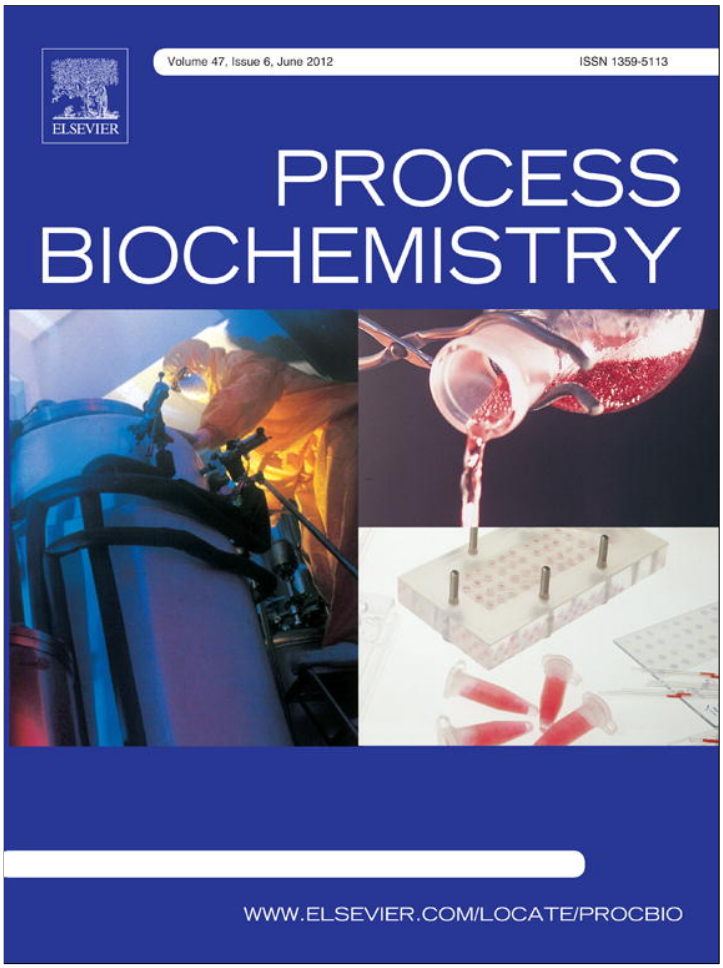

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N-Acetyl-D-glucosamine 2-epimerase from *Anabaena* sp. CH1 contains a novel ATP-binding site required for catalytic activity

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ABSTRACT

ATP is required as a structural activator for the reversible epimerization of *N*-acetyl-D-glucosamine to *N*-acetyl-D-mannosamine by *N*-acetyl-D-glucosamine 2-epimerase (AGE); however, the ATP-binding site on AGE has not been clearly identified. This study aimed to investigate the specific region of *Anabaena* sp. CH1 AGE (bAGE) that is required for ATP binding. In the absence of ATP, tryptic digest of bAGE resulted in the production of 2 segments of 17 and 26 kDa, while in the presence of 1 mM ATP, the enzyme was resistant to trypsin. ADP also displayed protective effects against trypsin digestion. A trypsin-mediated ATP-footprinting assay identified a deviant ATP-protected region, 156-GKYTK-160, which is located within the flexible loop of bAGE. Site-directed mutagenesis of residues in the loop region was performed, and both K151A and K160A variants greatly decreased the enzymatic activity as well as the ATP-binding ability of bAGE, indicating that residues K151 and K160 may be critical for ATP binding. This study demonstrated that the ATP-binding site (151-KDNPKGKYTK-160) of bAGE was a novel rather than a classical Walker motif A. This is the first ATP-binding site reported for AGEs.

1. Introduction

N-Acetyl-D-glucosamine 2-epimerase (AGE, EC 5.1.3.8) catalyzes the reversible conversion of *N*-acetyl-D-glucosamine (GlcNAc) to *N*-acetyl-D-mannosamine (ManNAc) and participates in the balance of the ManNAc pool [1,2]. ManNAc is a precursor of *N*-acetyl-D-neuraminic acid (NeuAc, sialic acid) in mammals and is important for mechanisms of cellular regulation, such as cell–cell recognition, cellular adhesion, signal transduction, leukocyte diapedesis, and B-cell activation [3,4]. NeuAc is also a constituent capsular polysaccharide in several pathogenic bacteria. In this role, NeuAc protects bacteria against host defenses, which may lead to infections, including neonatal meningitis and urinary tract infections [5].

AGEs from different organisms have been identified as adenosine triphosphate (ATP)-dependent epimerases, which require ATP as an activator for their activity [6–8]. The nucleotide-binding domain of AGEs has been preliminarily identified by the study of thermolysin hydrolysis, and a partial overlap is considered to exist between the thermolysin hydrolysis site and the ATP-binding region on AGEs [9]. Takahashi et al. [10] performed a domain-shuffling experiment between human AGE (hAGE) and rat AGE (rAGE) and demonstrated that a middle domain (fragment 96–305) of rAGE participates in the specificity and binding ability of nucleotides, and that these nucleotides are essential in the formation of the catalytic domain of the enzyme. To investigate the specific ATP-binding residues, Takahashi et al. [8] performed point mutations and identified residue S171 of rAGE as a critical residue for ATP binding. Another study on the characterization of porcine AGE (pAGE) indicated that pAGE exhibits a dimeric structure, and that each monomer is composed of an α_6/α_6-barrel [11]. Our previous study of AGE from *Anabaena* sp. CH1 (bAGE) revealed a similar α_6/α_6-barrel structure, which was suggested to form a disordered loop between residues 151 and 165 [4]. However, the exact region on bAGE that is required for ATP binding and catalytic activities remains to be elucidated.

Previously, we demonstrated that the epimerization activity of bAGE could be stimulated by different nucleotides, including ATP,

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ADP, dATP, and GTP [6]. In addition, we found that 5′-adenylyl imidodiphosphate (AMPPNP), an ATP analog with a non-hydrolyzable γ-phosphate, also exhibited an activation efficiency similar to and a binding ability almost equal to that of wild-type ATP, indicating that ATP hydrolysis might not be essential for bAGE activity [4,6]. Although the Mg^{2+} ion participates in the enzymatic reaction of most nucleotide-involved enzymes, such as F_1F_0 ATPase [12,13], our previous study developed a whole-cell catalyst method that demonstrated the production of NeuAc from GlcNAc and pyruvate without the addition of Mg^{2+} to the bAGE reaction [6]. Further studies to clarify the ATP-binding site of bAGE may provide useful information for the development of a desired bAGE with high ATP affinity for use in a cost-effective enzymatic process for producing NeuAc.

To verify the ATP-protected region in the flexible loop of bAGE, bAGE without ATP protection was hydrolyzed by trypsin into 2 segments, and the amino acid sequences of these 2 segments were determined by Edman degradation and MALDI-TOF mass analysis. Site-directed mutagenesis was performed to identify the residues in the ATP-protected region that play critical roles in ATP binding.

2. Materials and methods

2.1. Plasmid construction and site-directed mutagenesis

The coding sequence of bAGE from *Anabaena* sp. CH1 was previously cloned into the *Nde*I/*Xho*I sites of the *Escherichia coli* expression plasmid, pET32a [6], which encodes a C-terminal His_6-tag that allows affinity-chromatography purification of the recombinant bAGE protein by using a Ni-NTA resin column (Qiagen, Valencia, CA). Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis procedure according to the manufacturer's instructions (Stratagene, La Jolla, CA) with plasmid pET-bAGE as the template. Each of the mutated amplification products was transformed into *E. coli* BL21 (DE3) cells. The nucleotide sequence of each mutant construct was confirmed by DNA sequencing.

2.2. Expression and purification of His-tagged wild-type and mutant enzymes

The expression and purification of the wild-type and mutant bAGEs were performed according to the manufacturer's protocol (Qiagen). Briefly, a single colony of the transformed *E. coli* was cultured in Luria–Bertani (LB) medium supplemented with ampicillin (100 μg/mL) at 37 °C. Expression of the *age* gene was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside at 28 °C for 4 h. The cells were collected by centrifugation at 10,000 × *g* for 10 min, resuspended in sonication buffer (20 mM Tris–HCl [pH 8.0] and 100 mM NaCl), and then disrupted by sonication (Model XL-2020; Heat Systems, Plainview, NY). The cell extract was loaded onto a pre-charged nickel affinity column (Qiagen) for the elution of the His_6-tagged fusion proteins by sonication buffer containing 200 mM imidazole. Protein concentration was quantified using the Bio-Rad protein assay kit (Beverly, MA) with bovine serum albumin (BSA) as the standard.

2.3. Trypsin-mediated nucleotide-footprinting analysis

The purified bAGE (3 μg in sonication buffer) was pre-incubated at room temperature for 10 min with the addition of 1 mM of ATP, ADP, coenzyme A, or NADH, and 50 mM of GlcNAc or ManNAc in a total volume of 20 μL. Samples were treated with 1 μL of trypsin (50 ng) and reacted at room temperature for 3 min. The trypsin-digested samples were mixed with protein sample buffer (0.25 M Tris–HCl [pH 6.8] 10% SDS, 50% glycerol, 0.5% β-mercaptoethanol, and 0.005% bromophenol blue) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

2.4. N-terminal amino acid sequencing and MALDI-TOF mass analysis

The protein segments on the polyacrylamide gel were electrophoretically transferred to an Immobilon-P Transfer Membrane (Millipore Co., Billerica, MA) with 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) transfer buffer (10 mM CAPS [pH 11.0] and 10% methanol). The membrane was stained with Coomassie brilliant blue R-250, and the protein bands were excised and subjected to N-terminal amino acid sequencing by using an ABI Procise 494 protein sequencer (Applied Biosystems, Foster City, CA).For MALDI-TOF mass analysis, the target protein segment was excised from the SDS-PAGE gel, and the Coomassie blue dye and SDS were washed out from the granule gel with 50 mM NH_4HCO_3 containing 50% acetonitrile. The gels were ground with a pestle and suspended in 50 mM NH_4HCO_3 containing 5% acetonitrile and 0.1% trifluoroacetic acid (TFA). The gel solution was subjected to vigorous mixing and shaking with sonication and then centrifuged for 10 min at 12,000 × *g*. The suspended protein segments were collected, dehydrated, resuspended in deionized water containing 0.1% TFA, and desalted using a C18 Zip Tip column (Merck, Darmstadt, Germany). Finally, the samples were applied onto a MALDI-TOF mass spectrometer (AXIMA-CFR plus; Shimadzu/KRATOS, Manchester, UK).

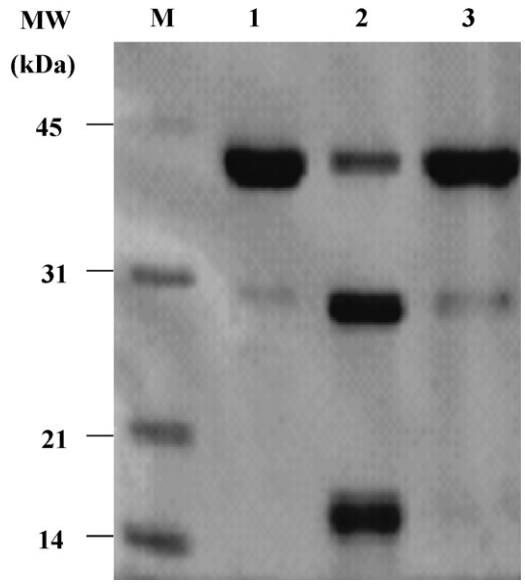

Fig. 1. Effect of ATP on the trypsin digestion of bAGE. bAGE proteins treated with trypsin were subjected to SDS-PAGE analysis. Lane M, protein size markers; lane 1, undigested bAGE; lane 2, tryptic digestion of bAGE; lane 3, tryptic digestion of bAGE plus ATP.

2.5. Enzyme assay

The bAGE activity was determined by monitoring the formation of ManNAc from the substrate (GlcNAc) by using high-performance liquid chromatography (HPLC; Waters, Milford, MA) with 2 connected aminex HPX-87H columns (Bio-Rad) at 65 °C. The absorbance at 210 nm was monitored. The mobile phase was a water solution containing 0.055% H_2SO_4, and the flow rate was 0.6 mL/min [14]. The retention times of GlcNAc and ManNAc were 22.16 and 23.21 min, respectively.

2.6. ATP-binding ability

The ATP-binding assay was performed according to the method of Lee et al. [6], which was originally described by Liou et al. [15]. Briefly, the reaction mixture (10 μL) containing 2 μCi of α-^{32}P ATP (3000 Ci/mmol) and 1 μg of bAGE in 0.1 M Tris–HCl buffer (pH 7.0) was mixed and incubated at 37 °C for 30 min. The mixture was spotted onto a piece of parafilm paper and incubated on ice for 15 min before irradiation for 8 min at 8 cm from the light source (0.78 J/cm^2) in a UV Stratalinker (Stratagene) equipped with 312-nm bulbs. The UV-cross-linked sample was mixed with 2 μL of protein sample buffer and boiled for 15 min, followed by electrophoresis on a 12% SDS-PAGE gel at 4 °C. The band intensity was visualized by both Coomassie brilliant blue R-250 staining and autoradiography on X-ray film.

3. Results and discussion

3.1. ATP footprinting of bAGE

The purified bAGE from *E. coli* BL21 (DE3) expressing the *age* gene of *Anabaena* sp. CH1 was subjected to trypsin digestion. As shown in Fig. 1, in the absence of ATP, the 43-kDa bAGE could be hydrolyzed by trypsin into 2 segments of 17 and 26 kDa (lane 2), while in the presence of 1 mM ATP (lane 3), the recombinant protein was resistant to trypsin, indicating that the binding of ATP could protect bAGE against tryptic digest.

A similar ATP-footprinting phenomenon has also been observed for some eukaryotic AGEs, including hAGE, rAGE, and pAGE [9]. Fig. 2 shows the structure-based alignment of the ATP-footprinting region in AGEs from prokaryotes (*Anabaena* sp. CH1 and *Synechocystis* sp. PCC 6803 bAGEs, sequences 1 and 2, respectively) and eukaryotes (rat, human, and porcine AGEs, sequences 3, 4, and 5, respectively). In the absence of ATP, these eukaryotic AGEs are susceptible to thermolysin digestion. For example, rAGE can be

Fig. 2. Structure-based alignment of the ATP-footprinting region of AGEs. 1, *Anabaena* sp. CH1 bAGE; 2, *Synechocystis* sp. PCC 6803 AGE; 3, rat AGE; 4, human AGE; 5, porcine AGE. The digestion targets of bAGE by trypsin and rAGE by thermolysin are indicated with an asterisk and a black circle, respectively. The secondary structural elements are shown above the sequence.

specifically digested at Leu170 by thermolysin (Fig. 2, black circle) to produce N-terminal (20 kDa) and C-terminal (29 kDa) segments [9]. Other eukaryotic AGEs have a similar flexible loop, such as the region W157–T176 of rAGE and W157–A176 of hAGE (Fig. 2). These regions also show protection against thermolysin digestion when the ATP is bound [9]. Because of the high structural homology between bAGE and other eukaryotic AGEs, the flexible loop R150–M168 of bAGE was predicted to be the location for ATP binding [4].

3.2. Effect of ATP-related compounds and substrates on bAGE hydrolysis by trypsin

Several nucleotides – such as ATP and ADP – can bind to and activate bAGE [6]. To determine the binding ability of ATP-related compounds to bAGE, we performed trypsin digestion of bAGE in the presence of ATP, ADP, coenzyme A (Co-A), NADH, GlcNAc, and ManNAc. As shown in Fig. 3, ADP protected bAGE from trypsin digestion in a manner similar to the level of protection provided by ATP, whereas Co-A and NADH were less protective. In the presence of GlcNAc and ManNAc, digestion of bAGE was similar to the trypsin-only control. A study on human, rat, and porcine AGEs also demonstrated that ATP, GTP, dGTP, or dTTP, but not the substrate ManNAc, could block the hydrolysis of AGE by thermolysin [9].

3.3. ATP-protected region of bAGE

To investigate the ATP-binding sites of bAGE, the N-terminal amino acid sequences of the protein segments produced by tryptic digestion of bAGE were identified by Edman degradation. The result showed that the 17-kDa segment started from the Gly2

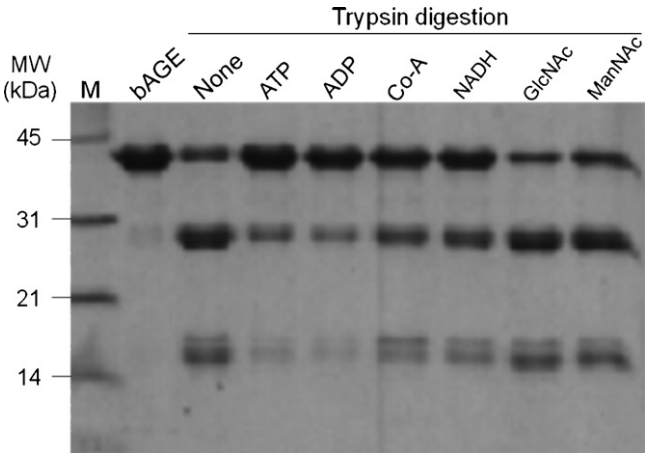

Fig. 3. Effect of ATP-related compounds and substrates on bAGE hydrolysis by trypsin. The bAGE proteins in the absence or presence of nucleotides or other related molecules were treated with trypsin and subsequently subjected to SDS-PAGE analysis.

residue of bAGE, while the 26-kDa segment started from Gly161 (Fig. 4A). The 17-kDa segment was collected and subjected to MALDI-TOF analysis to identify its C-terminal residue. As shown in Fig. 4B, the molecular masses of the 2 main peaks were 8930.68 Da and 17839.40 Da. The estimated molecular mass for the protein segment with C-terminal residue K155 was 17858 Da, whereas the masses for the segments ending with K151 and K157 were 17404 Da and 18043 Da, respectively. This result inferred that the first peak was a double charge, while the second peak was a single charge of the G2–K155 fragment; therefore, we hypothesized that the residue on the C-terminal of the 17-kDa segment was K155. The results shown in Figs. 1 and 4 suggest that trypsin can recognize lysine residues (e.g., K155, K157, and K160) in the disordered loop and then hydrolyze bAGE to yield 17-kDa and 26-kDa segments.

ATP-binding proteins are widely found in various organisms and play important roles in biological processes [16–18]. Structure-based studies on ATP-binding proteins indicate that 50% of the ATP/GTP-binding proteins contain the conserved sequence motif GXXXXGKT(S) (X represents any amino acid) [19–21]. This motif was first identified by Walker et al. in 1982 and has been called the Walker motif A (other names include Walker loop and P-loop) [19,21,22]. The common characteristic of this conserved sequence is glycine-richness, and it is located on a flexible loop structure between a β-strand and an α-helix. Because glycine residues lack a side chain, they provide ample binding space for the triphosphates. In addition, the lysine residues in this motif further improve ATP binding by interacting with negatively charged phosphates. Many enzymes contain the Walker motif A, including the α and β subunits of ATP synthase, several kinases, the ATP-binding proteins of active transporters (ABC transporters) [23,24], GTP-binding elongation factors (EF-Tu, EF1a, EF-G, EF-2, etc.) [25,26], the GTP-binding proteins of the Ras family (Ras, Rho, Ral, Ypt1, SEC4, etc.) [27–29], and DNA mismatch repair proteins of the mutS family [30].

As shown in Fig. 4, we identified a specific sequence, 156-GKYTK-160. While this sequence is not a classical Walker motif A, it may still act as an ATP-binding site for bAGE. In fact, many proteins use atypical Walker motifs A to bind ATP, such as the bacteriophage large terminase protein family (gp proteins) [31]. Only a few gp proteins bind ATP by using a classical Walker motif A. Most gp proteins use novel and deviant ATP-binding motifs, including KXXXXGA(Y/L/G/V/F) S/T and XXXXXA(Q/M/F/L/S)KS/T (where X is rich in glycine) [31]. In addition, a superfamily of ATPases with diverse functions contains either the classical or a deviant ATP-binding motif (KGGXXK[ST]) for ATP interaction [32]. The common property of the reported ATP-binding proteins is that they use a consensus lysine to interact with ATP, and this lysine is present in the motif that we identified in bAGE (K160).

3.4. K151 and K160 are required for ATP binding

We demonstrated that a flexible loop (residues 150–168) between the α-helix H5 (αH5) and S6 sheet of bAGE is an important region for ATP binding (Fig. 2). The positively charged residues

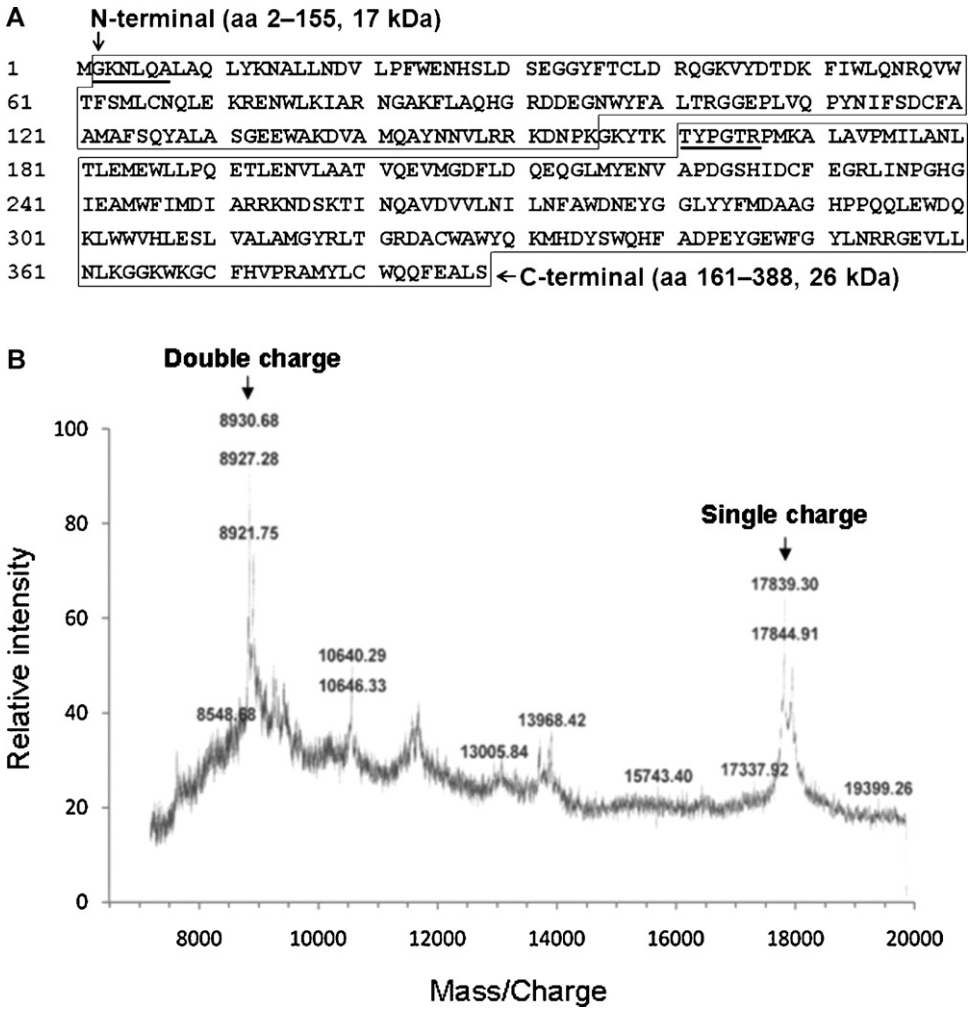

Fig. 4. Determination of the ATP-binding site of bAGE by N-terminal amino acid sequencing and MALDI-TOF mass analysis. (A) Amino acid sequence of bAGE. The peptide fragments produced by tryptic digestion of bAGE are boxed and their N-terminal amino acid sequences determined by Edman degradation are underlined. (B) MALDI-TOF mass analysis of the 17-kDa fragment produced by tryptic digestion of bAGE.

(such as arginine and lysine) and the glycine residues in proximity to the ATP-protected site may also participate in ATP binding. Therefore, the present study extended the region of ATP binding from residue 149 to residue 160, and the possible sites on bAGE for ATP binding were investigated by site-directed mutagenesis at R149, R150, K151, K155, G156, K157, and K160. All variants were expressed in soluble form in *E. coli*, were purified by affinity chromatography, and were 95% pure as judged by SDS-PAGE analysis (data not shown).

Fig. 5A shows the amount of each variant protein by SDS-PAGE analysis, and Fig. 5B illustrates the ATP-binding assay by using the autoradiographic image of α-^{32}P-ATP-labeled bAGE variants. The intensity of the autoradiographic image representing the level of ATP binding was calculated by normalizing the amount of proteins between the variants and the wild-type bAGE. The results show that with the exception of K155A, all variants have decreased ATP affinity. The variants K160A and K151A exhibited a significant decrease in ATP-binding ability (~8% and ~2% of the wild-type level, respectively). The variants R149A, R150A, G156S, and K157 showed 25–40% of the ATP-binding ability exhibited by the wild-type enzyme.

The enzymatic activity of bAGE with or without ATP was also determined. The wild-type and all the variants of bAGE exhibited very low enzymatic activity in the absence of ATP (Table 1). In the presence of ATP, the wild-type bAGE and the variant K155A exhibited high relative activity (100% and 99.8%, respectively), while K160A had very low activity (6.4%). Mutation of K151 decreased the activity to 32.0% compared with that of the wild-type bAGE. The other variants (R149A, R150A, G156S, and K157A) retained 76–94.4% of the specific activity in the presence of ATP

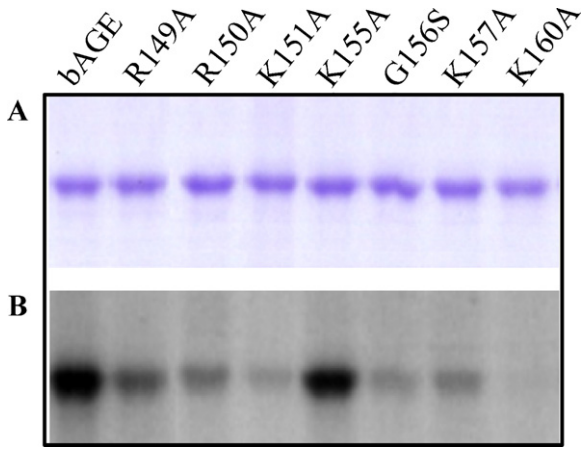

Fig. 5. Levels of ATP binding to bAGE variants. (A) SDS-PAGE analysis of variant proteins in each reaction. (B) Autoradiographic assay of α-^{32}P-labeled ATP binding to bAGE variants.

Table 1
Relative enzymatic activity (%) of bAGE variants with or without ATP.[a]

Variants	With ATP (1 mM)	Without ATP
Wild-type	100.0 ± 2.4	5.3 ± 0.8
R149A	94.4 ± 3.8	4.0 ± 0.5
R150A	76.3 ± 2.4	2.3 ± 0.8
K151A	32.0 ± 4.3	2.0 ± 0.7
K155A	99.8 ± 5.5	4.0 ± 1.1
G156S	85.7 ± 4.2	4.4 ± 0.9
K157A	89.3 ± 3.8	3.6 ± 0.6
K160A	6.4 ± 0.9	1.2 ± 0.4

[a] The specific activities of wild-type bAGE in the absence and presence of 1 mM ATP were 6.6 ± 1.0 U/mg protein and 123.8 ± 9.0 U/mg protein, respectively. The relative enzymatic activity of wild-type bAGE in the presence of 1 mM ATP was considered 100%. Data were expressed as the mean ± SE of at least three independent experiments.

compared with the wild-type enzyme. On comparing the ATP-binding ability (Fig. 5B) with enzymatic activity (Table 1) of these bAGE variants, we found that the ATP-binding affinity totally corresponded to the catalytic activity. All together, these observations suggest that in the flexible loop, from residue 150 to residue 168, the positively charged residues K160 and K151, but not K155, are critical for interaction with the negatively charged phosphate group of ATP; in addition, the residues R149, R150, G156, and K157 are also important for ATP binding. Although the residues R149, R150, and K151 are not located in the ATP-protected site (156-GKYTK-160) identified by the ATP-footprinting assay, they also influence the ATP-binding and bAGE activities, possibly because these residues are located at a site not accessible to trypsin.

4. Conclusion

This study showed that ATP binds to bAGE through a flexible loop region containing lysine, arginine, and glycine residues. The binding site was a novel rather than a classical Walker motif A, and the residues K160 and K151 play critical roles in ATP binding. Our previous investigation showed that an AGE with low ATP requirement for maximal catalytic activity was desired for the bioconversion of GlcNAc to ManNAc [6]. Further experiments will show whether the engineering of ATP-bound residues by site-directed mutagenesis can develop a bAGE with high ATP affinity for use in a cost-effective enzymatic process for the production of NeuAc from GlcNAc and pyruvate.

Acknowledgements

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