Biochimica et Biophysica Acta 1844 (2014) 759–766

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Engineering the allosteric properties of archaeal non-phosphorylating glyceraldehyde-3-phosphate dehydrogenases

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ARTICLE INFO

Article history:
Received 22 August 2013
Received in revised form 22 January 2014
Accepted 25 January 2014
Available online 31 January 2014

Keywords:
Allosteric enzyme
Archaea
K-type/V-type
Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN)
Positive cooperativity

ABSTRACT

The archaeal non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN, EC 1.2.1.9) is a highly allosteric enzyme activated by glucose 1-phosphate (Glc1P). Recent kinetic analyses of two GAPN homologs from Sulfolobales show different allosteric behaviors toward the substrate glyceraldehyde-3-phosphate (GAP) and the allosteric effector Glc1P. In GAPN from *Sulfolobus tokodaii* (*Sto*-GAPN), Glc1P-induced activation follows an increase in affinity for GAP rather than an increase in maximum velocity, whereas in GAPN from *Sulfolobus solfataricus* (*Sso*-GAPN), Glc1P-induced activation follows an increase in maximum velocity rather than in affinity for GAP. To explore the molecular basis of this difference between *Sto*-GAPN and *Sso*-GAPN, we generated 14 mutants and 2 chimeras. The analyses of chimeric GAPNs generated from regions of *Sto*-GAPN and *Sso*-GAPN indicated that a 57-residue module located in the subunit interface was clearly involved in their allosteric behavior. Among the point mutations in this modular region, the Y139R variant of *Sto*-GAPN no longer displayed a sigmoidal K-type-like allostery, but instead had apparent V-type allostery similar to that of *Sso*-GAPN, suggesting that the residue located in the center of the homotetramer critically contributes to the allosteric behavior.

1. Introduction

Allosteric regulation of enzyme activity is a fundamental strategy controlling the flux of cellular metabolism, including glycolysis, gluconeogenesis, and amino acid biosynthesis [1–5]. Classically, the concept of allosteric enzyme regulation is based on a ligand-dependent conformational change in which the conformation of an enzyme changes between a relaxed state (R-state) and a tense state (T-state) concertedly [6,7] or sequentially ("induced fit model") [8]. However, extensive studies have revealed that some allosteric enzymes diverge from this concept. These allosteric enzymes differ in the manner in which communications between the allosteric and catalytic sites are mediated [9–12]. The archaeal non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN), which is activated by glucose 1-phosphate (Glc1P), is an allosteric enzyme, the mechanism of allostery of which is not fully understood [13–16]. GAPN catalyzes the oxidation of glyceraldehyde-3-phosphate (GAP) by using $NADP^+$ as a co-substrate, producing 3-phosphoglycerate and NADPH. In contrast to the case with classical GAP dehydrogenase (GAPDH), the reaction catalyzed by GAPN is irreversible and does not require inorganic phosphate for oxidation of GAP. Archaeal GAPDH and GAPN play complementary roles as gluconeogenic and glycolytic enzymes, respectively, in central metabolism [14]. The crystal structures of GAPN from the thermophilic archaeon *Thermoproteus tenax* (*Tte*-GAPN) have been solved in the apo form and in complex with a number of ligands, including Glc1P. However, in these structures, no substantial conformational changes, such as transition between the T-state and the R-state, have been observed upon binding of the allosteric effector Glc1P. The mechanism through which effectors contribute to allosteric activation remains unclear [16].

Conventionally, there are two distinct types of catalytic behavior in allosteric enzymes: K-type and V-type. With a K-type allosteric enzyme, affinity for the substrate is altered through the binding of an allosteric effector to the enzyme. Often, positive cooperativity toward the substrate is exhibited in the absence of allosteric effectors. In this case, both homotropic and heterotropic controls are present in the enzyme. On the other hand, with a V-type allosteric enzyme, maximum velocity is altered by an allosteric effector, without a change in affinity (K_m value) for its substrate. In this case, only heterotropic control is present in the enzyme.

There have been numerous reports on the catalytic behavior (K-type or V-type) and molecular mechanisms of individual allosteric enzymes [17–22]. However, the molecular bases of the differences in these behaviors have not been sufficiently investigated. Recently, two

Abbreviations: GAP, glyceraldehyde-3-phosphate; GAPN, non-phosphorylating GAP dehydrogenase; Glc1P, glucose 1-phosphate

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http://dx.doi.org/10.1016/j.bbapap.2014.01.017

homologous GAPNs from the hyperthermophilic archaea Sulfolobales showed different allosteric behaviors despite their high amino acid sequence identity (66%) [13,15]. GAPN from *Sulfolobus tokodaii* (*Sto*-GAPN) shows positive cooperativity toward GAP, with a sigmoidal kinetic curve, and is activated by the addition of Glc1P with concomitant loss of cooperativity toward GAP, with apparent K-type allostery [13]. In contrast, GAPN from *Sulfolobus solfataricus* (*Sso*-GAPN) shows no positive cooperativity toward GAP, with hyperbolic kinetic features demonstrating apparent V-type allostery [15].

These two GAPNs from Sulfolobales are therefore good models for studying the molecular bases of different types of allosteric enzyme. We engineered the allosteric properties of *Sto*-GAPN by using chimeric and mutational analysis and identified the amino acid residues that determine allosteric behavior in archaeal GAPNs.

2. Materials and methods

2.1. Chemicals

D,L-Glyceraldehyde-3-phosphate (GAP) and α-D-glucose 1-phosphate were purchased from Sigma-Aldrich. $NADP^+$ was purchased from Oriental Yeast.

2.2. Strains and growth conditions

Escherichia coli strains XL10 GOLD, for cloning, and C43 (DE3), for expression, were cultured in Luria-Bertani (LB) medium.

2.3. Construction of expression plasmids

The *Sto*-GAPN expression plasmid was constructed previously [13]. The *Sso3194* gene from *S. solfataricus* was amplified by PCR and inserted between the *Nde*I and *Xho*I sites of the pET28a vector (Novagen) to construct an *Sso*-GAPN expression plasmid. R136K, K137E, L138T, Y139R, E141D, I158V, R160K, T170V, A198P, S230R, I444Q, M446F, K459R, and V502I mutant versions of *Sto*-GAPN were constructed by using a PrimeSTAR mutagenesis kit (Takara Bio) in accordance with the manufacturer's instructions. The TST and STS chimeric GAPNs were constructed using an In-Fusion HD cloning kit (Takara). All primers used in this study are summarized in supplementary Table S1.

2.4. Recombinant protein expression and purification

The recombinant *E. coli* C43 (DE3) strain was cultured at 37 °C in LB medium containing 0.1 mg/mL ampicillin until $OD_{600} = 0.6$, at which point 1 mM isopropyl-β-thiogalactoside was added. This was followed by further cultivation at 30 °C for 21 h. Recombinant *E. coli* cells (1 g wet weight) were suspended in 20 mM Tris–HCl (pH 7.5) containing 0.2 mM PMSF, disrupted by sonication, and centrifuged (10,000 *g* for 30 min at 4 °C). The resulting supernatant was incubated for 30 min at 80 °C and centrifuged (10,000 *g* for 30 min at 4 °C). The supernatant was applied to a HisTrap FF crude column (GE Healthcare) preequilibrated with 20 mM Tris–HCl (pH 7.5), and the column was washed with 40 mM imidazole and then eluted with 500 mM imidazole. The peak fraction was subjected to gel filtration on a Superdex 200 10/300 GL column (GE Healthcare) preequilibrated with 20 mM Tris–HCl (pH 7.5) containing 150 mM NaCl. The fractions containing the electrophoretically homogeneous enzyme were pooled and used for kinetic analysis. Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce) using bovine serum albumin as a standard.

2.5. Enzyme assays and determination of kinetic parameters

The standard enzyme assay was performed in 50 mM EPPS/NaOH (pH 8.0) with 1 mM $NADP^+$ and 10 μg of enzyme in a final volume of 400 μL at 60 °C. Reactions were initiated by the addition of 10 mM GAP after 100 s of preincubation. Enzymatic activity was measured by monitoring the increase in NADPH concentration at a wavelength of 340 nm. Kinetic parameters were calculated by iterative curve-fitting using the program IGOR Pro (HULINKS). The kinetic parameters of the GAPNs toward GAP were determined by using the following equation:

$$\frac{v}{V_{\max}} = \frac{1}{1 + \frac{K_S^{n_H}}{[S]^{n_H}} + \frac{[S]^x}{K_i^x}}. \quad (1)$$

Eq. (1) and its parameters are based on the modified Hill equation, with additional terms in the denominator for substrate inhibition, as proposed by LiCata and Allewell [23]. The exponent n_H is the Hill coefficient, and the other exponent x is a second Hill coefficient that allows for the possibility that binding of substrate in the inhibitory mode may also be cooperative. V_{max} is maximum velocity, and converted to k_{cat} using the molecular weight of the enzyme.

2.6. Modeling of Sto-GAPN structure

An initial monomer model of the structure of *Sto*-GAPN was constructed on the basis of homology modeling performed on the SWISS-MODEL web server, using the structure of *Tte*-GAPN (PDB ID: 1UXT) as a template. After creation of a tetramer model by symmetric operation, energy minimization and short-period molecular dynamics were applied, mainly to a region within 6 Å from the Tyr139 residues of the four subunits, including the glucose moiety of Glc1P molecules and solvents (Y139-6 Å region). The SANDER module of AMBER12 [24] with force-field parameters of ff99SB (protein) [25] and GLYCAM06h (sugar) were used. After addition of the hydrogen atoms, the tetramer molecule was immersed in 35,421 TIP3P water and 8 Na^+ molecules. The periodic boundary condition was applied with a non-bond cutoff radius of 8 Å. The Y139-6 Å region was subjected to 10,000-step energy minimization and then all atoms were subjected to another 1000-step energy minimization to relieve geometric strain and close contacts. Molecular dynamics simulations were conducted with the weak-coupling temperature-control algorithm [26] with a time step of 2 fs by fixing the remainder of the Y139-6 Å region. The lengths of the bonds involving hydrogen atoms were constrained by using the SHAKE algorithm [27]. Electrostatic interactions were calculated by using the particle mesh Ewald method [28]. After a molecular dynamics run with constant volume for 100 ps to increase the temperature to 300 K, a molecular dynamics run was made with constant temperature (300 K) and volume for 500 ps. The resultant final model was basically similar to the initial model. The root mean square deviation for all non-hydrogen atoms in the Y139-6 Å region was 1.24 Å. The root mean square deviation for Cα atoms was 0.499 Å in the Y139-6 Å region and 0.516 Å in the whole tetramer.

2.7. Circular dichroism measurement

Circular dichroism (CD) measurements were performed with a JASCO J820 spectropolarimeter using drum cells with 0.1- and 1-cm light paths at room temperature (25 °C). The protein concentrations were 0.11 mg *Sto*-GAPN/mL in 50 mM Tris–HCl (pH 7.5) for the far-UV region (190 to 260 nm) and 0.49 mg *Sto*-GAPN/mL or 0.25 mg *Sso*-GAPN/mL in 50 mM Tris–HCl (pH 7.5) for the near-UV region (260 to 410 nm). The averages of four scans are shown as molar ellipticity, using a residue number of 506 and molecular weight of 56,356 for *Sto*-GAPN and a residue number of 509 and molecular weight of 56,927 for *Sso*-GAPN. The α-helix and β-sheet contents were estimated as previously described by Yang et al. [29].