

## RESEARCH ARTICLE

Mechanistic studies on human *N*-acetylgalactosamine kinase

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**Abstract**

*N*-Acetylgalactosamine kinase (GALK2) is a small molecule kinase from the GHMP family which phosphorylates *N*-acetylgalactosamine at the expense of ATP. Recombinant GALK2 expressed in, and purified from, *Escherichia coli* was shown to be active with the following kinetic parameters: Michaelis constant for ATP,  $14 \pm 3 \mu\text{M}$ ; Michaelis constant for *N*-acetylgalactosamine,  $40 \pm 14 \mu\text{M}$ ; and turnover number,  $1.0 \pm 0.1 \text{ s}^{-1}$ . The combination of substrate inhibition by *N*-acetylgalactosamine and  $\alpha$ -methylgalactopyranoside acting as an uncompetitive inhibitor with respect to ATP suggested that the enzyme has an ordered ternary complex mechanism in which ATP is the first substrate to bind. The effects of pH on the kinetic parameters provided evidence for ionizable residues playing a role in substrate binding and catalysis. These results are discussed in the context of the mechanisms of the GHMP kinases.

**Keywords:** GHMP kinase; *N*-acetylgalactosamine; pH study; enzyme mechanism; galactokinase; GALK2

**Abbreviations:** GALK1, human galactokinase; GALK2, human *N*-acetylgalactosamine kinase; GalNAc, *N*-acetylgalactosamine; GHMP, galactokinase, homoserine kinase, mevalonate kinase, phosphomevalonate kinase;  $k_{\text{cat}}$ , turnover number;  $K_{\text{ic}}$ , competitive inhibition constant;  $K_{\text{iu}}$ , uncompetitive inhibition constant;  $K_{\text{is}}$ , substrate inhibition constant;  $K_{\text{m,ATP}}$ , Michaelis constant for ATP;  $K_{\text{m,GalNAc}}$ , Michaelis constant for GalNAc; LB, Luria–Bertani.

**Introduction**

The GHMP kinases are a group of small molecule kinases which are primarily involved in intermediary metabolism<sup>1,2</sup>. The name arises from some of the first members of the family identified—galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase. These enzymes have sequence and structural similarity<sup>1</sup>. On the basis of both sequence and structural alignments, the family has been extended to include a number of other enzymes (e.g. arabinose kinase and archaeal shikimate kinase<sup>3,4</sup>) and two non-enzymatically active proteins—the *Saccharomyces cerevisiae* transcriptional regulator Gal3p<sup>5</sup> and the *C. elegans* sex-fate determining protein XOL-1<sup>6</sup>.

*N*-Acetylgalactosamine kinase (GALK2, EC 2.7.1.157) shows high sequence and structural similarity to galactokinase (GALK1, EC 2.7.1.6)<sup>7,8</sup>. This mammalian enzyme was originally isolated from kidney<sup>9,10</sup> and shows greatest activity toward *N*-acetylgalactosamine with lower activity with galactosamine and galactose<sup>9,10</sup> (Figure 1a). Human galactokinase (GALK1) has no detectable activity against

*N*-acetylgalactosamine<sup>11</sup>. The structural basis of this specificity is the substitution of bulky methionine and cysteine residues in the active site of GALK1 with threonine and glycine respectively (Figure 1b)<sup>8</sup>. While GALK1 is known to function in the Leloir pathway of galactose catabolism<sup>12–14</sup>, the precise role of GALK2 *in vivo* is not known, but it has been suggested that it may play a role in glycoprotein metabolism<sup>10</sup>.

There is interest in developing inhibitors toward some members of the GHMP kinase family<sup>1</sup>. For example, inhibition of mevalonate kinase may provide an alternative means to statin drugs for the regulation of cholesterol synthesis<sup>1,15–17</sup> and as insecticides which target juvenile hormone biosynthesis<sup>18</sup>. There is also interest in inhibiting bacterial GHMP kinases for the development of novel antibiotics<sup>19</sup>, and galactokinase inhibition has been proposed as a therapy for type I galactosemia<sup>20</sup>. This severe, and currently untreatable, genetic disease results in irreversible mental and physical impairment during childhood<sup>21,22</sup>. These symptoms are believed to be caused by the build-up of the toxic metabolite galactose 1-phosphate<sup>23–25</sup>. Consequently it was

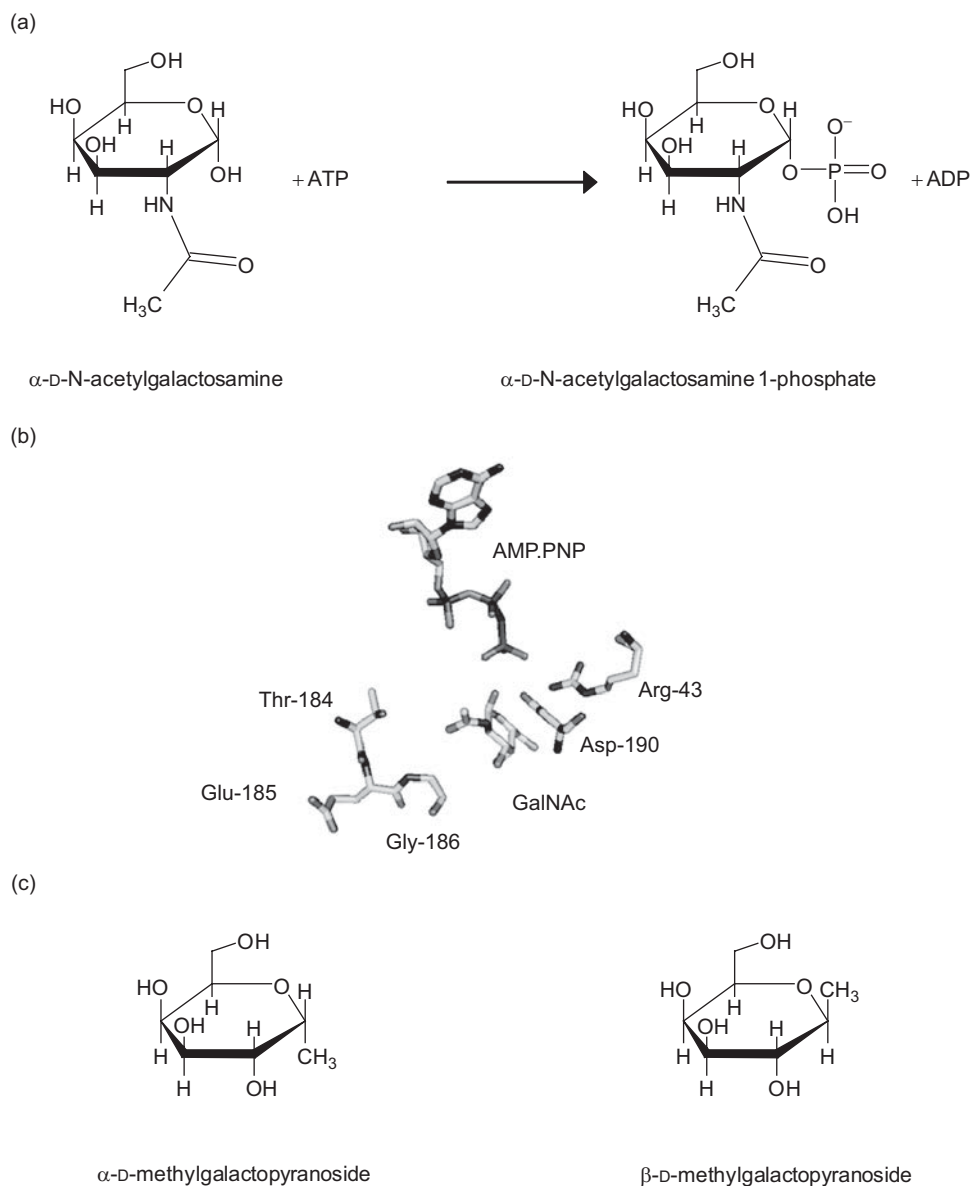
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**Figure 1.** (a) The reaction catalyzed by *N*-acetylgalactosamine kinase. (b) The active site of GALK2. Only the substrates and key residues are shown. The loop comprising Thr-184 to Gly-186 accommodates the *N*-acetyl group of *N*-acetylgalactosamine (GalNAc). In galactokinase, the threonine and glycine residues in this loop are substituted for bulkier methionine and cysteine side chains respectively. Asp-190 is located with the carboxyl group in between the C1-OH of the sugar and the  $\gamma$ -phosphate of the nucleotide. This residue is likely to be important in the catalytic mechanism of the enzyme and its ionization state is probably influenced by Arg-43. The figure was drawn using PyMol ([www.pymol.org](http://www.pymol.org)) and the PDB file 2A2D<sup>8</sup>. (c) Structures of *N*-acetylgalactosamine kinase inhibitors used in this study.

proposed that inhibition of galactokinase would reduce this build-up<sup>20</sup>. Although the build-up of galactose (and its reduced counterpart, galactitol) can also cause problems, the main symptom is early onset cataracts, which can be addressed by surgery. Recently a number of human galactokinase inhibitors were identified by high throughput screening<sup>26</sup>. One issue in the development of specific galactokinase inhibitors is that the high structural similarity between GALK1 and GALK2 means that there is a need to ensure that compounds can discriminate between the two enzymes. Currently, the enzymology of GALK2 is relatively poorly characterized. Here, we describe the inhibition of GALK2 by galactose analogs and the effects of pH on the

enzyme's kinetic parameters. From these data we make inferences about the enzyme's mechanism.

## Materials and methods

### *Expression and purification of human GALK2*

A GALK2 expression clone (generously provided by Professor Hazel Holden, University of Wisconsin, USA) was used to direct the expression of recombinant protein in *Escherichia coli*<sup>8</sup>. The plasmid was used to transform competent *E. coli* HMS174(DE3). A single colony was picked and grown in 5 mL Luria-Bertani (LB) medium supplemented with 100  $\mu$ g mL<sup>-1</sup> kanamycin, shaking overnight at 37°C. This

culture was transferred into 1 L of LB (supplemented with  $100 \mu\text{g mL}^{-1}$  kanamycin) which was then shaken at  $37^\circ\text{C}$  for 4 h. The cells were grown for a further 2 h at  $20^\circ\text{C}$ , by which time  $A_{600\text{nm}}$  was typically between 1.5 and 1.8. The expression of GALK2 was then induced by the addition of IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) to a final concentration of 2 mM. Induction was allowed to proceed for 18 h at  $20^\circ\text{C}$  and then the cells were collected by centrifugation (4200 g for 10 min), resuspended in cell resuspension buffer (50 mM HEPES-OH, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol), and frozen at  $-80^\circ\text{C}$ .

The cells were thawed and disrupted by sonication (three 30 s pulses at 100 W). Insoluble matter was removed by centrifugation (25,000 g for 15 min) and the supernatant applied to a 1 mL nickel agarose affinity column (His-Select, Sigma) which had been pre-equilibrated in washing buffer (50 mM HEPES-OH, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol). The supernatant was permitted to flow through by gravity and the column was then washed with 20 mL of washing buffer. Protein was eluted in three times 2 mL aliquots of elution buffer (washing buffer supplemented with 250 mM imidazole). Aliquots containing purified GALK2 were identified by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and dialyzed against cell resuspension buffer supplemented with 2 mM dithiothreitol (DTT). Following dialysis, protein concentrations were estimated using the method of Bradford<sup>27</sup>. The purified protein was stored frozen at  $-80^\circ\text{C}$  until required.

#### Assay for *N*-acetylgalactosamine kinase activity

The assay for *N*-acetylgalactosamine kinase activity was based on the coupled enzyme assay used for galactokinase<sup>28,29</sup> except that *N*-acetylgalactosamine was substituted for galactose. In this system, the production of adenosine diphosphate (ADP) is coupled to the oxidation of nicotinamide adenine dinucleotide (NADH) through the actions of pyruvate kinase and lactate dehydrogenase. Reactions were measured in a total volume of  $900 \mu\text{L}$  over a 10 min period at a temperature of  $37^\circ\text{C}$ . Each reaction contained 20 mM HEPES-OH, pH 8.0, 150 mM NaCl, 5 mM  $\text{MgCl}_2$ , 10% (v/v) glycerol, 1 mM NADH, 1 mM DTT,  $400 \mu\text{M}$  phosphoenolpyruvate, 7.5 U pyruvate kinase (Sigma), and 10 U lactate dehydrogenase (Sigma). Either GalNAc or ATP was maintained at a saturating concentration (5 mM or 0.5 mM, respectively) and the concentration of the other substrate was varied. Reactions were initiated by the addition of GALK2 (50–230 nM) and monitored by measuring the absorbance at 340 nm. Initial rates ( $v$ ) were calculated, converted to molar units by use of the extinction coefficient for NADH<sup>30</sup> ( $6220 \text{ L mol}^{-1} \text{ cm}^{-1}$ ) and divided by the enzyme concentration. These values were plotted against the concentration of the variable substrate and the data fitted to the equation  $v/[GALK2] = k_{\text{cat}} \cdot [S]/(K_m + [S])$  using non-linear curve fitting<sup>31</sup> as implemented in the program GraphPad Prism, version 3.02 (GraphPad Software, San Diego, USA). Where substrate inhibition was suspected on the basis of visual inspection of the data, a modified form of this

equation was used:  $v/[GALK2] = k_{\text{cat}} \cdot [S]/(K_m + [S] + [S]^2/K_{\text{is}})$ , where  $K_{\text{is}}$  is the substrate inhibition constant<sup>32</sup>. All points were weighted equally and values reported plus/minus the standard errors derived during this fitting process.

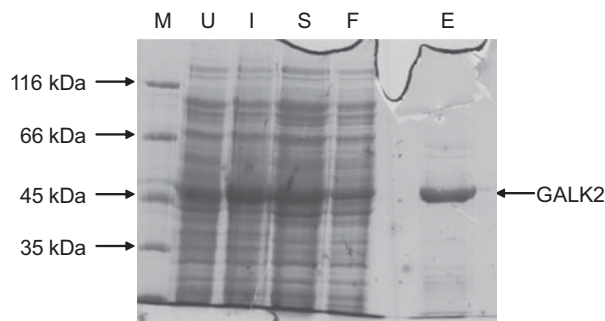
Inhibition studies were carried out over a range of substrate concentrations. At each concentration one substrate concentration was maintained at a saturating level (see above) and the other held at a sub-saturating level. These sub-saturating concentrations were chosen such that some were above and some below the  $K_m$  for that substrate. The rate was measured at these substrate concentrations for a range of inhibitor concentrations. Since methylgalactopyranosides have previously been shown to be poor inhibitors of GALK1<sup>29</sup>, a wide concentration range was used, with the highest concentrations (approximately 100 mM) dictated by the solubility of the compound in aqueous buffer.

The effects of pH were ascertained by measuring the kinetic parameters over the pH range 4.5–11. Since different buffer systems can also cause alterations in the rates of enzyme catalyzed reactions, the HEPES-OH buffer used in the experiments described above was substituted for a constant ionic strength buffer system of ACES (*N*-(2-acetamido)-2-aminoethanesulfonic acid) (100 mM)/Tris (50 mM)/ethanolamine (50 mM)<sup>33</sup>. The turnover number was plotted against pH and the data fitted (using GraphPad Prism) to the equation  $k_{\text{cat}} = k_{\text{cat,lim}} (1 + [\text{H}^+]/K_{\text{a1}} + K_{\text{a2}}/[\text{H}^+])$ , where  $k_{\text{cat,lim}}$  is the limiting value of  $k_{\text{cat}}$  and  $K_{\text{a1}}$  and  $K_{\text{a2}}$  are the acid dissociation constants of two different ionizable groups in the enzyme's active site<sup>34</sup>.

## Results

### Expression and purification of human GALK2

Recombinant human GALK2 could be expressed in, and purified from, *E. coli* (Figure 2). The overnight (approximately 18 h)  $20^\circ\text{C}$  incubation following induction was required to give good yields (typically 1–2 mg per liter of culture). The use of alternative induction conditions



**Figure 2.** Purification of recombinant human GALK2. Samples taken during the purification procedure (see methods) were analyzed by 10% SDS-PAGE. The sizes of molecular mass markers (M) are shown on the left. U, extract from cells prior to induction; I, extracts from induced cells prior to harvesting by centrifugation; S, protein solution extracted from cells by sonication and clarified by centrifugation; F, material which passed through the nickel-agarose column; E, protein eluted from this column in the presence of 250 mM imidazole.

(e.g. 3 h at 37°C, which has been reported for the bacterial expression of human galactokinase and UDP-galactose 4'-epimerase<sup>29,35</sup>) resulted in little, or no, protein after purification (data not shown).

#### Activity and specificity of recombinant human GALK2

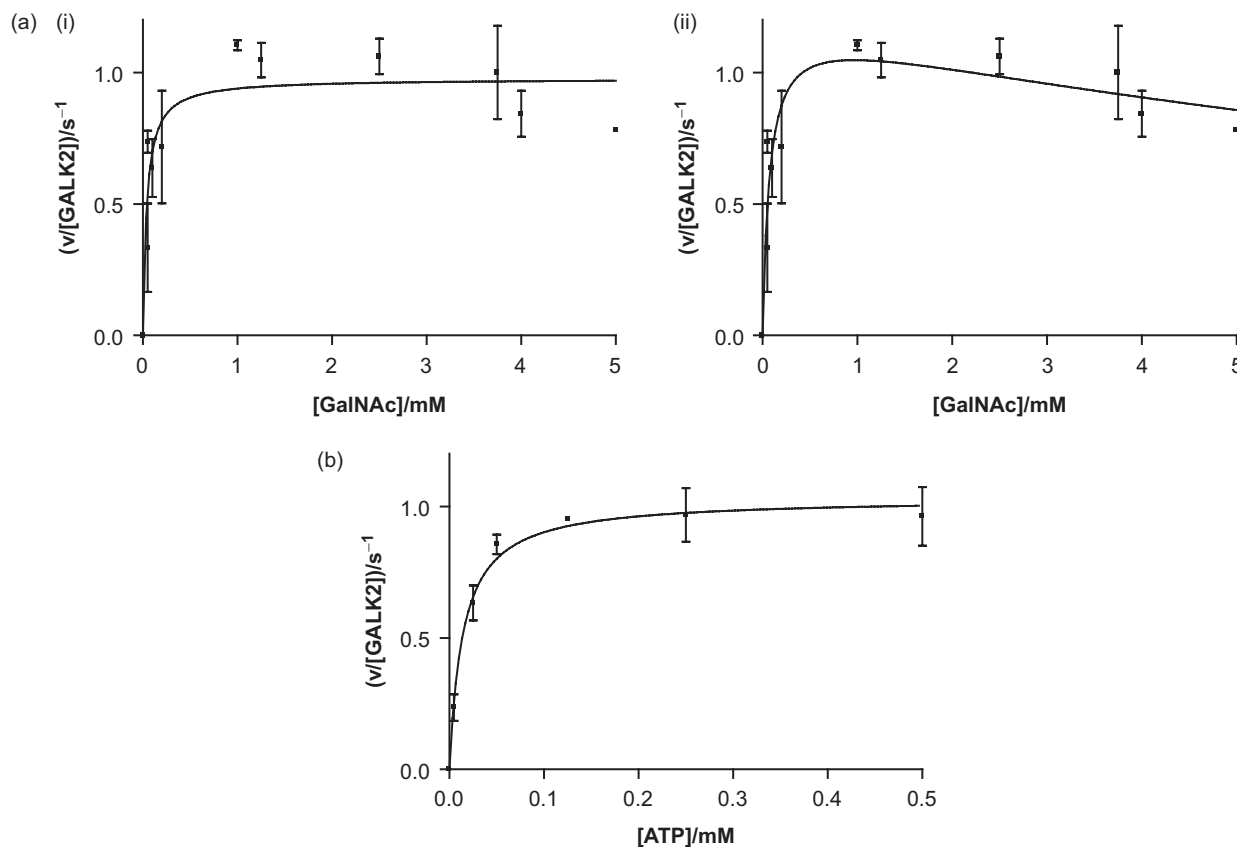
The purified recombinant protein was active and showed typical, saturable kinetics when either substrate was varied (Figure 3). Fitting of the data to the Michaelis-Menten equation resulted in values for the Michaelis constant for ATP ( $K_{m,ATP}$ ) of  $14 \pm 3 \mu\text{M}$ , the Michaelis constant for *N*-acetylgalactosamine ( $K_{m,GalNAc}$ ) of  $40 \pm 14 \mu\text{M}$ , and a turnover number ( $k_{cat}$ ) of  $1.0 \pm 0.1 \text{ s}^{-1}$ . These values compare well with those published for the enzyme purified from pig kidney<sup>9,10</sup>. When the *N*-acetylgalactosamine concentration was varied, high concentrations ( $>3.5 \text{ mM}$ ) appeared to result in a modest reduction in the rate. This may be due to substrate inhibition, a phenomenon which is well documented with galactose and galactokinase<sup>36,37</sup>. Therefore, the data were also fitted to a modified form of the Michaelis-Menten equation which accounts for substrate inhibition. This fit results in a value for the substrate inhibition constant,  $K_{is}$ , of  $13 \pm 8 \text{ mM}$  and modified values for  $K_m$  and  $k_{cat}$  of  $73 \pm 27 \text{ mM}$  and  $1.2 \pm 0.1 \text{ s}^{-1}$ . Low levels of activity were detectable with

galactosamine and galactose, but these were too small to permit meaningful kinetic analysis.

#### Inhibition by methylgalactopyranosides

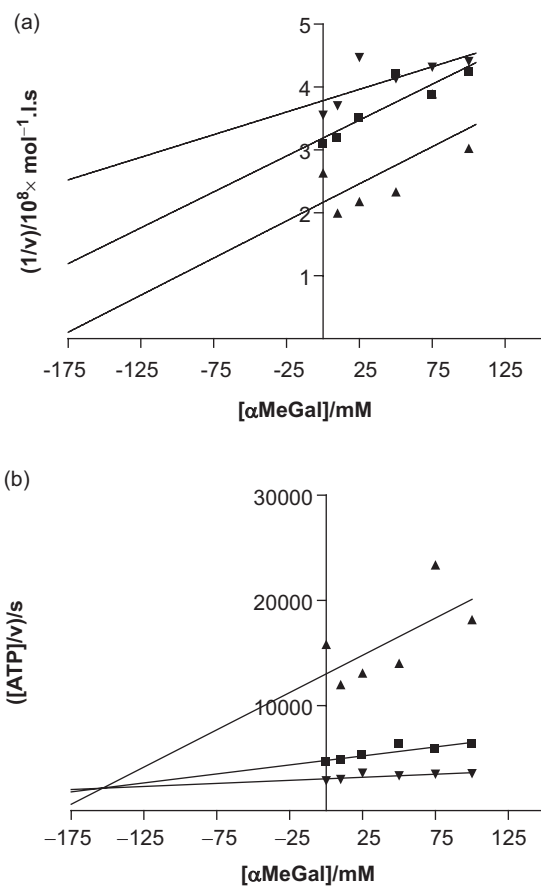
Since 1-methylpyranosides do not undergo anomeric inter-conversion in water, the  $\alpha$ - and  $\beta$ -methylgalactopyranosides (Figure 1c) are stable compounds in aqueous solution. Both were tested as potential inhibitors of human GALK2. Although  $\beta$ -methylgalactopyranoside was shown to reduce the rate of reaction catalyzed by GALK2, this effect was slight, and consequently was difficult to measure (data not shown). In contrast, reproducible measurements could be produced with  $\alpha$ -methylgalactopyranoside and, thus, further analysis was concentrated on this compound.

For a competitive inhibitor, a plot of reciprocal rate against inhibitor concentration (the so-called Dixon plot) results in a straight line at a constant substrate concentration. Repetition of the experiment with different substrate concentrations results in a family of straight lines which cross at a single point corresponding to the reciprocal  $V_{max}$  and minus the competitive inhibition constant,  $K_{ic}$ <sup>32,38,39</sup>. This was not observed under conditions where the GalNAc concentration was saturating, and three lower concentrations of ATP were used with a range of  $\alpha$ -methylgalactopyranoside



**Figure 3.** Recombinant human GALK2 (50 nM) is an active *N*-acetylgalactosamine kinase. In (a), the concentration of ATP was maintained at a constant level of 0.5 mM and *N*-acetylgalactosamine (GalNAc) was varied. The resulting data were fit (i) to the Michaelis-Menten equation (giving values of  $K_{m,GalNAc} = 40 \pm 14 \mu\text{M}$  and  $k_{cat} = 1.0 \pm 0.1 \text{ s}^{-1}$ ) and (ii) to a modified version of this equation which accounts for substrate inhibition (giving values of  $K_{is} = 13 \pm 8 \text{ mM}$ ,  $K_m = 73 \pm 27 \text{ mM}$ , and  $k_{cat} = 1.2 \pm 0.1 \text{ s}^{-1}$ ). In (b) the GalNAc concentration was maintained at 5 mM and the concentration of ATP was varied (giving values of  $K_{m,ATP} = 14 \pm 3 \mu\text{M}$  and  $k_{cat} = 1.0 \pm 0.1 \text{ s}^{-1}$ ). Each point represents the mean of two or three separate determinations and the error bars the standard deviations of these means.



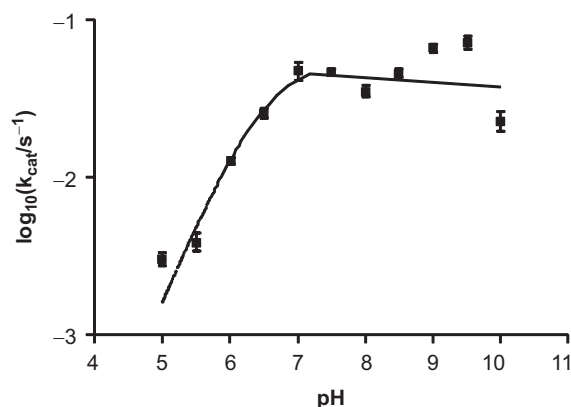


**Figure 4.** The inhibition of GALK2 (50 nM) by  $\alpha$ -methylgalactopyranoside. Inhibition was studied over a range of  $\alpha$ -methylgalactopyranoside concentrations and three different ATP concentrations: 8  $\mu$ M (inverted triangles,  $\nabla$ ), 15  $\mu$ M (squares,  $\blacksquare$ ), and 60  $\mu$ M (upright triangles,  $\blacktriangle$ ). (a) In a Dixon plot the lines do not converge, suggesting that the inhibition is not competitive. (b) The converging lines on a plot of [ATP]/rate against [inhibitor] suggest that the mode of inhibition is uncompetitive with respect to ATP. In all cases the *N*-acetylgalactosamine concentration was 1.0 mM.

concentrations (Figure 4a). In contrast, for uncompetitive inhibition, plots of substrate concentration divided by rate plotted against inhibitor concentration gives straight lines for each substrate concentration which intersect at  $K_m/V_{max}$  and minus the uncompetitive inhibition constant,  $K_{iu}$ <sup>39</sup>. This was observed with GALK2 (Figure 4b), and thus it can be concluded that  $\alpha$ -methylgalactopyranoside is an uncompetitive inhibitor with respect to ATP. Since  $K_{iu}$  is estimated to be approximately 150 mM, this compound is clearly a very poor inhibitor of GALK2.

#### Effects of pH on the turnover number

Careful analysis of the effects of pH on enzyme catalyzed reactions can reveal information about the chemical steps occurring in the enzyme's mechanism. Specifically, they can tell us about the role of ionizable residues in binding to the substrates and in acid-base catalysis. The  $k_{cat}$  showed a marked dependence on pH (Figure 5), with maximal activity being observed between pH 7 and 8. Fitting of the data (as described in "Materials and methods") to a simple model



**Figure 5.** The effects of pH on the turnover number,  $k_{cat}$ . The GALK2 concentration was 230 nM in these experiments. The line represents a fit to the equation  $k_{cat} = k_{cat,lim} (1 + [H^+]/K_{a1} + K_{a2}/[H^+])$ .

for pH effects on  $k_{cat}$ <sup>34</sup> was successful ( $R^2 = 0.7405$ ), yielding  $pK_a$  values of 6.5 and 10.3, although the standard errors in the estimation of the two  $K_a$  values were quite high (77% and 120% of the estimates).

## Discussion

Recombinant human *N*-acetylgalactosamine kinase is active and displays similar properties to native mammalian enzyme<sup>9,10</sup>. The observation of possible substrate inhibition by GalNAc combined with the uncompetitive nature of  $\alpha$ -methylgalactopyranoside inhibition argues strongly for an ordered ternary complex mechanism in which ATP is the first substrate to bind. Such a mechanism was also seen in some bacterial, pig, rat, human, and yeast galactokinases<sup>29,36,37,40-42</sup>, although not in *E. coli* galactokinase (where a random ternary complex mechanism was observed<sup>43</sup>) or plant galactokinases where galactose is the first substrate to bind in an ordered mechanism<sup>44,45</sup>. The greater potency of  $\alpha$ -methylgalactopyranoside as an inhibitor, compared to  $\beta$ -methylgalactopyranoside, suggests (but does not prove) that the enzyme, like galactokinase<sup>46</sup>, has a preference for the  $\alpha$ -anomer of its sugar substrate.

The interpretation of pH studies is complex, and care must be taken to distinguish specific effects resulting from the ionization of individual, catalytically important residues from generic effects on the protein (or the substrates) as a whole. The pH profile seen here is not the perfect bell-shaped curve that would be expected to arise from the ionization of two, catalytically important active site residues. Nevertheless, that a pH dependence was observed suggests that ionizable residues may be involved in catalysis, resulting in the observed alterations in  $k_{cat}$ .

There is considerable debate in the field of GHMP kinases about the role of active site acids and bases in catalysis. The crystal structures of galactokinase, *N*-acetylgalactosamine kinase, and mevalonate kinase<sup>7,8,47-51</sup> each show an aspartate residue in the active site appropriately positioned to abstract a proton from the substrate (see Figure 1b for a

depiction of the location of Asp-190, the residue proposed to act as an active site base in GALK2). In each case a positively charged residue (in the GALK2, Arg-43) is located close to the putative active site base and is believed to maintain the ionization state of the carboxylate side chain. In theory, this arrangement, which involves one acidic and one basic residue, should result in a bell-shaped pH dependence of  $k_{\text{cat}}$ . However, detailed pH and deuterium kinetic isotope studies on the yeast galactokinase, Gal1p, showed little effect of pH on  $k_{\text{cat}}$ <sup>42</sup>. Furthermore, the crystal structure of homoserine kinase reveals no obvious active site base, suggesting that GHMP kinases are able to catalyze their reactions by alternative mechanisms, perhaps involving transition-state stabilization<sup>52,53</sup>. It has been postulated that all GHMP kinases proceed not via acid-base catalysis but through an approximation mechanism<sup>8</sup>. In such a mechanism, the enzyme's role is to hold the substrates in a conformation which facilitates conversion to the transition state. The reaction would begin with partial breaking and lengthening of the  $\gamma$ -phosphorus-oxygen bond in ATP. Subsequent movement of the  $\gamma$ -phosphate group toward the hydroxyl group on the sugar would displace the proton from the hydroxyl. The proton may be picked up by Asp-190 in the active site<sup>8</sup>. An approximation mechanism would also show marked pH effects, similar to the ones observed here.

Given the relatively large errors, the estimated  $\text{p}K_{\text{a}}$  values determined for GALK2 should be treated with some caution (especially the higher value). However, assuming that they represent the unperturbed  $\text{p}K_{\text{a}}$  values of single amino acid side chains, reasonable assignments would be to a histidine residue for the lower value and either a lysine or tyrosine for the higher value<sup>34</sup>. Neither value is consistent with an aspartate residue, unless its  $\text{p}K_{\text{a}}$  has been raised by almost two orders of magnitude. Furthermore, such a raised  $\text{p}K_{\text{a}}$  would make the residue less able to abstract a proton from the sugar. Thus, the data presented here are more consistent with an approximation mechanism. However, further studies on this fascinating group of enzymes will be required to resolve these matters.

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