Thermodynamic dissection of large-scale domain motions coupled with ligand binding of enzyme I

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Abstract: Domain motions are central to the biological functions of many proteins. The energetics of the motions, however, is often difficult to characterize when motions are coupled with the ligand binding. Here, we determined the thermodynamic parameters of individual domain motions and ligand binding of enzyme I (EI) using strategic domain-deletion mutants that selectively removed particular motions. Upon ligand binding, EI employs two large-scale domain motions, the hinge motion and the swivel motion, to switch between conformational states of distinct domain–domain orientations. Calorimetric analysis of the EI mutants separated the free energy changes of the binding and motions, demonstrating that the unfavorable hinge motion ($\Delta G = 1.5 \text{ kcal mol}^{-1}$) was driven by the favorable swivel motion ($\Delta G = -5.2 \text{ kcal mol}^{-1}$). The large free energy differences could be explained by the physicochemical nature of the domain interfaces associated with the motions; the hinge motion employed much narrower interface than the swivel motion without any hydrogen bonds or salt bridges. The small heat capacity further suggested that the packing of the domain interfaces associated with the hinge motion was less compact than that commonly observed in proteins. Lastly, thermodynamic analysis of phosphorylated EI suggests that the domain motions are regulated by the ligand binding and the phosphorylation states. Taken together, the thermodynamic dissection approach illustrates how multiple motions and ligand binding are energetically connected during the functional cycle of EI.

Keywords: calorimetry; domain motions; free energy evolution; ligand binding; thermodynamics

Abbreviations: EI, enzyme I; EIN, N-terminal domain of enzyme I; EIC, C-terminal domain of enzyme I; ITC, isothermal titration calorimetry; PEP, phosphoenolpyruvate; PTS, phosphotransferase system

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Outline

Enzyme I employs a hinge motion and a swivel motion upon ligand binding to accomplish its autophosphorylation reaction. We have characterized the free energy changes of each motion and binding separately using calorimetric analysis of domain-deletion mutants. The thermodynamic parameters demonstrate the free energy loss and gain of individual motions in a quantitative manner, and illustrate how free energies evolve along a series of binding and motions in multidomain proteins.

Introduction

Domain motions coupled with ligand binding are closely related to the biological functions of many

Figure 1. The structures of EI with distinct domain–domain arrangements shown in a cartoon diagram for (A) the A state of free unphosphorylated EI,¹⁹ (B) the B state of phosphorylated EI quenched by oxalate,¹⁶ and (C) the dissection of the PEP binding and domain motions of EI during the A→B state transition shown in a schematic diagram. The A→B state transition is divided by the intermediate C and D states, each of which accounts for the PEP binding and individual motions. The postulated intermediate states are enclosed by the square brackets. The active site His189, phospho-His189, and oxalate are shown as space-filling models. The EIN^α domain is colored in cyan, the EIN^{αβ} domain in blue, the EIC domain in red, and the hinge loop and the swivel helix in green. The physical nature of the hinge and swivel motions required for the A→B state transition is depicted in between the cartoon structures of the A and B states. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

proteins.^{1–3} High-resolution protein structures and dynamics have been used to describe the physical nature and the time scales of motions that have functional implications.^{4,5} Thermodynamic information have also been accumulated to understand how the modulation of a particular motion impacts on the ligand binding.^{6–8} Less is known, however, about the thermodynamic nature of the motion itself separate from the binding, simply because the motion and the binding are tightly coupled in many cases. Calorimetry has been the method of choice for characterizing the thermodynamics of molecular interactions, as it provides the enthalpy and the entropy changes as well as the equilibrium constant of a given reaction.^{9,10} When a ligand binding is followed by a domain motion, calorimetry effectively measures the thermodynamic parameters of the overall reaction – the equilibrium binding and the accompanying domain rearrangement. This notion has led us to the idea that an engineered protein mutant that lacks a particular domain motion can be used to characterize the thermodynamics of the motion separate from the binding. Herein, we demonstrate the thermodynamic dissection of concerted domain motions and ligand binding of enzyme I (EI) and characterize the thermodynamics of the invisible intermediate states during the motion.

EI is the first signaling protein in the bacterial phosphotransferase system (PTS) for sugar translocation and phosphorylation.^{11–13} EI catalyzes two reversible reactions: an Mg²⁺-dependent autophosphorylation reaction using phosphoenolpyruvate (PEP) as a substrate, and a phosphotransfer reaction to the histidine-containing phosphocarrier protein HPr. EI has an N-terminal domain (EIN) comprising

a catalytic phosphohistidine subdomain (EIN^{αβ} domain) and an HPr-binding subdomain (EIN^α domain), and a C-terminal domain (EIC) that contains the PEP-binding site.^{14,15} The structures of EI show distinct conformational states that can explain the mechanisms underlying the two enzyme reactions.^{16–19} Figure 1(A) shows the solution structure of EI determined by NMR residual dipolar coupling and X-ray scattering data, in which EIN^α and EIN^{αβ} adopt the same orientation as that observed in the isolated EIN (A state). The structure of the A state is relevant to the phosphotransfer reaction between EI and HPr because the active site His15 of HPr bound to EIN^α is positioned close to the active site His189 in EIN^{αβ}.²⁰ Figure 1(B) shows the crystal structure of phosphorylated EI quenched by the inhibitor oxalate, in which the phospho-His189 of EIN^{αβ} points toward the oxalate bound to the PEP-binding site of EIC (B state). The structure of the B state captures the coordinate for the autophosphorylation reaction of EI by PEP.¹⁶ The conformational switch of EI between the A and B states upon PEP binding is supported by the following observations. First, both the solution and crystal structures demonstrated that free unphosphorylated EI existed predominantly in the A state conformation.^{18,19} Second, PEP binding coupled the thermal unfolding of the EIN and EIC domains in EI(H189A), which otherwise exhibited well-separate melting temperatures.²¹ Third, the PEP binding to EI(H189A) was three orders of magnitude stronger than the PEP binding to isolated EIC.^{22,23} Taken together, the PEP binding to EI triggers the A→B state conformational transition.

The transition between the A and B conformational states of EI requires large-scale domain

motions that can be modeled as two separate rigid-body motions. The 65° hinge motion between EIN^α and $\text{EIN}^{\alpha\beta}$ pulls back and disengages EIN^α from $\text{EIN}^{\alpha\beta}$ (Fig. 1). The 70° swivel motion of EIC around the swivel helix brings EIC and $\text{EIN}^{\alpha\beta}$ together. The hinge motion is energetically unfavorable as it disrupts the interaction between EIN^α and $\text{EIN}^{\alpha\beta}$. The swivel motion, on the other hand, can take place only after the hinge motion; otherwise a large steric overlap results between EIN^α and EIC. Hence, one can imagine that the two motions would occur in a concerted manner to achieve the $A \rightarrow B$ state transition of EI, yet how the free energies evolve along the series of the motions remains largely unknown. To explore the free energies of individual events, we divided the process of PEP binding and concomitant conformational changes between the A and B states into three steps [Fig. 1(C)]; (1) the binding between PEP and EIC (A state $\rightarrow C$ state), (2) the hinge motion between EIN^α and $\text{EIN}^{\alpha\beta}$ to make a room for EIC (C state $\rightarrow D$ state), (3) the swivel motion between EIC and $\text{EIN}^{\alpha\beta}$, resulting in the association between $\text{EIN}^{\alpha\beta}$ and EIC:PEP (D state $\rightarrow B$ state). Only the A and B states have been experimentally observed, and the C and D states in the square brackets in Figure 1(C) are postulated to be the intermediate states. The C state describes the EI:PEP complex immediately after PEP binding and prior to any domain motion. The D state describes the EI:PEP complex after the hinge motion of EIN^α and before the swivel motion of EIC:PEP.

Results and Discussion

We employed the domain-deletion mutations to remove particular domain motions of interest from EI. For example, $\text{EI}(\Delta\text{EIN}^\alpha)$ lacked the EIN^α domain required for the hinge motion, so that $\text{EI}(\Delta\text{EIN}^\alpha)$ could bind to PEP and proceed only with the swivel motion (transitions along $A \rightarrow C \rightarrow B$ states). On the other hand, the isolated EIC domain lacked both EIN^α and $\text{EIN}^{\alpha\beta}$ domains, and could only bind to PEP without any domain motions (transition of $A \rightarrow C$ states). The thermodynamic parameters were measured by calorimetry, and the active site H189A mutation was used to prevent the autophosphorylation of EI during the titration with PEP. The domain composition and the spanning residues of the mutants used in this study are described in detail in Supporting Information Table S1. The EI mutants maintained the secondary structures similar to EI from the circular dichroism spectra (Supporting Information Fig. S1). As EI forms a dimer via EIC and functions as a dimer,^{24,25} we employed the size exclusion chromatography to confirm that both $\text{EI}(\text{H189A})$ and $\text{EI}(\Delta\text{EIN}^\alpha, \text{H189A})$ formed a dimer (Supporting Information Fig. S2).

The overall ΔG of the transitions along $A \rightarrow C \rightarrow D \rightarrow B$ states in Figure 1(C) can be expressed

as the sum of the contributions from individual events:

$$\Delta G_{\text{EI}(\text{H189A}): \text{PEP}} = \Delta G_{\text{EIC:PEP}} + \Delta G_{\text{EI-hinge}} + \Delta G_{\text{EI-swivel}}, \quad (1)$$

where $\Delta G_{\text{EI}(\text{H189A}): \text{PEP}}$ accounts for the overall binding and domain motions of EI(H189A) with PEP, $\Delta G_{\text{EIC:PEP}}$ for the binding between EIC and PEP, $\Delta G_{\text{EI-hinge}}$ for the hinge motion, and $\Delta G_{\text{EI-swivel}}$ for the swivel motion. The equilibrium dissociation constant (K_D) for EI(H189A) and PEP was measured to be 430 ± 120 nM from ITC experiment [Fig. 2(A)]. The ΔG of the overall reaction between $\text{EI}(\Delta\text{EIN}^\alpha, \text{H189A})$ and PEP can be similarly expressed as Eq. (1), without the hinge motion term:

$$\Delta G_{\text{EI}(\Delta\text{EIN}^\alpha, \text{H189A}): \text{PEP}} = \Delta G_{\text{EIC:PEP}} + \Delta G_{\text{EI-swivel}}, \quad (2)$$

where $\Delta G_{\text{EI}(\Delta\text{EIN}^\alpha, \text{H189A}): \text{PEP}}$ accounts for the overall binding and domain motions of $\text{EI}(\Delta\text{EIN}^\alpha, \text{H189A})$ with PEP, and the other terms are defined as in

Figure 2. Measured binding and motion in a schematic diagram (top) and the integrated heats of injection (squares) and the least-squares fits (lines) derived from a one-site binding model from ITC measurement (bottom) for the interaction between (A) EI(H189A) and PEP, and (B) $\text{EI}(\Delta\text{EIN}^\alpha, \text{H189A})$ and PEP at 25°C. (C) The thermodynamic profiles of PEP binding and domain motions of EI based on the ITC measurements. The changes in Gibbs free energy (ΔG ; black), enthalpy (ΔH ; red), and entropy ($-T\Delta S$; blue) are shown in kcal mol^{-1} as described in Table 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3. Free energy level diagram showing the relative stabilities of individual conformational states of EI along the $A \rightarrow C \rightarrow D \rightarrow B$ transitions. PEP binding and domain motions are presented in a schematic diagram. The A state is arbitrarily assigned a free energy value of 0. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Eq. (1). We note that EI(Δ Ein $^{\alpha}$) remained functional for the autophosphorylation reaction by PEP,²⁶ indicating the integrity of its PEP binding and the swivel motion. This justifies the assumption that EI(Δ Ein $^{\alpha}$,H189A) proceeds with the transitions along $A \rightarrow C \rightarrow B$ states upon PEP binding as shown in Figure 2(B). The K_D for EI(Δ Ein $^{\alpha}$, H189A) and PEP was 37 ± 1.2 nM based on the ITC measurement. ΔG can be calculated from the measured K_D according to the equation $\Delta G = RT \cdot \ln K_D$, where R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), and T is the absolute temperature in Kelvin. The ΔG values for the overall binding and domain motions, $\Delta G_{\text{EI(H189A):PEP}}$ and $\Delta G_{\text{EI}(\Delta\text{Ein}^{\alpha},\text{H189A}):PEP}$ were $-8.7 \pm 0.2 \text{ kcal mol}^{-1}$ and $-10.1 \pm 0.02 \text{ kcal mol}^{-1}$, respectively. Subtracting Eq. (2) from (1) provides the ΔG for the hinge motion, $\Delta G_{\text{EI-hinge}}$. $\Delta G_{\text{EI-hinge}}$ was thus obtained as $1.5 \pm 0.2 \text{ kcal mol}^{-1}$, which amounts to the energetic cost of the uphill process for the $C \rightarrow D$ state transition (Fig. 3). Changes in the enthalpy (ΔH) and the entropy (ΔS) of the hinge motion can be obtained in a similar manner by subtracting the enthalpy and entropy terms of the individual reactions (Table 1). $\Delta H_{\text{EI-hinge}}$ was found to be $-2.9 \pm 0.2 \text{ kcal mol}^{-1}$, and $\Delta S_{\text{EI-hinge}}$ was found to be $-14.4 \pm 0.8 \text{ cal mol}^{-1} \text{ K}^{-1}$. It is notable that the hinge motion is energetically unfavorable due to the large entropic cost ($-T\Delta S = 4.3 \text{ kcal mol}^{-1}$) which dominates the favorable enthalpic gain ($\Delta H = -2.9 \text{ kcal mol}^{-1}$) as is illustrated in Figure 2(C).

We previously characterized the binding between the isolated EIC domain and PEP, yielding the $\Delta G_{\text{EIC:PEP}}$ of $-4.9 \pm 0.2 \text{ kcal mol}^{-1}$.²² From Eq. (1), $\Delta G_{\text{EI-swivel}}$ is calculated to be $-5.2 \pm 0.2 \text{ kcal mol}^{-1}$, which accounts for the energetic gain from the swivel motion. Note that the free energy change

Table 1. Thermodynamic Parameters for the PEP Binding and Domain Motions of EI, and for the Oxalate Binding and Domain Motions of Phosphorylated EI Obtained from Calorimetric Measurements

Transition of states	Type of events	Description	K_D^a	ΔG^b (kcal mol $^{-1}$)	ΔH^c (kcal mol $^{-1}$)	ΔS^d (cal mol $^{-1}$ K $^{-1}$)
EI and PEP						
A $\rightarrow$ C $\rightarrow$ D $\rightarrow$ B	Binding/motion	EI(H189A) + PEP	430 ± 120 (nM)	-8.7 ± 0.2	3.6 ± 0.1	41.2 ± 0.7
A $\rightarrow$ C $\rightarrow$ B	Binding/motion	EI(Δ Ein $^{\alpha}$, H189A) + PEP	37 ± 1.2 (nM)	-10.1 ± 0.0	6.5 ± 0.1	55.6 ± 0.3
A $\rightarrow$ C	Binding	EIC + PEP	260 ± 76 (μ M)	-4.9 ± 0.2	1.9 ± 0.4	22.7 ± 2.8
C $\rightarrow$ D	Motion	Hinge motion ($\Delta G_{\text{EI-hinge}}$)		1.5 ± 0.2	-2.9 ± 0.2	-14.4 ± 0.8
D $\rightarrow$ B	Motion	Swivel motion ($\Delta G_{\text{EI-swivel}}$)		-5.2 ± 0.2	4.6 ± 0.8	32.9 ± 2.8
Phospho-EI and oxalate						
A $\rightarrow$ C $\rightarrow$ D $\rightarrow$ B	Binding/motion	pEI + oxalate	210 ± 18 (μ M)	-5.0 ± 0.1	2.1 ± 0.3	23.7 ± 0.9
A $\rightarrow$ C $\rightarrow$ B	Binding/motion	pEI(Δ Ein $^{\alpha}$) + oxalate	34 ± 4.4 (μ M)	-6.1 ± 0.1	2.7 ± 0.1	29.4 ± 0.5
A $\rightarrow$ C	Binding	EIC + oxalate	660 ± 220 (μ M)	-4.3 ± 0.2	-0.6 ± 0.3	12.4 ± 1.1
C $\rightarrow$ D	Motion	Hinge motion ($\Delta G_{\text{pEI-hinge}}$)		1.1 ± 0.1	-0.6 ± 0.3	-5.7 ± 1.0
D $\rightarrow$ B	Motion	Swivel motion ($\Delta G_{\text{pEI-swivel}}$)		-1.8 ± 0.2	3.3 ± 0.3	17.0 ± 1.2

^a Apparent equilibrium dissociation constants (K_D) for the binding (and motions).

^b Free energy changes (ΔG).

^c Enthalpy changes (ΔH).

^d Entropy changes (ΔS).

Figure 4. Two-dimensional ^1H - ^{15}N heteronuclear single quantum correlation spectra of the 0.3 mM ^{15}N -labeled $\text{EIN}^{\alpha\beta}$ (H189A) in the presence (red) and absence (black) of 0.45 mM unlabeled EIC. Both samples were prepared in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM β -mercaptoethanol, 4 mM MgCl_2 , and 10 mM PEP. Residues with reduced intensity ($<10\%$) by line broadening are annotated in red, otherwise in black. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in favor of the swivel motion is much larger than the free energy cost of the hinge motion, which drives the $C \rightarrow D \rightarrow B$ state transition of EI upon PEP binding (Fig. 3). $\Delta H_{\text{EI-swivel}}$ and $\Delta S_{\text{EI-swivel}}$ can be similarly obtained by subtraction, yielding 4.6 ± 0.8 kcal mol $^{-1}$, and 32.9 ± 2.8 cal mol $^{-1}$ K $^{-1}$, respectively (Table 1). Thus the swivel motion is an energetically favorable process due to the large entropic gain ($-T\Delta S = -9.8$ kcal mol $^{-1}$). Given that the swivel motion involves the physical rotation of EIC around the swivel helix, the swivel helix may be used as a mechanical joint and undergoes a conformational change during the motion. To address the possible free energy contribution from the swivel helix, we measured the binding between $\text{EIN}^{\alpha\beta}$ (H189A) and EIC:PEP with the swivel helix disconnected. The ΔG for the domain–domain association between isolated $\text{EIN}^{\alpha\beta}$ and EIC:PEP was obtained as -4.9 ± 0.1 kcal mol $^{-1}$ ($K_D = 260$ μM). The free energy contribution from the swivel helix was thus -0.3 ± 0.2 kcal mol $^{-1}$; hence the main driving force for the $D \rightarrow B$ state transition was the domain–domain association between $\text{EIN}^{\alpha\beta}$ and EIC:PEP with a small contribution from the swivel helix. We note that isolated $\text{EIN}^{\alpha\beta}$ (H189A) interacted with EIC:PEP using the same binding interface as that observed in the B state conformation of EI. When ^{15}N - $\text{EIN}^{\alpha\beta}$ (H189A) was titrated with EIC in the presence of PEP, those residues at the interaction interface in the B state conformation showed line broadening (Fig. 4).

When we determined $\Delta G_{\text{EI-hinge}}$ and $\Delta G_{\text{EI-swivel}}$ from Eqs. (1) and (2), we implicitly assumed that

thermodynamic contributions from the interaction between EIN^{α} and EIC were negligible. We tested if this assumption was valid. The interaction interface between EIN^{α} and EIC spanned only a limited set of residues, featuring a few salt bridges. Specifically, Lys69 of EIN^{α} showed contacts with Glu312 of EIC in the B state conformation. In addition, Arg279 of EIC appeared to form salt bridges to Glu67 and Glu68 of EIN^{α} in the A state conformation. We prepared EI(H189A, E312A) and EI($\Delta\text{EIN}^{\alpha}$, H189A, E312A) mutants that removed the salt bridge between Lys69 and Glu312. ΔG values for PEP binding and domain motions of the mutants showed little changes by the E312A mutation (Supporting Information Table S2). We also prepared EI(H189A, R279A) and EI($\Delta\text{EIN}^{\alpha}$, H189A, R279A) that removed the salt bridge between Arg279 and Glu67/Glu68. Measured ΔG again indicated that ΔG values for the binding and motions were not perturbed by the salt bridge. Taken together, $\Delta G_{\text{EI-hinge}}$ and $\Delta G_{\text{EI-swivel}}$ were little affected by the interaction between EIN^{α} and EIC.

The large difference of free energy changes between the hinge motion and the swivel motion attributes to the physicochemical nature of the domain–domain interface. The interface between EIN^{α} and $\text{EIN}^{\alpha\beta}$ comprises 29 residues and buries ~ 500 $\text{\AA}^2$ of solvent accessible surface area (SASA) in the A state (Fig. 5). The interface is hydrophobic without any sign of hydrogen bonds or salt bridges (Table 2). The hinge motion results in a further reduction in the interaction interface between EIN^{α} and $\text{EIN}^{\alpha\beta}$ (eight residues and the burial of ~ 90 $\text{\AA}^2$ of SASA in the B state). The entropic cost of the hinge motion likely comes from the exposure of the hydrophobic residues (Phe65, Phe73, Met193, etc.) at these interfaces. We further obtained the heat capacity of the hinge motion from the temperature dependence of enthalpy changes upon PEP binding to EI mutants (Table 3). The heat capacity change (ΔC_p) of the hinge motion was unusually small (60 ± 14 cal mol $^{-1}$ K $^{-1}$) considering the SASA changes associated with the motion (Fig. 6).^{27–29} This raises the possibility that the packing between EIN^{α} and $\text{EIN}^{\alpha\beta}$ in the A state is less compact than that commonly observed in proteins.²⁹ The swivel motion closes EIC on $\text{EIN}^{\alpha\beta}$ via the interface comprising 62 residues and burying ~ 1000 $\text{\AA}^2$ of SASA in the B state (Fig. 7). The interface features eighteen hydrogen bonds and six salt bridges as well as numerous hydrophobic interactions (Table 4). The interface shrinks to only nine residues and ~ 100 $\text{\AA}^2$ of SASA in the A state, which explains the large energetic preference for the swivel motion. We note that the phosphoryl group provides a critical interaction interface between EIC and $\text{EIN}^{\alpha\beta}$, forming six hydrogen bonds on its own and a dense interaction network in its vicinity. The isolated $\text{EIN}^{\alpha\beta}$ did not

Figure 5. Comparison of the domain–domain interfaces between EIN^{α} and $EIN^{\alpha\beta}$ in the A and B conformational states of EI. The domains were defined as follows: EIN^{α} (residue 33–143), and $EIN^{\alpha\beta}$ (residue 2–19, 148–230). The interfacial residues between EIN^{α} and $EIN^{\alpha\beta}$ were calculated using a PDBePISA program for the A state (PDB code 3EZA) and the B state (PDB code 2HWG). The EIN^{α} and $EIN^{\alpha\beta}$ domains are shown in a cartoon diagram without the hinge loop and the swivel helix. EIN^{α} is colored in cyan, and the $EIN^{\alpha\beta}$ domain in blue. The interfacial residues of EIN^{α} are shown with space-filling models in yellow, and those of $EIN^{\alpha\beta}$ in orange. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

bind to EIC in the absence of PEP, reiterating the importance of the phosphoryl group at the interaction interface.

Lastly, we examined the motions of phosphorylated EI based on the observation that oxalate caused the conformational switch between A and B states of phosphorylated EI.¹⁶ Oxalate is an inhibitor of PEP-utilizing enzymes and can inhibit the autophosphorylation of EI.³⁰ ΔG corresponding to the overall oxalate binding and domain motions in phosphorylated EI and $EI(\Delta EIN^{\alpha})$ are expressed in Eqs. (3) and (4):

$$\Delta G_{pEI:Oxa} = \Delta G_{EIC:Oxa} + \Delta G_{pEI_hinge} + \Delta G_{pEI_swivel}, \quad (3)$$

$$\Delta G_{pEI(\Delta EIN^{\alpha}):Oxa} = \Delta G_{EIC:Oxa} + \Delta G_{pEI_swivel}, \quad (4)$$

where $\Delta G_{pEI:Oxa}$ accounts for the overall oxalate binding and domain motions of phosphorylated EI, $\Delta G_{EIC:Oxa}$ for the binding between EIC and oxalate, ΔG_{pEI_hinge} for the hinge motion of phosphorylated EI, ΔG_{pEI_swivel} for the swivel motion of phosphorylated EI, and $\Delta G_{pEI(\Delta EIN^{\alpha}):Oxa}$ for the overall oxalate binding and domain motions of phosphorylated $EI(\Delta EIN^{\alpha})$. The K_D of phosphorylated EI and oxalate was measured to be $210 \pm 18 \mu M$, and the K_D of phosphorylated $EI(\Delta EIN^{\alpha})$ and oxalate was $34 \pm 4.4 \mu M$ (Table 1). $\Delta G_{pEI:Oxa}$ and $\Delta G_{pEI(\Delta EIN^{\alpha}):Oxa}$ were then $-5.0 \pm 0.1 \text{ kcal mol}^{-1}$ and $-6.1 \pm 0.1 \text{ kcal mol}^{-1}$, respectively, yielding the ΔG_{pEI_hinge} of $1.1 \pm 0.1 \text{ kcal mol}^{-1}$ by subtraction (Supporting Information Fig. S3). From the $\Delta G_{EIC:Oxa}$ of $-4.3 \pm 0.2 \text{ kcal mol}^{-1}$, ΔG_{pEI_swivel} was obtained as $-1.8 \pm 0.2 \text{ kcal mol}^{-1}$. We note that the free energy of the swivel motion decreased as much as $3.4 \text{ kcal mol}^{-1}$ in phosphorylated EI ($\Delta G_{pEI_swivel} = -1.8 \text{ kcal mol}^{-1}$) compared to that of the unphosphorylated EI ($\Delta G_{EI_swivel} = -5.2 \text{ kcal mol}^{-1}$). Electrostatic repulsion between negative charges on the phosphoryl group and oxalate could be responsible for the decrease as the charges are brought in close proximity in the B conformational states. Similar observation has been reported between EIN and HPr, where ΔG for the

Table 2. Summary of Domain–Domain Interfaces Between EIN^{α} and $EIN^{\alpha\beta}$ in the A and B Conformational States of EI

Conformational states Domains	A state				B state			
	EIN^{α} (33–143)		$EIN^{\alpha\beta}$ (2–19 + 148–230)		EIN^{α} (33–143)		$EIN^{\alpha\beta}$ (2–19 + 148–230)	
Number of residues								
Interface	15	14%	14	14%	4	4%	4	4%
Surface	110	99%	100	99%	105	95%	92	91%
Total	111	100%	101	100%	111	100%	101	100%
Solvent accessible surface area ($\text{\AA}^2$)								
Interface	490	6.3%	480	8.6%	90	1.3%	90	1.8%
Total	7,740	100%	5,630	100%	6,870	100%	4,970	100%
No. interdomain interactions								
Hydrogen bonds	None		None		None		None	
Salt bridges	None		None		None		None	

The domains were defined as follows: EIN^{α} (residue 33–143), and $EIN^{\alpha\beta}$ (residue 2–19, 148–230). The interfaces between EIN^{α} and $EIN^{\alpha\beta}$ were calculated for the A state (PDB code 3EZA) and the B state (PDB code 2HWG). The interfacial residues were calculated and analyzed using the program PDBePISA. The EIN^{α} and $EIN^{\alpha\beta}$ domains without the hinge loop and the swivel helix were used for the calculation of the domain–domain interfaces.

Table 3. Apparent Equilibrium Dissociation Constants (K_D), Free Energy Changes (ΔG), Enthalpy Changes (ΔH), and Entropy Changes (ΔS) for the PEP Binding and Domain Motions of EI(H189A) and EI(Δ EIN $^{\alpha}$,H189A) at Different Temperatures

Description	ΔC_p (cal mol $^{-1}$ K $^{-1}$)	Temperature ($^{\circ}$ C)	K_D (nM)	ΔG (kcal mol $^{-1}$)	ΔH (kcal mol $^{-1}$)	ΔS (cal mol $^{-1}$ K $^{-1}$)
EI(H189A) + PEP	-520 ± 8.4	10	1040 ± 90	-7.7 ± 0.2	11.5 ± 0.2	67.8 ± 1.0
		15	600 ± 110	-8.2 ± 0.3	8.7 ± 0.2	58.6 ± 1.2
		20	390 ± 110	-8.6 ± 0.3	6.2 ± 0.2	50.6 ± 1.2
		25	430 ± 120	-8.7 ± 0.2	3.6 ± 0.1	41.2 ± 0.7
EI(Δ EIN $^{\alpha}$, H189A) + PEP	-590 ± 7.7	10	93 ± 30	-9.1 ± 0.4	15.2 ± 0.3	85.8 ± 1.7
		15	110 ± 37	-9.2 ± 0.4	12.3 ± 0.3	74.6 ± 1.7
		20	31 ± 18	-10.1 ± 0.3	9.2 ± 0.2	65.6 ± 1.2
		25	37 ± 12	-10.1 ± 0.0	6.5 ± 0.1	55.6 ± 0.7
Hinge motion	60 ± 14	10		1.4 ± 0.4	-3.7 ± 0.4	-18.0 ± 2.0
		15		1.0 ± 0.5	-3.6 ± 0.4	-16.0 ± 2.1
		20		1.5 ± 0.4	-3.0 ± 0.3	-15.0 ± 1.7
		25		1.4 ± 0.2	-2.9 ± 0.1	-14.4 ± 1.0

binding decreased by 2.3 kcal mol $^{-1}$ when phosphorylated EIN and HPr associated to make a biphosphorylated complex.³¹ On the other hand, the free energy change of the hinge motion was remarkably close when $\Delta G_{\text{pEI-hinge}}$ (1.1 kcal mol $^{-1}$) was compared with the $\Delta G_{\text{EI-hinge}}$ (1.5 kcal mol $^{-1}$). The free energy changes of motions obtained from phosphorylated EI bear biological relevance. Upon phosphorylation of EI, the product pyruvate on EIC is replaced by excess cellular PEP. When phosphorylated EI binds to PEP, the $A \rightarrow B$ state conformational transition is less likely owing to the electrostatic repulsion. Once EI transfers its phosphoryl group to HPr, unphosphorylated EI can immediately switch to the B state conformation to continue the phosphoryl transfer cascade reactions. We suppose that the conformational transition between A and B states is regulated under fine control of ligand binding and protein phosphorylation.

It is well known that conformational changes coupled with ligand binding are closely related to the protein allostery.³² Thermodynamic data as well as kinetic and structural information have been accu-

mulated to improve on the model for allostery.^{33,34} Uncoupling thermodynamics of binding and motions by rational mutant design can be useful to probe possible intermediate states during the allosteric response. Further, enthalpic and entropic contributions obtained by calorimetric measurement may provide better understanding of the driving forces behind the motions.

Figure 6. (A) Temperature dependence of ΔH for PEP binding and motions of EI(H189A) ($\circ$), EI(Δ EIN $^{\alpha}$,H189A) ($\square$), and the hinge motion of $C \rightarrow D$ state transition ($\diamond$). (B) Bar representation of heat capacity changes upon EI interaction with PEP.

Figure 7. Comparison of the domain–domain interfaces between phosphorylated EIN $^{\alpha\beta}$ and EIC in the B conformational state of EI. The domains were defined as follows: EIN $^{\alpha\beta}$ (residue 2–19, 148–230), and EIC (residue 260–575). The interfaces between EIN $^{\alpha\beta}$ and EIC were defined from the calculation using a PDBEPIA program for the A state (PDB code 2KX9) and the B state (PDB code 2HWG). The EIN $^{\alpha\beta}$ and EIC domains are shown in a cartoon diagram without the hinge loop and the swivel helix. EIN $^{\alpha\beta}$ is colored in blue, and the EIC domain in red. The interfacial residues of EIN $^{\alpha\beta}$ are shown with space-filling models in orange, and those of EIC in pink. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 4. Summary of domain–domain interfaces between $EIN^{\alpha\beta}$ and EIC in the A state, and between phosphorylated $EIN^{\alpha\beta}$ and EIC in the B state of EI

Conformational states Domains	A state				B state			
	$EIN^{\alpha\beta}$ (2–19 + 148–230)		EIC (260–573)		p $EIN^{\alpha\beta}$ (2–19 + 148–230)		EIC (260–573)	
Number of residues								
Interface	4	4%	5	2%	26	26%	36	12%
Surface	110	99%	289	92%	92	91%	273	87%
Total	111	100%	314	100%	101	100%	314	100%
Solvent accessible surface area ($\text{\AA}^2$)								
Interface	100	1.7%	100	0.7%	1020	20.5%	920	6.9%
Total	5,830	100%	14,250	100%	4,970	100%	13,280	100%
No. interdomain interactions								
Hydrogen bonds	None		None		18		18	
Salt bridges	None		None		6		6	

The domains were defined as follows: $EIN^{\alpha\beta}$ (residue 2–19, 148–230), and EIC (residue 260–573). The interfaces between $EIN^{\alpha\beta}$ and EIC were calculated for the A state (PDB code 2KX9) and the B state (PDB code 2HWG). The interfacial residues were calculated and analyzed using the program PDBEPIA. The $EIN^{\alpha\beta}$ and EIC domains without the swivel helix were used for the calculation of the domain–domain interfaces.

In summary, the calorimetry combined with selected domain-deletion mutants proved a useful strategy to dissect the thermodynamics of coupled binding and motions. Using this strategy, we demonstrated how the hinge and swivel motions are thermodynamically connected in EI in a quantitative manner. Our strategy can be generally applied to uncoupling the thermodynamic contributions of the binding and motions in multidomain proteins. The thermodynamic information about protein motions would complement the structural and dynamics information toward a better understanding of the energetics and driving forces of the motions.

Materials and Methods

Cloning, protein expression, and purification

The domain-deletion mutants of EI were cloned into a pET11a vector with an N-terminal His₆ tag. The plasmids were introduced into *Escherichia coli* strain BL21star(DE3) (Novagen), and the transformant was grown in either Luria Bertini or minimal media with $^{15}\text{NH}_4\text{Cl}$ and/or $^{13}\text{C}_6$ -glucose as the sole nitrogen or carbon sources. The culture was induced at an A_{600} of ~ 0.8 by the addition of 1 mM isopropyl- β -D-thiogalactopyranoside, and harvested by centrifugation after 4 h of induction. The cell pellet was resuspended in 50 mL (per liter of culture) of 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of protease cocktail inhibitor (Sigma-Aldrich, S8830 SIGMAFAST). The suspension was lysed by three passages through Emulsiflex (Avestin, Canada) after homogenizing and was centrifuged at 24,000g for 20 min at 4°C. The supernatant fraction was filtered and loaded onto a HisTrap column (GE Healthcare), and the proteins were eluted with a gradient of 500 mM imidazole. The

fractions containing the proteins were purified by gel filtration on a superdex200 column (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 2 mM β -mercaptoethanol and were then further purified by monoQ anion exchange column (8 mL; GE Healthcare) with a 160 mL gradient of 1M NaCl. $EIN^{\alpha\beta}$ (H189A) without a tag was purified by resuspending the cell pellet and lysing in 50 mL (per liter of culture) of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The supernatant fraction was loaded onto a DEAE column (GE Healthcare), and the proteins were eluted with a gradient of 1M NaCl. The fractions containing the proteins were purified by gel filtration on a superdex75 column (GE Healthcare) equilibrated 20 mM Tris-HCl, pH 7.4, 200 mM NaCl and were further purified on a monoQ column using 20 mM Tris-HCl, pH 7.4, with a gradient of 1M NaCl. All proteins were dialyzed against 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM β -mercaptoethanol, and 4 mM MgCl_2 (Buffer A) for the ITC and NMR measurements.

Isothermal titration calorimetry

Titration were performed using the ITC₂₀₀ microcalorimeter (GE Healthcare) at 25°C. For the PEP titration, 300 μM PEP was titrated into 30 μM EI(H189A) or EI(ΔEIN^{α} ,H189A) in Buffer A. For the oxalate titration, 10 mM oxalate was titrated into 300 μM EI or EI(ΔEIN^{α}) with 10 mM PEP in Buffer A. For the binding between isolated $EIN^{\alpha\beta}$ and isolated EIC, 3.5 mM EIC was titrated into 400 μM $EIN^{\alpha\beta}$ (H189A) with 10 mM PEP in Buffer A. Temperature dependence of the PEP interaction was measured for EI(H189A) or EI(ΔEIN^{α} , H189A) at 10, 15, 20, and 25°C. Twenty consecutive 2 μL aliquots of ligands or proteins were titrated into the cell. The duration of each injection was 4 s, and injections

were made at intervals of 150 s. The heats associated with the dilution of the substrates were subtracted from the measured heats of binding. ITC titration data were analyzed with the Origin version 7.0 program provided by MicroCal.

NMR spectroscopy

NMR spectra were recorded at 25°C on Bruker Avance 600 MHz and 900 MHz spectrometers equipped with *x,y,z*-shielded or *z*-shielded gradient triple resonance probes. To examine the binding interface of isolated EIN^{αβ} toward isolated EIC, two-dimensional ¹H-¹⁵N heteronuclear single quantum correlation spectra of the 0.3 mM ¹⁵N-labeled EIN^{αβ}(H189A) were obtained titrating with unlabeled EIC in the presence and absence of 10 mM PEP in Buffer A. The backbone assignment of EIN^{αβ}(H189A) was carried out using the three-dimensional triple resonance through-bond scalar correlation CBCACONH and HNCACB experiments. NMR spectra were processed using the NMRPipe³⁵ program and analyzed using the PIPP³⁶ and the NMRView³⁷ programs.

Calculation of the molecular interfaces

The domain interfaces were calculated using the PDBePISA program (<http://pdbe.org/pisa>).³⁸ The interfaces between EIN^α and EIN^{αβ} were calculated using the structures of the A state (PDB code 3EZA) and the B state (PDB code 2HWG). The interfaces between EIN^{αβ} and EIC were calculated using the structures of the A state (PDB code 2KX9) and the B state (PDB code 2HWG). EIN^α (33–143), EIN^{αβ} (2–19,148–230), and EIC (260–573) were used in the calculation to compare the same set of residues from different coordinates. The presence or absence of hydrogen atoms did not affect the calculated solvent accessible surface areas (SASA), allowing for a fair comparison of SASA between the NMR structure and the crystal structure.

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