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Dissecting the functional roles of the conserved NXXE and HXE motifs of the ADP-dependent glucokinase from *Thermococcus litoralis*

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ABSTRACT

The activity of the ADP-dependent glucokinase from *Thermococcus litoralis* (*Tl*GK) relies on the highly conserved motifs NXXE (i.e. Asn-Xaa-Xaa-Glu) and HXE (i.e. His-Xaa-Glu). Site-directed mutagenesis of residues Glu279 (HXE) and Glu308 (NXXE) leads to enzymes with highly reduced catalytic rates. The replacement of Glu308 by Gln increased the K_M for $MgADP^-$ and was activated by free Mg^{2+}. On the other hand, HXE mutants did not affect the K_M for $MgADP^-$, were still inhibited by free Mg^{2+}, and caused a large increase on K_M for glucose and an 87-fold weaker binding of glucose onto the non-hydrolysable *Tl*GK·AMP–AlF_3 complex. Our findings put forward the fundamental role of the HXE motif in glucose binding during ternary complex formation.

1. Introduction

Glucose phosphorylation into glucose-6-phosphate (glucose-6-P) constitutes the first step of glycolysis, the central metabolic pathway in all three domains of life. While most glucokinases use adenosine triphosphate (ATP) as the phosphoryl donor, several archaea of the *Euryarchaeota* possess an ADP-dependent glucokinase as part of a variety of modifications on their Embden–Meyerhof pathway [1]. Despite the lack of sequence similarity between these glucokinases and their ATP-dependent counterparts, the three-dimensional structure resolution of several of these ADP-dependent enzymes have allowed their classification as members of the ribokinase superfamily [2]. Structurally, these archaeal glucokinases are composed by two domains: a large α/β/α domain whose topology corresponds to a Rossmann-like fold [2,3] that constitutes the core structure for all members of the ribokinase superfamily [4]; and a small domain composed of a five-stranded β-sheet, which is embellished with several α-helices that are commonly present in monomeric members of this superfamily [5]. These domains are connected by four chain crossings due to an intertwined polypeptide topology, which has been shown to promote cooperativity across these domains in other members of the ribokinase superfamily [6].

Among the structures of ADP-dependent glucokinases that have been solved to date, belonging to the archaeal species *Thermococcus litoralis* [2,7], *Pyrococcus horikoshii* [8] and *Pyrococcus furiosus* [9], the enzyme from *T. litoralis* (*Tl*GK) corresponds to the best characterized model in terms of its structure, function and kinetic mechanism. Thorough initial velocity analysis of the phosphotransferase reaction catalyzed by *Tl*GK using variable concentrations of substrates, products and inhibitors allowed determination of its kinetic mechanism, corresponding to a sequential ordered Bi–Bi model where the metal–nucleotide complex is the first substrate to bind to the enzyme, while glucose binds only when the *Tl*GK·$MgADP^-$ complex is already formed [7]. In line with this kinetic mechanism, small angle X-ray scattering and structure solution of *Tl*GK in the presence of substrates and analogs have shown that the large and small domains of *Tl*GK experience a sequential open-to-closed conformational transition, where $MgADP^-$ binding elicits a semi-closed state and the subsequent binding of glucose leads to formation of the ternary complex,

Abbreviations: ADP, adenosine-diphosphate; ATP, adenosine triphosphate; GK, glucokinase; *Tl*GK, glucokinase from *Thermococcus litoralis*; glucose-6-P, glucose-6-phosphate; K_M, Michaelis constant; k_{cat}, turnover rate; K_i, inhibition constant; K_a, activation constant; K_D, dissociation constant

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triggering total domain closure [7]. Moreover, this sequential binding of substrates in *Tl*GK has been recently confirmed by single-molecule force spectroscopy experiments [10].

Although *Tl*GK can exert its activity with similar catalytic efficiencies using Mg^{2+}, Mn^{2+} or Co^{2+} as part of the metal–nucleotide complex that constitutes its true phosphoryl donor substrate, it is also inhibited by increasing concentrations of free divalent cations in the low millimolar range [11]. The need of a second divalent cation for the regulation of the activity of these enzymes has been largely stressed by its direct structural observation and by assessment of its functional impact in other enzymes of the ribokinase superfamily, such as human adenosine kinase [5,12], human pyridoxal kinase [13,14], *Escherichia coli* phosphofructokinase-2 [15–17] and others [18,19].

Even when the catalytic consequences of increasing the concentration of free divalent cations can vary between different enzymes, in some cases leading to activation instead of inhibition [17,20], it has been widely demonstrated that the glutamate of a highly conserved motif for all members of the ribokinase superfamily, known as NXXE (i.e. Asn-Xaa-Xaa-Glu, Fig. 1), is responsible of the catalytic and regulatory effect elicited by divalent metals in these enzymes [15,20]. However, recent sequence analysis and molecular dynamics of ADP-dependent kinases including *Tl*GK have also shown the presence of a second conserved motif, known as HXE (i.e. His-Xaa-Glu, Fig. 1), whose glutamate is thought to participate in the second coordination sphere of the ADP-complexed magnesium cation and is located in proximity to the phosphoryl acceptor substrate [11]. The functional role of this residue still remains to be ascertained.

In this work we first employed site-directed mutagenesis and enzyme kinetics assays to evaluate the functional role of residues Glu279 and Glu308 from *Tl*GK, belonging to the HXE and NXXE motifs, respectively. Although mutations of the glutamate of both motifs had a large impact on the turnover rate of *Tl*GK, HXE mutants primarily affected the Michaelis constant (K_M) for glucose while NXXE mutants increased the K_M for $MgADP^-$. Moreover, increasing concentrations of free Mg^{2+} activated a mutant of Glu308 by Gln (E308Q) while, unexpectedly, replacements of Glu279 by Asp (E279D), Gln (E279Q) or Leu (E279L) did not change the inhibitory effect exerted by this divalent cation in the wild type enzyme. Then, we performed intrinsic fluorescence assays in the presence of a $MgADP^-$ analog, AMP–AlF_3, to assess whether glucose binding to the *Tl*GK·$MgADP^-$ was affected by the HXE mutations, showing a 87-fold increase in the dissociation constant (K_D) for glucose on the E279Q and E279L mutants, in comparison to the wild type enzyme and E308Q mutant. These results allow us to establish that the HXE motif is involved in the formation of the ternary complex required for the proper catalytic activity of *Tl*GK rather than in regulation of its activity by free divalent cations.

2. Material and methods

2.1. Expression and protein purification of TlGK

Wild type *Tl*GK and E279D, E279Q, E279L and E308Q mutants were overexpressed in *E. coli* strain BL21 (DE3) pLysS using the pET-17b expression vector. Cells were cultured at 37 °C in Luria Bertani broth medium containing 50 μg/ml of ampicillin and 35 μg/ml of chloramphenicol until $OD_{600} \sim 0.5$. Isopropyl-β-D-thiogalactopyranoside was then added to the medium to a final concentration of 1 mM to induce *Tl*GK overexpression, and cells were cultured overnight. Proteins were purified as described in Merino et al. [11], with some modifications. After incubating the crude extract at 90 °C for 30 min, the denatured proteins were removed by centrifugation (47 850*g* for 15 min). The supernatant was saturated with 60% $(NH_4)_2SO_4$ for 30 min and later centrifuged to remove protein precipitates. The soluble fraction was loaded onto a Phenyl Sepharose HP column (GE Healthcare, 5 ml) and then eluted with a linear gradient of $(NH_4)_2SO_4$ (from 60% to 0%). Active fractions were pooled, dialyzed against buffer A (Tris–HCl 50 mM, 5 mM $MgCl_2$, pH 7.8), subjected to anion exchange chromatography on a HiTrap Q HP column (GE Healthcare, 5 ml) and eluted using a lineal gradient of KCl (0–1 M). Active fractions were concentrated and used as the purified enzyme. The protein concentration was quantified using the Bradford assay [21].

2.2. Enzyme activity

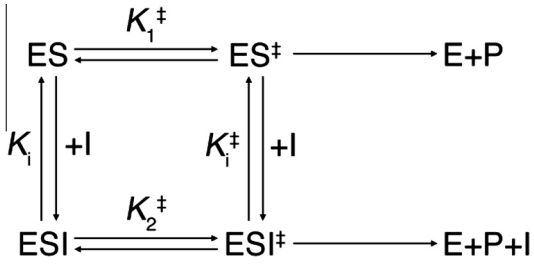
Glucokinase activity was measured spectrophotometrically at 40 °C as previously described [7,11], coupling the formation of glucose-6-P to the reduction of NAD^+. The reaction mixture contained 50 mM HEPES pH 7.8, glucose, adenosine-diphosphate (ADP), and divalent metal, 0.5 mM NAD^+ and 1.4 units of glucose-6-P dehydrogenase from *Leuconostoc mesenteroides*. The change in NADH concentration was followed spectrophotometrically at 340 nm using an extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$ [22].

Kinetic parameters were determined by measuring the enzyme activities as a function of the concentration of glucose and $MgADP^-$ respectively, using saturating concentrations of the corresponding co-substrate, which were experimentally determined for each enzyme. The concentration of free Mg^{2+} was held constant at 1 mM for all the kinetic characterizations. Specific activities were calculated from initial velocities of NAD^+ reduction and expressed in units per mg of protein. The kinetic parameters were obtained by fitting the experimental curves to the Michaelis–Menten model [23], excepting the E308Q and E279D mutants for which the Haldane model for substrate inhibition was used [24]. Experimental data were fitted using GraphPad Prism 5.0 (GraphPad Software, Inc.).

2.3. Inhibition by free divalent cations

We evaluated the effect of increasing the concentration of free Mg^{2+} on the activity of wild type *Tl*GK and single-point mutants of residues Glu279 and Glu308, using saturating $MgADP^-$ and glucose concentrations for each enzyme. Concentrations between 0.75 mM and 10 mM of free Mg^{2+} were assayed. The results are shown as relative activity, taking the highest activity observed as 100% for each enzyme. The concentration of free Mg^{2+}, ADP^{3-} and $MgADP^-$ were calculated from the total concentration of ADP^{3-} and Mg^{2+} as in Merino et al. [11], using the equilibrium $MgADP^- \leftrightarrow ADP^{3-} + Mg^{2+}$. The dissociation constant (K_D) of this process (676 μM) was obtained from the Critical Metal Complexes Database Version 5.0 (Texas A&M University).

The effect of free metal on the catalytic turnover was assessed using a formalism previously proposed in Merino et al. [11], which considers that both the ternary complex formed by *Tl*GK and its substrates in the absence (ES) and presence (ESI) of a second cation are productive, as shown below:



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