Purification and characterization of encystment-induced glucosamine 6-phosphate isomerase in *Giardia*

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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 (GlcN6P) [4,5]. In *G. intestinalis*, this reaction is catalyzed by GlcN6P isomerase (2-amino-2-deoxy-D-glucosamine 6-phosphate ketol isomerase, EC 5.3.1.10) which deaminates GlcN6P to F6P and NH₃ and aminates F6P with NH₃ 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* GlcN6P isomerase [3], some other eukaryotes [9-11], and an *Escherichia coli* K-12 mutant lacking GlcN6P synthase activity [12]. This paper describes the purification of GlcN6P isomerase from encysting *G. intestinalis* and represents the first characterization of a purified enzyme specifically induced during the encystment of a protozoan.

GlcN6P 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-

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¹ *Abbreviations:* F6P, fructose 6-phosphate; GalN, galactosamine; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; GlcNAc6P, N-acetylglucosamine 6-phosphate; GlcN6P, glucosamine 6-phosphate.
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 pH 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-HCl). The value for the deaminase activity is similar to that reported for the isocitrate 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 (means of values from at least three separate determinations per formed in duplicate) for quality of fit to a theoretical hyperbola using the non-linear regression analysis software program Enzfitter by R.J.
Leatherbarrow from Elsevier-Biosoft. These data also were used to calculate kinetic parameters by Cleland's program KINETICS [17]. *G. intestinalis* aminase exhibited a $V_{\text{max}}$ of $86.3 \pm 3.2$ pmol GlcN6P produced min$^{-1}$ mg protein$^{-1}$ with an apparent $K_m$ of $2.5 \pm 0.24$ mM for F6P and an apparent $K_m$ of $19 \pm 1.9$ mM for NH$_4$C1; deaminase exhibited a $V_{\text{max}}$ of $32.8 \pm 5.3$ pmol G1cN6P consumed min$^{-1}$ mg protein$^{-1}$ with an apparent $K_m$ of $0.38 \pm 0.16$ mM GlcN6P. *Giardia's* isomerase exhibits $K_m$ values for F6P and NH$_4$C1 which are considerably higher than that for GlcN6P, 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, respectively) 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. The *E. coli* isomerase also exhibits an apparent $K_m$ of 1.7 mM for F6P and an apparent $K_m$ of 31.4 mM for NH$_4$C1 [14] and these values are similar to those obtained for the *G. intestinalis* isomerase. $K_m$ values for F6P and NH$_4$C1 for dog kidney and *C. albicans* isomerases have not been reported [15,16].

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 role of the isomerase in amino sugar synthesis is considered minimal [4,5] and relatively little information has been reported for the aminase activity since G1cN6P synthesis from F6P is generally catalyzed by GlcN6P synthase in a glutamine-requiring, irreversible reaction [4,5]. However, *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 GalNAc 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 $V_{\text{max}}$ 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 GlcN6P N-acetylase [3] since the equilibrium of the isomerase reaction may be shifted toward GlcN6P synthesis as GlcN6P is converted to N-acetylglucosamine 6-phosphate (GlcNAc6P) 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 NH₄Cl was not observed. Likewise, neither L-glutamine (0.2 M) nor L-asparagine (0.2 M) served as ammonia donors to F6P. GlcNAc6P (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 GaIN synthesis, GlcNAc6P, 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-GlcNAc (3 mM), or UDP-GalNAc (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 domestica* [19], nor any of the intermediates in the GaIN 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-deoxyglucitol-6-phosphate (GlcN-tol-6P), an analogue of the straight-chain form of GlcN6P [18]. GlcN-tol-6P competitively inhibited the aminase = 2.0 x 10⁻⁸ M GlcN-tol-6P and the deaminase = 2.8 x 10⁻⁹ M GlcN-tol-1-6P as determined by double reciprocal plots yielding $K_{\text{app}}^\text{m}$ P used to solve for $K$, where $K_{\text{app}}^\text{m} = K_{\text{m}}(1 + [I]/K_I)$. 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, GlcN-tol-6P competitively inhibits the deaminase activity of the *E. coli* isomerase ($K_I = 2 x 10^{-7}$ M GlcN-tol-6P) [18]. By specifically inhibiting the *G. intestinalis* isomerase with GlcN-tol-1-6P 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-tol-6P (and its non-phosphorylated form) on *G. intestinalis* growth and encystment are being planned.

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References


