Archives of Biochemistry and Biophysics 607 (2016) 1–6

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

The effect of introducing small cavities on the allosteric inhibition of phosphofructokinase from *Bacillus stearothermophilus*☆

Amy M. Whitaker [1], Gregory D. Reinhart*

Department of Biochemistry and Biophysics, Texas A&M University and Texas AgriLife Research, 2128 TAMU, College Station, TX, 77843-2128, USA

ARTICLE INFO

Article history:
Received 2 March 2016
Received in revised form
7 June 2016
Accepted 25 June 2016
Available online 29 July 2016

Keywords:
Allosterism
Cavities

ABSTRACT

The allosteric coupling free energy between ligands fructose-6-phosphate (Fru-6-P) and phospho(*enol*) pyruvate (PEP) for phosphofructokinase-1 (PFK) from the moderate thermophile, *Bacillus stearothermophilus* (BsPFK), results from compensating enthalpy and entropy components. In BsPFK the positive coupling free energy that defines inhibition is opposite in sign from the negative enthalpy term and is therefore determined by the larger absolute value of the negative entropy term. Variants of BsPFK were made to determine the effect of adding small cavities to the structure on the allosteric function of the enzyme. The BsPFK Ile → Val (cavity containing) mutants have varied values for the coupling free energy between PEP and Fru-6-P, indicating that the modifications altered the effectiveness of PEP as an inhibitor. Notably, the mutation I153V had a substantial positive impact on the magnitude of inhibition by PEP. Van't Hoff analysis determined that this is the result of decreased entropy-enthalpy compensation with a larger change in the enthalpy term compared to the entropy term.

1. Introduction

The enzymes that catalyze the commitment steps of metabolic pathways are subject to intense regulation via allosteric mechanisms. However, the molecular basis of allosteric regulation is still not well understood in most cases. Due to the key role these enzymes play, the ability to manipulate allosteric control mechanisms holds promise for drug design [1–6]. For this reason, an enhanced understanding of the molecular mechanisms behind the regulation of allosteric enzymes is a prerequisite for rational drug design.

Prokaryotic phosphofructokinase-1 (PFK) is one such enzyme that has been extensively studied and characterized, resulting in an abundance of kinetic, structural, and thermodynamic information [7–19]. PFK from *Bacillus stearothermophilus* (BsPFK) in particular serves as model allosteric enzyme to explore the molecular mechanisms of allosteric regulation as well as the thermodynamic basis of the allosteric coupling.

From the X-ray crystallography structures of BsPFK, one can identify an extensive hydrogen-bonding network that stretches through the region between the allosteric site and the closest active site that are roughly 22 Å apart. The allosteric coupling between these sites has been previously shown to make the largest contribution to the overall heterotropic allosteric coupling free energy in BsPFK [18]. We recently probed the role of the residues in this region in the allosteric interaction by examining the influence of 3 chimeric substitutions on the allosteric coupling in PFK from the extreme thermophile *Thermus thermophilus* (TtPFK) [20]. Interestingly, restoring the entire network of hydrogen-bonded residues apparently linking the 2 sites enhanced the strength of the allosteric communication in TtPFK to levels comparable to, or even greater than, those of BsPFK. However, this overall enhancement was achieved as the sum of enhancements (in free energy terms) realized from each modification individually suggesting that the basis of the interaction is derived from properties that are more complex than a single, continuous chain of interacting residues might suggest. One possibility is that it is the ligand binding perturbations of the thermodynamic stability of the protein structure in this region that are important so that each amino acid substitution can influence the overall coupling essentially independently without the 'all or none' feature that a quasi-mechanical linkage would require.

To further probe the relationship between the structural

Abbreviations: PFK, phosphofructokinase; BsPFK, *Bacillus stearothermophilus* phosphofructokinase; EcPFK, *Escherichia coli* phosphofructokinase; Fru-6-P, fructose-6-phosphate; PEP, phospho(enol)pyruvate.

☆ This work was supported by National Institutes of Health Grant GM033216.

* Corresponding author.
E-mail address: gdr@tamu.edu (G.D. Reinhart).
[1] Current address: Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS, USA.

http://dx.doi.org/10.1016/j.abb.2016.06.022
0003-9861/

properties in this region and the strength of allosteric coupling, we investigated the sensitivity of the allosteric behavior of BsPFK to 3 isoleucine to valine substitutions at 3 different positions in this general region. The substitution effectively removes only a single methylene group, thus creating a small cavity at each of the positions. In this way we intended to modestly perturb the configurational entropy in the immediate vicinity of each substitution without otherwise altering the structure of the protein to a significant extent. We have previously observed that the coupling free energy between PEP and Fru-6-P in BsPFK, which quantitatively describes both the nature and the magnitude of the allosteric effect, is dominated by its constituent entropy component [21]. We have also discussed previously that ligand-induced perturbations in configurational degeneracy and/or protein dynamics may be a major cause of this type of allosteric behavior [22,23].

2. Materials and methods

2.1. Materials

All chemical reagents used in buffers for protein purification, enzymatic assays, and fluorescence assays were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ), or Research Products International (Mt. Prospect, IL). Deionized distilled water was used throughout. Lyophilized creatine kinase, the ammonium sulfate suspension of glycerol-3-phosphate dehydrogenase, and the potassium salt of phospho(*enol*)pyruvate were purchased from Roche (Indianapolis, IN). The ammonium sulfate suspensions of aldolase, the ammonium sulfate suspension of triosephosphate isomerase, the disodium salt of fructose-6-phosphate, and the disodium salt of phosphocreatine were purchased from Sigma-Aldrich (St. Louis, MO). The coupling enzymes were extensively dialyzed against 50 mM EPPS pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, and 0.1 mM EDTA before use. NADH and DTT were purchased from Research Products International (Mt. Prospect, IL). Mimetic Blue 1 A6XL resin used in protein purification was purchased from Promatic BioSciences (Rockville, MD). The Mono-Q HR anion exchange column used in protein purification was purchased prepacked for FPLC use from Pharmacia (currently GE Healthcare, Uppsala, Sweden). Amicon Ultra centrifugal filter units (spin concentrators) were from Millipore Corporation (Billerica, MA) and poly(ethylene glycol)-3000 was from Sigma-Aldrich (St. Louis, MO). Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis System from Stratagene (La Jolla, CA). Oligonucleotides were synthesized and purchased from Integrated DNA Technologies, Inc (Coralville, IA). DNA modifying enzymes and dNTPs were purchased from Stratagene (Cedar Creek, TX), New England Biolabs (Ipswich, MA), or Promega (Madison, WI).

2.2. Site-directed mutagenesis

The plasmid p-ALTER1/BsPFK contains the gene for BsPFK. Mutagenesis was performed on this plasmid following the protocol outlined in the QuikChange II Site-Directed Mutagenesis System from Stratagene. Two complementary oligonucleotides were designed to target the sequence surrounding the codon for each of the mutated amino acids; the template oligonucleotides are shown below:

I150V-BsPFK: 5′— ATA CGG TCA TTG ATG CCG TCG ACA AAA TCC GCG AC —3′.

I53V-BsPFK: 5′— GCC ATC GAC AAA GTG CGC GAC ACG G —3′.

I234V-BsPFK: 5′— GAC TTC GGC CGG CAA GTG CAG GAA G —3′.

2.3. Protein purification of wild type, I150V, I153V, and I234V-BsPFK

The plasmid p-ALTER1/BsPFK contains either the gene for BsPFK or the mutated genes. Expression from this plasmid occurs via the tac promoter. Wild-type BsPFK was expressed in *E. coli* RL257 cells [24], which is a strain lacking both the pfkA and pfkB genes. The purification of BsPFK was performed as described previously [25], with a few modifications. RL257 cells containing the plasmid p-ALTER1/BsPFK were grown at 37 °C for 16–18 h in Lysogeny Broth with tetracycline (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, and tetracycline 15 μg/mL). Induction of expression with 1 mM IPTG was carried out at the beginning of the growth. Cells were harvested by centrifugation and frozen at −20 °C for a minimum of 12 h. The cell pellet was resuspended in 60 mL of purification buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and sonicated using a Fisher 550 Sonic Dismemberator at 0 °C in 15-s pulses at setting six for 8–12 min. The crude lysate was centrifuged at 22,500 × g for 30 min at 4 °C. The clarified supernatant was incubated in a 70 °C water bath for 15 min, cooled on ice for 15 min, and centrifuged again at 22,500 × g for 30 min at 4 °C. The supernatant was diluted to at least 500 mL and then loaded onto a 100 mL Mimetic Blue 1 A6XL column that was previously equilibrated with purification buffer. The column was washed with purification buffer until the A_{280} reached a baseline, and the enzyme eluted with a 0–1.5 M NaCl gradient. Fractions containing enzyme activity were pooled and dialyzed into 20 mM Tris-HCl pH 8.5 and loaded to a Pharmacia/GE healthcare Mono-Q HR anion exchange column that was pre-equilibrated with the same buffer. The enzyme was eluted with a 0–1 M NaCl gradient, and fractions containing pure BsPFK were combined, concentrated with either a spin concentrator or poly(-ethylene glycol)-3000, and then dialyzed into EPPS buffer (50 mM EPPS pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, and 0.1 mM EDTA). The final enzyme was determined to be pure by SDS-PAGE, and stored at 4 °C. Protein concentration was determined by measuring absorbance at 280 nm ($\varepsilon = 18910\ M^{-1}cm^{-1}$) [16].

2.4. Protein purification of EcPFK

The purification of wild type EcPFK protein followed the protocol of Johnson et al. [11] with a few modifications, and is the same as above with the following exceptions. RL257 cells containing the p-ALTER1/EcPFK plasmid were grown to $OD_{600} = 0.6$ and then induced with 1 mM IPTG in Lysogeny Broth containing 100 μg/mL ampicillin at 37 °C. After induction the cells were grown until $OD_{600} = 1.2$ and harvested by centrifugation. Instead of a heat step, the supernatant after sonication and clarification was incubated with DNase at 37 °C for 15 min and then centrifuged for thirty minutes to remove the remaining cellular debris. The supernatant, containing EcPFK, was then further purified as above with a Mimetic Blue 1 A6XL column followed by an anion exchange step. Protein determined pure by SDS-PAGE was then concentrated and dialyzed into EPPS buffer (50 mM EPPS pH 8.0, 10 mM $MgCl_2$, 10 mM NH_4Cl, and 0.1 mM EDTA). Protein concentration was calculated by measuring absorbance at 278 nm ($\varepsilon = 0.6\ mg^{-1}cm^2$) [26].

2.5. Steady-state kinetic assays

Activity measurements for PFK were carried out using a coupled enzyme system [27,28] in a 0.6 mL reaction volume of EPPS buffer containing 50 mM EPPS pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 2 mM DTT, 0.2 mM NADH, 3 mM ATP, 250 μg aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, and 5 μg of triosephosphate isomerase at 25 °C unless otherwise noted. Creatine kinase (40 μg/mL) and phosphocreatine (4 mM) were added as