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Giardia intestinalis Glucosamine 6-Phosphate Isomerase: the Key Enzyme to Encystment Appears to be Controlled by Ubiquitin Attachment

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ABSTRACT. The cyst wall of the parasitic protozoan, *Giardia intestinalis,* is composed of a polymer of *N*-acetylgalactosamine, the precursor of which is synthesized by an inducible enzyme pathway. The first enzyme in this pathway, glucosamine 6-phosphate isomerase, is transcriptionally regulated. During encystment and in mature cysts this isomerase appears to be modified by ubiquitin attachment. Thus, it might be targeted for destruction by an ubiquitin-mediated pathway, suggesting that glucosamine 6-phosphate isomerase expression is tightly regulated.

Key Words. Competition inhibition, cyst wall, encystment, excystation, fusion protein, glutathione transferase, ubiquitin, ubiquitination, Western blot.

Cysts of the parasitic protozoan *Giardia intestinalis* are responsible for the transmission of infection from host to host. Cysts possess a rigid extracellular wall composed of both protein and carbohydrates (Luján et al. 1995; Manning et al. 1992). The filamentous structure of the cyst wall consists of a polymer of *N*-acetylgalactosamine, GalNAc) (Jarroll 1988, 2001). GalNAc is synthesized from endogenous glucose by a pathway induced when *Giardia* trophozoites encyst (Macechko et al. 1992). The first reaction unique to this biosynthetic pathway is catalyzed by glucosamine 6-phosphate isomerase (Gnp, EC 3.5.99.6, formerly EC 5.3.1.10, see http://www.chem.qmw.ac.uk/iubmb/enzyme/EC3/5/99/6.html). This enzyme catalyzes the reversible isomerization and amination of fructose 6-phosphate plus ammonium ion to glucosamine 6-phosphate. Because the isomerase is an inducible enzyme and because it catalyzes the first committed step of the cyst wall biosynthetic pathway, the gene encoding Gnp was cloned and its expression was analyzed (van Keulen et al. 1998). Two putative glucosamine 6-phosphate isomerase genes (*gnp1* and *gnp2*) (first named, incorrectly, *gpi*1 and *gpi*2 by van Keulen et al. (1998)) were found. Only one gene, *gnp1,* was believed to encode the inducible isomerase of *Giardia,* the other gene appeared to be inactive (van Keulen et al. 1998). This report describes the expression of both genes in *E. coli* to prove that their catalytic properties are that of the expected aminating isomerases. The results also suggest that both enzymes could be active. In addition, careful analysis has confirmed the initial observation that the enzyme is not present in trophozoites, contradicting the observation made by Knodler et al. (1999) and supporting the initial results of van Keulen et al. (1998). Detailed analysis showed that the turnover of Gnp in *Giardia* appeared to be by ubiquitination, which might cause the enzyme to be inactivated and degraded in cysts and non-encysting trophozoites.

MATERIALS AND METHODS

Immunoprecipitation. Trophozoites (Assemblage A, strain MR4), cysts, and protein samples were prepared as described (van Keulen et al. 1998). Isomerase from trophozoites was immunoprecipitated at different time intervals after induction of encystment with bile using antibodies raised against recombinant Gnp1 as described (anti-rGnp IgG; van Keulen et al. 1998). The enzyme was recovered by binding to protein-A sepharose beads (Davis et al. 1986), followed by the isomerase assay (see below), or by Western blot analysis using an antibody specific for ubiquitin (Sigma-Aldrich, St. Louis, MO). Gel electrophoresis and Western blot analysis was performed as described (van Keulen et al. 1998). Ubiquitinated proteins were immunoprecipitated in the same manner using this ubiquitin-specific antibody (0.1 µl per reaction), and the precipitated proteins were analyzed by Western blotting using anti-rGnp IgG (500-fold dilution). When the anti-rGnp IgG was used for immunoprecipitation using soluble lysates of trophozoites from cultures that had been induced to encyst for 0, 24 or 72 h, it was found that at 24 h the isomerase activity had reached its maximum value. This suggested that the antibody against the recombinant polypeptide precipitates a protein with isomerase activity and that the activity is inducible.

Construction of the expression vector pGEX-4T-1-*gnp*1. The complete reading frame of the *gnp*1 gene was amplified by the polymerase chain reaction (PCR) which added a 5′-*Eco*RI restriction site using the forward primer-5′-ATAgAatt ccCGTCCATCCACGTCTCC and a 3′-*Xho*I restriction site using a reverse primer-5′-CCATTCtCGaGT***TTA***AGCTTTTG CAGC. These included mismatches (lower case) to provide the restriction enzyme sites for cloning and the native stop codon (in **italics**). The amplified DNA was digested with *Eco*RI and *Xho*I and inserted in-frame into the pGEX-4T–1 expression vector by ligation to the corresponding sites of the *Eco*RI/*Xho*I-digested vector (Amersham Pharmacia Biotech, Inc. Piscataway, NJ). The resulting construct (pGEX-4T-1-*gnp*1) carrying the *Giardia gnp*1 gene insert was used to transform *E. coli* BL21(DE3) (Novagen, Madison, WI). Synthesis of Gnp1 was verified by Western blots using anti-rGnp IgG as probe. A single colony of transformed BL21(DE3) was grown overnight in 20 ml of LB medium containing 100 µg/ml of ampicillin. Gnp1 synthesis was induced by addition of isopropyl-β-D-thiogalactopyranoside (0.1 mM, final concentration) and the cells were allowed to grow for 4 h. Cells were harvested by centrifugation and lysed by lysozyme and DNase treatment followed by sonication. Cell debris was removed by centrifugation. Purification of the soluble recombinant protein was carried out on a Glutathione Sepharose 4B column as described by the manufacturer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Purified protein was stored with 10% glycerol at −70 °C.

Construction of pGEX-4T-1-*gnp*2. The ORF of the plasmid with the cloned second isomerase gene, *gnp*2, was amplified using the primer combination 5′-GTCTAAGGgAtccGTGAAG AAAAGCAACG, containing a *Bam*HI site for in-frame cloning and the reverse primer 5′-AT***TTA***TTCTGTAACGGTCTCGG CGATTC, which contained the native stop codon (in **italics**). The amplified DNA was purified and treated with T4 DNA polymerase to make it blunt-ended, then digested with *Bam*HI and ligated in *Bam*HI/*Sma*I digested pGEX-4T–1. The methods for cloning, expression and protein purification were the same as used for the GST-Gnp1 fusion protein.

Enzyme assay. Recombinant glucosamine 6-phosphate isom-

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erase activity was assayed colorimetrically in both the aminating and deaminating directions using a modification of the Morgan-Elson reaction as described by Levvy and McAllan (1959). In the aminating direction (formation of glucosamine 6-P (GlcN6P), activity was determined from a reaction mixture of 500 µl containing 0.1 M Tris-Base, 0.1 M ammonium chloride, 1 mM DTT, 0.1% Triton X-100 and variable amounts of D-fructose 6-phosphate, the final pH was 8.9. For the reverse reaction (deamination of GlcN6P), a reaction mixture of 500 µl containing 0.1 M Tris-HCl pH 8.9, 1 mM DTT, 0.1% Triton X-100 and variable amounts of GlcN6P was used. Reactions were initiated by addition of enzyme and mixtures were incubated at 37 °C for a time period such that activity was in the linear range (< 5% loss of substrate). Assays were terminated by heat (100 °C, 4 min) and the amount of GlcN6P formed (aminating) or consumed (deaminating) was measured spectrophotometrically at 585 nm by comparison with control tubes to which enzyme was added immediately prior to termination and with standards containing known amounts of GlcN6P but no enzyme.

RESULTS AND DISCUSSION

Glucosamine 6-phosphate isomerase becomes ubiquitinated. A recent report by Knodler et al. (1999) suggested that both enzymes Gnp1 and Gnp2 are present in trophozoites, but that only one, the equivalent of Gnp1 was inducible. In a prior study (van Keulen et al. 1998) neither enzyme was detected in uninduced trophozoites but Gnp1 was readily detected in induced trophozoites. This apparent contradiction might reflect a difference between the *Giardia* strains used in the two studies, as well as differences in culture conditions, e.g. age of the culture, cell density or culture medium. However, using the anti-rGnp IgG for Western blot analysis of uninduced trophozoites, we have occasionally observed an additional 37-kDa polypeptide antigen that did not correspond with the 29-kDa polypeptide predicted by the reading frame of *gnp1* and observed for the native enzyme (Steimle et al. 1997; van Keulen et al. 1998). On Western blots of lysates of uninduced or bile-induced trophozoites, the inducible 29-kDa protein antigen was invariably detected by 24 h (Fig. 1A, lane 7). However, the 37-kDa antigen could be observed at all examined time points and the amount increased, reaching a maximum at 72 h after induction (Fig. 1A, lanes 5 and 9). In addition, the 37-kDa antigen was the only one detected in cyst lysates obtained from cultures incubated with bile for 154 h (Fig. 1A, lanes 5 and 10).

Immunoprecipitation with anti-rGnp IgG followed by Western blot analysis and detection with a ubiquitin-specific antibody showed the same 37-kDa protein band, but not the 29-kDa one (data not shown). The reverse experiment, immunoprecipitation with the ubiquitin-specific antibody followed by Western blot analysis using anti-rGnp IgG showed the 37-kDa protein (Fig. 1A, lanes 1–5) in lysates obtained at time points of 24 h and longer after induction and in cysts. Ubiquitination of Gnp thus becomes apparent after induction and the total amount of modified protein increases beyond 24 h with virtually all of the antigen being ubiquitinated in mature cysts. On Western blots developed with anti-rGnp IgG, proteins isolated from cysts harvested from cultures incubated for 220 h with bile showed various small peptides not normally seen in protein samples obtained after a shorter period of incubation, indicating protein degradation in the cyst (Fig. 1B). Thus, while *gnp* can be detected immunochemically after 24 h of induction with bile and it continues to be detectable, the enzyme population becomes increasingly ubiquitinated until it is almost completely modified after 72 h of incubation. This results in cysts that contain a large amount of ubiquitinated isomerase. These data suggest that in uninduced trophozoites, which produce small amounts of this

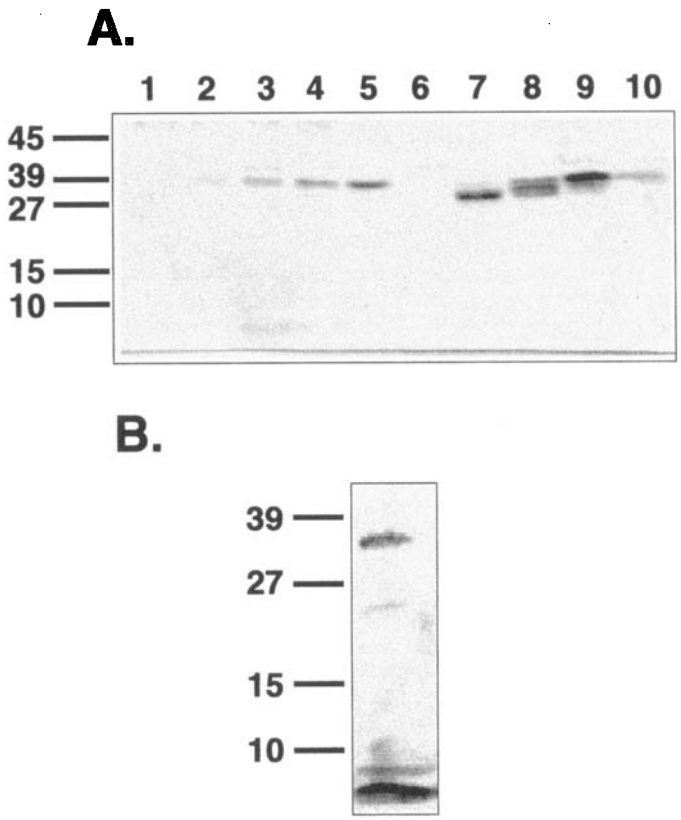

Fig. 1. Western blot analyses of *Giardia intestinalis* proteins probed with antibodies raised against recombinant Gnp1. **A.** Analysis of lysate proteins isolated from trophozoites that had been induced to encyst by incubation with bile for 0, 24, 48 or 72 h (lanes 1–4 and 6–9) or from cysts recovered 154 h after induction (lanes 5 and 10). Samples applied to lanes 1–5 were immunoprecipitated from 250 µg of lysate proteins using ubiquitin-specific antibody. Samples applied to lanes 6–10 were 100 µg samples of untreated lysate proteins. **B.** Analysis of total soluble cyst proteins (100 µg). The proteins were obtained by repeated freeze-thaw lysis of water-resistant cysts recovered from cultures after 220 h incubation with bile. The size in kDa of prestained markers, calibrated against non-stained markers, are indicated at the left sides of the blots.

enzyme (the first in the encystment pathway), cyst wall synthesis might be regulated by ubiquitination, and that the enzyme is degraded in cysts, making the newly excysting trophozoites devoid of active isomerase and so preventing premature cyst wall biosynthesis. It could not be determined which enzyme, Gnp1 or Gnp2, was present in these samples (the enzymes have a high degree of sequence identity) nor whether the ubiquitinated enzyme is enzymatically active or inactive. In many organisms ubiquitin targets proteins for destruction and after their ubiquitination, the modified proteins become associated with a proteasome complex which degrades them (Hershko and Ciechanover, 1998). Evidence that ubiquitin-mediated protein degradation might occur in *Giardia* is suggested by the existence within the genome of a gene for ubiquitin (Krebber et al. 1994), and also by the characterization of a 20S proteasome complex from *Giardia* trophozoites (Emmerlich et al. 1999). Genes encoding putative ubiquitin pathway enzymes are also evident within the *Giardia* genome (McArthur et al. 2000).

Both gene products Gnp1 and Gnp2 have the expected enzyme specificity. To prove that the cloned *gnp*1 gene indeed encodes glucosamine 6-phosphate isomerase, the coding se-

Table 1. Kinetic parameters[a] of native and recombinant isomerase. The data for the native enzyme are from Steimle et al. (1997).

	Native enzyme	Recombinant enzyme	Units
	Anabolic (substrate fructose 6-P)		
K_m	2.5 ± 0.24	20 ± 0.5	mM
V_{max}	86.3 ± 3.2	11.11 ± 1.2	μmol/min per mg protein
K_i[b]	2.0×10^{-8}	6.9×10^{-8}	M
	Catabolic (substrate glucosamine 6-P)		
K_m	0.38 ± 16	0.94 ± 0.01	mM
V_{max}	32.8 ± 5.3	7.7 ± 1.5	μmole/min per mg protein

[a] Values are means ± standard error, n = 6.
[b] For GlcN-tol-6P.

quence for this enzyme was amplified by PCR and cloned into the expression vector pGEX-4T–1, in order to obtain recombinant Gnp1 in soluble and functional form for enzyme assays. The glutathione-S-transferase (GST)-fusion protein exhibited enzyme activity in the presence of substrate, which enabled study of its enzymic properties. Enzyme kinetic data were determined in the anabolic (GlcN6P formation) and catabolic (GlcN6P deaminating) reaction and are summarized in Table 1.

To obtain definite proof that the *gnp*1 gene encodes the isomerase, 2-amino-2-deoxyglucitol 6-phosphate (GlcN-tol-6P), a reaction intermediate analog and competitive inhibitor of the isomerase from *E. coli* (Midelfort and Rose, 1977) was tested and found to inhibit the recombinant isomerase. The inhibition was found to be competitive for the aminating reaction with a K_i of 6.9×10^{-8} M using F6P as the variable substrate, indicating that the isomerase has a high affinity for this open-chain form of GlcN6P. The enzymatic properties of the recombinant enzyme and its inhibition by GlcN-tol-6P support the conclusion that the inducible isomerase is indeed encoded by the *gnp*1 gene from *Giardia*. The V_{max} and K_m of the native enzyme differ from those of the recombinant enzyme (Steimle et al. 1997; Table 1) possibly because the folding of the recombinant enzyme in *E. coli* differs from the folding of the native enzyme in *Giardia*. Cleavage of the isomerase from the GST fusion protein did not change the catalytic constants and therefore most kinetic measurements were done with the fusion protein. Differences with the native enzyme also were observed in the kinetic parameters of the deaminating reaction. In the deaminating direction the lower apparent K_m (0.94 ± 0.01 mM) for GlcN6P suggests that the enzyme has a higher affinity for the substrate for the catabolic reaction. This agrees with what had been observed with the native enzyme (Steimle et al. 1997). The second isomerase gene (*gnp*2) from *Giardia* was not detectably transcribed in trophozoites or during encystment. It differs from Gnp1 in its N- and C-terminal sequences, but it has retained the catalytically important amino acids (van Keulen et al. 1998). This gene was cloned and expressed in a fashion similar to *gnp*1 and its enzyme activity was determined. The recombinant protein showed isomerase activity in the aminating direction with a specific activity of 2.32 ± 0.12 μmole of GlcN6P produced/min per mg of protein. Therefore, even though the amino acid sequence of this enzyme differs considerably from Gnp1 in the N- and C-terminal ends, it possessed catalytic activity and may be active in its native form also.

General conclusion. Because sequence alignment and amino acid identity are no proof of enzyme function, especially in an organism such as *Giardia*, which has multi-functional enzymes (Sánchez 1998), proof for the identity of the *gnp* genes (*gnp*1 and *gnp*2) was obtained from enzyme kinetic parameters of recombinant enzymes produced by expression of the genes in *E. coli*. Both recombinant proteins exhibited all of the enzymic properties observed for the native enzyme. Furthermore, there was evidence that the enzyme is tightly controlled. In trophozoites induced to encyst, isomerase(s) could be detected as a 29-kDa and 37-kDa protein, the latter apparently ubiquitinated. This suggests that the enzyme is degraded via a ubiquitin-mediated pathway since in cysts only the 37-kDa protein and degradation products could be detected immunochemically. Excysted cells and uninduced trophozoites may therefore be safeguarded against cyst wall synthesis by removal and degradation of the isomerase until they are triggered to increase production of the enzyme by transcriptional activation.

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