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Effect of lysine to alanine mutations on the phosphate activation and BPTES inhibition of glutaminase

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Abstract

The *GLS1* gene encodes a mitochondrial glutaminase that is highly expressed in brain, kidney, small intestine and many transformed cells. Recent studies have identified multiple lysine residues in glutaminase that are sites of N-acetylation. Interestingly, these sites are located within either a loop segment that regulates access of glutamine to the active site or the dimer:dimer interface that participates in the phosphate-dependent oligomerization and activation of the enzyme. These two segments also contain the binding sites for bis-2[5-phenylacetamido-1,2,4-thiadiazol-2-yl]ethylsulfide (BPTES), a highly specific and potent uncompetitive inhibitor of this glutaminase. BPTES is also the lead compound for development of novel cancer chemotherapeutic agents. To provide a preliminary assessment of the potential effects of N-acetylation, the corresponding lysine to alanine mutations were constructed in the hGAC₁ plasmid. The wild type and mutated proteins were purified by Ni²⁺-affinity chromatography and their phosphate activation and BPTES inhibition profiles were analyzed. Two of the alanine substitutions in the loop segment (K₃₁₁A and K₃₂₈A) and the one in the dimer:dimer interface (K₃₉₆A) form enzymes that require greater concentrations of phosphate to produce half-maximal activation and exhibit greater sensitivity to BPTES inhibition. By contrast, the K₃₂₀A mutation results in a glutaminase that exhibits near maximal activity in the absence of phosphate and is not inhibited by BPTES. Thus, lysine N-acetylation may contribute to the acute regulation of glutaminase activity in various tissues and alter the efficacy of BPTES-type inhibitors.

Keywords

Glutaminase; Lysine N-acetylation; Alanine substitution; Phosphate activation; BPTES inhibition; Ni²⁺-affinity chromatography

1. Introduction

A unique catalytic property of the mitochondrial glutaminase encoded by the *GLS1* gene is its potent activation by phosphate and other polyvalent anions (Curthoys et al., 1976). The K_M for glutamine decreases in the presence of increasing phosphate concentration and phosphate activation correlates with the association of inactive dimers to form active tetramers (Godfrey et al., 1977; Morehouse and Curthoys, 1981) and larger oligomers

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(Ferreira et al., 2013). BPTES, bis-2[5-phenylacetamido-1,2,4-thiadiazol-2-yl]ethylsulfide, functions as a very specific and potent uncompetitive inhibitor (K_i of 0.2 μ M) with respect to glutamine (Hartwick and Curthoys, 2011). BPTES blocks the allosteric activation caused by phosphate binding, promotes the formation of an inactive tetramer, and prevents the formation of larger phosphate-induced oligomers (Robinson et al., 2007). Therefore, BPTES is a potent inhibitor of this isoform of glutaminase that binds to an allosteric site and prevents a conformational change that is required for activity.

The *GLS1* gene contains 19 exons and uses alternative splicing and different polyadenylation sites to form multiple mRNAs (Porter et al., 2002). The kidney-type glutaminase (KGA) mRNA (Shapiro et al., 1991) is derived from exons 1–14 and 16–19 and is terminated at alternative polyadenylation signals to produce a more abundant 4.7-kb and a less abundant 3.4-kb mRNA that encode the same 74-kDa precursor (Fig. 1). The first 16 amino acids of the KGA sequence form an amphipathic α -helix that functions as a mitochondrial targeting sequence (Shapiro et al., 1991). Following translocation into mitochondria, the matrix processing protease generates the mature 66-kDa subunit by removal of the N-terminal 72-amino acids (Srinivasan et al., 1995). A variant of the KGA cDNA, termed GAC, was initially cloned from a human carcinoma cDNA library (Elgadi et al., 1999). The GAC mRNA is derived from exons 1–15 and contains a unique C-terminal coding sequence and 3'-UTR. The shorter GAC precursor protein is also translocated into the mitochondria and similarly processed to produce a 58-kDa subunit. The central core region of either *GLS1* gene product forms a highly conserved structure that is characteristic of all crystallized forms of glutaminase (Brown et al., 2008; Cassago et al., 2012; DeLaBarre et al., 2011; Thangavelu et al., 2012).

The structure of human glutaminase (hGAC_{71–598}) bound to BPTES was recently determined (DeLaBarre et al., 2011). The hGAC forms a highly symmetrical tetramer containing two molecules of BPTES that are positioned at the dimer:dimer interfaces (PDB: 3UO9). The N-terminal region (residues 71–135) contains an amino sequence of low complexity. This region and the unique C-terminal segment (residues 547–598) are not evident in the X-ray crystallographic structure, suggesting that they are highly flexible or disordered. Residues 137–224 form small helical domains of unknown function that are positioned on the sides of the tetramer opposite from the dimer:dimer interfaces. The catalytic core of the hGAC (residues 224–546) forms a compact globular structure that is composed of two domains. One domain is entirely α -helical and the other contains both α -helices and β -sheets. The two domains form a pocket, which contains the active site serine residue. A co-crystallized glutamate molecule was tightly bound within this grove and appropriately positioned adjacent to the active site serine. Each BPTES interacts in a highly symmetrical fashion with residues in a loop sequence (residues 309–334) and α -helix-13 (residues 386–399), which forms the interface between two dimers. The loop sequence is normally unstructured, but it adopts a specific conformation upon binding of BPTES. The reported structure provides a detailed molecular model of how BPTES promotes the formation of an inactive tetramer. Additional structures suggest that the loop segment plays an important role in mediating the conformational changes that are essential for glutaminase activity (Cassago et al., 2012; Thangavelu et al., 2012).

Recent studies indicate that many of the enzymes of carbohydrate metabolism, the TCA cycle, β -oxidation, and amino acid oxidation are regulated by acetylation of specific lysine residues (Choudhary et al., 2009; Zhao et al., 2010). Previous mass spectrometric screens identified K₃₁₁ (Choudhary et al., 2009), K₃₂₀ (Weinert et al., 2013), K₃₂₈ (Wisniewski et al., 2010), and K₃₉₆ (Still et al., 2013) as sites of lysine acetylation in hGAC. All of these sites occur within the loop sequence or the α -helix-13 that bind BPTES. Mutations of residues within these segments have pronounced effects on oligomerization and activation of human glutaminase. For example, a K₃₂₀A mutant of GAC forms extended oligomers in the absence of phosphate and exhibits a 400-fold increase in catalytic efficiency (Ferreira et al., 2013). In addition, K₃₉₆ forms a salt bridge with D₃₈₆ in the complementary α -helix that contributes to the dimer:dimer association (DeLaBarre et al., 2011). Thus, acetylation of this lysine residue would prevent this interaction and reduce formation of active tetramers. Thus, N-acetylation of specific residues in glutaminase is very likely to regulate its activity. To provide a preliminary assessment of the potential effects of N-acetylation, the corresponding lysine to alanine mutations were expressed and their phosphate activation and BPTES inhibition profiles were analyzed.

2. Materials and methods

2.1. Cloning

The hGAC₁ sequence, which lacks the sequence encoded by exon 1 of the GLS1 gene (Kenny et al., 2003), was cloned into the pET-15b plasmid. The QuikChange Lightning Site-Directed Mutagenesis Kit was used to introduce specific lysine to alanine mutations. The mutated PCR products were transformed into XL10 Gold competent cells and plated on LB media containing 100 μ g/ml ampicillin. Plasmid DNA was purified using Wizard Plus SV Minipreps DNA Purification System and sequenced to establish that the DNA contained the correct mutation. This DNA was then transformed into BL-21(DE3)-pRARE cells and plated on LB/agar plates containing 100 μ g/ml ampicillin and 50 μ g/ml chloramphenicol.

2.2. Protein expression

Single colonies from the LB/agar plates were grown overnight in 5 ml 2xYT medium containing 100 μ g/ml ampicillin and 50 μ g/ml chloramphenicol. A 1 ml aliquot of the overnight culture was inoculated into 500 ml of medium containing the same antibiotics. The cell cultures were grown on a 37 °C shaker for 4–5 hours to reach an O.D._{600 nm} of 0.8–1.0. The cultures were then cooled on ice before adding 2.5 ml of 100 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The cultures were then incubated overnight on an 18 °C shaker to induce expression of hGAC₁. The cells were then pelleted by centrifugation at 5000 $\times$ g for 15 min at 4 °C.

2.3. Protein purification

The cells were resuspended in 20 ml of lysis buffer containing 300 mM potassium chloride, 10 mM potassium phosphate, 10 mM Tris chloride, 10 mM imidazole, 10% glycerol and Protease Inhibitor Cocktail (Roche Diagnostics), pH 8.0. The K₃₂₀A protein was solubilized by sonicating the resuspended cells for seven 1-min cycles at 50% duty with an output setting of 7. The remaining cell samples (wild type hGAC₁, K₃₁₁A, K₃₂₈A, K₃₉₆A) were

disrupted by passing through a micro fluidizer 5 times with a pressure of 100 psi. Cell debris was removed by ultracentrifugation at $128,000 \times g$ for 30 min at 4 °C. The supernatant was then filtered through 0.22 μ m syringe filter. A 5-ml HiTrap Chelating HP nickel column was used to purify the His₆-tagged hGAC₁. The column was charged with 0.1 M nickel sulfate and equilibrated with lysis buffer. The protein sample was loaded on the column at a flow rate of 3 ml/min and the flow through was collected. The column was then washed with 24 ml lysis buffer and the wash was collected as a separate sample. The column was subsequently washed with 24 ml lysis buffer containing 70 mM imidazole and then eluted with 24 ml of lysis buffer containing 400 mM imidazole. The column eluent was collected as sixteen 3-ml fractions and assayed for glutaminase activity. The purified protein was dialyzed versus 25 mM Tris chloride, 300 mM potassium chloride, 10% glycerol, and 2 mM dithiothreitol.

2.4. Glutaminase assay

Glutaminase activity was determined using an endpoint assay that involves the hydrolysis of glutamine to glutamate and the subsequent quantification of glutamate using purified bovine liver glutamate dehydrogenase to form α -ketoglutarate and NADH (Curthoys and Weiss, 1974). Phosphate activation profiles were determined by varying the potassium phosphate concentration from 0–150 mM. BPTES inhibition profiles were determined by varying the BPTES concentration from 0 to 0.1 mM BPTES with 10, 30, or 150 mM potassium phosphate. Assays were performed in quadruplicate and the normalized data were plotted using KaleidaGraph software to perform a non-linear least squares fit of the mean of the data points to the Hill equation.

3. Results

Phosphate activation profiles – For the current study, various lysine to alanine mutations of the hGAC₁ construct were expressed from a pET-15b plasmid and purified by Ni²⁺-affinity chromatography. The resulting proteins contain amino acids 124–598 of the human GAC protein. The purified wild type hGAC₁ exhibits a sigmoidal phosphate activation profile with a K_{0.5} of 40 mM (Fig. 2). The activation profiles for the K₃₁₁A, K₃₂₈A, and K₃₉₆A mutants are shifted to the right and become even more sigmoidal with Hill coefficients greater than 2. Therefore, the 3 mutated proteins require significantly greater concentrations of phosphate to produce half maximal activation. By contrast, the fourth mutation (K₃₂₀A) exhibits near maximal activity in the absence of phosphate and is only slightly activated by increasing concentrations of phosphate. Previous studies suggest that the mitochondrial concentration of phosphate is approximately 16 mM (Akerboom et al., 1978). When compared at this concentration (boxed area in Fig. 2), the mutations produce either a pronounced activation or significant inhibition of glutaminase activity.

BPTES inhibition – BPTES inhibition of wild type hGAC₁ is dependent upon the phosphate concentration (Fig. 3A). When assayed with 10–30 mM phosphate, BPTES is a very effective inhibitor with a K_i less than 1 μ M. However, the BPTES inhibition is overcome when the hGAC₁ is assayed with 150 mM phosphate. By contrast, the K₃₁₁A, K₃₂₈A, and K₃₉₆A proteins are significantly inhibited by BPTES even when assayed with

150 mM phosphate (Fig. 3B). On the other hand, the activity of the K₃₂₀A mutant is not inhibited by BPTES regardless of the phosphate concentration.

4. Discussion

Recent X-ray crystallographic structures (Cassago et al., 2012; DeLaBarre et al., 2011; Thangavelu et al., 2012) strongly implicate the importance of the loop segment (residues 309–334) and α -helix-13 (residues 386–399) in the catalytic mechanism and the phosphate-dependent oligomerization and activation of hGAC. The loop segment apparently regulates access of glutamine to the active site (Thangavelu et al., 2012). This segment contains 3 of the amino acids (L321, F322 and L323) that form binding interactions with BPTES. Individual alanine mutations of each of these amino acids significantly reduce catalytic activity and BPTES binding (Thangavelu et al., 2012). This segment also contains 3 lysine residues (K311, K320 and K328), which are known sites of N-acetylation (Choudhary et al., 2009). Therefore, acetylation of the lysine residues in the loop segment may affect oligomerization and activation of GA. Antiparallel alignment of the α -helices-13 from 2 subunits allows for the formation of Y393/F389 π -stacking and D386/K396 ionic interactions that constitute the primary binding interactions at the dimer:dimer interface of hGAC (DeLaBarre et al., 2011; Thangavelu et al., 2012). This segment contains Y394 that also interacts with BPTES. Mutation of the latter tyrosine also reduces hGAC activity and prevents BPTES binding (DeLaBarre et al., 2011; Thangavelu et al., 2012). Finally, the lysine residue in the liver glutaminase, which is encoded by the *GLS2* gene, that corresponds to K396 in hKGA is significantly deacetylated in mice within 2 h following feeding after an overnight fast (Still et al., 2013). Acetylation of K396 would prevent its interaction with D386 and would significantly decrease the overall binding energy that drives formation of active tetramers. Therefore, deacetylation of this residue is likely to enhance glutaminase activity.

When expressed in *E. coli*, the full-length recombinant rat KGA forms inclusion bodies (Shapiro et al., 1991). The N-terminal segment, containing the mitochondrial targeting signal and a region of low complexity, is encoded by exon 1 (Fig. 1). A construct that lacks this sequence (KGA₁) is readily expressed in *E. coli* as a soluble and fully active protein (Kenny et al., 2003). This form of purified recombinant rKGA was used to perform the initial characterization of BPTES inhibition (Robinson et al., 2007). More recently, we cloned the hKGA₁, hGAC₁, and hGA_{124–551} segments into the pET-15b expression vector, which adds an N-terminal His₆-tag. The latter construct lacks the unique C-terminal sequences from either isoform (Fig. 1). All three proteins were purified by Ni²⁺-affinity chromatography and shown to retain full activity, indicating that neither C-terminal domain is essential for catalysis. Kinetic experiments established that all three purified proteins exhibit a phosphate activation profile and a K_M for glutamine (2.5 mM) that are identical to the native enzyme (Hartwick and Curthoys, 2011).

To test the potential role of lysine acetylation, we introduced K to A mutations in the hGAC₁ plasmid and compared the phosphate activation profiles of the purified wild type and four mutated proteins (Fig. 2). Three of the mutations (K₃₁₁A, K₃₂₈A, and K₃₉₆A) require significantly greater concentrations of phosphate to produce half maximal activation

and exhibit an increased Hill coefficient (greater cooperativity). The fourth mutation (K₃₂₀A) is highly active in the absence of phosphate and is only slightly activated by increasing concentrations of phosphate. When compared at physiological concentrations of mitochondrial phosphate (Akerboom et al., 1978), the mutations produce either a pronounced activation or significant inhibition of glutaminase activity. These data strongly suggest that lysine acetylation may constitute a potential mechanism to acutely regulate glutaminase activity. A previous study established that the K₃₂₀A mutation enhances oligomerization and greatly activates hGAC in the absence of phosphate (Ferreira et al., 2013). This form of hGAC₁ is also significantly more stable than the wild type enzyme. Thus, this mutation may lock the enzyme in a conformation that mimics the phosphate-activated form of glutaminase. All of the previously reported structures of hGAC are thought to model the inactive form of the enzyme (DeLaBarre et al., 2011; Thangavelu et al., 2012). Therefore, it would be interesting to crystallize and solve the structure of the K₃₂₀A mutant.

The inhibition of the wild type hGAC₁ by BPTES is reduced with increasing concentrations of phosphate. Thus, the observation that the K₃₁₁A, K₃₂₈A, and K₃₉₆A mutations produce increased sensitivity to BPTES is consistent with their decreased response to phosphate. By contrast, the K₃₂₀A mutant, which is highly active in the absence of phosphate, is resistant to BPTES inhibition. BPTES was the lead compound for the development of a more potent glutaminase inhibitor, CB-839, that is currently in clinical trials as a potential cancer chemotherapeutic agent (Gross et al., 2014). Thus, it will also be important to determine if acetylation of specific lysine residues affects the sensitivity of hGAC to this class of inhibitors.

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Fig. 1.

Structures of the human *GLS1* gene and the hKGA and hGAC isoforms of human glutaminase. The h*GLS1* gene encodes 19 exons that are alternatively spliced to encode the two isoforms that contain unique C-termini. The initial precursors of both hKGA and hGAC are cleaved at two sites (arrows) by the matrix processing protease following translocation into the mitochondria. The $\Delta 1$ constructs lack the sequence encoded by exon 1.

Fig. 2.

Phosphate activation profiles of the purified recombinant hGAC₁ and the K₃₁₁A, K₃₂₀A, K₃₂₈A, and K₃₉₆A mutations of the GAC isoform of human glutaminase. The enzymes were assayed at various phosphate concentrations and the activities were normalized to the maximal activity measured with 150 mM phosphate and 20 mM glutamine. The relative maximal activities of the enzymes were 1.0, 2.1, 1.2, 0.46 and 0.23, respectively. The boxed area reflects the mitochondrial concentration of phosphate measured in isolate hepatocytes. The data points are the mean $\pm$ S.E. of 4–8 determinations.

Fig. 3.

BPTES Inhibition of hGAC₁ and the K₃₁₁A, K₃₂₀A, K₃₂₈A, and K₃₉₆A mutations of human glutaminase. Panel A. Effect of increasing BPTES concentration on the relative activities of purified recombinant hGAC₁ measured with 10, 30 and 150 mM phosphate. Panel B. Effect of increasing BPTES concentration on the relative activities of various lysine to alanine mutations of hGAC₁ measured with 150 mM phosphate. The data points are the mean $\pm$ S.E. of 4–8 determinations.