A Thesis

For the Degree of Master of Science

Thermodynamic Characterization of Domain motions coupled with Ligand Binding of Enzyme I in the Bacterial Phosphotransferase System

대장균의 당 인산화 단백질 **Enzyme I**의
리간드 결합과 도메인 운동에 대한
열역학적 특성 규명

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By

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Abstract

Thermodynamic Characterization of Domain motions coupled with Ligand Binding of Enzyme I in the Bacterial Phosphotransferase System

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Enzyme I (EI) of the bacterial phosphoenolpyruvate: sugar phosphotransferase system can be auto-phosphorylated using PEP and transfers the phosphoryl group to the phosphocarrier protein HPr. Domain motions are central to biological functions of many proteins. EI which is the first protein to initiate a series of phosphotransfer reactions consists of two domains connected by the linker helix: the N-terminal domain (EIN) which is composed of a catalytic $EIN^{\alpha\beta}$ subdomain, and an HPr binding EIN^{α} subdomain, and the C-terminal domain (EIC) which is a PEP binding domain. Here we employed different domain-deletion constructs to dissect and characterize the domain-domain motions coupled with ligand binding of unphosphorylated EI using isothermal titration calorimetry (ITC) .

We demonstrated that the free energy of the hinge motion ($\triangle G$ = 1.5kcal/mol) is

unfavorable energy, which can be overcome by the free energy of the swivel motion. The domain motions are entropy-driven, which is caused by the changes in the inter-domain interfaces upon ligand binding and domain motions.

In addition, PEP binding and HPr binding did not crosstalk with each other. The fact that two substrates can bind independently suggests that EI:PEP:HPr ternary complex can be easily formed during phosphotransfer reactions. Our results will help understand the thermodynamics of large domain-domain motions associated with protein-ligand interactions.

Key Words: Enzyme I, calorimetry, domain motions, ligand binding, thermodynamics

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LIST OF ABBREVIATION

CD: circular dichroism

DNA: deoxyribonucleic acid

EI: Enzyme I

IPTG: isopropyl beta-D-1-thiogalactopyranoside

ITC: isothermal titration calorimetry

K_D: dissociation constant

n: stoichiometry

NMR: nuclear magnetic resonance

OD: optical density

PCR: polymerase chain reaction

PEP: phosphoenolpyruvate

PMSF: phenylmethanesulfonyl fluoride

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

UV: ultraviolet

β-ME: beta mercaptoethanol

Introduction

The phosphoenolpyruvate: sugar phosphotransferase system (PTS) is a key signal transduction pathway with sequential phosphorylation cascade via a series of bimolecular protein-protein. The initial two steps of the PTS are same to all branches of the pathway, and involve the autophosphorylation of enzyme I (EI) by phosphoenolpyruvate (PEP) in the presence of Mg^{2+}, followed by phosphoryl transfer from His189 of EI to His15 of the histidine phosphocarrier protein HPr [Fig. 1]. EI is a ~128 kDa dimer that consists of two domains: an N-terminal phosphoryl transfer domain (EIN, residues 1-254) and a C-terminal dimerization domain (EIC, residues 261-575) that included the PEP binding site. Isolated EIN can reversibly transfer a phosphoryl group to HPr (~9.5 kDa) but cannot be autophosphorylated by PEP [Mariana N.Dimitrova et al. 2003].

Especially a protein domain is consist of protein sequence and structure that can evolve, function, and exist independently of the rest of the protein chain. Each domain forms a compact three-dimensional structure and often can be independently stable and folded. Many proteins consist of several structural domains and then the presence of multiple domains in proteins gives rise to a great deal of flexibility and mobility, leading to protein domain dynamics. Domain motions are important for: catalysis, regulatory activity, transport of metabolites and formation of protein assemblies [Bu Z, Biehl R, et al. 2005].

These domain motions represent in many proteins, and are significant to the biological functions. Protein dynamics and structure has applied to appear the physical nature and time scale of motions with functional implications.

On the other hand, thermodynamic information about enthalpy and entropy changes are related to domain motions has been limited. Attempts have been made to know how

modulation of domain motions are relevant to the thermodynamics of ligand binding. In many cases, multiple binding sites for a given ligand are non-equivalent and in this study which used calorimetry methods allows the characterization of the thermodynamic binding parameters for each individual binding site.

Less is known, however, about the thermodynamic nature of the domain motion itself apart from the ligand binding. So calorimetry has been method of choice to characterize the thermodynamics of molecular interaction, through free energy, enthalpy, and entropy of reaction. When a domain motions coupled with ligand binding, calorimetry measures are very effective. This concept shows that mutated proteins that delete domains can be used to understand the thermodynamics of the binding and the motions. We proved our presumption using Enzyme I (EI) as a model protein.

Here, the almost studies used the measurements of ITC. Isothermal titration calorimetry (ITC) is commonly used to determine the thermodynamic parameters associated with the binding of a ligand to a host macromolecule. ITC has some advantages over common spectroscopic approaches for studying ligand interactions. For example, the heat released or absorbed when the two components interact is directly measured. Thus the binding enthalpy and the association constant (Ka) are directly obtained from ITC data, and can be used to compute the entropic contribution [Lee A. Freiburger et al. 2011].

For this experiments I used domain-deletion protein sample. It is important to use confirmed mutated protein, needed containing secondary structure of proteins using CD spectroscopy. Circular dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light which arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum which can contain both positive and negative signals.

Circular dichroism spectroscopy is particularly good for: determining whether a protein

is folded, and if so characterizing its secondary structure, tertiary structure, and the structural family to which it belongs and comparing the structures of a protein obtained from different sources or comparing structures for different mutants of the same protein and demonstrating comparability of solution conformation and/or thermal stability after changes in manufacturing processes or formulation, studying the conformational stability of a protein under stress, thermal stability, pH stability, and stability to denaturants and how this stability is altered by buffer composition or addition of stabilizers and excipients. determining whether protein-protein or protein-ligand interactions alter the conformation of protein [2000-2013, Alliance Protein Laboratories Inc].

This experiment was used in same cuvette, buffer, temperature and protein concentration, for checking sample in comparison wild-type EI.

In this study, I focus on characterizing the calorimetry investigation of individual domain motion coupled with ligand binding. Also I wonder that whether HPr can impact on the domain motions with PEP or not and PEP can influence on the domain motions with HPr or not during the phophotransfer process,. It can expected thermodynamics of binding domains each others through experiments such as ITC, CD spectroscopy.

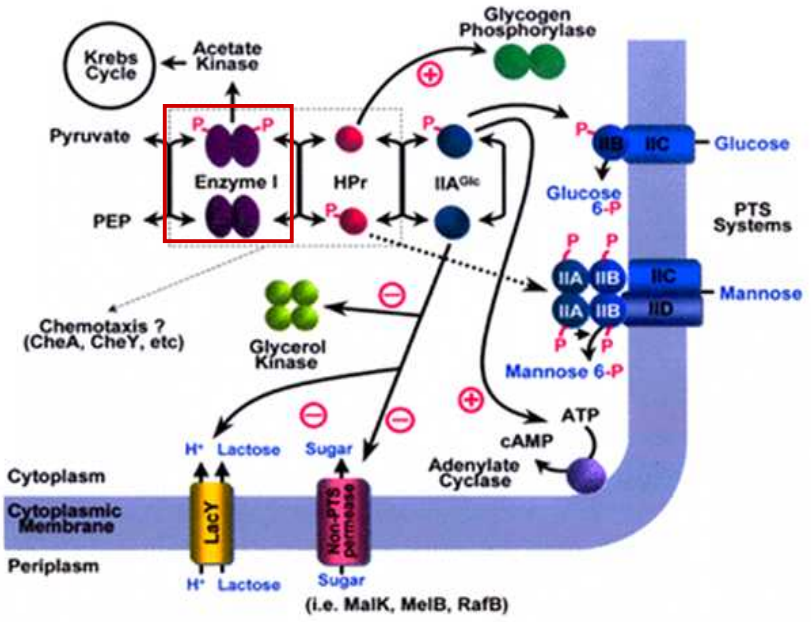


Fig. 1. Enzyme I (EI) of the bacterial phosphoenolpyruvate: sugar phosphotransferase system can be auto-phosphorylated using PEP (phosphoenolpyruvate) and transfers the phosphoryl group to the phosphocarrier protein HPr. This complex group translocation system present in many bacteria and transport sugars (such as glucose, mannose, mannitol) into the cell. Especially Enzyme I (EI) which is the first protein to initiate a series of phosphotransfer reactions consists of two domains

connected by linker helix. [Garrett, D.S et al. 1997]

CHAPTER 1.

Domain motions coupled with ligand in unphosphorylated Enzyme I

Introduction

EI is the first signaling protein in bacterial phosphotransferase system (PTS) for sugar translocation and phosphorylation. EI catalyzed two reversible reactions: magnesium dependent autophosphorylation reaction using phosphoenolpyruvate (PEP) and a phosphotransfer reaction to phosphocarrier protein HPr.

This protein has an N-terminal domain (EIN) comprising a catalytic domain($EIN^{\alpha\beta}$ subdomain) , and an HPr binding domain called (EIN^{α} subdomain), and the C-terminal domain (EIC) which is a PEP binding domain.

EI has two available structure states of A and B, first, the A state shows the structure of EI determined by NMR and X-ray data. The structure in the A state is relevant to the phosphotransfer reaction between EI and HPr. Second, the B state shows the crystal structure of phosphorylated EI quenched by the inhibitor oxalate. Especially first is the 65 degree hinge motion between EIN^{α} and $EIN^{\alpha\beta}$ which pulls back and disengages EIN^{α} from $EIN^{\alpha\beta}$. Second is the 70 degree swivel motion of EIC around linker helix, which brings EIC and $EIN^{\alpha\beta}$.

In this chapter, the experiments were carried out in the background of A state which related to unphosphorylated Enzyme I.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli strain Top10 was used for plasmid amplication and the plasmids were introduced into *Escherichia coli* strain BL21(DE3) (Novagen).

Enzyme I (residue 1-575), EI mutated protein and coding sequences were PCR amplified using DNA *pfu* tag polymerase (Stratagene) and modified pET-11a vector as the template. The PCR products cloned into the modified pET-11a vector with an His_6 tag. The sequences, domain composition, active site of EI,EI mutants and HPr(H15N): full length EI wild type (1-575, EIN^{α}+$EIN^{\alpha\beta}$+EIC, His189), full length EI(H189A) (1-575, EIN^{α}+$EIN^{\alpha\beta}$+EIC, Ala189), EI(△EIN^{α}) (1-21,146-575, $EIN^{\alpha\beta}$+EIC, His189), EI(△EIN^{α},H189A) (1-21,146-575, $EIN^{\alpha\beta}$+EIC, Ala189).

Protein expression and purification

The EI and EI mutants plasmids were introduced into *Escherichia coli* strain BL21star(DE3) (Novagen) and transformant was grown in either Luria Bertini or minimal media. The cell were cultured at 37℃ an A_{600} ~0.8 by 1mM isopropyl-beta-D-thiogalactopyranoside, and express for 4-6 hours.

The cells were harvested by centrifugation and for purification of EI(H189A) and EI(△EIN^{α},H189A), the cell pellet were resuspended with 50ml(per liter of culture)of 50mM Tris-HCl, pH 7.4, 200mM NaCl, 2mM beta-mercaptoethanol,

1mM phenylmethysulfonyl fluoride, and 1 tablet of protease cocktail inhibitor(Sigma-Aldrich, S8830 SIGMAFAST). The suspension was lysed by Emulsiflex after homogenizing and centrifuged at 24000g for 20min at 4℃.

The supernatant fraction was filtered and loaded onto a Ni^{2+}-NTA column (GE Healthcare) and the proteins were eluted with gradient from 0 to 500mM Imidazole. The proteins of fraction were purified by gel filtration on a superdex200 column (GE Healthcare) through 20mM Tris-HCl, pH 7.4, 200mM NaCl and 2mM beta-mercaptoethanol and through fractions were concentrated by Amicon ultra centrifugal filter (Millipore, UFC901096). After concentration, the proteins were further purified by monoQ ion exchange column (GE Healthcare) with a gradient of 1M NaCl. Through these process, using SDS-PAGE gel, the status of protein purity can be showed. If protein purity is not clear, the monoQ step was used one more time. [Fig. 2] [Fig.3]

Enzyme I (H189A)

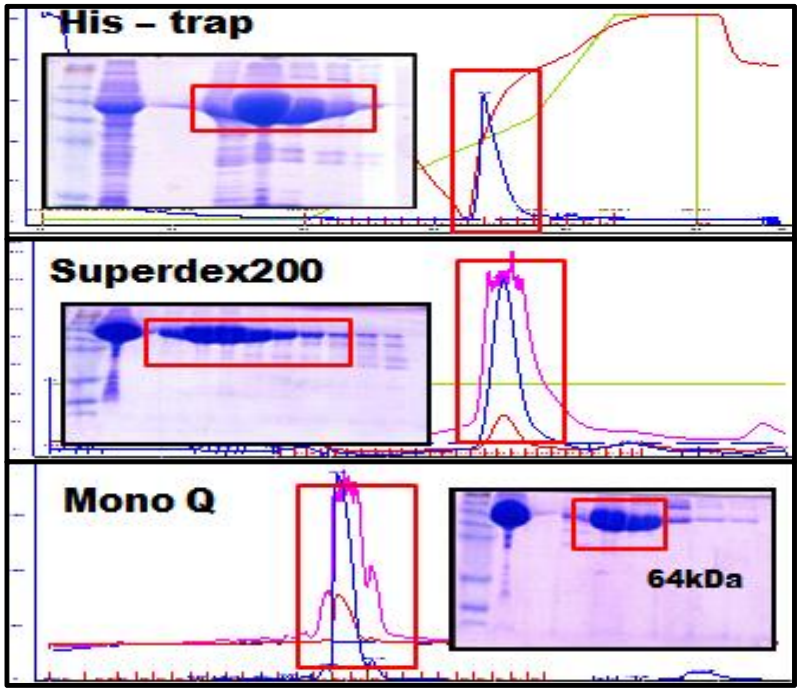


Fig. 2. The purification process of EI (H189A). The supernatant fraction was loaded onto a Ni^{2+} -NTA column (GE Healthcare) and the eluted with linear gradient of buffer composed of 500mM Imidazole. The fractions containing the protein were further by gel filtration through a superdex-200 column (GE Healthcare) equilibrated with 20mM Tris-HCl(pH 7.4), 200mM NaCl, 2mM beta-mercaptoethanol. After gel filtration, protein were purified by monoQ column (GE Healthcare) with gradient of buffer composed of 20mM Tris-HCl (pH 7.4), 1M NaCl, 2mM beta-mercaptoethanol.

Enzyme I (△EIN$^{\alpha}$,H189A)

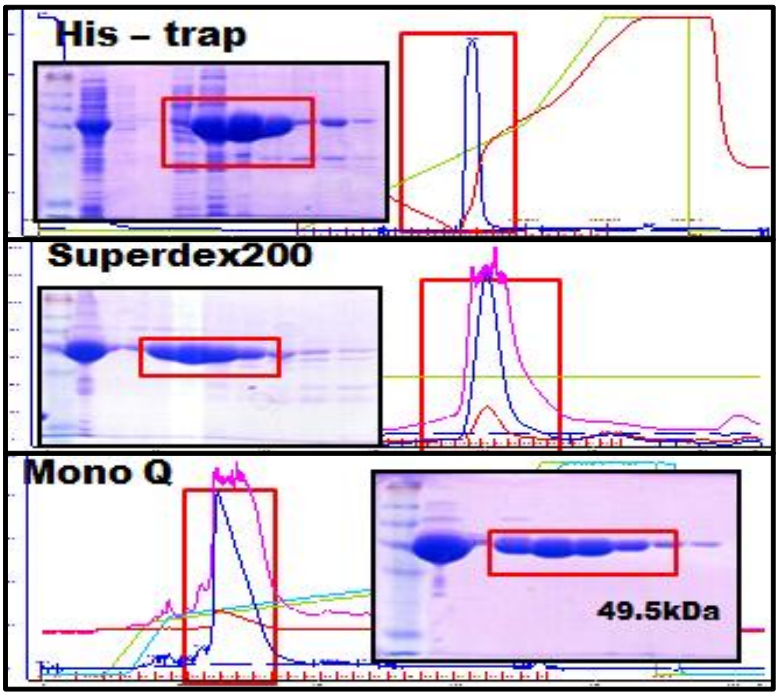


Fig. 3. The purification process of EI (△EIN$^{\alpha}$,H189A). The supernatant fraction was loaded onto a Ni^{2+} -NTA column (GE Healthcare) and the eluted with linear gradient of buffer composed of 500mM Imidazole. The fractions containing the protein were further by gel filtration through a superdex-200 column (GE Healthcare) equilibrated with 20mM Tris-HCl (pH 7.4), 200mM NaCl, 2mM beta-mercaptoethanol. After gel filteration, protein were purified by monoQ column (GE Healthcare) with gradient of buffer composed of 20mM Tris-HCl(pH 7.4), 1M NaCl, 2mM beta-mercaptoethanol.

Isothermal Titration Calorimetry

ITC is a physical technique used to determine the thermodynamic parameters of interactions in solution. It is a quantitative technique that can directly measure the binding affinity (K_a), enthalpy changes (ΔH), and binding stoichiometry (n) of the interaction between two or more molecules in solution. From these initial measurements Gibbs energy changes (ΔG), and entropy changes (ΔS), can be determined using the relationship.

So I used this method. Titration were carried out using the ITC_{200} microcalorimeter (GE Healthcare) at 25℃. The PEP titration, 300uM PEP was injected into the cell containing 30uM EI (H189A) or EI ($\triangle EIN^{\alpha}$,H189A) in 20mM Tris-HCl, pH 7.4, 100mM NaCl, 2mM beta-mercaptoethanol, 4mM $MgCl_2$.. All protein and ligand samples must originate from the same stock solutions, to minimize random sample-to-sample fluctuations in concentration. Before main titration between ligand and protein, implemented titration between ligand and buffer to remove dilution heat of buffer.

First, setting up the syringe with 300uM PEP of 40㎕. Gently slide the pipette injector into the injection syringe. Make sure the plunger tip is fed directly into the hole of the syringe. Once fully inserted, screw the locking collar of the syringe holder into the pipette injector.

Second, loading the sample with 30uM EI (H189A) or EI ($\triangle EIN^{\alpha}$,H189A). Wash sample cell with running buffer, and remove any remaining liquid by using a long-needled glass syringe. And slowly draw a minimum of 200㎕ of protein sample solution into the clean and dry long-needled syringe. Be careful not to introduce any bubbles. Carefully insert needle in sample cell and gently touch the bottom of the cell. Raise tip slightly and gently inject EI (H189A) or EI ($\triangle EIN^{\alpha}$,H189A) solution into

the cell until excess liquid is visible above the top of the sample cell. Slowly raise the needle about 1 cm while ensuring liquid remains in the overflow. Quickly withdraw and inject a small amount of solution to remove any trapped bubbles in the sample cell. Remove all solution overflow. This is achieved by gently sliding the needle along side of overflow into the sample cell. The syringe tip will hit a ledge, this is the desire height for the running solution. Remove all liquid which sits above this line [Lee A. Freiburger et al. 2011].

The duration of each injection was 4 sec, and injection were made at intervals of 150 sec. The heats is related to the dilution of the substrates were subtracted from the measured heats of binding. ITC titration data were analyzed through the Origin version 7.0 program by MicroCal.

Circular dichroism spectroscopy

Circular Dichroism (CD) is a spectroscopic technique used for the evaluation of the conformation and stability of proteins in several environmental conditions like temperature, ionic destructive, relatively easy to operate, requires small amount of sample. CD spectroscopy demonstrates the secondary structure of proteins because the peptide bond is asymmetric and molecules without a plan of symmetry show the phenomenon of circular dichroism. The amide chromoto phore of the peptide bond dominates the CD spectra of proteins between 190nm to 250 nm strength, and presence of solutes or small molecules [Daniel H. A. Corrêa et al.2009]. This study was related CD spectroscopy measurement was performed using Chirascan^{TM}-plus CD spectrometer.

UV CD spectra were measured in 20mM Tris-HCl, pH 7.4, at 25℃. Far CD spectra were scaned from 190 to 250nm using a protein concentration of 10uM EI(H189A) and EI ($\triangle EIN^{\alpha}$,H189A) with a path length 0.2mm cuvette. Each UV CD spectrum was obtained from an average of three scans and the results were showed the mean residue ellipticity (deg cm^2/dmol) at each wavelength. [Fig. 4]

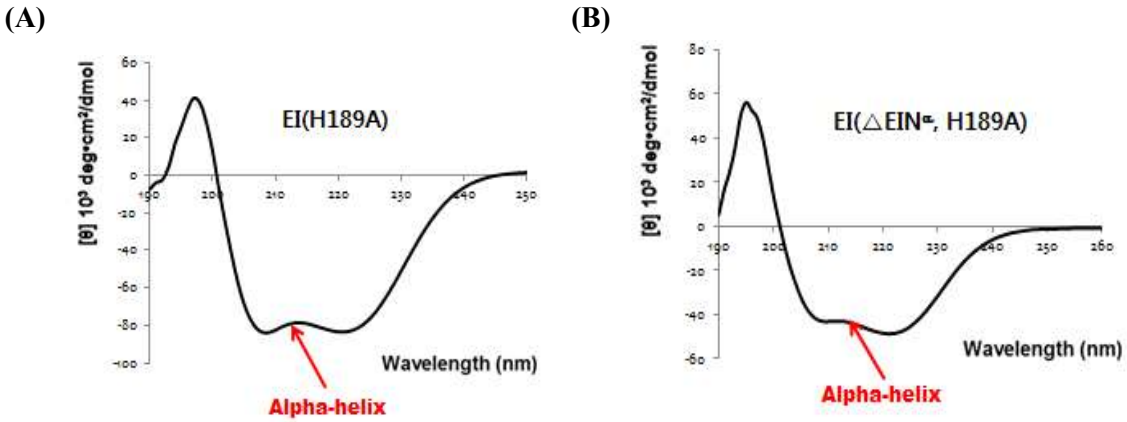


Fig. 4. (A) CD spectroscopy spectrum of EI (H189A) in 20mM Tris-HCl at pH 7.4. (B) CD spectroscopy spectrum of EI (△EIN$^{\alpha}$,H189A) in 20mM Tris-HCl at pH 7.4.

Result

Cloning of EI(H189A), EI(△EIN$^{\alpha}$,H189A)

The studies used EI and its active-site or domain-deletion mutants. DNA fragments of EI were PCR amplified with primers and contained the following. PCR assembly was performed in reactions of 50㎕ reaction volume which was composed of pfu, forward-reverse primer, template of EI(H189A), dNTPs, 10X pfu polymerase buffer and distilled water. This PCR product were used and the reaction vessels transferred to thermal cycler and subjected to the following cycling program: 95℃(2') - [95℃(30'')-55℃(30'')-73℃(14')]$_{18}$ cycles- 72℃(20') (BIORAD). And then using DpnI, stored in incubation for 1 hour at 37℃. The PCR product of collect size was purified by agarose gel extraction using the quick Gel Extraction kit (Invitrogen) and stored until use at −20℃.

EI protein sequences: full length EI(H189A) (1-575, EIN$^{\alpha}$+EIN$^{\alpha\beta}$+EIC), EI(△EIN$^{\alpha}$,H189A) (1-21,146-575, EIN$^{\alpha\beta}$+EIC). They were cloned into the modified pET-11a vector. After cloning, EI mutants cloned into pET-11a were overexpressed in E.coli strain BL21. The active site H189A construct was introduced to prevent the autophosphorylation reaction of EI during calorimetric titration using PEP, as ligand chemical reaction would cause a reaction heat. The domain-deletion mutants were designed by choosing one domain for removing particular domain motions of unphosphorylated EI. The deletion of EIN$^{\alpha}$ would remove the hinge motion of EI. [Fig. 5]

(A)

(B)

Name of construct	Domain composition	Residues	Active site
Wild type EI	EIN$^{\alpha}$+EIN$^{\alpha\beta}$+EIC	1-575	His189
EI (H189A)	EIN$^{\alpha}$+EIN$^{\alpha\beta}$+EIC	1-575	Ala189
EI(△EIN$^{\alpha}$)	EIN$^{\alpha\beta}$+EIC	1-21, 146-575	His189
EI(△EIN$^{\alpha}$, H189A)	EIN$^{\alpha\beta}$+EIC	1-21, 146-575	Ala189
EIN	EIN$^{\alpha}$+EIN$^{\alpha\beta}$	1-249	His189
EIC	EIC	231-575	-

Fig. 5. (A) The schematic diagram of domain structure of full-length Enzyme I (EI). EI is consists of two domains connected by linker helix: the N-terminal domain (EIN) which is composed of a catalytic domain called EIN$^{\alpha\beta}$ subdomain, and an HPr binding domain called EIN$^{\alpha}$ subdomain, and the C-terminal domain (EIC) which is a PEP binding domain. (B) The name of construct, domain composition, residues, and the active site residue in EIN$^{\alpha\beta}$of wild type and its mutants concluding the active site and domain-deletion mutations used in this study.

Dimerization of EI(H189A), EI(△EIN$^{\alpha}$,H189A)

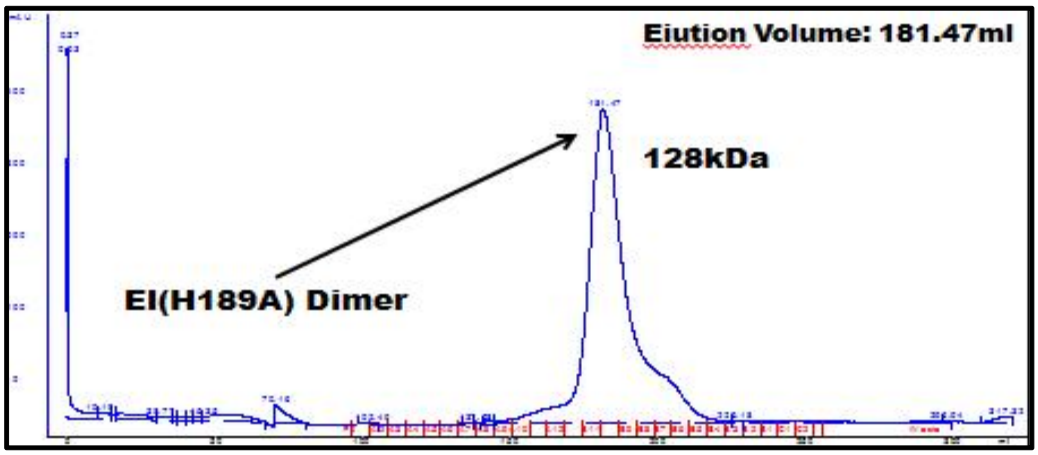
Previous studies have demonstrated that phosphorylation of H189 or substitution of Glu for His189 in the N-terminal domain of EI destabilizes this domain and increases by 10=fold the $K_{1,2}$' for dimerization [Nosworthy et al. 1998; Ginsburg et al. 2000; Dimitrova et al. 2002], which occurs through C-terminal domain interactions [Zhu et al. 1999; Brokx et al. 2000; Ginsburg and Peterkofsky 2002].

The present result shows a correlation between the coupling of N- and C-terminal domains during unfolding/refolding by effectors binding to the C-terminal domain and the dimerization constant for EI and EI(H189A). In the presence of 2 mM Mg2+, PEP binds with high-affinity, markedly stabilizes both C- and N-terminal domains of EI(H189A), and promotes essentially complete dimerization of the protein by preferential binding to the dimer. Thus, physiological concentrations of Mg2+, PEP regulate the dimerization of EI and thereby the activity of EI in catalyzing the first reaction of the phosphoenolpyruvate: sugar phosphotransferase system. Once phosphorylated, the dimerization constant of enzyme I is increased 10-fold [Dimitrova et al. 2002] and phospho-EI is poised for reversible phosphotransfer to HPr.

As EI is known to active as a dimer, I checked whether the EI mutants formed a dimer in the experimental state. I The experiment was carried out Superdex200 size exclusion chromatography for EI(H189A) and EI(△EIN$^{\alpha}$,H189A) to examine the dimerization state of the EI mutants. The chromatogram of serial dilution showed that both EI(H189A) and EI(△EIN$^{\alpha}$,H189A) were fully dimeric at 20uM. If one estimate that >90% of the protein was dimeric at 20uM. This experiment indicates that the domain-deletion mutation did not harm the dimerization of EI. Since the addition of PEP or HPr promotes the dimerization of EI, EI and its mutants maintained dimeric

state at 30uM and higher concentration used in the titration.[Fig. 6]

(A)



(B)

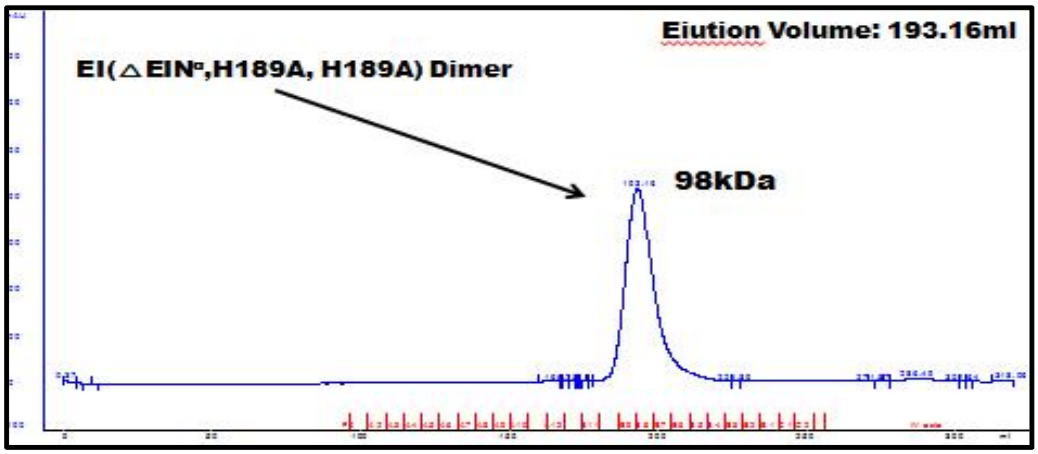


Fig. 6. (A) Analytical size exclusion chromatography. EI(H189A) loaded at a concentration of 20uM elutes as a dimer (Elution volume: 181.47ml). Size of EI(H189A) monomer is 64kDa. (B) Analytical size exclusion chromatography. EI(△EIN$^{\alpha}$,H189A) loaded at a concentration of 20uM elutes as a dimer (Elution volume: 193.16ml). Size of EI(△EIN$^{\alpha}$,H189A) monomer is 49.5kDa.

Domain-domain arrangements of the A state and B state

EI which is the first protein to initiate a series of phosphotransfer reactions consists of two domains connected by linker helix: the N-terminal domain (EIN) which comprises a catalytic domain called $EIN^{\alpha\beta}$ subdomain and an HPr binding domain called EIN^{α} subdomain, and the C-terminal domain (EIC) which is a PEP binding domain. $EIN^{\alpha\beta}$ domain is closely located to EIN^{α} domain (A state), but $EIN^{\alpha\beta}$ domain comes close to EIC domain with reorientation of EIN^{α} domain close to the $EIN^{\alpha\beta}$ domain through hinge motion during the auto-phosphorylation reaction (B state). [Fig. 7]. In the crystal structure of phosphorylated EI, there is large 65 degree reorientation of the alpha and alpha-beta subdomains of EIN domain relevant to that showed in the structure of isolated EIN, both free in solution and in the crystal state, as well as complexed with HPr [Jeong-Yong Suh et al. 2008].

The experiment was analyzed thermodynamic properties about the reorientation of EIN^{α} domain prevent to the $EIN^{\alpha\beta}$ domain during the phosphorylation reaction using the domain-deletion structure of EI(△HD, H189A) which is removed EIN^{α} domain. Isothermal titration calorimetry figures showed that the binding between EI(△HD,H189A) and PEP was stronger about 11.5 times and the free energy was decreased about 1.5kcal/mol in compared to EI(H189A).

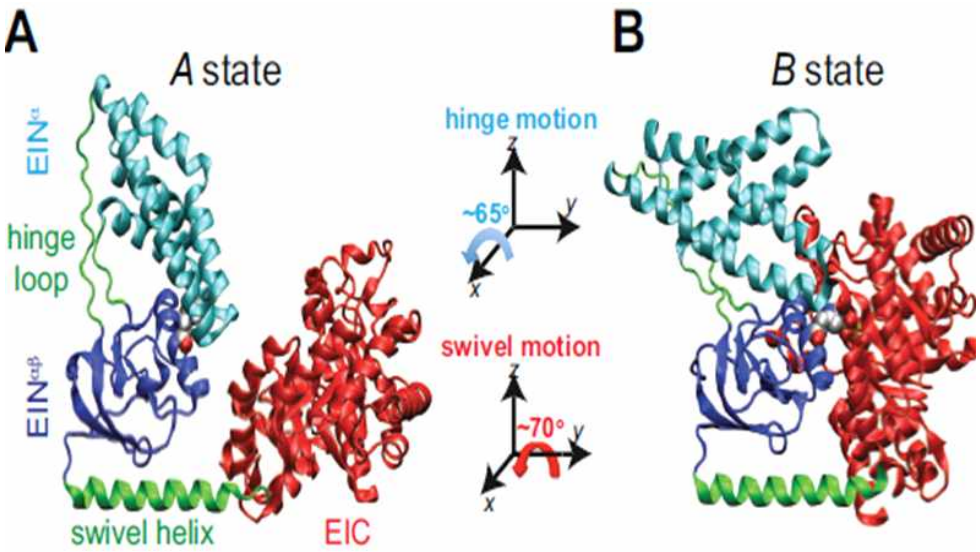


Fig. 7. Diagram representing the distict domain-domain arrangements of the A and B states of EI. (A) The crystal structure of unphosphorylated EI (B) The crystal structure of phosphorylated EI quenched by the inhibitor oxalate. The HPr binding EIN^{α} domain (cyan), the catalytic $EIN^{\alpha\beta}$ domain (blue), and the hinge loop and swivel helix (green).

Domain motions coupled with ligand binding in EI

The next attemption is to justify ligand binding and individual domain motions in EI using calorimetry measurement. The assumption that EI exists in the A state upon PEP binding switches the conformation into B state is showed by the following experiments. As the results were showed before section, there are the conformational change of EI(H189A) between the A state the B state upon PEP binding. [Fig. 8]

The PEP binding and conformational change between A and B states of EI can be separated into three reactions. a binding between PEP and EIC (A state → C state) a hinge motion between EIN^{α} and $EIN^{\alpha\beta}$ to make a space for EIC (C state → D state) a swivel motion between EIC and $EIN^{\alpha\beta}$, accompanied by the domain association between $EIN^{\alpha\beta}$ and EIC:PEP (D state → B state). Only the A and the B states have been experimentally observed, and C and D states are guessed as intermediate states. The C state showed the EI:PEP complex immediately PEP binding before any domain motions. The D state showed the EI:PEP complex hinge motion of EIN^{α} before the swivel motion of EIC:PEP. The △G of the whole reaction between EI(H189A) and PEP can be calculated ad the sum from four events respectively.

$$\triangle G_{EI(H189A):PEP} = \triangle G_{EIC:PEP} + \triangle \mathbf{G_{EI\_hinge}} + \triangle G_{EI\_swivel} (\triangle G_{EIN\alpha\beta:(EIC:PEP)}) \quad [1]$$

where $\triangle G_{EI(H189A):PEP}$ is the whole binding and domain motions of EI(H189A) with PEP, $\triangle G_{EIC:PEP}$ is the binding between EIC and PEP, $\triangle G_{EI\_hinge}$ is the hinge motion, $\triangle G_{EI\_swivel}$ is the swivel motion, $\triangle G_{EIN^{\alpha\beta}:(EIC:PEP)}$ is the domain association between $EIN^{\alpha\beta}$ and EIC:PEP

The △G for the PEP binding of EI(△EIN$^{\alpha}$,H189A) can be represented in a similar as Equation[1] except for the hinge motion:

$$\triangle G_{EI(\triangle EIN^{\alpha}H189A):PEP} = \triangle G_{EIC:PEP} + \triangle G_{EI\_swivel}(\triangle G_{EIN\alpha\beta:(EIC:PEP)}) \quad [2]$$

where $\triangle G_{EI(\triangle EIN^{\alpha}H189A):PEP}$ is the whole binding and domain motions of EI(△EIN$^{\alpha}$,H189A) with PEP and the other △G's figures are calculated as Equation[1]. The substraction between Equation [1] and [2] shows the △G of the hinge motion, $\triangle G_{EI\_hinge}$. [Fig. 8] [Table. 1]

The △G's figures from whole binding and domain motions, Through ITC titration, $\triangle G_{EI(H189A):PEP}$ and $\triangle G_{EI(\triangle EIN^{\alpha}H189A):PEP}$ are calculated as -8.7 ± 0.2 kcal/mol and -10.1 ± 0.02 kcal/mol, respectively. $\triangle G_{EI\_hinge}$ is 1.5 ± 0.2 kcal/mol. [Fig. 9]

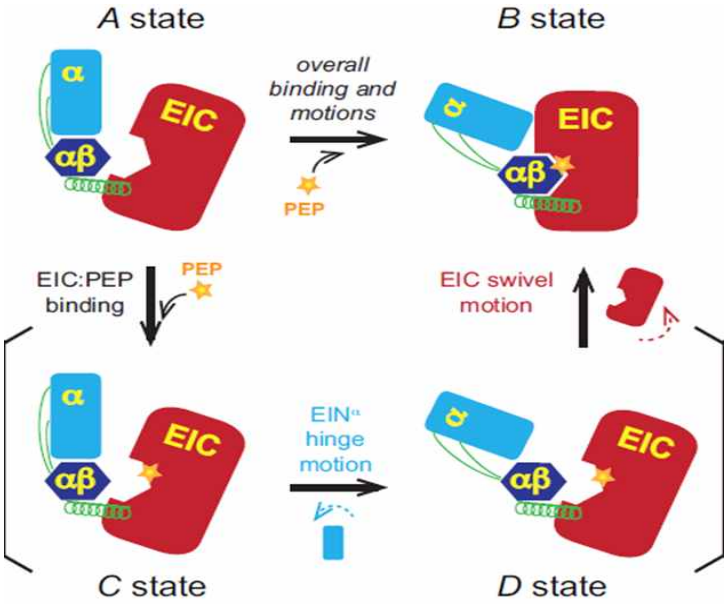


Fig. 8. Schematic diagram for the dissection of the ligand binding and domain motions during the autophosphorylation reaction of EI by PEP. The domain-domain rearrangements relevant to the transition between the A and B states can be divided by the intermediate C and D states, each of which accounts for the individual binding and motions.

(A) **(B)**

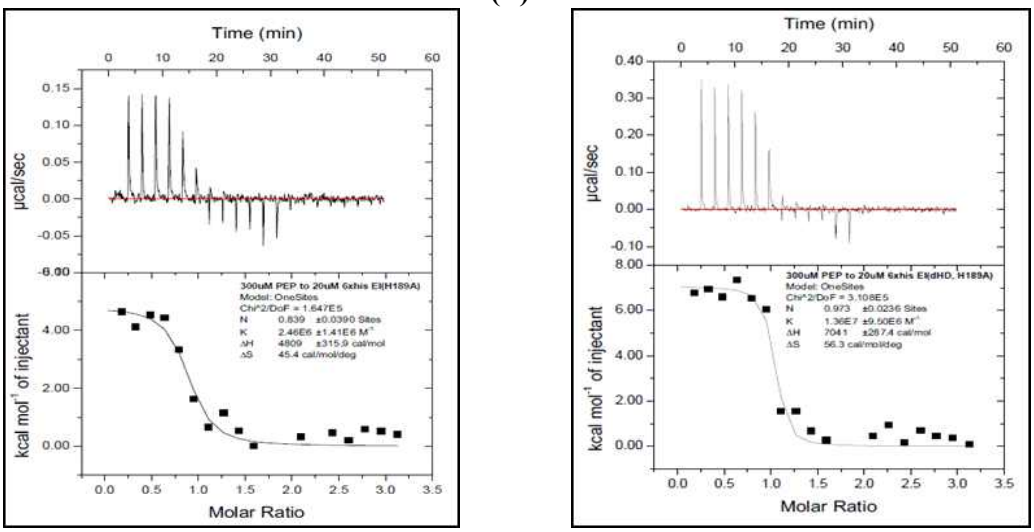


Fig. 9. Raw ITC data and integrated heats of injection for the interaction between (A) EI(H189A) and PEP, and (B) EI(△EIN$^{\alpha}$,H189A) and PEP. This figures show the experimental data, and represent best-fit curves derived for a one-site binding model.

*** PEP to EI (H189A) or EI (EIN^{α}, H189A)**

Buffer	protein (mM)	substrates (mM)	*n* (*stoichiometry*)	*Ka* (M^{-1})	$\triangle H$ (*kcal/mol*)	$\triangle S$ (*cal/mol/deg*)	$\triangle G$ (**kcal/mol**)
Tris-HCl (pH7.4)	EI (H189A) 0.03	PEP 0.3	0.84 ± 0.04	$2.46 \pm 1.4 \times 10^{6}$	3.6 ± 0.1	41.2	**-8.7**
	EI (EIN^{α} H189A) 0.03	PEP 0.3	0.97 ± 0.02	$1.36 \pm 0.1 \times 10^{7}$	6.5 ± 0.1	55.6	**-10.1**

Table. 1. Thermodynamic parameters obtained from ITC data for the interaction between PEP and EI(H189A), its domain deletion mutants

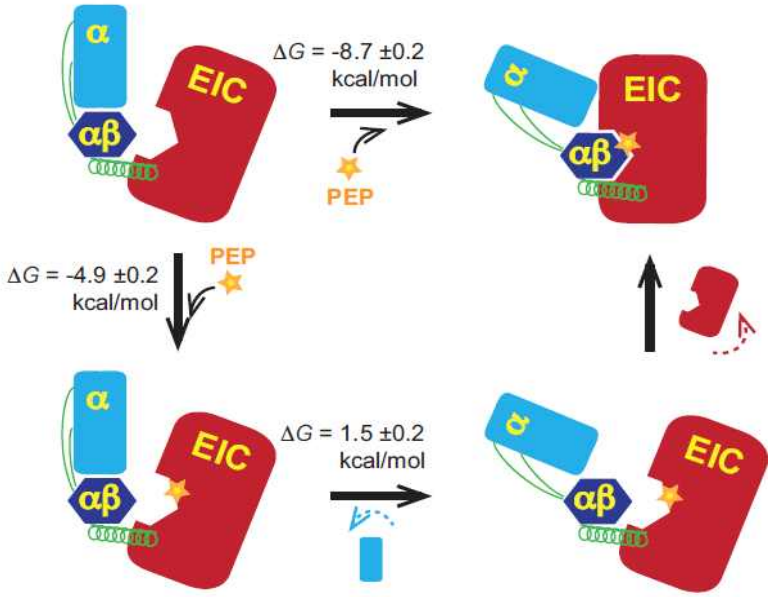


Fig. 10. Free energy changes (△G) relevant to ligand binding, domain motions and interaction between EI(H189A) and PEP. △G figures for whole binding and domain motions, for the ligand binding alone, and for the individual domain motions are separately determined for unphosphorylated EI.

Discussion

Thermodynamic figures relevant to a series of ligand binding and domain motions during system of EI were characterized by calorimetry measurements.

EI(H189A) in the presence of 4mM Mg^{2+} was titrated with PEP in cell buffer with 4mM Mg^{2+} at 20℃ in isothermal titration calorimetry (ITC) experiments. From titrations, an average stoichiometry of 0.98 ± 0.03 for PEP binding to a subunit of EI(H189A) with an affinity constant of 1.0 ± 0.1 x 10^6 M^{-1} (△G = -8.05 ± 0.05 kcal/mole) are obtained. And then the average enthalpy for binding PEP to EI(H189A) in the presence of 4mM Mg^{2+} at 20℃ is +3900 ± 150 cal/mol, and T△S= 12.0 ± 0.1kcal/mol. [Mariana N. Dimitrova et. al. 2003].

In comparison this past study, our experiment results showed similar data. EI(H189A) was contained 20mM Tris-HCl (pH 7.4), 100mM NaCl, 4mM $MgCl_2$, 2mM β-ME with PEP in cell buffer with same components at 25℃. From titration, an stoichiometry of 0.84 ± 0.04 for PEP binding to a subunit of EI(H189A) with an affinity constant of 2.46 ± 1.4 x 10^6 M^{-1} (△G = -8.7 ± 0.2 kcal/mole) are obtained. And then the enthalpy for binding PEP to EI(H189A) was contained 4mM Mg^{2+} at 25℃ is +3600 ± 100 cal/mol, and T△S= 12.2 ± 0.1kcal/mol.

On the other hand, through these measurements of same conditions, isothermal titration calorimetry results demonstrated that the interaction between EI(△HD,H189A) and PEP was stronger about 11.5 times and the free energy was decreased about 1.5kcal/mol in compared to EI(H189A). This free energy of 1.5 kcal/mol was derived from hinge motion which was connected by EIN^{α} and $EIN^{\alpha\beta}$ domain.

Second. the △G's figures from whole binding and domain motions, through ITC titration, $\triangle G_{EI(H189A):PEP}$ and $\triangle G_{EI(\triangle EIN^{\alpha}H189A):PEP}$ are demonstrated as −8.7 ± 0.2 kcal/mol

and −10.1 ± 0.02 kcal/mol, respectively. For substraction, $\triangle G_{EI\_hinge}$ is 1.5 ± 0.2 kcal/mol [Fig. 10].

CHAPTER 2.

Crosstalk between PEP binding and HPr binding

Introduction

The structure and thermodynamic relevant to phosphorylation on the interaction of the N-terminal domain of enzyme I (EIN) and histidine phosphocarrier protein (HPr), the common proteins of the bacterial phosphotransferase system. In addition, the phosphorylation state of individual components of the PTS act as swithes in the regulation of diverse cellular proceses. PEP is the phosphoryl donor in a Mg^{2+} -dependent autophosphorylation of enzyme I on the N3 atom of His189, Phospho-enzyme I reversibly transfer its phosphoryl group to the N1 atom of His15 of HPr [Meadow et al. 1990]. Phosphorylated HPr can donate its phosphoryl group to sugar-specific enzyme II, which ultimately phophorylate and translocate various sugars across the membrane [Postma et al. 1996].

Especially, the PEP binding site of EIC and the HPr binding site of EIN^{α} are away from each other in A and B states of EI. Thereby the binding of one substrate can not prevent the binding site of the other. Less is known, however, whether one substrate binding can impact on the domain motions and change the binding of the other. Hpr binding domain of EIN^{α} is opened to HPr for binding both in A and B states of EI.

So in this chapter the experiments were carried out whether HPr can impact on the domain motions with PEP or not and PEP can influence on the domain motions with HPr or not during the phophotranfer process by calorimetry measurement using ITC.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli strain Top10 was used for plasmid amplication and the plasmids were introduced into *Escherichia coli* strain BL21(DE3) (Novagen).

HPr(H15N) (residue 1-85), HPr mutated protein and coding sequences were PCR amplified using DNA *pfu* tag polymerase (Stratagene) and modified pET-11a vector as the template. The PCR products cloned into the modified pET-11a vector with an His_6 tag. The sequences, domain composition, active site of HPr(H15N): HPr wild type (1-58, histidine 15 to asparagine) and full length EI(H189A) (1-575, EIN^{α}+$EIN^{\alpha\beta}$+EIC, Ala189),

Protein expression and purification

The EI (H189A) plasmids were introduced into *Escherichia coli* strain BL21star(DE3) (Novagen) and transformant was grown in either Luria Bertini or minimal media. The cell were cultured at 37℃ an A_{600} ~0.8 by 1mM isopropyl-beta-D-thiogalactopyranoside, and express for 4-6 hours.

The cells were harvested by centrifugation and for purification of EI(H189A) and the cell pellet were resuspended with 50ml(per liter of culture)of 50mM Tris-HCl, pH 7.4, 200mM NaCl, 2mM beta-mercaptoethanol,

1mM phenylmethysulfonyl fluoride, and 1 tablet of protease cocktail

inhibitor(Sigma-Aldrich, S8830 SIGMAFAST). The suspension was lysed by Emulsiflex after homogenizing and centrifuged at 24000g for 20min at 4℃.

The supernatant fraction was filtered and loaded onto a Ni^{2+}-NTA column (GE Healthcare) and the proteins were eluted with gradient from 0 to 500mM Imidazole. The proteins of fraction were purified by gel filtration on a superdex200 column (GE Healthcare) through 20mM Tris-HCl, pH 7.4, 200mM NaCl and 2mM beta-mercaptoethanol and through fractions were concentrated by Amicon ultra centrifugal filter (Millipore, UFC901096). After concentration, the proteins were further purified by monoQ ion exchange column (GE Healthcare) with a gradient of 1M NaCl.

For purification of HPr(H15N), the pellet was resuspended and lysed in 50ml (per liter of culture) of 20mM Tris-HCl, pH 8.0, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride. The supernatant fraction was filtered and loaded onto a DEAE column (GE Healthcare) equilibrated 20mM Tris-HCl, pH 7.4, 200mM NaCl and further purified by superdex-75 column through 20mM Tris-HCl, pH 7.4, 200mM NaCl. The eluted fractions containing proteins were checked by SDS-PAGE to analyze the mass and purity of samples. And all proteins were changed by 20mM Tris-HCl, pH 7.4, 100mM NaCl, 2mM beta-mercaptoethanol, 4mM $MgCl_2$. [Fig. 11]

HPr (H15N)

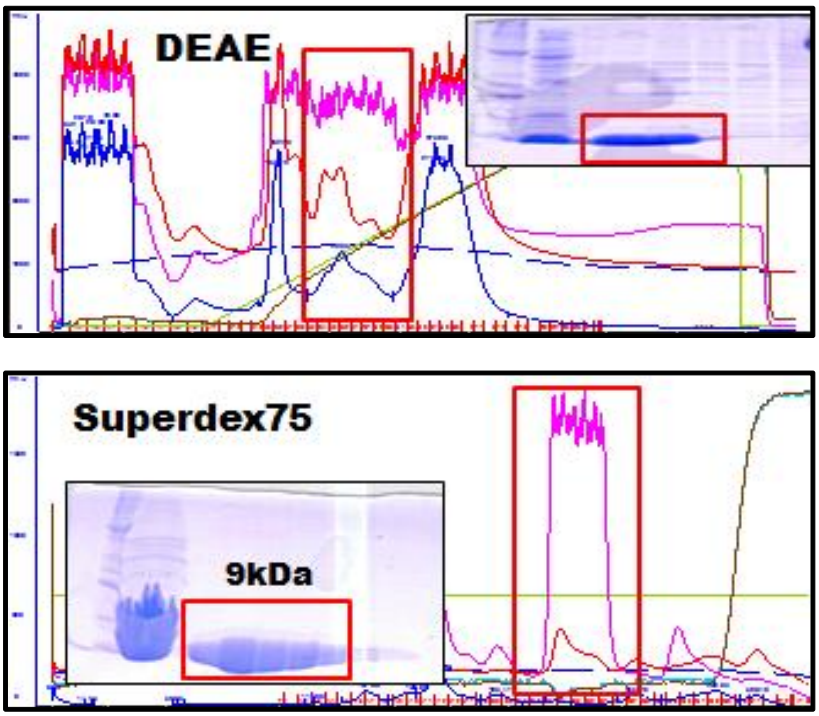


Fig. 11, The purification process of HPr(H15N). The supernatant fraction was loaded onto a DEAE column (GE Healthcare) and the eluted with linear gradient of buffer composed of 1M NaCl. The fractions containing the protein were further by gel filtration through a superdex-75 column (GE Healthcare) equilibrated with 20mM Tris-HCl(pH 7.4), 200mM NaCl.

Isothermal Titration Calorimetry

Calorimetry measurement was used by ITC. Titration were carried out using the ITC_{200} microcalorimeter (GE Healthcare) at 25℃. The HPr titration, 1mM HPr(H15N) or PEP was injected into the cell containing 100uM HPr(H15N) or PEP with EI(H189A). All protein and ligand samples must originate from the same stock solutions, to minimize random sample-to-sample fluctuations in concentration. Before main titration between ligand and protein, implemented titration between ligand and buffer to remove dilution heat of buffer.

First, setting up the syringe with 1mM HPr(H15N) or 1mM PEP of 40㎕. Gently slide the pipette injector into the injection syringe. Make sure the plunger tip is fed directly into the hole of the syringe. Once fully inserted, screw the locking collar of the syringe holder into the pipette injector.

Second, loading the sample with 100uM HPr(H15N) or 1mM PEP with 100uM EI(H189A). Wash sample cell with running buffer, and remove any remaining liquid by using a long-needled glass syringe. And slowly draw a minimum of 200㎕ of protein sample solution into the clean and dry long-needled syringe. Be careful not to introduce any bubbles. Carefully insert needle in sample cell and gently touch the bottom of the cell. Raise tip slightly and gently inject 100uM HPr(H15N) or 1mM PEP with 100uM EI(H189A) solution into the cell until excess liquid is visible above the top of the sample cell. Slowly raise the needle about 1 cm while ensuring liquid remains in the overflow. Quickly withdraw and inject a small amount of solution to remove any trapped bubbles in the sample cell. Remove all solution overflow. This is achieved by gently sliding the needle along side of overflow into the sample cell. The syringe tip will hit a ledge, this is the desire height for the running solution. Remove all liquid

which sits above this line. [Lee A. Freiburger et al. 2011]

The duration of each injection was 4 sec, and injection were made at intervals of 150 sec. The heats is related to the dilution of the substrates were subtracted from the measured heats of binding. ITC titration data were analyzed through the Origin version 7.0 program by MicroCal.

Result

Cloning of HPr(H15N)

HPr wild type was mutated active-site of histidine 15 to asparagine. DNA fragments of HPr were PCR amplified with primers and contained the following. PCR assembly was performed in reactions of 50㎕ reaction volume which was composed of pfu, forward-reverse primer, template of EI(H189A), dNTPs, 10X pfu polymerase buffer and distilled water. This PCR product were used and the reaction vessels transferred to thermal cycler and subjected to the following cycling program: 95℃(2') - [95℃(30'')-55℃(30'')-73℃(14')]$_{18}$ cycles- 72℃(20') (BIORAD). And then using DpnI, stored in incubation for 1 hour at 37℃.

The PCR product of collect size was purified by agarose gel extraction. using the quick Gel Extraction kit (Invitrogen) and stored until use at −20℃.

Free energy changes for binding of PEP to EI:HPr and HPr to EI:PEP

The PEP binding site of EIC and the HPr binding site of EIN$^{\alpha}$ are away from each other in A and B states of EI. Thereby the binding of one substrate can not prevent the binding site of the other. Less is known, however, whether one substrate binding can impact on the domain motions and change the binding of the other. Hpr binding domain of EIN$^{\alpha}$ is opened to HPr for binding both in A and B states of EI.

In the A state, EIN domain pulls back from EIC to give a room for HPr binding, and then HPr enter between EIN^{α} and EIC. Whereas in the B state, HPr binding site of EIN^{α} is opened for binding. I The experiment was carried out the HPr binding to EI in the existence of PEP or not to understand the binding thermodynamics in the A and B states.

The titration between EI(H189A) and HPr wild type with PEP showed unusual reaction heats which came from phosphotransfer reaction between contaminant of wild type EI and HPr. So I used HPr(H15N) which has the active site His15 changed by the isosteric asparagine residue, the background heats disappeared.

Discussion

Phosphorylated HPr can donate its phosphoryl group to sugar-specific, membrane-associated enzymes II, which ultimately phosphorylate various sugars [Postma et al. 1996]. The amino terminal domain of *Escherichia coli* enzyme I (EIN) has been cloned and purified by [Seok et al. 1996] who demonstrated, in agreement with the results of [LiCalsi et al. 1991] that EIN can be reversibly phosphorylated in vitro by phospho-HPr but is not autophosphorylated by PEP. X-ray crystallographic [Liao et al. 1996] and NMR solution [Garrett et al. 1997] structures of EIN show that the non-phosphorylated, active site His189 is buried near the interface between two subdomains: an alpha-beta-domain (residues 1−20 and 148−230) and an alpha-domain (residues 33−143). Upon phosphorylation, His189 rotates toward the surface with only small structural changes detected [Garrett et al. 1998].

This chapter study showed that the binding thermodynamics of EI(H189A) and HPr(H15N) was similar with the binding of EI(H189A) and HPr with PEP. Through calorimetry measurements, these △G's figures are −5.7 ± 0.1 kcal/mol and −5.8 ± 0.1kcal/mol, respectively.

The experiment was also carried out the PEP binding to EI with HPr to know of the HPr binding affects the hinge motion of EIN^{α} and change the PEP binding. These △G's figures are −8.7 ± 0.2kcal/mol which PEP binding to EI as past experiment result and −8.8 ± 0.4kcal/mol which PEP binding to EI with HPr , respectively. Through this study, the HPr binding of EI little affected the PEP binding [Fig. 12].

In short, the free energy that the binding thermodynamics of EI(H189A) and HPr(H15N), the binding of EI(H189A) and HPr with PEP are −5.7 ± 0.1kcal/mol and −5.8 ± 0.1kcal/mol, respectively. On the other hand, free energy figures are −8.7 ±

0.2kcal/mol which PEP binding to EI and −8.8 ± 0.4kcal/mol which PEP binding to EI with HPr, respectively. The thermodynamics of the whole binding and domain motions were unchanged [Fig. 13].

(A) (B) (C)

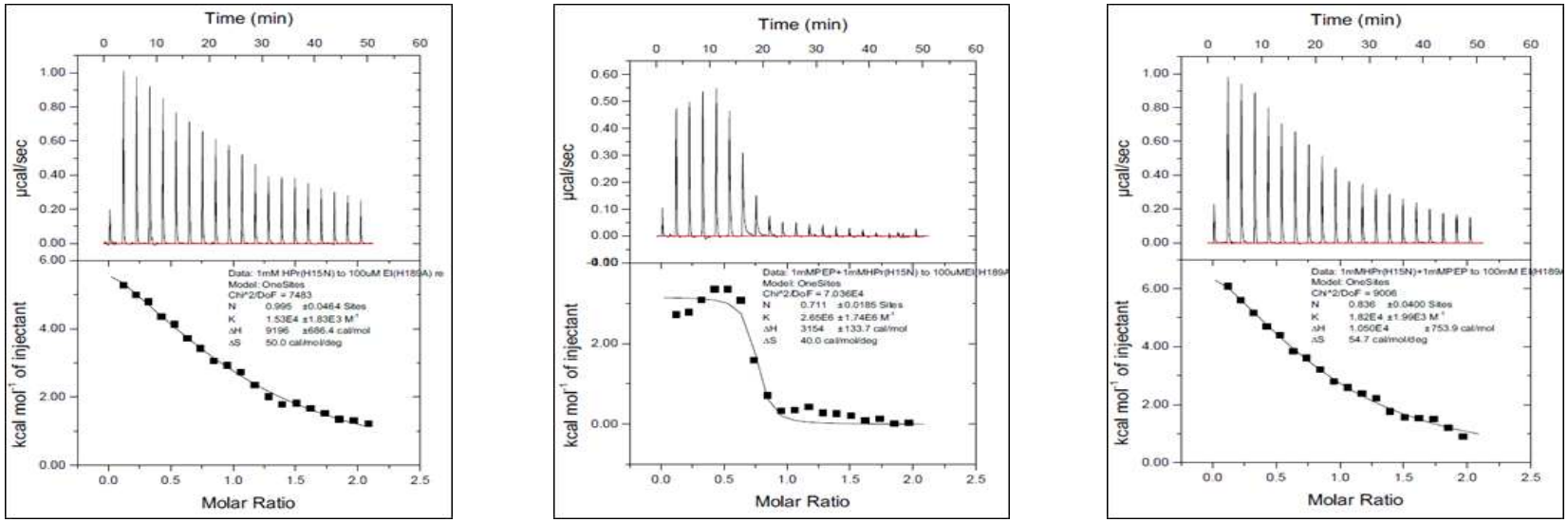


Fig. 12. Raw ITC data and integrated heats of injection for the interaction between (A) EI(H189A) and HPr(H15N), (B) EI(H189A):HPr(H15N) and PEP, and (C) EI(H189A):PEP and HPr(H15N). This figures show the experimental data, and represent best-fit curves derived for a one-site binding model.

*** HPr(H15N) or PEP to EI (H189A) + HPr or PEP**

Buffer	protein (mM)	substrates (mM)	*n* (stoichiometry)	*Ka* (M^{-1})	$\triangle H$ (kcal/mol)	$\triangle S$ (cal/mol/deg)	$\triangle G$ (kcal/mol)
Tris-HCl (pH7.4)	EI (H189A) 0.1	HPr 1	0.99 ± 0.04	1.53 ± 0.2 x 10^4	9.1 ± 0.7	50	-5.7
	EI (H189A) 0.1:HPr 1	PEP 1	0.71 ± 0.02	2.65 ± 1.7x 10^6	3.15 ± 0.1	40	-8.8
	EI (H189A) 0.1:PEP 1	HPr 1	0.84 ± 0.04	1.82 ± 0.2 x 10^4	10.5 ± 0.7	54.7	-5.8

Table. 2. Thermodynamic parameters obtained from ITC data for the interaction between HPr or PEP and EI(H189A) with HPr, PEP

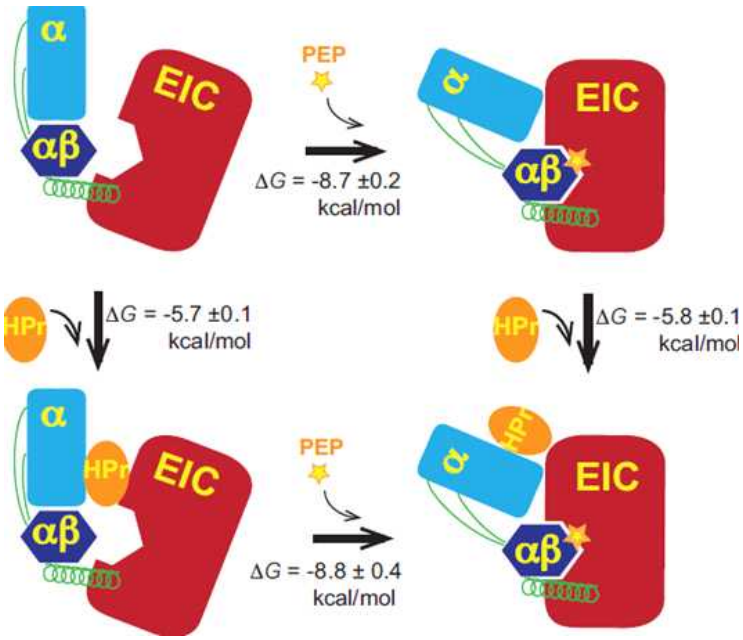


Fig. 13. Free energy changes (△G) for the binding of PEP to EI in the HPr or not, and for the binding of HPr to EI in the PEP or not.

Conclusion

From A state to B state during phosphorylation process of EI, the reorientation of EIN $^{\alpha}$ domain prevent to the EIN$^{\alpha\beta}$ domain. For this reason, the calorimetry figures showed that between EI(△HD,H189A) and PEP was stronger than EI(H189A) as 11.5 times. In other words, EIN$^{\alpha}$ domain can not assist EIN$^{\alpha\beta}$ domain during whole binding and motions.

To know difference of △G as 1.5kcal/mol, using two equation. First, $\triangle G_{EI(H189A):PEP} = \triangle G_{EIC:PEP}+\triangle G_{EI\_hinge}+\triangle G_{EI\_swivel}(\triangle G_{EIN\alpha\beta:(EIC:PEP)})$. Second, $\triangle G_{EI(\triangle EIN^{\alpha}H189A):PEP} = \triangle G_{EIC:PEP}+\triangle G_{EI\_swivel}(\triangle G_{EIN\alpha\beta:(EIC:PEP)})$. From subtraction were demonstrated that free energy of 1.5kcal/mol was derived from hinge motion. In short, the own domains can not influence on the binding of domain coupled with ligand binding strongly than hinge motion. In short, through the figures of whole binding and motions, especially, that free energy of the hinge motion (△G = 1.5kcal/mol) and expected this free energy of hinge motion is unfavorable energy can be overcome by the swivel motion through whole binding and domain motion. In short, this results were expected that ligand binding and protein phosphorylation can impact on the energetics of the domain motions by changing the inter-domain interfaces.

In this part, for confirming our expectation, the studies would carried out more about free energy of swivel motion by the domain association between isolated EIN$^{\alpha\beta}$ and isolated EIC:PEP.

In another part, PEP binding and HPr binding did not crosstalk with each other, leaving the thermodynamics whole binding and domain motions unchanged. In short, the fact that two substrates can bind independently and EI:PEP:HPr ternary complex can be simply formed during phosphotransfer reactions. The ternary complex can maintain EI to

active dimer form.

To the best of our results, this is the first thermodynamics studies that characterized concerted large-scale domain motions coupled with ligand binding. The strategic mutant design and calorimetric measurement can demonstrate the thermodynamic dissection of ligand binding and domain motions.

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국문초록

대장균의 당 인산화 단백질 Enzyme I의 리간드 결합과 도메인 운동에 대한 열역학적 특성 규명

Enzyme I (EI)이 포함된 당 인산화 시스템은 phosphoenolpyruvate (PEP)를 사용하여 자동 인산화를 할 수 있고 특히 EI은 인산기를 Hpr 이라는 인산기 전달 단백질에 수송한다. 이미 알려진 바로써 많은 단백질 안에서의 도메인 간의 운동은 중요한 역할을 담당하고 있다. EI은 연속된 당 인산화 전달 반응에 관여하는 첫 번째 단백질로서, N-말단 및 C-말단 도메인이 linker helix로 연결되어져 있다. N-말단도메인은 인산화 활성 부위를 가진 $EIN^{\alpha\beta}$ 도메인과 HPr과 결합하는 EIN^{α} 도메인으로 구성되어 있고, C-말단 도메인은 PEP 결합 부위를 가진다.

본 연구에서는 isothermal titration calorimetry를 사용하여 리간드와 결합 및 도메인 사이 상호작용에 대한 열역학적 특성에 대한 분석을 하였고, 이를 위하여 각 도메인들을 선택적으로 제거시킨 EI을 사용하여 관측하였다. 이를 통해 hinge 운동의 Gibbs free energy 값을 측정하였고 ($\triangle G$ = 1.5 kcal/mol), 이 에너지 측면에서 불리한 free energy가 더 유리한 swivel 운동에 의해 극복되어질 것이라고 예상하였다. 또한 hinge 운동들은 EIN^{α} 도메인과 $EIN^{\alpha\beta}$ 도메인 사이의 결합을 약하게 하여 에너지 면에서 불리하며 이는 결합 엔트로피에서 오는 것을 밝혔다.

마지막으로, EI의 리간드인 PEP의 결합과 다음 인산기 전달 단백질인 HPr 결합은 각각의 결합 반응에 있어서 서로 방해를 하지 않는다. 즉, 이 두 개의 기질은 결합 부위가 따로 떨어져 있을뿐 아니라, 두 결합이 열역학적으로 독립적어 있어 반응 중에 삼중 복합체를 쉽게 형성하며 반응 효율을 높일 것으로 예측된다. 본 결과들은 리간드 결합에 의한 큰 도메인들 운동의 열역학적 특성에 대한 규명을 이해

하는데 있어 도움을 줄 것이다.

주요어 : Enzyme I, 리간드 결합, 도메인 운동, 열역학, 열량 측정

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감사의 글

관악 땅 220동에 들어선지도 어느덧 2년이 다 되어갑니다. 연구실 분위기가 이제 좀 익숙해지나 했더니 졸업을 하게 되었습니다. 실험도 사람들도 뭔가 감이 잡히고 정이 들어가는데 떠나야 한다는 아쉬움이 저를 괴롭힙니다. 저의 삶은 언제나 맛만 보고 넘어가는 그런 식인가 봅니다. 새로운 곳에서 다시 새로운 마음가짐으로 열심히 하겠다는 스스로의 다짐으로 그런 슬픔을 잠시 잊어야겠습니다.

먼저 이루 말할 수 없는 무한한 사랑으로 저를 믿어주시고 키워주신 사랑하는 아버지와 어머니, 내 착한 동생에게 감사의 마음을 전하면서 이 부족한 논문을 바칩니다. 이제는 이러한 가족들의 은혜에 조금이나마 보답할 수 있도록 노력하겠습니다.

그리고 석사 과정 동안 연구에 매진할 수 있도록 아낌없는 격려와 지도를 해주시고 인격적으로 대해주신 서정용 지도교수님께 진심으로 감사드립니다. 또한 저의 논문 심사를 맡아주시고, 소중한 충고와 조언을 해주셨던 김민규 교수님, 배의영 교수님께 감사를 드립니다.

2년여 간 함께했던 생물물리 및 나노생물학 실험실 식구들에게 고마움의 말을 전하고 싶습니다. 실험실의 제일 큰 형님으로 지금도 실험실 사람들을 잘 챙겨주시는 마음 따뜻한 부산 사나이 펩시 태경 형님 (소개팅 기대하세요..^^), 지금의 실험실이 있기 까지 초석을 놓아주셨던 독일 매니아 무경 누나, 모든 문제에 분석적으로 접근하시만 가끔 엉뚱한 모습으로 웃음을 자아내시는 잠실 동방불패 고은 누나, 무슨 문제이든 다 해결해주시던 해결사이자 오창 전도사이신 착하신 택배 익태 형님, 지금까지 살아오면서 처음 접

해본 스타일이지만 정도 많고 마음이 따뜻한 친구인 분당 백화점 아줌마 지연이, 항상 많은 대화도 나누고 장난도 치면서 힘들 때 큰 힘이 됐던 친동생 같은 엉뚱발랄시끌 승아, 특히 2년 동안 부족한 저를 물심양면 뒤에서 도와주시고 이끌어주신 너무나도 고마운 현모양처 포포 엄마 영주 누나 (경준이 형이 누나한테 정말 잘해주셔야 합니다) 까지 결코 한 사람 한 사람과의 행복하고 즐거운 추억들 결코 잊지 못할 것입니다. 우리 실험실 식구들의 도움이 없었다면 저는 졸업하지 못했을 것입니다. 지금도 새로운 환경들과 사람들을 접하고 있지만 석사과정 동안 함께 했던 BPNB 식구들은 결코 영원히 잊지 못할 것입니다.

마지막으로 나의 모든 인생 가운데 언제나 동행하여 주시고 힘주시는 그리고 앞으로도 그러하실 살아계신 하나님 아버지의 사랑과 은혜에 감사의 기도를 드립니다.

2년여 간 실험실 식구들에게 받은 조건없는 사랑 어딜가나 기억하며 베풀며 살도록 노력하겠습니다. 다시 한 번 서정용 교수님과 실험실 식구들에게 진심으로 감사드립니다. 사랑합니다.