# Purification and characterization of encystment-induced glucosamine 6-phosphate isomerase in *Giardia*[1]

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*Giardia intestinalis* encystment results in the incorporation of galactosamine (GalN) (a cyst- wall specific sugar) into outer cyst wall filaments [1,2]. GalN is synthesized during encystment from endogenous glucose by an inducible enzyme pathway [3] and the first reaction unique to GalN synthesis is the conversion of fructose-6-phosphate (F6P) to glucosamine 6-phosphate (G1cN6P) [4,5]. In *G. intestinalis,* this reaction is catalyzed by G1cN6P isomerase (2-amino-2-deoxy-D-glucosamine 6-phosphate ketol isomerase, EC 5.3.1.10) which deaminates G1cN6P to F6P and $NH_3$ and aminates F6P with $NH_3$ to produce GlcN6P [3,6,7]. These activities as well as those of the other GalN synthetic enzymes of *G. intestinalis* [3] are induced when trophozoites encyst in the presence of bile. In bacterial and yeast systems, the isomerase is regarded as a catabolic enzyme involved in the degradation of amino sugars [7,8]. In contrast, an in vivo anabolic role has been suggested for *Giardia* G1cN6P isomerase [3], some other eukaryotes [9-11], and an *Escherichia coli* K-12 mutant lacking G1cN6P synthase activity [12]. This paper describes the purification of G1cN6P isomerase from encysting *G. intestinalis* and represents the first characterization of a purified enzyme specifically induced during the encystment of a protozoan.

G1cN6P isomerase from *G. intestinalis* was assayed colorimetrically at 30°C in the aminase and deaminase directions [13]. The isomerase's aminase and deaminase activities were purified 505-

[1] *Abbreviations:* F6P, fructose 6-phosphate; GalN, galactosamine; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; G1cNAc6P, N-acetylglucosamine 6-phosphate; G1cN6P, glucosamine 6-phosphate.

Table 1
Purification of glucosamine 6-phosphate isomerase (aminase) from *Giardia intestinalis*[a]

| Purification step | Total protein (mg) | Total activity (units) | Specific activity (Units/mg) | Purification factor (fold) | Yield (%) |
|---|---|---|---|---|---|
| Cell Homogenate | 799 | 487 | 0.61 | 1 | 100 |
| Soluble (*S*) Fraction | 238 | 189 | 0.79 | 1.3 | 39 |
| Gel filtration HPLC | 8.6 | 33 | 3.8 | 6.2 | 7 |
| Cellulose phosphate (NaCl gradient elution) | 0.25 | 14 | 56 | 92 | 3 |
| Cellulose phosphate (substrate elution) | 0.06 | 5.6 | 93 | 152 | 1 |
| DEAE HPLC | 0.012 | 3.7 | 308 | 505 | 0.8 |

[a] One unit of enzyme activity catalyzes the formation of 1 $\mu$mol of glucosamine 6-phosphate/min. Specific activity is in units (1/mg protein). Protein was estimated by the method of Bradford [20]. Trophozoites (strain MR4) were cultivated, encysted and fractionated to yield a soluble (cytosolic) fraction (*S*-fraction) [3]. An Affi-Gel 501 (organomercurial column, Bio-Rad, Melville, NY) ($1.7 \times 7.0$ cm$^2$) was washed with dd$H_2O$ followed 50 mM Tris–HCl (pH 7.3) containing 1 mM EDTA. *S*-fraction was applied to this column at a flow rate of 1.0 ml/min. The sample was allowed to bind for 30 min and protein was eluted isocratically with the same buffer containing 30 mM DTT (data not shown, see text). These and all subsequent column eluates were concentrated by Amicon Centriprep-10 concentrators prior to application to the subsequent column. Activity recovered from the Affi-501 column was applied to a Waters Protein Pak 300 sw (7.5 mm × 30 cm) gel filtration-HPLC column at a flow rate of 0.5 ml/min. This column was equilibrated and protein eluted with 50 mM Tris–HCl (pH 7.3) buffer containing 0.1 M NaCl, 1 mM EDTA, and 5 mM DTT (added to all subsequent buffers to maintain enzyme stability during purification). Native molecular weights of proteins eluted from the column were determined by comparing their elution profiles to those of Bio-Rad gel filtration standards. Gel filtration fractions exhibiting the highest isomerase activity were pooled, diluted with 0.1 M sodium acetate buffer, the pH was adjusted to 5.6 with 0.1 M acetic acid. A pre-cycled cellulose phosphate (CP) [14–16] column was equilibrated with 0.1 M sodium acetate buffer pH 5.6 and the column was loaded at a flow rate of 0.7 ml/min with the isomerase containing sample from gel filtration (at pH 5.6) and the enzyme was allowed to bind to the column for 30 min at 4°C. The protein was eluted with a linear salt gradient (0–0.3 M NaCl) in 0.1 M sodium acetate buffer (pH 5.6). Fractions with the highest isomerase activity were pooled and desalted. CP chromatography (substrate elution) was performed on the desalted sample containing isomerase activity recovered from the first CP column by preparing a second CP column exactly as the first. Sample was allowed to bind for 30 min at 4°C and then was eluted isocratically at 0.7 ml/min with sodium acetate buffer (pH 5.6) containing 30 mM fructose 6-phosphate. The highest activity fractions were pooled and desalted. Buffer from this concentrated sample was exchanged with 50 mM Tris–Base and the pH was adjusted to 8.9. This sample (pH 8.9) was applied to a Waters Protein Pack DEAE-HPLC column (7.5 mm × 7.5 cm) at a flow rate of 1.0 ml/min. Protein was eluted with a linear gradient of NaCl (0–0.3 M NaCl) in 50 mM Tris–Base (pH 8.9).

and 400-fold, respectively (Table 1), with both activities co-purifying. The isomerase was stabilized by adding DTT (5 mM) to storage buffer and activity was enhanced (3.8-fold) when 1 mM DTT was added to the assay reaction mix. Omitted from Table 1 is the step involving elution from an organomercurial agarose column since DTT at high concentrations interferes with the assay color reaction making an accurate assessment of the enzyme activity impossible. For cellulose phosphate chromatography (substrate elution), specific elution was achieved by washing the column with buffer containing 30 mM F6P, but not with buffer containing 30 mM NaCl. Purified isomerase was labile to freezing and thawing but was stable for up to 2 months when stored in 50% glycerol at — 20°C.

The purified enzyme was subjected to two-dimensional gel electrophoresis (Fig. 1) revealing what appear to be at least two isoforms of the isomerase with pI values of ca. 7.1 and 7.3. *G. intestinalis* isomerase exhibits a denatured molecular mass (29 kDa) similar to that reported for the catabolic isomerases of *E. coli* (29.7 kDa), dog kidney (30.4 kDa) and *C. albicans* (28 kDa) [14-16]. The isomerase exhibited a native molecular mass of ca. 29 kDa as determined from gel filtration HPLC. The native molecular mass of the *G. intestinalis* isomerase, while resembling that of the *C. albicans* isomerase (43 kDa), differs considerably from that of the *E. coli* (178 kDa) and dog kidney (180 kDa) isomerases which are oligomers of six subunits [14-16].

Aminase and deaminase activities were optimal at pH 8.9 (range of 8.5-9.0 in 0.2 M Tris-Base to 0.2 M Tris-HCI). The value for the deaminase activity is similar to that reported for the isomcrase deaminase activity from dog kidney (pH 8.8), but is higher than those reported for C. *albicans* (pH 7.5-7.8) and *E. coli* (pH 7.6-8.2) deaminases [15,16]. pH optima for isomerase aminating activities have not been reported in other systems.

Enzyme kinetics for each substrate were assessed over a range of substrate concentrations (at optimal pH) and then analyzed (mcans of values from at least three separate determinations per formed in duplicate) for quality of fit to a theorctical hyperbola using the non-linear regression analysis software program Enzfitter by R.J.

Leatherbarrow from Elscvier-Biosoft. These data also were used to calculate kinetic parameters by Cleland's program KINETICS [17]. *G. intestinalis* aminase exhibited a $_{Vmax}$ of 86.3 ± 3.2 pmol GIcN6P produced min$^{-1}$ mg protein $^{-1}$ with an apparent $K_m$ of 2.5 ± 0.24 mM for F6P and an apparent $K_m$ of 19 ± 1.9 mM for $NH_4C1$; deaminase exhibited a $_{Vmax}$ of 32.8 ± 5.3 pmol G1cN6P consumed min $^{-1}$ mg protein -$^{1}$ with an apparcnt $K_m$ of 0.38 ± 0.16 mM GIcN6P. *Giardia's* isomerase exhibits $K_m$ values for F6P and $NH_4C1$ which are considerably higher than that for GIcN6P, suggesting that the enzyme has a higher affinity for GlcN6P. In comparison, the catabolic isomerases of *C. albicans* and dog kidney exhibit affinities *($K_m$* values 0.10 and 0.25 mM, respcc-tively) for G1cN6P reflecting their catabolic role. A higher apparent $K_m$ (2.2 mM) for G1cN6P was reported for *E. coli* isomerase even though this enzyme is more active in the catabolic direction, exhibiting deaminase activity which is four-fold higher than that for the anabolic reaction. Thc *E. colt* isomerase also exhibits an apparent $K_m$ of 1.7 mM for F6P and an apparent $K_m$ of 31.4 mM for $NH_4C1$ [14] and these values are similar to those obtained for the *G. intestinalis* isomerase. $K_m$ values for F6P and $NH^4C_l$ for dog kidney and *C. albicans* isomerases have not been reported [15,16].

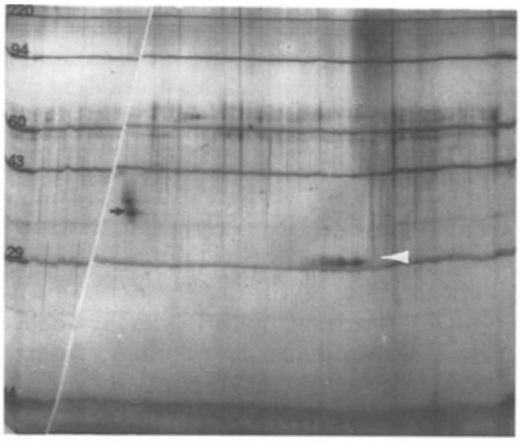


Fig. 1. Two-dimensional polyacrylamide gel electrophoresis (PAGE) of *Giardia intestinalis* GlcN6P isomerase. The white arrow indicates the purified isomerase (2 $\mu$g of starting protein) and the black arrow indicates tropomyosin (40 ng), the internal standard. Two-dimensional electrophoresis was performed according to the method of O'Farrell [21] by Kendrick Labs, (Madison, WI). Isoelectric focusing was carried out in glass tubes (2.0 mm inner diameter) using 2% pH 4–8 BDH ampholines (Hoefer Scientific, San Francisco, CA) for 9600 volt-h. After equilibration for 10 min in buffer containing 10% glycerol, 50 mM DTT, 2.3% SDS and 0.625 M Tris–HCl (pH 6.8), each tube gel was sealed to the top of a stacking gel overlying a 10% acrylamide slab gel (0.75 mm thick) and SDS slab gel electrophoresis was carried out for about 4 h at 12.5 mA/gel. Molecular weight standards were added to the agarose which sealed the tube gels to the slab gels: myosin (220 000); phosphorylase A (94 000); catalase (60 000); actin (43 000); carbonic anhydrase (29 000); and lysozyme (14 000). The slab gels were fixed in a solution of 10% acetic acid/50% methanol overnight. Proteins standards appear as horizontal lines in the silver stained [22] gel.

Isomerases from *E. coli* [14], dog kidney [15] and *C. albicans* [16] have been well characterized with regard to their deaminase activities. In contrast, the rolc of the isomerase in amino sugar synthesis is considered minimal [4,5] and relativcly little information has been reported for the aminase activity since G1cN6P synthesis from F6P is generally catalyzed by GIcN6P synthase in a glutamine-requiring, irreversible reaction [4,5]. How-cver, *G. intestinalis* lacks detectable G1cN6P synthase activity *[3]* and thus G1cN6P isomerase appears to play an important anabolic role by catalyzing synthesis of G1cN6P to be used in Ga1NAc synthesis for cyst wall formation. Despite resembling the other catabolic isomerases with respect to its apparent substrate affinities, *Giardia's* isomerase exhibits a higher rate of anabolic activity with a $_{Vmax}$ for the aminase reaction

that is 2.3-fold higher than for the deaminase. Furthermore, GlcN6P aminase activity in *Giardia* may be enhanced when the isomerase is coupled with G1cN6P N-acetylase [3] since the equilibrium of the isomerase reaction may be shifted toward G1cN6P synthesis as G1cN6P is converted to N-acetylglucosamine 6- phosphate (G1cNAc6P) by the acetylase [14,18].

Substrate specificity experiments revealed that when F6P was replaced with D-glucose 6-phosphate (32 mM), D-mannose 6-phosphate (32 mM), or D-galactose 6-phosphate (32 mM), amination by $NH_4C1$ was not observed. Likewise, neither L-glutamine (0.2 M) nor L-asparagine (0.2 M) served as ammonia donors to F6P. G1cNAc6P (2 mM), N-acetylglucosamine (GlcNAc) (2 mM), glucosamine (GlcN) (2 mM), and GalN (2 mM) did not serve as substrates for the deaminase. An intermediate in the pathway to Ga1NAc synthesis, G1cNAc6P, allosterically activates the *E. coli* and dog kidney isomerases [7,14,15]. However, GlcNAc6P (up to 2.5 mM) does not activate the purified *G. intestinalis* isomerase and in this respect, it resembles the isomerase from *C. albicans* [7,14]. The isomerase was not affected by GlcNAc 1-phosphate (3 mM), UDP-G1cNAc (3 mM), or UDP-Ga1NAc (0.5 mM). The absence of allosteric regulation may be related to the nonoligomeric nature of the *Giardia* isomerase as was suggested for the *C. albicans* isomerase [16]. Since neither glucose 6-phosphate (up to 2.5 mM), an activator of this isomerase in *Drosophila virilis* [12] and *Musca domestics* [19], nor any of the intermediates in the Ga1NAc synthetic pathway seem to activate *G. intestinalis* isomerase, it appears that this isomerase is not allosterically regulated in *Giardia's* GaIN synthetic pathway.

*G. intestinalis* aminase activity was inhibited 68% by iodoacetamide (20 mM) and 61% by N-ethylmaleimide (20 mM); these compounds also inhibited deaminase activity ( > 95%). Aminase and deaminase activities were inhibited by 2-amino -2-deoxyglucito1-6-pho sphate (G1cN-tol6P)), an analogue of the straight-chain form of G1cN6P [18]. GlcN-tol- 6P competitively inhibited the aminase = 2.0 x $10^{-8}$ M GlcN-to1-6P) and the deaminase = 2.8 x 10 — M G1cN-to1-6P) as determined by double reciprocal plots yielding $K_m^{app}$ P used to solve for *K,* where $K_m^{app}$ = $K_m(1 + [I]/K_1)$,. The potent inhibition of the *G. intestinalis* isomerase by GlcN-tol-6P suggests that the isomerase has high affinity for the open chain form of GlcN 6-phosphate. Likewise, G1cN-to1-6P competitively inhibits the deaminase activity of the *E. coli* isomerase *(K,* = 2 x $10^{-7}$ M GlcN-tol-6P) [18]. By specifically inhibiting the *G. intestinalis* isomerase with G1cN-to1-6P in vivo, GaIN synthesis may be blocked during encystment and more importantly, the formation of viable cysts may be inhibited. Future studies examining the effects of GlcN-to1-6P (and its non-phosphorylated form) on *G. intestinalis* growth and encystment are being planned.

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