. Various concentrations ranging from 2 µM to 1 mM were used for the incubation with cells, cellular protein lysates, and recombinant protein.

Indirect immunofluorescence – Trophozoites and cells induced to encyst for different times were harvested and washed. Indirect immunofluorescence was performed according to the procedure of Repasky et al. (1982) with modifications. Washed cells were diluted until slightly turbid in 3 % Formalin-PBS. The Micro slides (Corning Incorporated, Corning, NY) were coated with 0.01% poly-L-lysine, allowed to sit for 10 min, and washed with Phosphate - buffered saline (PBS). Cells in formalin-PBS solution
were transferred to the treated slide and incubated for 20 min at room temperature. The adherent cells were washed in PBS with 2% Triton X-100 and blocked with calf serum for 15 min at room temperature. Following that slides were washed twice in PBS-Triton X-100 and incubated with 20 - 30 µl of primary antibodies at appropriate dilution for 1 h at 37 °C in a wet chamber. The slides were washed twice in PBS-Triton X-100 solution and twice in PBS. The incubations with the secondary antibodies were also performed for 1 h at 37 °C in a moist chamber. For better contact incubation was performed under a cover glass that was later removed. After secondary antibody treatment, slides were washed three times with PBS-Triton X-100 solution, once with PBS, and once with distilled water. The 0.17 mm cover glass (Corning) were mounted on to the slides using Vectashield®Hard Set Mounting Medium with DAPI (Vector laboratories, Burlingame, CA). The slides were sealed using nail polish and dried in the dark. Cells were observed with a Zeiss Pascal confocal microscope through a Plan-Apochromat 63 x 1.4 N.A. water immersion objective. For details see Chapter III and IV.

Measurements of GNP Activity — GNP enzyme activity was determined in the aminating direction in a final volume 50 µl with 0.016 M of F6P as substrate. Reactions were carried out at 37 °C for 30 min, unless otherwise specified. The p-dimethylamino-benzaldehyde (DMAB) colorimetric method was used for assays (Lopez et al., 2002). Reactions were terminated by heating at 100 °C, which was followed by a colorimetric reaction read at 585 nm. Each assay was performed in duplicate and repeated three or more times. The results presented are means of these assays.
CHAPTER III

POST-TRANSLATIONAL MODIFICATION OF GNP AND ITS EFFECT ON THE ENZYME’S CATALYTIC PROPERTIES

INTRODUCTION

The enzyme glucosamine 6-phosphate isomerase (GNP) is the first enzyme in the encystment pathway (Fig. 5). It has been identified in several animal, fungal, as well as bacterial species and purified from *Escherichia coli*, *Bacillus subtilis*, *Candida albicans* and dog kidney (Fontes et al., 1995; Bustos-Jaimes and Calcagno, 2001). In *E. coli*, GNP as well as glucosamine 6-phosphate synthase (GlcN6P synthase) participate in aminosugar metabolism. GlcN6P synthase is responsible for D-glucosamine 6-phosphate (GlcN6P) production, while GNP catalyzes the reversible isomerization and subsequent deamination of GlcN6P into D-fructose 6-phosphate (F6P) and ammonium ion. Due to this fact, the enzyme’s name was changed to GlcN6P deaminase, E.C 3.5.99.6 (Fontes et al., 1995). Unlike the bacterial enzyme, *Giardia* GNP (gGNP) catalyzes the unique conversion and simultaneous amination of F6P to GlcN6P (Steimle et al., 1997). Thus, in *Giardia* GNP is an anabolic (synthetic), rather than a catabolic enzyme.
Figure 5. The pathway of giardan synthesis.
From Jarroll et al. (2001).

The same GNP aminating activity was described for some other eukaryotes and an E. coli K-12 mutant lacking GlcN6P synthase (Steimle et al., 1997). However, this is not the only difference between the E. coli and Giardia enzymes.

GNP of E. coli (EcoGNP) is an allosterically controlled hexamer with a native molecular mass of 178 kDa, while gGNP is a monomer of ca. 29 kDa (Steimle et al., 1997; Altamirano et al., 1993). EcoGNP is composed of identical polypeptide chains of 266 residues with one catalytic (amino acids 141-148) site and one allosteric (amino acids 151-161) site. Amino acid residues 163-183 form the active site lid (Cisneros et al., 2004; Horjales et al., 1999) and enclose the active site. The active site and the lid sequence are highly conserved among the organisms, especially in amino acids critical
for catalysis; these are: D 72, H 141, E 148, and R 172 (Fig. 6) (Cisneros et al., 2004, Horjales et al., 1999). Each *E. coli* monomer has four cysteine residues at positions 118, 219, 228, and 239. The disulfide bond at C 219 along with hydrogen bonds at the C-end and van der Waals contacts are responsible for the monomer’s interaction (Altamirano et al., 1993; Oliva et al., 1995). On the other hand, C 118 and C 239, located in the area with high flexibility scores (as predicted by crystallography), are important for enzyme catalytic activity (as shown by mutational studies). Residues C 219 and C 228 are predicted to be in a hydrophobic area and buried inside the deaminase structure. However, one of them, C 228 was also shown to be an important residue for full enzyme activity (Altamirano et al., 1992). gGNP has 13 cysteine residues that are unevenly spread through the molecule, with a higher concentration at the N- and C-termini. Residue C 118 is conserved in the *Giardia* and *B. subtilis* (position 113 in both organisms) enzymes (Fig. 6). The *Giardia* enzyme, but not the *B. subtilis* one, also has cysteines at positions 215, 219, and 230, which is close to the *Eco*GNP cysteine residue positions (Fig. 6).

Being a monomer gGNP is more related to the *B. subtilis* and *C. albicans* (molecular mass ca. 27 kDa and 43 kDa, respectively) than to the *E. coli* enzyme (Steimle et al., 1997). However, it has ~40% identity with the enzymes from these three organisms (Knodler et al., 1999).

Two-dimensional gel-electrophoresis of native gGNP revealed two isoforms with pI values of ca. 7.1 and 7.3, and denatured molecular masses of around 29 kDa (Steimle et al. 1997).
Figure 6. Alignments of GNP from *Giardia* (GiaGNP), *B. subtilis* (BsuGNP), and *E. coli* (EcoGNP).

* conserved residues, : conserved substitution, . semi-conserved substitution. The amino acids critical for catalysis (as well as their numbering) are shown in red, cysteine residues are indicated with turquoise color, the numbering is in black. Allosteric site and active site lid are labeled in yellow and green blocks. *E. coli* cysteine residues and critical for catalysis H and E are indicated with arrows, relevant *Giardia* residues are indicated with arrow heads.

The gGNP has a $V_{\text{max}}$ of 86.3±3.2 µmol. min$^{-1}$. mg protein$^{-1}$ GlcN6P produced, which is 2.3 fold higher than that for the deaminase (32.8±3.5 µmol. min$^{-1}$. mg protein$^{-1}$ GlcN6P consumed) (Table 2). Although gGNP’s $K_m$ is 2.5±0.24 mM for F6P and only 0.38±0.16 mM for GlcN6P, it is highly specific towards F6P (Steimle et al., 1997). The
absence of allostERIC regulation (due to its monomeric structure) is compensated by coupling GNP with GlcN6P N-acetylase activity (Jarroll et al., 2001).

**Table 2. Comparative properties of native and recombinant gGNPs.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;, mM</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; µmole of product. min&lt;sup&gt;-1&lt;/sup&gt; . mg protein&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aminase activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native enzyme</td>
<td>2.5±0.24 (F-6-P)</td>
<td>86.3±3.2</td>
</tr>
<tr>
<td></td>
<td>19±1.9 (NH₄Cl)</td>
<td></td>
</tr>
<tr>
<td>GNP1</td>
<td>3.7±0.34</td>
<td>204±34</td>
</tr>
<tr>
<td>GNP2</td>
<td>4.6±2.0</td>
<td>63±22</td>
</tr>
<tr>
<td><strong>Deaminase activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native enzyme</td>
<td>0.38±0.16 (F-6-P)</td>
<td>32.8±5.3</td>
</tr>
<tr>
<td>GNP1</td>
<td>0.31±0.15</td>
<td>151±20</td>
</tr>
<tr>
<td>GNP2</td>
<td>0.68±0.27</td>
<td>63±9.1</td>
</tr>
</tbody>
</table>

*The numbers for the native gGNP are from Steimle et al. (1997); those for the recombinant enzymes are from Lopez et al. (2002).

*Giardia* has two single-copy genes coding for GNP: *gnp1* and *gnp2*. Both of them are present on chromosome 3. The predicted protein sequences of both genes have 70% identity with high similarity in the catalytically important area and higher variations in the N- and C- termini (van Keulen et al., 1998; Knodler et al., 1999). *Gnp1* has an open reading frame (ORF) of 798 bp encoding a 29,421 Da polypeptide with a calculated pI comparable to that determined for the purified enzyme (Steimle et al., 1997).

*Gnp2* has an ORF of 789 bp with two possible start codons 54 bp apart. Neither of them gives a product with molecular mass and pI comparable with that of the purified native enzyme. One ORF would produce a protein with 263, the other with 244 amino acid residues (van Keulen et al., 1998). *Gnp2* appears to be derived from a recent gene
duplication. The closest example of a similar organization is *B. subtilis*, whose genome also encodes two monomeric glucosamine 6-phosphate isomerases sharing only 49% identity (Knodler et al., 1999). In contrast the *E. coli* genome encodes a hexameric (*nagB*) and a tetrameric (*nagA*) protein (Altamirano et al., 1992; Vincent et al., 2005). Inducible expression of *gnp1* with the product detected first at 6 h after induction was shown using RT – PCR and Northern blot analysis (van Keulen et al., 1998; Sun et al., 2003). Transcription of *gnp2* mRNA in encysting or non-encysting cells was not detected, which is consistent with the sequence analysis data (van Keulen et al., 1998). However, in 1999 Knodler et al. demonstrated that both genes were expressed: *gnp1* is inducible (appearing in encysting cells), while *gnp2* is constitutive (expressed at a constant level in trophozoites and throughout encystment). The apparent contradiction was explained by a difference between the strains and cultivation methods used (Lopez et al., 2002).

Both genes have been cloned and sequenced by van Keulen et al. (1998). The product of *gnp1* expression has typical glucosamine 6-phosphate isomerase activity. Monospecific polyclonal antibodies produced against expressed GNP1 recognized the purified gGNP (van Keulen et al., 1998). Western blot analysis with these antibodies identified a protein of approximately 29 kDa in induced trophozoites. In addition a 37 kDa protein was also observed at all examined time points (24, 48, 72, and 154 h after encystment was induced). Its amount increased, reaching a maximum at 72 h after induction. The same molecular weight protein was occasionally detected in non-induced trophozoites (Lopez et al., 2002). Immunoprecipitation studies demonstrated that the 37 kDa polypeptide is a ubiquitin-bound GNP. It was proposed that GNP is ubiquitinated
throughout encystment in order to be degraded by the proteasome. This hypothesis was supported by Western blot analysis, which demonstrated various small peptides, representing degradation products in cultures incubated for 220 h after induction of encystment (Lopez et al., 2002). It could not be established which enzyme, GNP1 or GNP2, was present in these samples. It also was not possible to examine if the ubiquitinated enzyme had activity (Lopez et al., 2002). Evidence of ubiquitin-mediated degradation was suggested by the existence of an ubiquitin gene in the genome (Krebber et al., 1994) as well as characterization of a 20S proteasome complex within Giardia trophozoites (McArthur et al., 2000). In 2011, Elligio-Garcia et al. showed that ubiquitin expression was initiated 6 h after stimulation with bile along with GNP1 and CWP2 (cyst wall protein 2) expression. It is worth-mentioning, however, that the authors unintentionally used *gnp2*- instead of *gnp1*-specific primers in their study and they measured expression of a ubiquitin carboxy-terminal hydrolase instead of the ubiquitin gene. A recent re-evaluation of transcription of GNP2 by semi-quantitative RT - PCR showed a low level of constitutive expression (van Keulen, personal communication).

In summary, GNP belongs to the (2-R) aldose-ketose isomerase class of proteins. In human, mouse and *E. coli*, GNP homologs form a superfamily (Pfam PF01182) of three clusters. The first branch shares quaternary structure and allosteric mode of regulation by GlcNAc6P and contains human and *E. coli* enzymes. The second branch comprises the monomeric enzymes, and the third one is predicted to be phosphoglucolactonases (6-phospho-D-glucono-1, 5-lactone lactonohydrolase, EC 3.1.1.31), which catalyzes the second step in the pentose phosphate pathway (Vincent et al., 2005).
We hypothesize that due to its importance for the cyst wall synthesis, GNP is post-
translationally regulated via nitrosylation in addition to the transcriptional activation. The
question addressed here is the nitrosylation status of GNP and its effect on the enzyme.

MATERIALS AND METHODS

Two-dimensional gel electrophoresis (2-D gel) — Non-encysting trophozoites and
trophozoites induced to encyst for 24 h were collected and processed according to the
protocol provided by the manufacturer (FOCUS™ – Soluble & Insoluble Protein
Extraction, GBiosciences, Maryland Heights, MO). The obtained soluble fractions were
treated using Perfect-FOCUS™ (GBioscience). Protein concentrations were determined
using the Bio-Rad (Bio-Rad, Hercules, CA) method with Bovine Serum Albumin (BSA)
as the standard. Proteins were first separated by isoelectric focusing (IEF) using the
ZOOM IPGRunner system (Invitrogen, Carlsbad, CA), pH 3-10, non-linear, 7 cm IPG
strips and rehydration buffer (RB, containing 7 M urea, 2 M thiourea, 4% NP-40, 50 mM
DTT, 1 % ampholytes and 0.002% Bromophenol Blue) according to the manufacturer’s
instructions. Approximately 50 μg of the sample were diluted with RB to give a final
volume of 160 μl. Samples were first applied in the mode of in-gel RB of the IPG strips
and passive rehydration was carried out overnight at room temperature. Prior to
rehydration, 50 mM DTT and Bromophenol Blue (3.2μl /160μl RB) were added to the
buffer. IEF was performed at room temperature for 20 min at 200 V, 15 min at 450 V, 15
min at 750 V and 1 h at 2000 V. The total volt-hours applied in the IEF step was 250.
Prior to the second dimension analysis, the strips were equilibrated for 15 min in 10 ml
equilibration solution (1 ml of 10 x sample reducing agent mixed with 9 ml of 1 x LDS
sample buffer (Invitrogen) and subsequently for another 15 min in 10 ml of 125 mM
alkylation solution (232 mg of iodoacetamide in 10 ml of 1x LDS sample buffer). The 2-
D gels were blotted onto a PVDF membrane (Fisher Scientific), which was blocked
overnight in 5% dry non-fat milk in TBS-T (Tris-Buffered Saline plus Tween-20: 20 mM
Tris-HCl, pH 7.6, 150 mM NaCl and 0.5% Tween-20) and probed with GNP-specific
serum using a 1: 750 dilution.

Western blot analysis — Giardia trophozoites were collected, washed, suspended in
the encystment medium and aliquots were placed into tubes. Encystment was carried out
for 6, 12, 18, 24, 30, 36, 48, and 72 h. At the indicated time points encysting cells as well
as trophozoites were collected and washed in 0.25 M sucrose. Aliquots of 10 µl were
aspirated, suspended in formalin-PBS (as described earlier in Materials and Methods,
Chapter II), and the cell number and percentage of fully formed cysts were determined
using a light compound microscope. After that, cellular suspensions were centrifuged at
12,000 x g for 2 min to remove supernatants and cells were lysed in 0.2% Triton X-100
with protease inhibitor (GBioscience). Protein concentrations were measured using the
Bio-Rad method. Protein lysates (15 µg of total protein per well (Invitrogen)) were
loaded onto gradient 4 - 20 % Tris - glycine gels and separated electrophoretically. Gels
were blotted onto PVDF membranes (Fisher Scientific), blocked overnight, and probed
with anti-GNP serum using a 1: 750 dilution (Lopez et al., 2002).

Enzyme assay — Lysates (6 µl) prepared from trophozoites and induced cells,
collected at different time points after induction, were used to measure GNP activity as
was described earlier (see chapter II). Assays were carried out at 37 ºC for 30 min to
measure enzyme activity (Lopez et al., 2002). The activity units obtained were
normalized to the activity measured in the blank (0.2 % Triton X-100) and adjusted to 15 µg of total protein.

**Biotin – Switch Method** – Cell pellets from 70 ml cultures were lysed in 5 volumes of buffer A plus blocking reagent as described in the S-Nitrosylation Protein Detection Assay Kit (Cayman Chemical Company, Ann Arbor, MI). The entire protocol described in the Assay Kit was followed exactly. Non-encysting trophozoites and trophozoites induced to encyst for 6, 12, and 24 h were pelleted and the pellets lysed, blocked and subjected to the biotin - switch method. One sample of non-encysting trophozoites was used as control for detection of possible internal biotin-labeled proteins and treated as the other samples except it was not subjected to the reduction/ biotinylation step, but incubated in the same buffer without the required reagents. After acetone precipitation the final pellets were dissolved in 200 µl wash buffer. Protein concentration was measured using the Bio-Rad method.

For analysis of nitrosylated proteins, 20 µg of proteins were subjected to polyacrylamide gel electrophoresis; gels were transferred to PVDF membranes, blocked with TBS plus 2% BSA and developed with the avidin-horseradish peroxidase, supplied with the kit. In addition, an avidin capture was done using avidin-agarose (Pierce, Rockford, IL) and 100 µg of proteins per assay, using non-encysting cells and 6, 12 and 24 h encysting cells. The treatment and following washes were as described by Martínez-Ruiz and Lamas (2007). Captured proteins were subjected to gel electrophoresis and transferred to PVDF membranes. Blots were developed using enhanced chemiluminescence (Pierce).
Cloning and protein expression — The GNP was cloned in PGEX-4T-1 as described (Lopez et al., 2002) and expressed in BL21(DE3) (Novagen, Madison, WI) cells. Mutant GNPs were constructed using two mutagenic primers, one to amplify the N-end and a complementary one to amplify the C-end of the ORF to replace the cysteine in combination with the sense and antisense primers used to amplify the entire gene, see Table 3 for a list of the primers. In addition, unique PstI or SalI sites were introduced in some cases for easy detection of mutated DNA.

TABLE 3. Primers to introduce mutations by PCR in recombinant glucosamine 6-phosphate deaminase. Mutations are indicated in italics in the C-end primer only; the following restriction enzyme sites were introduced: mutant 1: SalI, 2: SalI, 3: PstI, 4: SalI, 6a: SalI, 5 and 6b: none.

<table>
<thead>
<tr>
<th>Mutant #</th>
<th>Position of mutation</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant 1</td>
<td>C219</td>
<td>C-end: TTAAAGGAAGAACGTGCACCAATTCGCTGTTGCCCAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-end: CCGGGCAATGGGTGCTGACTTTCTCTTTAA</td>
</tr>
<tr>
<td>Mutant 2</td>
<td>C118</td>
<td>C-end: AAAAGGAGATCGAGCAGCTATGAGCAGAG</td>
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<tr>
<td></td>
<td></td>
<td>N-end GCCTCTGCTCATAGCGCGTCGACTCTCTTTT</td>
</tr>
<tr>
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<td>C131</td>
<td>C-end: TTGAGCCCTGAGATGTCTGCTGTTTCTGAG</td>
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<td></td>
<td></td>
<td>N-end CCCAGAAAGCCAGACATCTCGACAGG</td>
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<tr>
<td>Mutant 4</td>
<td>C161</td>
<td>C-end: GCGTCGTGCACCTGACCCAGACAGCAGATC</td>
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<tr>
<td></td>
<td></td>
<td>N-end CGATCGTCCTGGGTGAGGTGAGGTG</td>
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<tr>
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<td>C234</td>
<td>C-end: AACATCCTCCTCAGGCTTTTCTATGTCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-end GTGACATAGAAACGAGAGGTAGAGGT</td>
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<tr>
<td>Mutant 6a</td>
<td>C223</td>
<td>C-end: AGACCGATAGCCCGGCCTCTTTCCTCATG</td>
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<td></td>
<td></td>
<td>N-end CATGGAGGAGGAGACGTGAGCATGGG</td>
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<td>Mutant 6b</td>
<td>C223</td>
<td>C-end: AGACCCATAGCCGGGCCCTTTCCTCCATG</td>
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<tr>
<td></td>
<td></td>
<td>N-end CATGGAGGAGGAGGCCCAGGTATGGG</td>
</tr>
</tbody>
</table>

The polymerase chain reaction (PCR) was used with each of the mutagenic primers and corresponding primer for the 5’ or 3’ end of the gene using conditions as described (Lopez et al., 2002). The PCR generated DNAs were fractionated on a 1% agarose gel,
excised and purified using spin columns (Invitrogen). About 10% of each fragment was used for a second PCR reaction using the primers specific of expression of the entire gene. DNAs were denatured first for 10 min and the first DNA synthesis reaction was for 10 min at 72 °C, followed by standard PCR as described. The resulting DNA was purified as indicated above, cloned in pGEM-T Easy for analysis of the mutated DNA and then after excision of the insert with EcoRI and XhoI inserted into pGEX-4T-1 as described for GNP (Lopez et al., 2002). Successful mutation was verified by sequence analysis of the pGEX clones (DNA Analysis, LLC, Cincinnati, OH).

For isolation of recombinant enzyme overnight cultures (20 ml) with optical density (at 600 nm) between 0.6 – 1.0 were diluted with an equal amount of fresh medium and grown for 1 h in LB medium. Then, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM and incubation continued for 4 h. E. coli proteins were purified by lysing cells using B-PER™ Reagent (Pierce) and lysozyme followed by adjusting the Mg²⁺ concentration to 10 mM with MgCl₂ and addition of DNase I. Lysates were cleared by centrifugation in a microcentrifuge for 10 min at 12,000 RPM. Glutathione-S-Transferase (GST)-fusion proteins were purified on glutathione-agarose columns (Pierce) according to the manufacturer’s instructions. The fusion protein was then subjected to gel filtration over an Excellulose DF-5 desalting column (Pierce) in 50 mM Tris-HCl, pH 8, to remove the glutathione. The collected proteins were then digested overnight at 20 °C with 200 μl thrombin-agarose (Sigma). The thrombin-agarose was collected by centrifugation and the supernatant was then incubated with glutathione-agarose for 15 min after which the glutathione-agarose was poured into a column and the eluant collected, and recycled over the column three times. These proteins were subjected
to gel electrophoresis to determine if the removal of the GST partner was successful. Protein concentrations were measured as described above. By approximation of the proteins on the gel, at least 90% of the GNP was recovered as GST-free protein.

**Enzyme assays with the recombinant proteins** — For GNP enzyme assays, proteins were stored with 10% glycerol at -80 °C. Enzyme activity was determined in the aminating direction in 50 µl assays. Reactions were carried out at 37 °C for 30 s to measure initial velocity, using 0.2 µg enzyme per assay. The p-dimethylamino-benzaldehyde (DMAB) colorimetric method was used for assays (Lopez et al., 2002). Standard curves were produced using glucosamine 6-phosphate; reactions were terminated by heating at 100 °C, which was followed by the addition of the color reagent and read at 585 nm. Each assay was performed in duplicate and repeated three or more times; the results presented are means of these assays. Representatives of numerous assays were used for data analysis because of the large variations of this method, as has been observed by others (Šolar et al., 2008). $K_m$ values were measured with GraFit software (Erithacus software version 7, Surrey, UK) and the enzyme kinetics template. Eight assays were averaged to produce a value. $V_{max}$ was determined from the same assays and was converted to µmol.min$^{-1}$ and used to calculate the $K_{cat}$ values using the enzyme concentration in the assay and a molecular mass of 29 kDa. For measurement of the effect of each mutation Lineweaver Burk plots were constructed using Microsoft Excel.

In vitro nitrosylation of GNP was performed using 5 min preincubation with cys-NO and the entire reaction mixture on ice. Each reaction was started at 15 s time intervals so that each assay received the same time on ice and at 37 °C.
RESULTS

Expression and modification of GNP during encystment: Two-dimensional gel electrophoresis followed by Western blot analysis using GNP-specific serum identified a single spot of approximate molecular mass 66 kDa and pI 3.0 in trophozoites (Fig. 7). Proteins from cells induced to encyst for 24 h showed on the blot two additional spots and a large streak on the blot. One spot represents a protein of approximately 37 kDa with isoelectric point around 4 (Fig. 7, black arrow).

![Figure 7. Western blot analysis of glucosamine 6-phosphate in trophozoites and 24 h - encysting cells.](image)

Proteins from (trophozoites and cells induced for 24 h were separated by two-dimensional gel electrophoresis and blotted onto PVDF membranes. The blots were probed with a 1:750 dilution of GNP-specific serum. Black arrow indicates 37 kDa protein in the trophozoites; white arrow indicates 29 kDa protein in 24-h encysting cells.)
The other spot and the streak represented proteins of the same molecular mass, of approximately 29 kDa and different isoelectric points, ranging from 5 to 6 (indicated with a white arrow).

*Giardia* trophozoites were induced to encyst and collected after 0, 6, 12, 18, 24, 30, 36, 42, 48, and 72 h in encystment medium. Cell concentrations and the total percentage of fully formed cysts were determined by counting. Cells were lysed and soluble protein fractions were analyzed using Western blots with GNP-specific serum (Fig. 8, panel A) and each fraction was assayed for GNP activity (Fig. 8, panel B). Polypeptides of 37 and 50 kDa were recognized in trophozoites, the first one corresponding to the previously identified ubiquitinated GNP (Lopez et al., 2004). Additional polypeptides were identified by GNP-specific serum in samples obtained after different encystment periods. A 29 kDa polypeptide, representing an inducible GNP was first identified after 6 h of encystment. The strength of the signal increased after 12 h and, with slight variation, was present throughout the entire period of encystment. The size of the inducible GNP shows minor changes throughout encystment, suggesting protein modification. Additional polypeptides of lower molecular weight (15 kDa and below) were identified at the 12, 30, 42, and 72 h time points, suggesting protein degradation. It is worth to mention that their appearance corresponds to the highest percentage of cysts detected in the cultures: 34% at 30 h, 39% at 42 h, and 57% at 72 h. The exception is 12 h which had only 2.2% of fully-formed cysts. A polypeptide of 50 kDa was also detected at the 18, 24, 36, 42, 48, and 72 h time points, with the strongest signal at 36 h. A polypeptide of 82 kDa was recognized at the 24, 30, 36, and 42 h time points, with the strongest signal at 36 h. These two polypeptides, 50 and 82 kDa, - were consistently present in the same samples.
Figure 8. Western blot and enzymatic analysis of glucosamine 6-phosphate.

(A) Analysis of lysate proteins isolated from trophozoite (0 h) and cells that had been induced to encyst for 6, 12, 18, 24, 30, 36, 42, 48, and 72 h. Time in the encystment medium and percentile of fully formed cysts at the end of the incubation are indicated below the image. (B) GNP activity measured per 1µg of total protein in the samples analyzed in panel A.
Two groups of peptides, those with low molecular masses and those with 50 - 82 kDa, were not detected in the same samples. The 15 kDa and below polypeptides increased in amount while those of 50 - 82 kDa decreased. GNP activity detected per 1 µg of total protein increased throughout the encystment period with an insignificant drop at 30 and 42 h (the same time points that had low molecular weight polypeptides present). This experiment was repeated twice using two different strains of *G. intestinalis*. The results were consistent with an insignificant time-dependant variations between two strains.

**Figure 9. Western blot analysis of glucosamine 6-phosphate using trophozoites incubated with different concentrations of NO-donor.**

*Giardia* cells were incubated with 1 mM (panel A) or 0.4 mM (panel B) cys-NO for 24 h. Cells were lysed and protein lysate was separated using SDS-PAGE along with the lysates of trophozoites and cells induced for cyst production. Western Blots were analyzed with GNP-specific serum in a 1:750 dilution. 1-trophozoites; 2-cells induced to encyst for 24 h; 3-trophozoites incubated with cysteine-NO for 24 h.

Comparative Western blot analysis of *Giardia* trophozoites, induced to encyst for 24 h, and cells incubated with the NO donor cysteine-NO (cys-NO) for 24 h was performed next (Fig. 9). The anti-GNP serum recognized polypeptides of 37 and 50 kDa in the
trophozoites and cells incubated in the presence of 1 mM cys-NO and the 29 kDa polypeptide in the cells induced for 24 h (panel A). At the same time no polypeptides were identified in trophozoites or cells incubated in the presence of 0.4 mM cys-NO, while the 29 and 15 kDa polypeptides were recognized in cells undergoing encystment (panel B).

**GNP is nitrosylated in vivo:** Non-encysting trophozoites and cells induced to encyst for 6, 12, and 24 h were used to analyze their nitrosylation state by the biotin-switch assay (for the details see Materials and Methods, chapter III) (Fig. 10). Biotinylated proteins were captured using avidin-agarose. Following extensive washing of the agarose beads, the samples of captured proteins, as well as the biotinylated non-captured proteins, were heated for 3-5 min at 80°C in the presence of loading buffer and SDS and subjected to gel electrophoresis followed by Western blot analysis of the gels. The blots were developed using avidin-HRP and showed capture of biotinylated proteins (Fig. 10, lanes 1 - 4) that looked essentially as samples before capture (not shown). A duplicate set of the blots was developed with GNP-specific serum and showed induction of GNP as a result of encystment (Fig. 10, lanes 5 - 8) and the presence of GNP in the avidin-captured samples, indicating that GNP was nitrosylated in the cells. To control for specific capture of biotinylated GNP both sets of blots were analyzed using a serum directed against N-acetyl-glucosamine 6-phosphate mutase, the third enzyme in the encystment pathway, which has no specific (consensus) nitrosylation site. Mutase (panel A) as well as an anonymous cross-reacting low molecular weight protein (panel C) were present in total protein samples (lanes 5 - 8), but not in the captured samples (lanes 1 - 4). The mutase increased in amount during encystment, as expected. Both, inducible (29 kDa) and
ubiquitinated (37 kDa) GNP are present in total and avidin-captured samples, indicating nitrosylation of the enzyme in vivo upon synthesis. Several bands with molecular weight ranging from 30 to 34 kDa were also present. The obtained data were reproducible upon repeat.

![Figure 10. Nitrosylated proteins captured with avidin-agarose.](image)

Proteins from trophozoites (lanes 1 and 5) and cells encysting for 6 (lanes 2 and 6), 12 (lanes 3 and 7) and 24 h (lanes 4 and 8) were subjected to the biotin-switch assay. Fifty µg of each assay were used for capture with avidin-agarose and loaded after binding and washing on lanes 1 – 4. Twenty µg of the treated proteins were loaded directly on the gel without capture, lanes 5 – 8. After electrophoresis, the gel was blotted onto a PVDF membrane which was developed with anti *Giardia* mutase (AGM) serum, panel A; deaminase (GNP) panel B; and AGM, showing a cross-reacting protein panel C. The arrows in panel B indicate the 37 kDa ubiquinated GNP and 29 kDa induced GNP.
Detection of nitrosylated cysteine residues: GNP has a putative consensus nitrosylation motif of GKCE (Stamler et al., 1997) in position 213 - 216 (Fig. 11). In order to assess whether this site can be nitrosylated or not, the recombinant GNP (Lopez et al., 2002) was purified and the GST-fusion partner removed by thrombin digestion and affinity-chromatography. In addition, a mutant GNP gene was constructed with serine replacing cys at position 215, and then cloned and expressed in E. coli as a GST-fusion protein. This mutant recombinant GNP$^{215\text{cs}}$ was purified and its GST-partner removed as was done for “wild type” GNP.

The amino acid sequence of GNP is shown with the mutated cysteine residues indicated by a red color and their position number. The three modified residues that are possible nitrosylation sites are marked with *. The catalytic region is underlined and the flexible lid is indicated in italics.

Figure 11. Sequence of glucosamine 6-phosphate with cysteine sites to be mutated indicated in red.
Mutants and wild type GNPs were incubated in the presence of increasing concentrations of cys-NO (0 - 0.005 mM) for 2 min. Following the incubation in vitro nitrosylation was detected using the biotin-switch method. Equal amounts of wild type and mutant GNPs of each treatment were separated by PAGE and then transferred to a PVDF membrane. The membrane was developed with avidin-HRP (Fig. 12). Wild type GNP (panel A) as well as the mutant GNP\textsuperscript{215cs} (panel B) were biotinylated in the absence (lane 1) as well as presence of the cys-NO donor (lanes 2-4). The intensity of the signal, however, increased along with the increase in concentration of cysteine-NO (cys-NO) up to 0.05 mM (lanes 2, 3). The results indicate that both wild type and mutant GNP can be nitrosylated in vitro.

Figure 12. Comparative biotin-switch analysis of wild type and mutated GNP.
Wild type (panel A) and mutant CS215 (panel B) GNP were incubated with increasing concentration of cysteine-NO (lane 1-0 mM; 2-0.5 mM; 3-0.05 mM; 4-0.005 mM). Recombinant proteins nitrosylation was assayed using the biotin-switch method.

To evaluate the effect of nitrosylation on GNP the kinetic parameters $K_m$ and $V_{max}$ of the enzyme incubated in the presence or absence of cys-NO were measured. The $K_m$ of the recombinant enzyme (without cys-NO treatment) was $3.5 \pm 0.18$ mM with fructose 6-phosphate as substrate. Upon 5 min preincubation with cys-NO (0.4 - 0.002 mM) the
values of the $K_m$ changed depending on the NO donor concentration (data not shown). The $K_m$ increased up to 7.7 mM at high cys-NO concentrations (0.4 mM), but decreased to 1.9 - 1.12 mM with 0.2 mM to 0.002 mM cys-NO. For routine comparison, 0.002 mM (2 µM) cys-NO was selected. The average $K_m$ of several independent measurements was 1.5 ± 0.85 mM. The $V_{\text{max}}$ did not change much in these assays. Without nitrosylation the $V_{\text{max}}$ was 90 µmol/min/mg and was 80 µmol/min/mg in the presence of 2 µM cysteine-NO. A typical Lineweaver-Burk plot is shown in Fig. 13. The $K_m$ in the absence of cys-NO was 2.54 mM in this particular experiment and 0.74 mM in the presence of 2 µM of cys-NO.

**Figure 13.** Effect of nitrosylation on catalytic parameters of recombinant GNP. Recombinant GNP (0.4 µg) was assayed for 30 s in 50 µl at 37°C with fructose 6-phosphate (F6P) as substrate (0.5 – 32 mM) without (circles) or with 2 µM Cys-NO (triangles). The results are presented as a Lineweaver-Burk plot. The vertical axis ($1/V$) is in arbitrary units, the horizontal axis is in $1/[S]$ in 1/mM F6P.
Figure 14. Effect of nitrosylation on catalytic parameters of recombinant mutant GNP. Mutant GNPs (0.4 µg) were assayed for 30 s in 50 µl at 37°C with fructose 6-phosphate (F6P) as substrate (0.5 – 32 mM) without (circles) or with 2 µM Cys-NO (triangles). The data are presented as representative Lineweaver-Burk plots. A: mutant C113; B: mutant C156 and C230. The vertical axis (1/V) is in arbitrary units, the horizontal axis is in 1/[S] in 1/mM F6P.
The effect of nitrosylation on mutant GNP_215cs was investigated in the same manner. The $K_m$ was $2.5 \pm 0.75$ mM. In the presence of cys-NO the $K_m$ decreased to $1.2 \pm 0.3$ mM, which suggests that nitrosylation has the same effect on the $K_m$ of the mutant as it had on the $K_m$ of the wild type GNP. This result suggested strongly that mutant GNP_215cs is not nitrosylated at position 215, as the biotin - switch experiment already indicated since this mutant was biotinylated as strongly as the wild type GNP.

Therefore, to evaluate the possibility that other cysteines could be nitrosylated, several separate mutations were introduced in positions: 113, 126, 156, 219, and 230. These positions represent cysteine residues flanking the catalytic region of GNP (Fig. 11). In addition a double mutant (position 113 plus 230) was constructed. All mutants were cloned and expressed in *E. coli* and the expressed proteins were purified and treated as described above. The kinetic parameters of all these mutants were measured in the presence and absence of 2 µM of cys-NO. Representative Lineweaver-Burk plots for all mutants are shown in Fig. 14. The ratio of $K_m$ in the absence and presence of 2 µM cys-NO was used as an indication of the effect of the mutation. A ratio close to one will indicate the loss of the effect of nitrosylation on the catalytic properties of GNP, because of a loss of a cysteine that could be nitrosylated. The values for $K_m$ and $V_{max}$ were calculated as an average of 5 - 10 assays for each mutant.

A summary of the results, including the ratio of $K_m$ values in the absence and presence of cys-NO is presented in Table 4. Mutants GNP_215cs and GNP_126cs showed $K_m$ ratios similar to wild type GNP, namely $2.3 \pm 0.28$ and $3.9 \pm 1.75$ similar to wild type, $2.9 \pm 1.36$. Their Lineweaver-Burk plots were similar as well as shown in Fig. 13. However, mutants GNP_156cd and GNP_230cs lost their response to nitrosylation. Their $K_m$
ratios with and without cys-NO are 1.0 ± 0.08 and 1.2 ± 0.16 and representative Lineweaver-Burk plots show a minimal effect on $K_m$ values and are shown in Fig. 14 (panel B). To a lesser extent mutant GNP$^{113cs}$ showed some effect of nitrosylation with a $K_m$ ratio of 1.2 ± 0.17 and a representative Lineweaver-Burk plot is shown in Fig. 8 (panel A).

![Figure 15. Effect of nitrosylation on catalytic parameters of recombinant mutant C219 GNP.](image)

Mutant C219b GNP (0.4 µg) was assayed for 30 s in 50 µl at 37°C with fructose 6-phosphate (F6P) as substrate (0.5 – 32 mM) without (circles) or with 2 µM Cys-NO (triangles). The data are presented as Lineweaver-Burk plots. The vertical axis ($1/V$) is in arbitrary units, the horizontal axis is in $1/[S]$ in mM M6P.

Mutation of the cysteine in position 219 of GNP to a valine and the proline of position 220 to aspartate, to introduce a restriction site in the mutant for easy selection, however abolished enzyme activity completely at the concentrations used for the assays. It had a very low activity that could only be measured when a 10 fold higher amount of
enzyme compared to the standard assay was incubated for 10 min. Because this mutation introduced a negatively charged residue, another mutation was made changing C219 into S219. This still resulted in an enzyme with very low activity (Fig. 15) and kinetics that made measurement of a $K_m$ impossible. The GNP mutant carrying a double mutation in cysteine 113 and 230 behaved similar to their single mutants (Fig. 16).

![Figure 16. Effect of nitrosylation on catalytic parameters of recombinant GNP carrying double mutation at positions C113 and C230.](image)

Double mutant GNP (C113 and C230) was assayed for 30 s in 50 µl at 37°C with fructose 6-phosphate (F6P) as substrate (0.5 – 32 mM) without (circles) or with 2 µM Cys-NO (triangles). The data are presented as representative Lineweaver-Burk plots. The vertical axis ($1/V$) is in arbitrary units, the horizontal axis is in $1/[S]$ in 1/mM F6P.
Table 4. Comparison of kinetic parameters of recombinant and mutant glucosamine 6-phosphate.

The ratio of $K_m$ + and – cys-NO is per pair-wise comparison of each individual assay. The averages are of duplicates repeated 3 times for C126 and C156 to 6 times for the other mutants. GNP wt represent wild type enzyme.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mutation position</th>
<th>$K_m$, mM</th>
<th>$V_{max}$ μmol/min/mg</th>
<th>$K_m$ -NO-cys</th>
<th>$V_{max}$ μmol/min/mg</th>
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<tr>
<td>GNP wt</td>
<td>112</td>
<td>3.5 ± 1.1</td>
<td>137 ± 53.5</td>
<td>115 ± 38.9</td>
<td>2.9 ± 1.36</td>
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<tr>
<td>Mutant 2</td>
<td>C113</td>
<td>2.8 ± 0.96</td>
<td>136 ± 48.0</td>
<td>91 ± 29.7</td>
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<td>Mutant 3</td>
<td>C126</td>
<td>3.3 ± 0.98</td>
<td>187 ± 102.6</td>
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<td>3.9 ± 1.75</td>
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<td>Mutant 4</td>
<td>C156</td>
<td>1.3 ± 0.32</td>
<td>198 ± 48.0</td>
<td>282 ± 22.1</td>
<td>1.0 ± 0.08</td>
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<tr>
<td>Mutant 1</td>
<td>C215</td>
<td>2.5 ± 0.75</td>
<td>215 ± 139.1</td>
<td>212 ± 113.1</td>
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<tr>
<td>Mutant 5</td>
<td>C230</td>
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<td>Mutant 2+5</td>
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<td>118 ± 64.3</td>
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<td>0.8 ± 0.20</td>
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<td>Mutant 6</td>
<td>C219</td>
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Effect of nitrosylation on the catalytic parameters of GNP: As shown in the previous section, GNP can be nitrosylated and two cysteines (156 and 230) identified as candidates for nitrosylation. The relevance of these positions will be discussed later. To see what the effect of nitrosylation might be a more accurate determination of $K_m$ and $V_{max}$ values is needed than the use of double reciprocal plots. These were useful to detect the effect of mutations but for an accurate measurement of the catalytic parameters the assays done with wild type GNP were analyzed by using the GraFit program which calculates $K_m$ and
$V_{\text{max}}$ using the Michaelis Menten equation. The values obtained were used to calculate $K_{\text{cat}}$ and $K_{\text{cat}}/K_m$ of recombinant GNP in the absence or presence of an NO donor.

**Table 5. Catalytic parameters of wild type GNP**

<table>
<thead>
<tr>
<th></th>
<th>In the absence of NO</th>
<th>In the presence of NO (2 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$</td>
<td>2.7 ± 0.44 mM</td>
<td>1.2 ± 0.45 mM</td>
</tr>
<tr>
<td>$K_{\text{cat}}$</td>
<td>42 ± 11 s$^{-1}$</td>
<td>31 ± 6.7 s$^{-1}$</td>
</tr>
<tr>
<td>$K_{\text{cat}}/K_m$</td>
<td>15.55 mM$^{-1}$.s$^{-1}$</td>
<td>25.83 mM$^{-1}$.s$^{-1}$</td>
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</tbody>
</table>

In addition assays were performed as described above using a 5 min preincubation with cys-NO and also by treating GNP with cys-NO followed by removal of cys-NO by gel filtration. Both treatments had similar effects. The obtained values of $K_{\text{cat}}/K_m$ are shown in Table 5. The ratio of $K_{\text{cat}}/K_m$ in the presence of nitrosylation and without nitrosylation is on average 2. Also the ratio $V_{\text{max}}/k_m$ was calculated as the physiological catalytic efficiency as used by Moreno-Sánchez et al., (2008). Again the ratio of these constants before and after nitrosylation is on average 2. This indicates that the enzyme becomes twice as efficient after nitrosylation.

**DISCUSSION**

Glucose is needed for energy and cyst wall production. GNP is the first and the key enzyme in the *Giardia* encystment pathway. The substrate of the enzyme is F6P, which
also is an intermediate of glycolysis, one, of at least two energy producing pathway in *Giardia* trophozoites. GNP is regulated at the gene level by transcriptional activation (Lopez et al., 2003) and converts F6P and NH4+ (supplied by the arginine dihydrolase pathway) to glucosamine-6-phosphate (GlcN-6-P) (Macechko et al., 1992; Morrison et al., 2007). GlcN-6-P, the product of the first reaction in cyst wall synthesis, also serves as an allosteric regulator for the fourth enzyme in the encystment pathway: UDP-N-acetylglucosamine pyrophosphorylase (Fig. 5), which is constitutively expressed in *Giardia* trophozoites (Bulik et al., 2000). Glycolysis is ubiquitous and a highly conserved metabolic pathway: its main control point in most organisms is the enzyme phosphofructokinase (PFK), which catalyzes a rate-limiting step of F6P phosphorylation, the third irreversible reaction in this pathway. The enzyme is allosterically regulated by AMP/ATP concentrations in most organisms (Bagyan et al., 2005). In contrast the glycolytic pathway in *Giardia* relies on a pyrophosphate (PPi)-dependent PFK, which makes this reaction reversible (Li and Phillips, 1995; Mertens, 1991). However, the Kcat/Km for PPi is two orders of magnitude larger than the Kcat/Km for P, (the reverse reaction), namely 2075 mM⁻¹.s⁻¹ for PPi versus 75 mM⁻¹.s⁻¹ for P (Li and Phillips, 1995).

Both *Giardia* enzymes, constitutively expressed PFK and transcriptionally activated GNP, use the same substrate, F6P. The source of F6P is glucose obtained from endogenous reserves as shown by Macechko et al. (1992), who also showed that glucose is diverted towards glucosamine during encystment. *Giardia* PFK has a Km of 0.25±0.0196 mM (Li and Phillips, 1995) while GNP has a Km of 2.5±0.24 mM (Steimle et al., 1997). The Kcat/Km ratio calculated by Phillips is 332 mM⁻¹.s⁻¹ for PFK and 18 mM⁻¹.s⁻¹ for GNP (Phillips, personal communication), thus the catalytic efficiency is 18 times
better for PFK. This clearly is a paradox, so the regulatory question becomes how is the switch from the use of glucose for glycolysis to its use in the encystment pathway for giardan synthesis accomplished biochemically? Note that the increase in GlcN-6-P during encystment as the result of GNP activity is approximately 5 fold (Şener et al., 2009). Previously it was suggested that the difference in $K_m$ is simply overridden by the drastically increased amount of GNP (Jarroll et al., 2001). In this case glycolysis will have to slow down or maybe even stop to accommodate the high demand on glucose in the form of F6P for encystment. Therefore, energy production in the form of ATP synthesis must come from another pathway, supposedly from ADHP (this will be discussed later). In conclusion, down-regulation of glycolysis downstream from F6P would be expected if the catalytic efficiency of GNP is not sufficient to redirect F6P towards cyst wall biosynthesis. Since PFK itself is not allosterically regulated in Giardia another enzyme might be involved.

Could nitrosylation be the key regulatory mechanism? The logical consequence of NO production by Giardia (observed by Harris et al., 2006) is modification of parasitic proteins by a post - translational addition of an NO moiety to cysteine. This modification has been shown to alter protein activity or function. The best examples are hemoglobin, several caspasas, dimethylarginine dimethylaminohydrolase, but what might pertain to the situation in Giardia, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hess et al., 2001; Leiper et al., 2002; Shenton and Grant, 2003; Kunciewicz et al., 2003) is another possibility. The example of human GAPDH modification is well documented (Mohr et al., 1996; Mohr et al., 1999; Mannick, 2007). By analyzing protein databases Stamler et al. (1997) proposed a putative motif composed of acidic and basic amino acids
to predict cysteines prone to nitrosylation. The postulated consensus sequence is a cysteine surrounded by proton donor and acceptors that looks like this: ([G,S,T,C,Y,N,Q] [K,R,H,D,E] C [D,E]). In a later publication, the same author modified the idea of a consensus to a more general acid/base motif in which the tertiary structure also is needed to provide the acids/bases (in aquaporin, for instance) and the cysteine might even be in a non-polar sequence, as is the case for the ryanodine receptor (Hess et al., 2001). The idea of a consensus sequence also has been challenged later by Marino and Gladyshev (2009) using a comparison of known nitrosylated proteins. The immediate flanking of acidic residues appeared not to be critical although distant acidic groups still might play a role (Marino and Gladyshev, 2009). Based on the above, the working hypothesis is that GAPDH might be involved in the control of glycolysis through inactivation by nitrosylation.

Is GNP nitrosylated? The possibility of GNP modifications that might affect protein activity and localization was demonstrated by two-dimensional gel electrophoresis. Earlier it was shown that Giardia proteins undergo post - translational modifications by phosphorylation (Alvarado and Wasserman, 2010; Ellis et al., 2003; Slavin et al., 2002), glycosylation and palmitoylation (Hiltpold et al., 2000; Webera et al., 1997), isoprenylation (Luján et al., 1995), ubiquination (Lopez et al., 2002), and SUMOylation (Touz et al., 2008). Originally two native isoforms of GNP were purified; both had molecular masses of 29 kDa, one with pI of 7.1 and 7.3 (Steimle et al., 1997). However, more isoforms, including the one with a higher molecular weight are annotated in the Giardia data base (Morrison et al., 2007). The spot of 66 kDa, identified by GNP-specific serum in Giardia lysates separated by 2-D gel electrophoresis (Fig. 7) could be a result of
serum cross-reactivity with one of the high molecular weight GNP-like protein (this one
has a calculated pI of 5.56; note that the IEF gels used were not linear and determination
of the pI is not accurate). The protein spots with molecular weights 29 and 37 kDa
respectively, correspond to the inducible GNP and GNP modified by ubiquitin
attachment. The streak that was observed shows that GNP is abundant in the induced
cells and also is subjected to a different kind of modification. Phosphorylation as well as
nitrosylation would cause protein mobility shifts. Usually the shift is large when the pI of
a protein is 7 or above and negligible when the pI is around 5 (Zhu et al., 2005).
Calculation of the pI of GNP using the program of Halligan (2009) revealed a pI of 6.69,
which changes to 6.51 with one phosphate added, 6.36 with two and 6.23 with three
phosphates. Phosphorylation with a high score (≥0.8-0.9) was predicted for GNP1 at
serine positions 107, 146, 150, 202, and 210, and for threonine at position 187. The GNP
2 sequence also showed the possibility of phosphorylation with a high score at serine
positions 13, 161, 165, and 217, and threonine positions 202. Analysis was performed
using NetPhos 2.0 server on gGNP2 (Blom et al., 1997).

Nitrosylation can shift the pI 0.6 units higher than the pI without modification as
shown by Kim et al. (2004) for Iron Regulatory Protein 2 which shifted from a pI of 6.4
to 7.0 after nitrosylation. Giardia proteins show a high degree of modification shown by
a proteomics analysis (Su et al., 2007). GAPDH was present as two spots on 2-D gels,
one with a pI of 6.9, which is the calculated pI, one with a pI of 7.5 (calculated using the
gels shown in the authors’ figure). This could be the result of nitrosylation. Other proteins
present as two spots were phosphoglycerate kinase, glutamate dehydrogenase, and
ornithine carbamoyl transferase. Phosphoglycerate kinase shows an extra spot with a
lower pI, suggesting phosphorylation; the other two enzymes both show a second spot with a higher pI than the calculated ones. When the *Giardia* database (Morrison et al., 2007) was searched for the consensus nitrosylation motif as described above ornithine carbamoyl transferase as well as GNP and a number of other metabolic enzymes (in the pentose-phosphate pathway and in the ADHP) exhibited them. The putatively identified nitrosylation consensus sequence in GNP was a cys at position 215, in the sequence GKCE.

The method of choice for detecting nitrosylated proteins, the biotin – switch, was developed by Jaffrey et al. (2001). This method is based on blocking free thiol groups by methylation, followed by reducing a nitrosylated cysteine to a thiol which is then labeled with activated biotin. This method was applied to *Giardia* lysates and many proteins were biotinylated suggesting that they originally contained nitrosylated cysteine residues. One problem reported by others is that some thiol groups might not be accessible to the blocking reagent. One way to overcome this problem is denaturation of proteins in the presence of SDS, which can also destroy the unstable nitrosothiols (Forrester et al., 2009). However, under these stringent conditions still many proteins in *Giardia* were biotinylated suggesting that nitrosylation of *Giardia* proteins indeed takes place (data are not shown). Capture of the biotinylated proteins with avidin-agarose and their analysis using GNP-specific antiserum identified GNP among these nitrosylated proteins.

In summary, the ubiquitinated (constitutive, modified) enzyme appears to be nitrosylated in vivo and the induced enzyme can be artificially nitrosylated in vitro.
Is GNP nitrosylation a part of the metabolic switching mechanism? GNP contains the consensus nitrosylation sequence proposed by Stamler (1997) and appeared to be nitrosylated in vivo. Nitrosylation of GNP was postulated as a mechanism to help regulate GNP activity. Such modification could account for F6P redirection from glycolysis to cyst wall synthesis. To test this hypothesis, in vitro nitrosylation of recombinant GNP was performed and activity was measured at various concentrations of the substrate F6P in the presence or absence of cys-NO. Initial assays showed that nitrosylation had little if any effect on the enzyme activity, therefore, fixed amounts of cys-NO were tested with F6P concentrations ranging from 0.5 – 32 mM in order to measure the effect of nitrosylation on the \( K_m \) of the enzyme. High concentrations of cys-NO (0.2 and 0.4 mM) increased the \( K_m \), indicating a lower affinity for the substrate. However, a low concentration of cys-NO of 2 \( \mu \)M decreased the \( K_m \) of the enzyme from about 2.5 mM to 0.7 mM in one typical assay, indicating an increased affinity for the substrate. The implication is that the enzyme could reach \( V_{\text{max}} \) at a much lower substrate concentration, making it plausible that it could redirect F6P usage towards cyst wall polysaccharide biosynthesis at a fixed F6P concentration. The fact that the NO concentration increases during early encystment (Timothy Paget, personal communications) would support this conclusion (see Fig. 20, Chapter IV). The largest effect on GNP \( K_m \) was found when 2\( \mu \)M cys-NO was used for 5 min. While it is not possible to say at this time which NO concentration would be the closest to the natural one, physiological concentrations of NO are quite low as described by Hall and Gartwaite (2009) and ranges from as low as 100 pM to 5 nM. The results of a large number of assays show consistently that the GNP \( K_m \) becomes smaller upon nitrosylation. The effect
of nitrosylation on the $V_{\text{max}}$ of the reactions was quite variable, but on average it was lower. The resulting $K_{\text{cat}}/K_m$ ratio increases only by a factor of 2.

GNP nitrosylation resulted in a twofold increase in catalytic potential. Taking into account that the amount of enzyme also increases because of transcriptional activation, this might be sufficient to account for the switch to the encystment pathway. Because there are no data available on metabolite flow and especially the concentration of F6P in the cells, this conclusion might be premature. Maybe the increase in enzyme quantity is already sufficient to account for the above mentioned switch. In addition, the down-regulation of GAPDH might slow glycolysis sufficiently to assist in redirection of carbons toward cyst wall production. Then the question arises, why nitrosylation? One possibility is that nitrosylation is used to control the amounts of GNP. Nitrosylation could be the signal to start the ubiquination of the enzyme. From the results of the 2-D gels, it is clear that the enzyme is modified during encystment. In fact the “smeary pattern” could be due to nitrosylation and/or phosphorylation. In either case this modification could serve as a signal for the ubiquination observed previously (Lopez et al., 2002). It is not clear why the enzyme is ubiquitinated. The data provided by the above mentioned authors do not indicate if the enzyme is inactivated.

Why is GNP ubiquitinated? In 2002, Lopez et al. demonstrated that GNP is ubiquitinated upon induction of encystment. Ubiquitinated GNP (37 kDa protein) along with immuno-reactive small peptides was the only form of the enzyme present in encysting cells for longer periods of time (48 h - 72 h) and in formed cysts. Ubiquitination typically targets enzymes for proteasomal degradation. Although poly-ubiquination (Fang and Weissman, 2004; Winget and Mayor, 2010) is usually required
for recognition by the proteasomal complex, mono-ubiquination was demonstrated as sufficient signal for degradation in some systems (Kravtsova-Ivantsiv et al., 2009).

Ubiquitinated GNP was occasionally detected in non-induced Giardia trophozoites. Such inconsistency could be explained by the absence of synchronization in Giardia cell cultures and the observation that some trophozoites encyst even when they are not in ideal encystment conditions (Sulemana et al., personal communication). The other possibility is that ubiquination followed by degradation is a way to regulate/neutralize the effect of a continuous but low level of transcription present in Giardia (Vanacova et al., 2003). The biotin-switch method showed that both the ubiquinated as well as the unmodified enzyme were nitrosylated. Additionally, some other forms of the enzyme, possibly partially degraded proteins, were also present as nitrosylated. This assay does not allow for quantitation of the amount of nitrosylation or for measurement of the percentage of nitrosylated enzyme in the total lysate, so the effect of nitrosylation on enzyme activity is not easily measurable.

Lysine residues, prone to ubiquitin attachment, could also be targeted by SUMO. The resulting SUMOylation usually protects the protein from ubiquitin-dependent proteasomal degradation and the modified protein acquires a novel function. Touz et al. (2008) demonstrated SUMOylation of Giardia’s arginine deiminase (gADI). It (gADI) is the first enzyme in the ADHP and catalyzes the irreversible catabolism of arginine to citrulline (Schofield et al., 1990; Schofield et al., 1992). Additionally, gADI serves as a peptidylarginine deiminase that converts protein-bound arginine to citrulline and by that regulates antigenic shift in Giardia (Touz et al., 2008). Finally, gADI is SUMOylated and translocated to the nucleus in the cells induced to encyst.
The exact lysine, targeted by ubiquitin attachment in GNP was not detected. Using ubiquination prediction software, K 111 and K 265 (Fig. 6) were identified as possible modified residues, K 111 with a low and K 265 with a moderate confidence level (Radivojac et al., 2010). Different lysine residues were identified by SUMOylation prediction software. Among them K 95, that carries the consensus sequence Ψ-K-X-E, and two lysines at positions K 203 and K 265 as non-consensus sequences (Ren et al., 2009). Analysis of the protein sequence of GNP2 did not identify a site for ubiquination, however, it showed a very high score (3.19) for SUMOylation at position K 110 (consensus sequence Ψ-K-X-E).

In summary, GNP appears to be regulated via nitrosylation in addition to the previously reported regulation by transcriptional activation and ubiquitin attachment (Lopez et al., 2002; Lopez et al., 2003). The finding that both forms of the enzyme appear to be nitrosylated suggests that this modification precedes ubiquination. The recombinant GNP can be expressed as an active enzyme with the correct specificity, which made it possible for use in vitro nitrosylation experiments to predict the effect of nitrosylation on the native enzyme. In vitro nitrosylation using cys-NO, an NO donor that exchanges the NO group from soluble cys-NO to thiol groups in proteins assumes that a similar reaction takes place in vivo. Nitrosylation reduces gGNP’s $K_m$ for F6P two-fold. A change in $K_m$ and increase in the amount of enzyme during encystment could increase the flow of F6P from glycolysis to the encystment pathway. It is not clear if in vitro nitrosylation mimics precisely in vivo nitrosylation, but nitrosylation of the native and recombinant enzymes in the presence of cys-NO was demonstrated by the biotin - switch method. Additionally GNP could be modified also by phosphorylation and SUMOylation as is suggested by the
shift in the pI in 2-D gel electrophoresis and the modification prediction software used. The exact mechanism and consequences of the demonstrated modifications are not known. As suggested by the results of Western blot analysis performed on the cells undergoing encystment, the amount inducible gGNP (29 kDa) activity increased throughout the “active” encystment phase (6 - 24 h). At the same time the protein molecular mass increases due to ubiquitin attachment. After 24 h, when most cells in the culture have finished encysting, the products of protein degradation appear along with a decrease in the amount of ubiquitinated GNP.

Is the cysteine in the consensus sequence nitrosylated? The question of which cysteine(s) is/are modified was addressed by in vitro mutation of cysteine residues close to the active site in the E. coli enzyme. Cysteine does not play a role in the catalytic mechanism of the E. coli enzyme, although two cysteine residues (C 118, comparable to C 113 in Giardia and C 239) are involved in allosteric control (Lucumí-Moreno et al., 2005; Oliva et al., 1995). C 239 is not conserved in the Giardia enzyme, but C 230 is close to the C 239 in the E. coli enzyme. First position C 215 was mutated because it is part of the consensus sequence suggested by Stamler et al. (1997) and was replaced with a serine. The mutant enzyme behaved essentially the same as the non-mutated enzyme, indicating that other residues must be nitrosylated and this also suggested that this consensus sequence is not critical, as has been shown by others (Marino and Gladyshev, 2009). Therefore, a number of other cysteine residues were mutated (Fig. 11). These are close to the active site and one, C 230, is close to the C 239 involved in allosteric regulation in the E. coli enzyme. Mutations in C 126 showed no effect, the enzyme behaved as the wild type enzyme. Mutation of C 230 made the enzyme not responding to
nitrosylation, suggesting that this is a major controlling residue. Some effect was observed in the mutant that had C 113 modified. A double mutant with both C 113 and 230 modified completely abolished response to cys-NO. In addition, modification of C 156 abolished the effect of nitrosylation, so it appears that C 156 and C 230 are the main target sites for nitrosylation with C 113 as a tertiary site. MS data support the notion that between two and four sites can be nitrosylated with a certain degree of variability (Timothy Paget, personal communications). Modification of C 219 could not be studied because it resulted in complete loss of enzyme activity when the C was mutated to a V followed by a D replacing the P 220. Another mutant that had the C 219 replaced for a serine only was active but showed a low activity and unusual kinetics. This suggests that C 219 might be involved in the catalytic mechanism. Modeling of the enzyme using SWISS-MODEL (Fig. 17) showed that this C 219 is close to the catalytic H 138 and E 143 in the active site, which residues are involved in a proton relay in the E. coli enzyme (Horjales et al., 1999) and are conserved in the Giardia enzyme as well (van Keulen et al., 1998). The mechanism by which nitrosylation affects the enzyme kinetics might be through a conformational change in the enzyme (Lucumí-Moreno et al., 2005; Oliva et al., 1995). The Giardia enzyme is active as a monomer (Steimle et al., 1997) in contrast to the E. coli enzyme which is a hexameric enzyme that shows allosteric control through the allosteric effector N-Acetyl-glucosamine 6-phosphate (Oliva et al., 1995).

However, the gGNP might have retained the ability to undergo conformational changes. In the E. coli enzyme E 148, D 141 and H 143 form a proton relay (Oliva et al., 1995). E 148 neutralizes positive charges in the allosteric site, but after the allosteric
effector binds, the phosphate in it takes over this role and makes the E available for the proton relay (Horjales et al., 1999).

Figure 17. Model of the GNP active site before mutation (panel A) and after mutation of C-P to V- A in the positions 219-220 (panel B).

Aminoacids His 138 and Glu 143 involved in the catalysis are shown (Altamirano et al., 1992; Oliva et al., 1995).

The charge change after nitrosylation of recombinant gGNP might have a similar effect and, in that manner, changes the $K_m$ of gGNP. It is intriguing to speculate that nitrosylation of C 156 and C 230 are critical because both are localized close to the allosteric site identified in the E. coli enzyme’s crystal structure (Oliva et al., 1995). C156, in particular, is interesting because it is located in the flexible lid, covering the active sites, identified in the E. coli enzyme as two antiparallel β-strands connected by a loop-helix-loop structure (Lucumí-Moreno et al., 2005) from position 158-187. This corresponds to region 153 - 183 in gGNP. The allosteric site made up of L 160 and T 161 (Lucumí-Moreno et al., 2005) in this flexible lid is in Giardia GNP identical to V 155 and C 156. C 113 on the other hand is on the surface of the enzyme (Altamirano et al., 1992; Oliva et al., 1995), which might be the reason it can be nitrosylated.
In summary, despite the monomeric nature gGNP behaves like an allosteric enzyme. This agrees with the notion that allostery is the result of a conformational change in a protein due to binding of a ligand or any change in the protein, such as a covalent modification. The effector binds at a site away from the active site (allosteric site) and affects the catalytic rate of the enzyme. This is an intrinsic property of all monomeric proteins and thus also the one subunit enzyme such as GNP falls in this category as has been described by Goodey and Bekovic (2008) in their review on allosteric regulation. The major finding is, therefore, that nitrosylation could act as an allosteric control mechanism in *Giardia*. 
CHAPTER IV

ANALYSIS OF NO-SYNTHESIZING ACTIVITY IN GIARDIA

INTRODUCTION

The importance of NO and protein modification by NO attachment was discussed earlier (see Chapter I). Here the question is: What is the source of NO in Giardia? There are two ways that NO may be produced: enzymatically and non-enzymatically (Fig. 18). Non-enzymatically, NO is produced from nitrite by reducing compounds, such as NADPH, L-cysteine, ascorbate, reduced glutathione and other thiols. Enzymatically NO is produced from L-arginine by NO synthase (NOS) in the presence of oxygen. The enzyme catalyses formation of NO as well as transfer of NO from S-nitrosocysteine (Ischiropoulos, 2005). Three classes of NOS have been identified in mammals: neuronal (nNOS), or NOS 1, epithelial (eNOS) or NOS 3, and inducible (iNOS) or NOS 2. The first two enzymes are produced in animal tissues constitutively and regulated by the intracellular Ca\(^{2+}\) level as well as phosphorylation. At the same time iNOS is a Ca\(^{2+}\) independent enzyme produced by macrophages upon stimulation with lipopolysaccharides or other potentially damaging stimuli. It is regulated transcriptionally or via nitrosylation (Hausel et al., 2006; Gaston et al., 2003; Mitchell et al., 2005).
The endogenous concentration of NO is $10^{-12} - 10^{-6}$ M and is strongly affected by the extracellular redox state. The amount of NO produced by iNOS is higher and may reach up to 10 μM, the toxic level for the cell (Hernández-Campos et al., 2003). The biological half life of NO varies from milliseconds to hours, depending on the concentration and presence of thiols. For protection, nitrosothiols must be stored in lipophilic protein folds, vesicles and interstitial spaces. For example, activation (denitrosylation) of caspases occurs upon release from mitochondria to the cytosol (Gaston et al., 2003; Mitchell et al., 2005). Typical NOS is a homodimer, consisting of an oxygenase or oxygen-binding and a reductase domain (Fig. 19) (Mitchell et al., 2007). The catalytic domain has an arginine-, tetrahydrobiopterin- (whose role remains elusive) and a zinc tetrathiolate-binding site. The reductase domain has a FMN, FAD, and NADPH – binding site. Ca$^{2+}$ /Calmodulin activates transport of electrons from the reductase domain to the N-terminal oxygenase domain (Mitchell et al., 2007).
Figure 19. Domain structure of human iNOS (courtesy of Alderton et al., 2001).

Oxygenase and reductase domains are indicated by solid boxes with the amino acid residue number shown at the start/end of the domain. The cysteine residue that ligates the Haem and Calmodulin (CaM) –binding site are indicated in black. Zink (Zn)-binding site is indicated in grey.

Nonconventional NOS-like enzymes have been found in plants, for instance in Arabidopsis thaliana, as well as in protozoa such as P. falciparum, E. histolytica, Leishmania donovani, Toxoplasma gondii, Trypanosoma sp. (Basu et al., 1997; Crawford, 2006; Gutierrez-Escobar et al., 2008; Hernández-Campos et al., 2003; Paveto et al., 1995).

As was mentioned in Chapter I, the Giardia database (McArthur et al., 2000) has a gene annotated as iNOS, however, it encodes only the reductase-like domain of the typical human iNOS. The active consumption of external arginine resulting in 89 % conversion into ornithine within 4 sec was documented by Knodler et al. (1995). The evident consumption of arginine via ADHP by Giardia was demonstrated by Schofield et al. (1990). This pathway converts arginine to ornithine and ammonium with a concomitant ATP production via substrate level phosphorylation (Schofield et al., 1990). ADHP is present in many prokaryotes, while Giardia and Trichomonas are the only eukaryotes identified so far that have the pathway (Linstead and Cranshaw, 1983;
Schofield et al., 1992). ADHP operates via three enzymes: arginine deiminase (ADI), ornithine transcarbamoylase (OTC), and carbamate kinase (CK). The activity of all three of these enzymes resulted in an 80-fold higher arginine flux through *Giardia* than in *Trichomonas* (Schofield et al., 1992). OCT was proposed as a candidate for the rate controlling step in the overall pathway. The ATP yield from 1 mM of arginine was 30 nmol.min⁻¹.mg protein⁻¹ compared to 2 nmol.min⁻¹.mg protein⁻¹ from 1 mM glucose in glycolysis (Schofield et al., 1992). Furthermore, arginine catabolism is not in conflict with glycolysis and mainly operates during the active proliferative stage (Schofield et al., 1992). The ADHP function during encystment has never been studied, although additional properties of ADI, the first enzyme in the pathway, in encysting and non-encysting trophozoites were recently identified (Touz et al., 2008). In non-encysting trophozoites, ADI also appeared to function as a peptidylarginine deiminase performing citrullination of variant-specific surface proteins. This activity plays an important role in *Giardia* antigenic shift which allows the parasite to evade the host’s immune system (Touz et al., 2008). In encysting cells, ADI is SUMOylated and translocated to the nuclei. Downregulation of CWP 2 expression was registered in the cells overexpressing ADI (Touz et al., 2008). The authors speculate that there are two possible explanations for ADI SUMOylation: 1) downregulation of the enzyme activity through sequestration from the cytoplasm; 2) transcription regulation through histone citrullination (Touz et al., 2008). OCT on the other hand seems to be nitrosylated as was discussed earlier (see Chapter III). Besides, it seems to be regulated through the same signaling pathway as CK, GAPDH and CWP 1-3 as suggested by Su et al. (2007). The present hypothesis is that *Giardia* actively uses the ADHP in the initial hours of encystment (first 6 h), later the
parasite might switch to the use of arginine for NO synthesis, but this is speculation. The ADHP is regulated through post-translational modification of ADI and OCT. The former is SUMOylated and sequestered from the cytoplasm as it was demonstrated by Touz et al. (2008). The latter is probably subjected to nitrosylation as is suggested by the presence of a putative nitrosylation motif and by the findings of Su et al. (2007). The switch between arginine conversion to citrulline and arginine use for NO production could be differentiated on the basis of the presence of NADPH. None of the ADHP reactions require NADPH, while arginine conversion to NO is NADPH dependent (see Fig. 3).

In summary, it was demonstrated that *G. intestinalis* produce NO, however so far there is no evidence of active NOS. The goal of this section of the dissertation is to study the NO-synthesizing activity in trophozoites and cells induced to encyst.

**MATERIALS AND METHODS**

**Detection of Nitric Oxide** — NO production by encysting and non-encysting *Giardia* trophozoites was estimated spectrophotometrically as formed nitrite (NO$_2^-$) using the Griess Reagent System (Promega). Briefly, cells were treated as indicated (see Chapter II) then washed three times in PBS and resuspended at a concentration of $10^7$ cells ml$^{-1}$ in PBS with arginine (0.5 mM) and incubated for 1 h at 37°C. Cells were then removed by centrifugation at 2500 x g. To measure nitrite formed, 500 μl of the supernatant were incubated with 500 μl of Griess reagent at room temperature for 10 min, then the absorbance was measured at 543 nm using a Perkin Elmer 1420 Multilabel Counter.
microtiter plate reader. The nitrite content was determined from a calibration curve using NaNO₂ performed in parallel with the samples.

**In vivo NO detection** — In order to detect NO production in vivo 2,3-Diamino Rhodamine (DAR-1 (Sigma)) was used. Harvested and washed trophozoites from normal growth medium with or without arginine (5 mM) and NADPH (100 nM) or trophozoites encysting for 6 h (treated the same way) were incubated with DAR-1 at a final concentration 10 µM for 40 min. A separate group of 6 h-induced and non-induced cells was incubated with 7.7 µM cys-NO and used as a positive control. Cells were processed as discussed earlier (see Chapter II) and observed with a Deltavision deconvolution microscope using Olympus 10X0.40 optics and Rhodamine-Texas Red- Phycoerythrin (RD-TR-PE) and 4',6-diamidino-2-phenylindole (DAPI) specific filters. All manipulations with cells labeled with DAR-1 were performed at reduced light.

**IFA performed on induced or cys-NO treated cells using Cyst Wall specific serum and Jacalin** — Non-induced or cells induced for 6, 10, 18, 24, 30, and 36 h as well as cells induced to encyst for 6 h in the presence of 50 µM cys-NO were harvested and processed as discussed earlier (Chapter II). Cells attached to a slide were incubated with Cyst Wall specific serum (1:1000 dilution) (Erlandsen et al., 1990). After rinsing, the slide was incubated with a 1:1000 dilution of secondary antibody conjugated to tetramethyl rhodamine isothiocyanate (TRITC). After that the slide was rinsed and then incubated with fluorescein isothiocyanate (FITC)-conjugated Jacalin (Vector laboratories) at a final concentration 10 µg.ml⁻¹. All the antibodies/Jacalin incubations were performed for 1 h at 37 °C. All the manipulations with the cells followed incubation with the primary antibodies were performed under reduced light. Cells were observed
with a Deltavision Deconvolution microscope using Olympus 10X0.40 optics and RD-TR-PE and FITC-specific filters.

Detection of intracellular NO and protein nitrosylation levels — NO production by encysting and non-encysting *Giardia* trophozoites was estimated spectrophotometrically as formed nitrite (NO$_2^-$) using the Griess Reagen system (Promega). To measure the intracellular level of nitrite, cells were resuspended in HEN buffer (Schonhoff and Benhar, 2001) to a final volume of 1 ml, 10 µl of suspension was aspirated and fixed in a total volume 1 ml of formaldehyde-PBS (Repasky at al., 1982) to account for the cyst percentage. Cells were then broken using Molecular Grinding Resin and pestles (GBioscience). The lysate obtained was centrifuged at 12000 x g for 2 min and 50 µl of supernatant was used per assay. Assays were performed in quadruplicate. Each supernatant aliquot was incubated with 50 µl of sulfanilamide solution with or without 0.2% HgCl$_2$ (Schonhoff and Benhar, 2001). After 5 min incubation 50 µl of NED solution was added to the mixture and following 5 min incubation the absorbance of the formed azocompound was measured with a Perkin Elmer 1420 Multilabel Counter microtiter plate reader with a 570 nm filter for 1 sec. Reactions were carried at room temperature with reduced light. The protein concentration was measured using the Bradford reagent (Amresco). The readings obtained were normalized using a NaNO$_2$ calibration curve (the standards were tested in parallel with the samples) and protein concentrations. The formed nitrite amount was presented as µM per 1 mg of total protein.

Conversion of arginine — For the conversion of arginine to citrulline by the ADHP, the NOS Activity Assay Kit from Cayman Chemical Company was used. Radiolabeled arginine, 250 µCi of L-(2,3,4,5 [$^3$H]) arginine-HCl was obtained from GE Healthcare
(Piscataway, NJ). The kit measures the conversion of arginine to citrulline in crude protein extracts because the arginine is selectively bound by a resin and removed by using spin columns. The remaining radioactivity as citrulline was collected and counted in a Beckman scintillation counter. The assay was tested by using the supplied recombinant human iNOS and a specific iNOS inhibitor NG-nitro-L-Arginine (L-NNA). In the control reactions, performed as described by the manufacturer, recombinant iNOS converted 90% of the arginine supplied to citrulline but only 10% in the presence of the inhibitor. Protein lysates were prepared as described in Chapter II. Non-induced, 6 h-induced and 24 h-induced cells were used and 10 μg of protein was used per assay. The assay consisted of 25 μl 2 x reaction buffer, 1 μCi arginine in a total volume of 50 μl and reactions were incubated at 37 °C for 30 min. The reactions were terminated by adding 400 μl stop buffer and processed as suggested by the manufacturer of the test kit. The percentage of conversion to citrulline was calculated as cpm reaction – cpm blank divided by the total cpm input times 100.

Measurement of NO-synthesizing activity — Giardia trophozoites and cells induced to encyst for 6 h were lysed in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl and 0.2% Triton X-100 with protease inhibitor (GBioscience). The mixed lysates were fractioned on a Sephadex G-150 column with 50 mM Tris-HCl pH 8 as buffer and fractions of 500 μl were collected. Separated fractions were measured for NO-synthesizing activity using 4,5-Diaminofluorescein (DAF-2) (Cayman). Originally assays were performed in the presence of NADPH, however due to the absence of an effect of NADPH it was omitted from following assays. Briefly 10 μl of each fraction was incubated in 200 μl assay mixture containing 25 mM Tris-HCl pH 8 and 0.1 μl of DAF-2
(5 mM in DMSO) for 30 min at 37 °C. Fluorescence was measured with a Perkin Elmer 1420 Multilabel Counter microtiter plate reader and filter setting of 485/535 nm, the value with assay buffer only was subtracted. After initial fluorescence detection the most “active” fractions were pooled and separate aliquots were heat-treated 10 min at 90 °C or assayed in buffers with different pH values.

**RNA isolation and analysis** — RNA was isolated and subjected to RT-PCR as described by van Keulen et al. (2008). The primers were designed based on the DNA sequence of giNOS, GNP and glutamate dehydrogenase (GDH) (as loading control (Touz et al., 2008)) to amplify the entire ORF. Reverse transcription coupled polymerase chain reaction (RT-PCR) was used to synthesize cDNA from total RNA (0.5 μg) using each gene specific antisense primer, and Moloney Murine Leukemia virus reverse transcriptase (Epicentre, Madison, WI) according the manufacturer's protocol in a 20 μl reaction.

**Table 6. giNOS- and GDH-specific primers.**

<table>
<thead>
<tr>
<th>PRIMERS</th>
<th>FORWARD</th>
<th>REVERSE</th>
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<tbody>
<tr>
<td><strong>giNOS</strong></td>
<td>5’ AAAGGGGAATTTCTGAGCATAAGTGATG</td>
<td>5’ CTAATATGCCTCGAGGTGAACTCTATTC</td>
</tr>
<tr>
<td><strong>GDH</strong></td>
<td>5’ ATGCCTGCCAGACGATCAATCGA</td>
<td>5’ GAGCCAGAAAGAAGGACGTT</td>
</tr>
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</table>
The cDNA obtained was heated for 15 min at 90 °C and then treated with 1 μl RNase (Invitrogen) for 30 min at 37 °C. Then 10% of the cDNA was subjected to the polymerase chain reaction (PCR) using these gene specific primers and Taq DNA polymerase as described (van Keulen et al., 1998).

Semi-quantitative RT-PCR (comparison of 18, 25 and 32 cycles of the PCR reaction) using RNA from non-induced and 6 h-induced cells was used to investigate whether mRNA was present or up- or down-regulated. The PCR-generated DNA was analyzed on a 1% agarose gel and stained with ethidium bromide. The primers for giNOS and GDH are shown in the Table 6.

The same primers also were used for cloning of giNOS. Cloning was performed as previously described for GNP (see Chapter III). For GNP amplification primers previously used for enzyme cloning in pGEX-4-T-1 were used (Lopez et al., 2002).

RESULTS

Detection of NO production throughout encystment: The preliminary results obtained by Dr. Timothy A. Paget (personal communication) showed that non-encysting trophozoites of MR4 produced NO at approximately 15 nmol/min/10^6 cells. During encystment (6 - 12 h) this number significantly increased up to 23. By 24 h, NO production had significantly diminished to one-fifth the maximum NO produced (Fig. 20). Upon 1 h incubation with 0.5 mM arginine the final yield of NO increased 4 - 5 fold.
NO production in trophozoites and cells induced to encyst for 6 h was also examined using 2,3-Diamino Rhodamine (DAR-1).

![Graph showing NO production in trophozoites during encystment.](image)

**Figure 20. NO production by *Giardia* trophozoites during encystment.**

NO production by cells induced to encyst for 0, 6, 12, and 24 h was measured using the Griess assay. Cells were incubated with or without 5mM arginine for 1 h and total NO production measured. The percentage of encystment after 24 h induction was 56%. Black boxes represent NO production in the presence of 5 mM arginine; white boxes represent NO production without arginine. The insert shows the increase of percentage of encystment during this time period. The bars represent standard deviation of the mean (SD) (Courtesy of Dr. Timothy A. Paget).

The amount of NO in non-induced trophozoites was below detection level (Fig. 21; A), but a significant increase in the signal was measured and mostly concentrated in the perinuclear space when cells were induced to encyst for 6 h (Fig. 21; E). Upon
incubation with 5 mM arginine or arginine in combination with 100 nM NADPH, an NO-generated signal appeared in non-induced cells and increased in induced cells (Fig. 21; B-C, F-G). The captured signal had a punctate pattern spread throughout the cell and concentrated in the perinuclear space. Non-induced trophozoites incubated with an NO donor (cys-NO) generated a signal similar to that produced by induced cells and cells incubated with arginine plus NADPH (Fig. 21; D). When induced trophozoites were incubated with cys-NO the strength of the signal increased (H).

Figure 21. Detection of in vivo produced NO using DAR-1.

*Giardia* cells (panels A and B) were incubated with DAR-1 for 40 min. Panel A represents *Giardia* trophozoites; panel B represents *Giardia* trophozoites induced to encyst for 6 h. Additionally cells were incubated with 5 mM arginine alone, or in combination with 100 nM NADPH and with 7.7 µM cysteine-NO.
NO induces morphological changes in *Giardia*: In order to study the in vivo effect of NO, *Giardia* trophozoites were incubated with various concentrations of cys-NO. Cells were analyzed using cyst wall specific serum (1:100) and Jacalin (10 µg/ml) using IFA. The images were compared with the images generated from non-induced

![Images of Giardia trophozoites with varying induction times](image)

**Figure 22. Indirect immunofluorescence of induced and cysteine-NO treated cells using cyst wall specific serum and Jacalin.**

*Giardia* trophozoites non-induced (A) or induced to encyst for 5-10 (B), 18-24 (C), 30-36 (D) h, and induced to encyst for 6 h as well as incubated in the presence of 50 µM cys-NO (E) were subjected to IFA using cyst wall specific serum at 1:1000 dilution (detected with TRITC at 1:1000 dilution) and FITC-conjugated Jacalin at 10 µg/ml.
trophozoites or cells induced to encyst for various lengths of time after reaction with the same serum and the lectin. Cyst wall specific antiserum did not generate any signal in non-induced trophozoites (Fig. 22); a few dots recognized by Jacalin appeared to be non-specific. The signal showing the punctate pattern spread throughout the cytosol and concentrated to the periphery when the assay was done on cells obtained after incubation of 5 - 10 h in the encystment medium. The Jacalin-generated signal was spread throughout the cytosol. After 18 - 24 h in the encystment medium the signal generated by the cyst wall serum and Jacalin overlapped in the center of the forming cyst’s body, and only the cyst wall specific signal was detected on the periphery of the cyst (Fig. 22; C). After 30 - 36 h in encystment medium the signal obtained with Jacalin was almost lost, while anti-cyst wall serum produced a typical image of fully formed cyst with a solid surface and a single “tail” (Fig. 22; D). Cells incubated in maintenance medium with various concentrations of cys-NO (the result of only one concentration: 50 µM is shown) resembled fully-formed cysts after a 6 h induction period. The “cysts” produced as result of incubation with 50 µM cys-NO (Fig. 22; E) had atypical shapes and a “swollen” tail pattern. The pattern obtained with the cyst wall serum and the Jacalin-generated signal was also different. The signal generated with Jacalin was mostly concentrated in the central part (as in the case of 5 - 10 h of induction), while both signals overlapped on the periphery of the “cyst” body and the “tail”.

Analysis of protein nitrosylation throughout encystment: To evaluate the amount of nitrosylated proteins in *Giardia* throughout the encystment period, non-induced (0 h) and induced trophozoites (6, 16, and 24 h) were harvested and lysed to obtain soluble proteins. Cells were broken using Molecular Grinding Resins and pestles and identical
soluble fractions were treated with the Griess reagent in the presence and absence of HgCl$_2$, a reagent that reduces S-nitrosothioles into nitrites (Schonhoff and Benhar, 2011).

Figure 23. Protein nitrosylation throughout encystment.

_Giardia_ trophozoites non-induced (0 h) or induced for 6, 16, and 24 h were broken using Molecular Grinding Resins and pestles. Nitrosothioles were reduced to NO$_2^-$ using HgCl$_2$, total nitrite level was determined using Griess reagent. The percent of fully formed cysts present in the culture after each time point is indicated on the top of the bars in the panel. White bars represent NO present in the medium as nitrite, black bars represent bound NO that was reduced to nitrite. MM-maintenance medium, EM-encystment medium. The experiment was performed three times in triplicate and bars represent SD.

The amount of nitrosylated proteins was estimated as the difference between the nitrite amounts detected in the presence versus absence of HgCl$_2$. The results obtained were quantified using a nitrite standard curve and normalized by protein concentrations in the samples. The amount of nitrosylated proteins per 1 mg of total proteins increased
from 72.9±2.5 to 102.3±6.7 µM immediately upon transferring cells from maintenance to the induction medium (Fig. 23). In the first 6 h of encystment this number increased to 155.9±2.7 µM), and stayed almost the same between 6 and 24 h induction.

**Arginine conversion in *Giardia* during encystment:** In order to detect arginine conversion by giNOS an assay method that uses radioactive arginine was employed (Moncasa and Higgs, 1993; Nathan and Xie, 1994). Addition or omission of NADPH was used to differentiate between giNOS and ADI activities. The arginine conversion in *Giardia* lysates is presented as percent of citrulline obtained compared to the initial arginine input. Arginine conversion did not depend on NADPH and was 41.8 ± 8.1 % in trophozoites, while increased up to 80.6 ± 8.7 % during the first 6 h of encystment. Towards 24 h of encystment this number dropped to 66 ± 5.8 % (Fig. 24).

![Figure 24. Arginine conversion in *Giardia* trophozoites and cells induced to encyst for 6 and 24 h.](image)

The final amount of radiolabeled citrulline was measured in lysates of *Giardia* trophozoites and cells induced to encyst for 6 and 24 h. The amount converted is shown as an average of 8 assays for each time point, the bars represent SD.
Detection of NO-synthesizing activity: In order to detect NO-synthesizing activity, *Giardia* protein lysates were subjected to gel-fractionation on a Sephadex G-150 column. The fractions were assayed for NO-producing activity, using DAF-2 reagent, which emits fluorescence only upon reaction with NO.

**Figure 25. Effect of pH and heat inactivation on *Giardia* NO-synthesizing activity.**

*Giardia* lysates of trophozoites and cells induced to encyst for 6 h were subjected to fractionation on Sephadex G-150. Fractions were assayed for NO-producing activity. The active fractions were pooled and NO-synthesizing activity was measured using DAF-2 after a variety of treatments in the presence (lanes 3, 5, 7, 9) or absence (lanes 2, 4, 6, 8) of arginine (0.5 mM) and compared to the blank (lane 10). Lane 1- heat inactivated for 10 min at 90°C; lanes 2, 3- pH 6.8; lanes 4, 5-pH 7.3; lanes 6, 7-pH 8.0; lanes 8, 9- pH 9.0.

The fractions showing NO-producing activity were pooled and either treated with heat (90 °C) for 10 min or incubated in buffers with a different pH (pH of 6.8; 7.3; 8.0; and 9.0 were selected). The intensity of fluorescence, corresponding to NO-producing activity, was measured in the treated fractions and normalized with respect to the blank. NO synthesis was sensitive to heat inactivation and had a distinct pH optimum of 8.
also was evident from the results that the NO-producing activity did not depend on the presence of arginine (Fig. 25).

**Analysis of putative *Giardia* NOS**: Anti-giNOS serum was generated using the immunogenic C-terminal peptide CGPANDAVDTSNKVI (Fig. 26). The peptide was selected by the manufacturer. Pre-immune and giNOS-specific sera were tested on Western blots and by indirect immunofluorescence analyses (IFA). *Giardia* lysates (trophozoites and encysting cells) were subjected to differential centrifugation to separate soluble (S) and two particle-associated fractions (P1, (unbroken cells, nuclei, fragments of flagella and adhesive disks and P2, large particle fraction (Lindmark, 1988)). *E. coli* BL21 cells expressing GST-giNOS and His-tagged giNOS were lysed and the recombinant GST and His-tagged proteins were purified as described (see materials and methods). *Giardia* trophozoites (total lysate, fractions S, P1, and P2) and 24 h-encysting cells (fraction P1) samples as well as purified GST-giNOS were separated using SDS-PAGE and transferred to PVDF membranes. The membrane was probed with preimmune (data not shown) and anti-giNOS sera in a 1:1000 dilution (Fig. 27; panel A). Preimmune serum was tested along with anti-giNOS serum in each assay and did not give non-specific signals (Fig. 28). The predicted molecular masses of giNOS, GST-fused giNOS and His-tagged giNOS are 66.6 kDa, 92.6 kDa, and 69.1 kDa, respectively.

The giNOS-specific serum showed a polypeptide of approximate molecular mass of 84 kDa, which agrees with the size of the recombinant GST-giNOS and some minor bands of 50 - 60 kDa (Fig. 27; panel A; lane 1). When total *Giardia* proteins were subjected to Western blot analysis the anti-serum reacted with a few polypeptides with molecular masses of 30, 44, 46, and 50 kDa (lane 2).
Figure 26. Sequence alignment of *Giardia* annotated inducible NOS (giNOS) and human inducible NOS (NOS 2).
Identical amino acid are indicated by “*”, conserved substitutions by “:”, semi-conserved by “.”. The oxygenase domain is indicated as a bold box, while the reductase domain is indicated by colored boxes. The sequences responsible for binding of FMN and FAD/NADPH are indicated by grey and orange colors, respectively (Based on Alderton et al., 2001). The immunogenic peptide used for giNOS serum production is identified by red font.

Figure 27. Western blot probed with giNOS specific serum.

*Giardia* trophozoites and encysting cells were lysed and subjected to differential centrifugation to obtain soluble (S), and two particle-associated fractions (P1 and P2). The obtained fractions as well as the lysate of bacterial cells expressing GST-fused giNOS and purified recombinant giNOS were separated using SDS-PAGE and transferred to PVDF membranes. The membrane was probed by giNOS specific serum at 1:1000 dilution (Panel A) and 1:2000 dilution (Panel B). **Panel A:** 1-purified GST-giNOS; 2- *G. intestinalis* trophozoites, total lysate; 3- S fraction; 4- P1 fraction; 5- P2 fraction; 6- *G. intestinalis*, 24 h induction of encystment, P1 fraction. **Panel B:** P2 fraction of *Giardia* trophozoites.
Some additional bands showed a low-intensity signal but disappeared when the serum was diluted 2000 fold (Fig. 27; panel B). Two of the above mentioned bands (50 and 46 kDa), with a lower intensity, were seen in the soluble fraction (lane 3). The same bands as well as an additional one of 30 kDa were present in the P1 (lane 4) and P2 (lane 5) fractions and gave a strong signal.

The anti-giNOS serum recognized an additional polypeptide of 70 kDa in fraction P2, this band was present in lower intensity in the S fraction, but absent from the P1 fraction. In the particulate fraction of 24 h-encysting cells (lane 6), the serum reacted with bands of 46 kDa and 37 kDa (specific for 24 h). To ascertain the specificity of the antibodies, a competition experiment was performed by using the peptide to which the antiserum was generated (Fig. 28). The anti-giNOS-specific serum was incubated with increasing concentrations of the immunogenic peptide (panels C-H) prior to incubation with the membranes. The strength of the signal obtained was compared to the signal of preimmune serum (panel A), and anti-giNOS serum with no peptide added (panel B). The bands recognized by the anti-serum in the lanes containing recombinant GST-giNOS and His-tagged-giNOS (lanes 1 and 2) were decreasing in intensity with increasing concentrations of the peptide and disappeared when the serum was competed with 100 µg peptide.

The giNOS specific serum gave a signal of a punctate pattern, that was mostly concentrated to the periphery of cells in trophozoite and spread throughout the cytosol in cells undergoing encystment for 6, 12, and 18 h (Fig. 29). The strength of the signal increased during encystment and at 24 h was also localized to the periphery of the cells. Preimmune serum was tested along with the giNOS-specific one and occasionally also
would give a weak signal with a punctate-pattern (12 h, panel A). In a competition study (Fig. 30) giNOS-specific serum was incubated with increasing concentrations of the immunogenic peptide prior to IFA. It was shown that the strength of the signal changed a little but did not depend on the peptide concentration.

Figure 28. Western blot analysis of giNOS with serum previously incubated with the immunogenic peptide.

Purified GST-giNOS (lane 1) and HIS-tagged giNOS (lane 2) as well as the lysates of 0 (lane 3) and 6 h (lane 4) induced Giardia cells were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with preimmune serum (panel A), and giNOS specific serum at 1:2000 dilution (panels B - H), previously incubated with different concentrations of the immunogenic peptide. Peptide concentrations: panel A – 0 µg; B - 0 µg; C - 100 µg; D – 10 µg; E – 1 µg; F - 0.1 µg; G - 0.01 µg; and H - 0.001 µg.
Figure 29. Indirect immunofluorescence of *Giardia* trophozoites and encysting cells using preimmune serum (panel A) and giNOS specific serum (panel B).
Figure 30. Indirect immunofluorescence of encysting *Giardia* cells using giNOS specific serum preincubated with immunogenic peptide.

*Giardia* cells undergoing encystment for 12 h were probed with giNOS-specific serum previously incubated with different concentrations of the immunogenic peptide: panel A – 0 µg; B - 0.001 µg; C - 0.01 µg; D - 0.1 µg; E – 1 µg; F – 10 µg; and G – 100 µg.
Semi-quantitative RT-PCR analysis of giNOS expression: To analyse expression of giNOS in trophozoites and induced to encyst for 6 h cells, we performed RT-PCR using GDH and GNP as an example of a genes expressed in constitutive and inducible manner. The results demonstrate that giNOS is expressed in a very low level in trophozoites (Fig. 31: panel A (top), lane 3) with a slight increase upon encystment (Fig. 31; panel B (top), lanes 2-3). As expected, GDH was constitutively expressed. Surprisingly, GNP expression was detected in trophozoites when higher amount of amplification cycles were used (panel C (top), lanes 2-3). However, in comparison to panel D (top), lane 1 showed that GNP expression is induced upon encystment. The absence of tight regulation during expression in Giardia was discussed earlier (Vanacova et al., 2003).

Figure 31. Comparative expression analysis of giNOS, GDH, and GNP using RT-PCR.

RNA from non-induced trophozoites (panel A and C) and trophozoites induced to encyst for 6 h (panels B and D) were used in RT-PCR with the primers specific to the following genes: giNOS (panel A-B; top), GDH (panel A-B; bottom); GNP (panel C-D; top). For the reverse transcriptase control we used the following parimers: giNOS (panel C; bottom), and GDH (panel D; bottom). Different number of cycles was used in the PCR: lane 1: 18, 2: 25, and 3: 32 cycles.
DISCUSSION

Nitric oxide production in *Giardia*: NO is an important physiological messenger, involved in the homeostasis of cardiovascular tissue and regulation of neuronal signal transduction and immune response in higher eukaryotes (Bredt and Snyder, 1994; Dawson and Dawson, 1995). In prokaryotes NO is involved in the regulation of gene expression (Cutruzzola, 1999; Spiro, 2006; Zumft, 2002). While NO production was discovered in such protozoa as *P. falciparum, E. histolytica, L. donovani, T. gondii, Trypanosoma* sp. and recently in *Giardia intestinalis* and *T. vaginalis*, its effect/function is not well understood (Basu et al., 1997; Crawford, 2006; Gutierrez-Escobar et al., 2008; Harris et al., 2006; Hernandez-Campos et al., 2003; Paveto et al., 1995). The half life of NO is measured in milliseconds which makes its detection extremely difficult. One means to investigate NO formation is to measure nitrite ($\text{NO}_2^-$), which is one of two primary and stable products of NO breakdown (Schonhoff and Benhar, 2011). Using the Griess Reagent System, Timothy A. Paget showed NO production in non-encysting trophozoites ($15 \text{ nmol.min}^{-1}.10^{-6} \text{ cells}$) with a significant increase (up to $23 \text{ nmol.min}^{-1}.10^{-6} \text{ cells}$) in the first 6 h of encystment. This is the period of active synthesis of the protein involved in cyst wall synthesis and its regulation. This is also the time when GNP transcripts were detected first (van Keulen et al., 1998). DAR-1 staining in indirect immunofluorescence showed that the amount of NO increased after 6 h of encystment. DAR-1 is a membrane-permeable fluorescent indicator based on the rhodamine chromophore. Upon interaction with NO, DAR-1 is converted to a fluorescent trizol (McQuade, L.E., Lippard, S.J. (2010)). The presence of a strong fluorescent signal in the perinuclear area of undergoing encystment cells indicates nitrosylation of newly synthesized proteins. The fluorescent
signal increased when non-induced cells were incubated with arginine alone, or arginine with NADPH, which indicates that arginine serves either as a substrate for NO production or affect NO production indirectly through the ADHP. Incubation of non-induced trophozoites with cys-NO also resulted in a strong fluorescent signal and was used as a positive control.

The Griess reagent also was used to evaluate the intracellular level of NO in the form of nitrite. In order to assess the amount of NO present in the form of nitrosyl groups HgCl₂ was used to reduce nitrosothioles and convert the bound NO into nitrite. The level of nitrosylated proteins was evaluated as a difference of the presence of NO₂⁻ without and with HgCl₂. To monitor encystment the total cell amount and the amount of fully formed cysts in the samples were calculated. The level of nitrosylated proteins increased immediately after cells were transferred to the encystment medium, while the level of free NO did not change significantly. This indicates that the switch in culture medium induces NO production and this NO is used to nitrosylate the proteins present. Further during the first 6 h of encystment both cellular NO and nitrosylated protein levels continue to increase (Fig. 20, 23). This observation agrees with the data obtained earlier by Timothy A. Paget. During the first 6 h of encystment Giardia cells actively synthesize proteins involved in cyst wall production; this is the time when transcripts of GNP and CWP 1 - 3 are first detected (Luján and Touz, 2003). This finding along with the IFA results indicate that Giardia NO-synthesizing activity increases in the first 6 h of encystment and the NO is produced used to nitrosylate several proteins. During the next 10 h of encystment the level of nitrosylated proteins as well as the total NO level
fluctuate insignificantly (155±2.7 - 160±5.1 µM) showing a slight increase at 24 h (181±15.1 µM). At this time nitrosothiols were present in a low amount.

**What is the function of internal NO in Giardia?** According to the results obtained so far, *Giardia* actively synthesize NO at the initial stage of encystment and use it as a signaling mechanism during encystment. GNP, the first enzyme in the giardan biosynthesis, is one of the proteins suggested to be nitrosylated.

To evaluate the possibility of encystment as a result of incubation with cys-NO, cyst wall specific serum and a lectin specific for (β-1, 3) N-acetylgalactosamine (Roque-Barreira and Campos-Neto, 1985) were used in indirect immunofluorescence. The morphological changes observed were compared to the transformations of cells induced to encyst for 5 - 10, 18 - 24, and 30 – 36 h. It is interesting to observe the change in the pattern of the signals produced by the cyst wall-specific serum and Jacalin throughout the process of encystment. At the earlier stage (5 - 6 h, not shown) the cyst wall signal has a punctate pattern that increases in strength and spreads towards the periphery when encystment comes closer to the 10 - 12 h mark (Fig. 22; B). This signal at this time point coincides with CWP 1 - 3 synthesis, which are delivered to the encysting cell periphery by encystment specific vesicles (ESV (see Fig. 4; Chapter 1)), as was demonstrated earlier (Luján and Touz, 2003). The signal obtained with Jacalin is mostly spread throughout the cell cytoplasm, which is in agreement with localization of the cyst wall synthesizing activity to the cytosolic fraction (Luján and Touz, 2003). At the later stage of encystment (18 - 24 h) an overlap of Jacalin and cyst-wall specific signals was observed. These findings are in agreement with earlier demonstrated strong interactions between polysaccharides and proteins in the actively growing cyst wall (Chavez-Munglia
et al., 2007). The Jacalin signal is diminished in the fully formed cysts (Fig. 22, D). This is probably explained by the unavailability of free (β-1, 3) N-acetylgalactosamine on one hand and outcompeting action of cyst-wall specific serum on the other hand. The opposite picture can be observed in the cells treated with cys-NO for 6 h. The Jacalin signal is strong and spreads throughout the cytosol as well as to the periphery of the cell and the “tail”. The cyst wall specific signal is less strong and localized to the periphery if present (Fig. 22, E). The cyst “body” seems less compact and is irregular. Most probably cys-NO induces the synthesis of the carbohydrate moiety of the cyst wall, but not the protein one (concluded from the absence of ESV-associated signals). This results in defective cysts.

In summary, NO production is detected in *Giardia*. The amount of produced NO dramatically increases during the initial 6 h of encystment, which suggests the importance of this molecule for the process of encystment. It is still not clear if NO directly participates in regulation of encystment-specific gene expression, however, the increase in the amount of nitrosylated proteins suggests regulation of protein activity via post-translational modification and may be change in intracellular localization.

**Is giNOS responsible for the NO production?** Originally NO production in *Giardia* trophozoites was documented and measured by Harris et al. (2006). They estimated NO-producing activity as 4 nmoles.mg⁻¹ protein.h⁻¹ and attributed it to the NOS (ORF 91252) annotated as such in the *Giardia* database.

The gene for giNOS was cloned and expressed in two vectors: PGEX-4T-1 (as a GST-fusion protein) and in pET (as a HIS-tagged protein). giNOS specific serum
(generated against an immunogenic C-terminal peptide) recognized the GST-giNOS fusion and His-tagged giNOS as proteins of approximate molecular masses of 90 and 77 kDa. These numbers match the predicted molecular masses of 92.6 and 69.1 kDa for the above mentioned (fusion) proteins. These facts confirm that the produced serum is specific for the postulated *Giardia* NOS. The serum reacted with several proteins in *Giardia* protein lysates. The strongest signal showed bands of approximately 30, 44, 46, and 50 kDa in trophozoites (Fig. 27). The band of 46 kDa was also present in the protein lysate of cells encysting for 24 h. In addition there was also a band of approximately 37 kDa specific for 24 h-induced cells. The signal generated by this anti-serum was mostly associated with the particulate fraction of the protein samples. The weak bands observed in Western blots were lost when the serum was diluted 2000 fold (Fig. 27).

However, none of these numbers match the predicted molecular mass for giNOS, 66.6 kDa. IFA using giNOS-specific serum resulted in a punctate pattern signal that was spread in the cytosol of the cells encysting for 6, 12, and 18 h and localized mostly to the periphery of the trophozoites and 24 h-encysting cells. Incubation of giNOS-specific serum with varying concentrations of the immunogenic peptide out-competed the signal generated by the GST fusion- and His-tagged-giNOS, but not the signal in *Giardia* protein samples. The results obtained demonstrate that the signal registered by anti-giNOS serum is specific for the cloned ORF but cross reacts with *Giardia* protein samples and cells in a non-specific manner. It is possible that the level of expression of the protein is so low that it is below the detection level. The use of semi-quantitative RT-PCR with the giNOS specific primers showed a very low level of expression when compared to the expression of the inducible GNP and the constitutively expressed GDH
used as a standard (Fig. 31). The radioactive assay based on arginin econversion to citrulline did not detect NOS activity in *Giardia* protein lysates. The conversion of arginine was the same with or without NADPH and insensitive to the addition of a NOS inhibitor. Therefore, the assay detected only activity obtained from arginine deaminase. It is possible that the gene encoding the protein in non-functional, which is characteristic of many *Giardia* genes (Jerlström-Hultqvist et al., 2010; Vanacova et al., 2003). Precise analysis of the ORF annotated in *Giardia* data base as giNOS revealed that it encodes only the reductase domain of a typical iNOS. A Blast search of the Universal Protein Resource Knowledgebase (UniProtKB) with *Giardia* “NOS” sequence identified Pyruvate: oxidoreductase and NADPH-cytochrome P450 reductase as the closest analogous proteins (Jain et al., 2009).

Does NO-synthesizing activity in *Giardia* come from a protein? The presence of an NO-synthesizing activity in *Giardia* was investigated using DAF-2 to measure NO production in protein samples. To optimize the search for such an activity, a mixed protein lysate of trophozoites and cells induced to encyst for 6 h was fractionated using Sephadex G-150. The NO-synthesizing activity in the fractions was measured and appeared to elute at a relatively low molecular weight of the proteins (not shown). The fractions showing the highest activity were pooled to evaluate if the activity was protein-based: heat sensitive and a distinct pH optimum suggested protein involvement. The intriguing observation was that the activity was not dependant on external arginine. This observation is in conflict with an observation earlier showing an arginine-dependent increase in NO production using the Griess reaction (Timothy A. Paget, personal communications).
So far proteins associated NO production was demonstrated in *Giardia* trophozoites and encysting cells; however, the protein/proteins responsible for this activity have not been identified. The gene annotated as giNOS in the *Giardia* database encodes the reductase domain only. Examples of NO-producing activity with NOS carrying only oxygenase domains are known in bacteria. Some bacterial cells can use a cellular redox partner (for instance flavoprotein reductase) to cooperatively produce NO (Gusarov et al., 2008; Zemojtel et al., 2003). However, the RT-PCR registered only a low level of expression of this so called “giNOS” in trophozoites and encysting cells, suggesting a low expression level of the gene. The crude protein fractionation showed NO production independent of arginine. This could be explained by a tight binding of internal arginine to the enzyme/enzymes complex. The other possibility is that the enzyme is using internal arginine and converts it to citrulline, which was shown earlier for ADI by Touz et al. (2008). The function of the giNOS gene has to be studied in more detail and the protein/protein complex responsible for the NO production in *Giardia* has to be purified further. Preliminary observations have indicated that DEAE-Sephadex fractionation and hydroxylapatite column chromatography purifies this activity further and keeps the activity intact (not shown).
CHAPTER V

GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

INTRODUCTION

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a key enzyme in glucose metabolism. It catalyses the reversible oxidation of D-glyceraldehyde 3-phosphate into 1, 3-bisphosphoglycerate (Harris and Water, 1976). Due to its central role in metabolism the GAPDH-coding gene is considered a housekeeping gene, hence constitutively expressed (Yang et al., 2002). As an active glycolytic enzyme, GAPDH is isolated as a tetramer comprised of four 37 kDa subunits. Each monomer has a binding site for NAD$^+$ (amino acids 1-150) and glyceraldehyde-3-phosphate (amino acids 151 - 335) (Sirover, 1999). Catalytic activity of the enzyme is dependent on the amino acid cysteine (position 150) that acts in conjunction with a histidine in position 176. The catalytic cysteine is highly conserved from one species to another. It could be found at position 149 in E. coli, 150 in rat, and 152 in human (Souza and Radi, 1998; Hara and Snyder, 2006). E. coli has two gap genes, designated: gap A and gap B. The Gap A protein is the only active enzyme in E. coli, and it is more similar to eukaryotic than eubacterial GAPDH. The gap B gene is
expressed at a very low level and its protein product has D-erythrose 4-phosphate dehydrogenase (E4PDH) activity (Yang et al., 2002). In the last decade it became clear that multiple copies of gap genes in higher eukaryotes exist, that earlier were overlooked, in fact they have various intracellular activities beyond its role in glycolysis. Among them are membrane-membrane fusion, modulation of the cytoskeleton, binding and transportation of heme, DNA repair and replication, apoptosis, transcriptional activation and tRNA transport (Tisdale et al., 2007; Brüne and Lapetina, 1995; Chakravartti et al., 2010). For instance, human liver GAPDH has protein kinase and ADP-ribosyl transferase activities, while GAPDH in human lung carcinoma cells is involved in telomere maintenance (Alvarez et al., 2007). The localization and differential expression of GAPDH in higher eukaryotes is dependent on the developmental stage of the cell (Tisdale et al., 2004). Recent analysis of the GAPDH sequence reveals several motifs with partial homology to a nuclear localization signal (KKVVK, amino acids 259-263), and to a nuclear export domain (ALQNIIP, amino acids 202-208) (Sirover, 1999).

Newly described activities of GAPDH depend on the change in cellular localization (possibly due to structural signals) and its post-translational modification due to interaction with compartment-specific proteins (Sirover, 1999). Indeed GAPDH is potentially phosphorylated, glutathionylated, nitrated, mono-, and poly-ADP-ribosylated. As an example, phosphorylation at Tyr 41 in human GAPDH (modification occurs upon recruitment to the ER-Golgi intermediate compartment) allows it to bind to Ras-related small GTPase Rab2 and protein kinase C and secure the latter to the membrane. This recruitment plays an important role in membrane trafficking between the ER and the Golgi apparatus (Tisdale et al., 2004; Tisdale, 2007). GAPDH was identified as a major
S-glutathionylated protein in activated monocytes and endothelial cells undergoing oxidative stress (Mohr et al., 1999). Possibility of nitration of Tyr 311 and Tyr 317 upon oxidative stress with subsequent loss of glycolytic activity was also demonstrated (Palamalai and Miyagi, 2009).

The catalytic cysteine makes GAPDH vulnerable to oxidation. Its activity is down-regulated by oxidative stress conditions, H$_2$O$_2$, NO, as well as by interaction with NO intermediates (i.e. peroxynitrite anion) and S-nitrosothiols (i.e. S-nitrosoglutathione) (Mohr et al., 1999; Padgett and Whorton, 1995; Souza and Radi, 1998). Interaction of GAPDH with NO could result in direct oxidative modification, NO binding (S-nitrosylation), or NO-dependent binding of NAD$^+$. All NO-dependent modifications occur on the catalytic thiol group with subsequent enzyme inactivation (Alvarez et al., 2007; Brüne and Lapetina, 1995; McDonald et al., 1993; Mohr et al., 1999; Sirover, 1999; Souza and Radi, 1998). Studies on S-nitrosylated GAPDH demonstrated modulation of its interaction with cellular membranes (Sirover, 1999). The outcome of GAPDH S-nitrosylation in neuronal cells is its contact and stabilization of E3-ubiquitin ligase (Siah) that leads to nuclear translocation of both molecules with selective protein degradation (Hara and Snyder, 2006). It was shown to be important for neutrophil invagination during foreign particle engulfment and for inflammation control through the loss of its ability to bind and insert heme into inducible NO-synthase (iNOS) (Chakravarti et al., 2010). GAPDH was also identified among S-nitrosylated proteins in *Arabidopsis thaliana* (Lindermayr et al., 2005).

Various functions dependent on the covalent modifications of GAPDH were also registered in protozoa and bacteria. For example, in *E. histolytica*, enteropathogenic *E.*
coli, and Streptococcus pyogenes, GAPDH was secreted upon ADP-ribosylation (Alvarez et al., 2007). Furthermore, GAPDH, secreted by E. histolytica trophozoites, binds extracellular NO and by means of that protects the parasite from being killed by oxidative stress (Alvarez et al., 2007). A similar function of GAPDH was registered in Leishmania where this protein is the main target of oxidation by NO. Amastigotes, the life stage of the parasite that is highly resistant to NO, over-express GAPDH (Alvarez et al., 2007). In some pathogens such as Candida albicans, Mycoplasma genitalium, Schistosoma bovis, Staphylococcus and Streptococcus sp., GAPDH was localized to the cell surface and declared to be responsible for binding extracellular matrix, playing a role of immunogen (Yang et al., 2002; Alvarez et al., 2007).

Giardia has two putative gap genes, gap1 and gap2 (Yang et al., 2002). The ORF of gap1 encodes a typical GAPDH, while the gap2-encoded protein has only limited homology to it. Their protein products gGAP1 and gGAP2 have only 51% identity. Expression studies showed that gap1 is constitutively expressed in trophozoites and throughout encystment. At the same time gap2 expression was below detectable level in trophozoites, however, it dramatically increased during encystment with a maximum at 12 h post induction. Complementation tests with E. coli deficient in GAPDH activity showed that gGAP1 has both GAPDH and E4PDH activities (Yang et al., 2002).

Glyceraldehyde 3-phosphate dehydrogenase is inhibited by nitrosylation: When nitrosylated GAPDH binds the Siah-1 protein, which contains a nuclear localization signal (Hara and Snyder, 2006). This complex moves to the nucleus and GAPDH acts as a NO donor in transnitrosylation reactions (Kornberg et al., 2010), a totally new and unexpected function of GAPDH. Also, GAPDH inactivation by nitrosylation has been
reported (Mohr et al., 1999; Padgett and Whorton, 1995; Souza and Radi, 1998). Nitrosylation of GAPDH is possible because the catalytic cysteine in the active site (position 152 in human GAPDH) is close to a histidine, which acts as a proton acceptor (Mohr et al., 1999). As a result the thiol group involved in the catalytic events becomes more acidic, which makes it a good target for nitrosylation (Mohr et al., 1999).

In summary, GAPDH is a pleiomorphic molecule whose function depends on the post-translational modification. Nitrosylation resulting in loss of dehydrogenase activity of the enzyme was demonstrated so far in many systems. The question we address here is whether gGAPDH is nitrosylated and if this modification can be responsible for the control of switch between glycolysis and cyst wall synthesis.

**MATERIALS AND METHODS**

**GAPDH enzyme assay** — To detect GAPDH activity, we used lysates of known protein concentrations from non-induced trophozoites (0 h) and trophozoites induced to encyst for 6, 8, 10, and 24 h. The reactions were carried out at room temperature for 20 s in a final volume 800 µl. Reaction mixtures contained 10 mM Tris-HCl, pH 8.5, 10 mM arsenate, 1 mM NAD⁺ (Velick, 1955). The effect of NO on GAPDH activity was addressed by incubation of non-induced trophozoite lysates with 1, 10, and 100 nM cys-NO for 2, 5, and 10 min. In this case, reactions were preincubated at room temperature and initiated by addition of 0.2 mM of substrate: glyceraldehyde 3-phosphate (Sigma). Cys-NO was synthesized fresh daily as described earlier (see Chapter II) and diluted with water to the indicated concentrations. The reagent was kept on ice in the dark prior to the
as control, the same amount of reagents was used without the sodium nitrite and
diluted the same way as the cys-NO.

Initial velocity was determined as means of accumulation of NADH at 340 nm using
a Beckman DU-64 spectrophotometer with an enzyme kinetics package. To standardize
the reaction we used yeast GAPDH (Sigma). For enzyme kinetics, different substrate
concentrations (0.04 - 0.2 mM) were used. Specific activity of the enzyme was
determined using the extinction coefficient for NADH as described in the protocol for
yeast GAPDH (Sigma).

Western blot analysis of Giardia proteins with GAPDH-specific serum — Anti-
serum against a C-terminal Giardia GAPDH peptide (GYANKLVELAKYVGSKGCQ)
was generated by Open Biosystems, Inc. (Huntsville, AL). Serum dilutions were tested
(data are shown) and a dilution of 1:1000 was chosen for further assays. Samples
subjected to the biotin - switch method, proteins recovered after capture with avidin –
agarose as described earlier (see Chapter III), and protein lysates that were prepared fresh
from trophozoites and cells induced to encyst for 6 h were loaded (20 µg per well) onto
SDS-polyacrylamide gels and separated by electrophoresis. Protein samples were blotted
to a PVDF membrane (as described in Chapter II) and probed by the GAPDH specific
serum.

RESULTS

GAPDH activity decreases throughout encystment: Protein samples were prepared
by lysing cells induced to encyst for 0, 6, 8, 10, and 24 h and each was assayed for
GAPDH activity as described in the Materials and Methods section. The results (Fig. 32) showed that the specific activity of the enzyme for the conversion of glyceraldehyde 3-phosphate decreases significantly during the first 6 – 8 h of encystment and remains at a low level throughout the remaining time of encystment.

**Nitrosylation effect on GAPDH activity:** All organisms studied to date have GAPDH with a catalytic cysteine and histidine (indicated with red circles on the figure) that are highly conserved (Fig. 33). The histidine acts as a proton acceptor and changes the thiol group of the catalytic cysteine into a nucleophile that attacks the carbonyl carbon of the substrate glyceraldehyde 3-phosphate and produces a thioester bond. The gGAPDH has a similar conserved catalytic site but also has a putative nitrosylation site at position C 39 (indicated with a blue circle). The catalytic cysteine (position 152 in human GAPDH and also position 152 in the *Giardia* enzyme) is a well known target of nitrosylation (Mohr et al., 1999; Padgett and Whorton, 1995; Souza and Radi, 1998). To investigate whether the gGAPDH is susceptible to nitrosylation, its activity was measured in *Giardia* lysates in the presence of increasing concentrations of cys-NO. The activities measured were compared to the activity in the absence of cys-NO. The decrease in enzyme activity is presented as percent inhibition (Fig. 34). Addition of 1 and 10 nM of cys-NO showed a similar degree of inhibition: 42.8 ± 13.2 and 46.4 ± 7.2 %, respectively, while addition of 100 nM of cys-NO inhibited 76.4 ± 14.8 % of the activity (Fig. 34). Higher concentrations of cys-NO (1 mM) showed a complete inhibition of gGAPDH activity (data not shown). Preincubation of the assay mixture (excluding substrate) with different concentrations of cys-NO for 2, 5 and 10 min showed significant inhibition of gGAPDH in a time-dependent manner even at low concentrations of the NO
donor. Addition of 1 nM inhibited 52.7 ± 3.8 % of the enzyme activity after 2 min, 75.5 ± 18.5 % after 5 min, and 95.7 ± 7.4% after 10 min of preincubation (Fig. 35). The kinetic parameters of gGAPDH in the presence or absence of cyst-NO were determined as well. A significant decrease in $V_{\text{max}}$ of gGAPDH was demonstrated in the presence of the NO donor (based on the average of three independent measurements). Without nitrosylation the $V_{\text{max}}$ was 0.171 μmol.min$^{-1}$.mg$^{-1}$ while in the presence of 0.01 mM of cys-NO it was 0.035 μmol.min$^{-1}$.mg$^{-1}$. Measurement of the $V_{\text{max}}$ of gGAPDH at higher concentrations of cys-NO was not possible due to complete inhibition of the enzyme activity (not shown).

Analysis of Giardia GAPDH: To see if Giardia GAPDH is nitrosylated in vivo, Western blot analysis of Giardia proteins with anti-serum generated against a peptide of GAPDH was performed on protein samples subjected to the biotin - switch method (Fig. 36; lanes 1 - 4), non - treated cell lysates (lanes 5 and 6) and proteins recovered after capture with avidin – agarose. The blot shows a strong signal with a protein of the expected size (37 kDa) and also that the amount of enzyme is the same in non – induced as well as in cells induced to encyst for 6 to 24 h, suggesting that the amount of enzyme is constant. Lanes 7 – 10 show that the same protein can be recovered after capture with avidin – agarose, indicating that the enzyme must have been nitrosylated in vivo. The protein samples were the same as used to shown nitrosylation of GNP as shown in Fig. 10, Chapter III. Note also that the protein in lanes 5 and 6 shows a slight mobility shift as compared to the protein in lanes 1 to 4, which were used in the biotin – switch protocol and had their modification replaced by biotin.
Figure 32. Specific activity of *G. intestinalis* GAPDH during encystment.

*Giardia* cells induced to encyst for 0, 6, 8, 10, and 24 h were collected, resuspended in 0.2% Triton X-100 and broken using Molecular grinding resin and pestles and 0.005 ml of each lysate was assayed to detect GAPDH activity. Total protein concentration in each lysate was detected separately. The measurements were converted to specific activity units, using NADH milimolar extinction coefficient. The presented data are an average of 3 assays for each time point, the error bars represent SD.
Figure 33. Alignments of human, bacterial, and *Giardia* GAPDH.

Catalytic cysteine and histidine are enclosed in red circles, another putative nitrosylation site in *Giardia* GAPDH is enclosed with a blue circle.
Figure 34. Inhibition of *Giardia* GAPDH activity by different concentrations of cysteine-NO. Cysteine-NO at final concentrations 1, 10, and 100 nM were added to the reaction mixture prior to addition of the substrate. The enzyme activity was compared to the gGAPDH activity in the absence of cysteine-NO and the percentage inhibition was calculated. The presented data are an average of 3 assays, the error bars represent SD.

Figure 35. Effect of time on the inhibition of *Giardia* activity by fixed concentration of cysteine-NO. Cysteine-NO at a fixed concentration of 1 nM was incubated with the reaction mixture (excluding the substrate) for 2, 5, or 10 min. The gGAPDH activity was measured and compared to the activity in the absence of NO donor and percentage of inhibition was calculated. The presented data are an average of 3 assays, the error bars represent SD.
Figure 36. Western blot of *Giardia* proteins screened with anti-GADPH serum.

Lanes 1 - 4, and 7- 10 were loaded with cell lysates treated according to the biotin-switch protocol, while lanes 5 and 6 with non-treated cell lysates. Lanes 1 - 4 were loaded with 20 μg of total protein from non-encysting trophozoites cultivated in medium with no bile (1) or low bile (2); trophozoites induced to encyst for 12 h (3) and 24 h (4). Lanes 5 and 6 were loaded with 20 μg proteins from fresh cell lysates of non-induced trophozoites (5) and trophozoites induced to encyst for 6 h (6). Lanes 7 – 10 contain proteins recovered by capture with avidin-agarose. Lane 7, standard medium, lane 8, 6 h-, lane 9, 12 h- and lane 10, 24 h- encysting cells. The blot was screened with a 1:1000 dilution of serum generated against a small peptide of GAPDH.
DISCUSSION

What do we know so far about GAPDH? In the last decade the view of GAPDH as a classical glycolytic enzyme has been changed dramatically. The changes in the protein functions have been associated with the change in the protein localization and/or post-translational modification, one of which is nitrosylation (Mohr et al., 1999; Padgett and Whorton, 1995; Souza and Radi, 1998). Snyder and Hara (2006) showed that nitrosylated GAPDH binds the Siah-1 protein, which contains a nuclear localization signal, the complex than moves to the nucleus and GAPDH acts as a NO donor in transnitrosylation reactions (Kornberg et al., 2010), a totally new and unexpected function of GAPDH. Also, GAPDH inactivation by nitrosylation has been reported (Hara and Snyder, 2004; Mohr et al., 1999; Sirover, 1999). Nitrosylation of GAPDH is possible because the catalytic cysteine in the active site (position 152 in human GAPDH) is close to a histidine, which acts as a proton acceptor (Mohr et al., 1999). As a result the thiol group involved in the catalytic event becomes more acidic, which makes it a good target for nitrosylation (Mohr et al., 1999).

Giardia GAPDH: In Giardia this enzyme is encoded by two genes: gap1 and gap2. The gap1 gene encodes a protein with a typical dehydrogenase sequence and is constitutively expressed, while gap2 expression was below detection level in trophozoites and increased during encystment with a peak at 12 h and had D-erythrose 4-phosphate dehydrogenase activity (Yang et al., 2002). The function of this GAP2 enzyme remains elusive. Activity of GAPDH throughout encystment has never been studied before.
The findings in this studied show that the GAPDH specific activity declined after cells are present 6 - 8 h in encystment medium. Semi-quantitative RT-PCR showed that the enzyme is expressed in a constitutive manner (data not shown), which is supported by the results obtained by Western blot analysis with the specific anti-\textit{Giardia} GAPDH serum. Thus, the decline in specific activity is rather associated with either a change in GAPDH intracellular localization or a post-translational modification. GAPDH susceptibility to nitrosylation with the following loss of activity in other systems suggests that the \textit{Giardia} enzyme also could be affected by nitrosylation. A \textit{Giardia} Database search identified a nitrosylation motif (Stamler et al., 1997) on the GAP1 protein at C 39, which is unique to \textit{Giardia}. However, conserved in the \textit{Giardia} enzyme are the equivalents of the catalytic C 152 and H 176 (Sirover, 1997). Addition of cys-NO to cell lysates inhibited gGAPDH activity ($V_{\text{max}}$) in a dose-dependent manner and in a time-dependent manner even at low cys-NO concentrations. So far it is not clear if both C 39 and C 152 are modified, but the latter one seems most likely.

As shown above, the specific activity of GAPDH goes down when the cells are induced to encyst. In vitro nitrosylation with cys-NO showed clearly that nitrosylation is able to inhibit enzyme activity as has been observed with this enzyme in many other systems. The results of Western blot analysis with the specific anti-serum showed very clearly that: 1) the amount of enzyme stays constant during encystment, in agreement with semi-quantitative RT-PCR, which shows that the level of GAPDH mRNA stays constant as well during encystment (data not shown); 2) the protein shows a slight mobility shift when the enzyme is compared to the one that was treated with the biotin – switch method, indicating the presence of a charged group that interferes with binding.
SDS before removal of this group and replacement with biotin; and 3) that the enzyme can be bound to avidin-agarose when it was biotinylated. Note that the protein samples were the same as was used to capture GNP, which was discussed in Chapter III. Thus GAPDH shows constitutive expression during encystment, appears to be nitrosylated in vivo and loses its activity. The conclusion must be that the enzyme’s catalytic ability is changed by post – translational modification and that just as is the case for GNP, nitrosylation acts as an allosteric control mechanism of the enzyme. The implication is that glycolysis will slow down at the point of the redox reaction catalyzed by this enzyme. Control of glycolysis at this level has been shown in ischemic kidneys. Inhibition of GAPDH inhibited glycolysis and anaerobic ATP production (Devalaraja-Narashimha and Padanilam, 2009). In the case of Giardia the ADHP is present as an alternative source of ATP. Still the control at the level of GADPH is unusual; in studies that measure metabolic flux, the couple glyceraldehyde 3-phosphate dehydrogenase and 1,3 bisphoglycerate kinase always appears to be close to equilibrium and the least likely to form a point of regulation (Canelas et al., 2011). Metabolic flux studies in Giardia would form an excellent way to study control at a branch-point of two major pathways, glycolysis and the encystment pathway.
CHAPTER VI

SUMMARY AND FUTURE RESEARCH

The long term goal of this research is to investigate the mechanism(s) of regulation of *Giardia intestinalis* encystment. The facts that glucose in the form of F6P is used for energy production via glycolysis in trophozoites, uptake of glucose ceases by 6 h into the encystment process, and the endogenous glucose is used as a substrate for cyst wall synthesis in the cells undergoing encystment raises the regulatory question of how does *Giardia* regulate the flux from glucose’s bioenergetics use to its synthetic use during encystment. The recent discovery that *Giardia* synthesize NO, coupled with the role of this molecule in the cell cycle regulation of other systems, suggests that NO involvement in the regulation of this switch is possible. To date we know that *Giardia* synthesize NO and use it to modify GNP. GNP is the first enzyme in the giardan synthetic pathway. In vitro nitrosylation of recombinant GNP resulted in a two fold increase in the enzyme’s catalytic performance and there is MS evidence that GNP is nitrosylated. It is difficult to extrapolate these findings to the in vivo situation, however, if it is the case than
nitrosylation along with the increased amount of protein (due to induced expression) is enough to redirect F6P from glycolysis toward the cyst wall synthesis.

There is also preliminary evidence that GAPDH is nitrosylated and down regulated during encystment. Findings in other systems as well as the results presented in this dissertation suggest that nitrosylation of GAPDH might lead to the inactivation of the enzyme and thus slowing down of glycolysis.

ADHP is up-regulated during the first 6 h of encystment – the same time when NO production increases and protein nitrosylation is observed. It is also the time when GNP is first detected. The fact that ADHP is an alternative pathway for ATP production along with the other findings suggests that Giardia can switch, at least in part, from glycolysis to ADHP as an energy producing pathway during encystment. The switch between glycolysis and cyst wall synthesis could certainly be regulated through GAPDH inactivation and GNP activation by means of nitrosylation along with increased expression of GNP.

Even though the source of NO in Giardia is still not clear, the putative enzyme detected in our fractionation is heat labile, has a pH optimum around 8 and does not depend on external arginine. The so-called giNOS, annotated in the Giardia database, cannot be responsible for NO production itself because the enzyme is missing the oxygen and arginine binding domains.

The logical continuation of the current study would be purification of the NO-synthesizing activity. This could be approached by using classical biochemical procedures followed by mass spectrometry of the purified protein(s).
nitrosylated GAPDH has to be studied further. This protein plays a role as transcriptional regulator in some systems as well as a guard against oxidative stress in another microaerophile – *Entamoeba*. Primarily, the localization of the protein during encystment has to be determined to explore the function of nitrosylated GAPDH. In addition, the protein should be studied by over-expression and down-regulation in transfection studies.

Although GNP is transcriptionally activated and appears to be regulated by a single ubiquitin attachment, the further fate of nitrosylated and uniquitinated GNP is not known. The increased amount of peptides reacting with GNP-specific serum in the cells induced to encyst for long periods of time suggests proteasomal degradation. Studies inhibiting proteasomal activity could give an answer to the question of degradation and its effect. In addition it is possible that the protein is subjected to phosphorylation and/or SUMOylation. This question could be addressed by application of SUMO-specific antibodies and antibodies specific for phosphorylated serine and threonine in Western blot analysis. Upon confirmation of any of the mentioned modifications the following step would be to establish if modification precedes or competes with a single ubiquitin attachment.

The *Giardia* database shows three single ubiquitin- and one SUMO-coding gene. In preliminary studies (data not shown), all four genes are expressed. However, comparative expression of all four genes upon encystment was never addressed. The proteasomal activity was also never studied in encysting cells. Due to the fact that ubiquination along with SUMOylation are often used in gene/protein regulation it is interesting to assess these modifications and their role in the encysting cells.
In conclusion, because *Giardia* is a relatively simple organism as discussed in Chapter I and has a minimal genome, some of the questions raised about metabolic flow and redirection of pathways by enzyme amounts and protein modifications make this organism ideal to study those questions.
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