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On the conformational stability and dimerization of phosphotransferase enzyme I from *Escherichia coli*

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Abstract

The activity of enzyme I (EI), the first protein in the bacterial PEP:sugar phosphotransferase system, is regulated by a monomer–dimer equilibrium where a Mg^{2+}-dependent autophosphorylation by PEP requires the homodimer. Using inactive EI(H189A), in which alanine is substituted for the active-site His189, substrate binding effects can be separated from those of phosphorylation. Whereas 1 mM PEP (with 2 mM Mg^{2+}) strongly promotes dimerization of EI(H189A) at pH 7.5 and 20 °C, 5 mM pyruvate (with 2 mM Mg^{2+}) has the opposite effect. A correlation between the coupling of N- and C-terminal domain unfolding, measured by differential scanning calorimetry, and the dimerization constant for EI, determined by sedimentation equilibrium, is observed. That is, when the coupling between N- and C-terminal domain unfolding produced by 0.2 or 1.0 mM PEP and 2 mM Mg^{2+} is inhibited by 5 mM pyruvate, the dimerization constant for EI(H189A) decreases from $>10^8$ to $<5 \times 10^5$ or $3 \times 10^7\ M^{-1}$, respectively. With 2 mM Mg^{2+} at 15–25 °C and pH 7.5, PEP has been found to bind to one site/monomer of EI(H189A) with $K'_A \cong 10^6\ M^{-1}$ ($\Delta G' = -33.7 \pm 0.2\ kJ\ mol^{-1}$ and $\Delta H = +16.3\ kJ\ mol^{-1}$ at 20 °C with $\Delta C_p = -1.4\ kJ\ K^{-1}\ mol^{-1}$). The binding of PEP to EI(H189A) is synergistic with that of Mg^{2+}. Thus, physiological concentrations of PEP and Mg^{2+} increase, whereas pyruvate and Mg^{2+} decrease the amount of dimeric, active, dephospho-enzyme I.

Keywords: Enzyme I; *E. coli* phosphoenolpyruvate:sugar phosphotransferase system; Active-site mutant [EI(H189A)]; Phosphoenolpyruvate; Pyruvate; Mg^{2+}; Differential scanning calorimetry; Trp fluorescence; Sedimentation equilibrium; Dimerization; Isothermal titration calorimetry

1. Introduction

The bacterial phosphoenolpyruvate (PEP):sugar phosphotransferase system is composed of two cytosolic, sugar non-specific, proteins (enzyme I and HPr) and many sugar-specific components (enzymes II). PEP is the phosphoryl donor in a Mg^{2+}-dependent autophosphorylation of enzyme I on the N3 atom of His189 and pyruvate is a product of the reaction [1]. Phospho-enzyme I reversibly transfers its phosphoryl group to the N1 atom of His15 of HPr [1]. Phosphorylated HPr can donate its phosphoryl group to sugar-specific enzymes II, which ultimately phosphorylate and translocate various sugars across the membrane [2].

Chauvin et al. [3] have proposed that regulation of the PTS involves a monomer–dimer equilibrium of enzyme I, since only the dimer form of enzyme I is active in the Mg^{2+}-dependent autophosphorylation by PEP [4–8]. PEP binds to the C-terminal domain of enzyme I [9,10] and this domain contains the contacts necessary for the dimerization of EI [10,11]. The availability of the inactive, active-site mutant EI(H189A) [12] has allowed separation of the effects of PEP and Mg^{2+} binding on the dimerization of enzyme I from those of phosphorylation. Previously, 1 mM PEP in the presence of 2 mM Mg^{2+} has been shown to strongly pro-

Abbreviations: PEP, phosphoenolpyruvate; Pyr, pyruvate; PTS, phosphoenolpyruvate:sugar phosphotransferase system; EI, enzyme I (575 amino acid residues) of the PTS; EI(H189A), EI in which the active-site His189 has been replaced by alanine; HPr, histidine-containing phosphocarrier protein (∼9 kDa); ME, 2-mercaptoethanol; Hepes, *N*-(*2*-hydroxyethyl)piperazine-*N*′-2-ethane-sulfonic acid; Trp fluorescence, intrinsic tryptophanyl residue fluorescence; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; $K_{1,2}$, dimerization constant expressed per mol monomer of EI

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mote dimerizaton of EI(H189A) and coupling between the N- and C-terminal domains in unfolding studies [13].Here, we summarize recent results from studies by Dimitrova et al. [14] on the opposing effects of PEP and pyruvate alone (with 2 mM Mg^{2+}) and in mixtures of 0.2–1.0 mM PEP and 5 mM pyruvate (physiological concentrations [15,16], with 2 mM Mg^{2+} present) on both the dimerization constant and domain coupling of enzyme I.

2. Experimental

2.1. Protein and other materials

Mutageneses of the active-site His189 of enzyme I (EI) to Ala or Glu has been described as have the expression and purification of wild type EI, and EI(H189A) [12,17]. Purified proteins were homogeneous in SDS-PAGE [18] and were stored at −80 °C in the 10 mM Tris–HCl/100 mM NaCl, pH 7.5 buffer used for purification. For the present studies, proteins were dialyzed overnight at 4 °C against several changes of 20 mM potassium phosphate (buffer A) containing 100 mM KCl ± 2 mM ME (pH 7.5) using Slide-A-Lyzer Cassettes [10,000 MW cutoff; Pierce, Rockford, IL]. For ultracentrifugation and fluorescence measurements, buffers contained 2 mM ME. For DSC scans, proteins were equilibrated against the same degassed buffer without added ME. For equilibration of proteins with effectors present, gel filtration through a PD-10 [G25M Sephadex; Pharmacia, Uppsala, Sweden] column at room temperature was used. Before each measurement, the protein spectrum and concentration were determined spectrophotometrically using the specific absorption coefficient $A_{280\,nm,1\,cm} = 0.40\,cm^2/mg$ determined by Waygood [19].

Phosphoenolpyruvate (Roche), pyruvate (Sigma), and other commercially-available chemicals were of the highest purity available. All solutions were prepared with deionized and filtered water from a Milli-Q Plus system.

2.2. Differential scanning calorimetry

DSC measurements were performed with the VP-DSC calorimeter of Plotnikov et al. [20] from MicroCal, Inc. (Northampton, MA) without feedback and calibrated as previously described [21]. Samples were pre-equilibrated at 15 °C before the first scan and between scans for 45–60 min. A scan rate of 30 K h^{-1} was used in all the experiments, since this was found to be a good compromise between scan-rate independence and minimizing the time that proteins stay at high temperatures [17]. DSC data were corrected for instrument baselines (determined by buffer versus buffer scans) and normalized for scan rate and protein concentration (∼3–5 μmol enzyme I subunit). For experiments involving effectors, scans were conducted with the effectors added to the buffer in the reference cell. Data conversion and analysis were performed using Origin software (MicroCal, Inc.). Excess heat capacity (C_p) is expressed in kJ K^{-1} (mol monomer)$^{-1}$ where 1.000 cal = 4.184 J.

2.3. Isothermal titration calorimetry

The VP-ITC microcalorimeter (MicroCal, Inc., Northampton, MA) was used as recommended by the manufacturer. Titrations were performed at 15, 20, and 25 °C. In experiments, 20 consecutive 5 μL aliquots of 0.3 mM PEP in buffer A with 2 mM Mg^{2+} were injected into the cell (1.4061 mL) containing 3–6 μmol EI(H189A) subunit in buffer A + 2 mM Mg^{2+}. The duration of each injection was proportional to the volume added (2 s/μL injectant) and injections were made at intervals of 10 min. The protein solution was maintained at constant stirring speed (300 rpm) to ensure proper mixing after each injection of PEP. A first injection of <10% of later injection volumes was made for each titration to avoid artifacts arising from bubbles and/or diffusion from the syringe tip during temperature equilibration. Dilution heats of PEP into the protein solution (obtained at the end of titrations) were subtracted from observed heats. Titrations of EI with 10 mM pyruvate in buffer A + 2 mM Mg^{2+} were conducted at 20 °C as described above. ITC titration data were analyzed with the Origin version 5.0 program provided by MicroCal Inc.

2.4. Fluorescence spectroscopy

Fluorescence measurements were performed in a SLM Aminco-Bowman Series 2 spectrofluorometer as previously described [14] with excitation at 295 nm and emission measured at 342 nm, the intensity maximum for folded EI forms. Temperature was controlled by a programmable Neslab RTE-111 water bath using water-jacketed fluorescence cuvettes (1 mL, 1 cm path length). The sample cuvette temperature was monitored directly by inserting a micro-thermocouple (Omega Inc., Stamford, CT). Progress curves for Trp exposure were analyzed by the two-state thermodynamic analysis program of Kirchhoff [22].

2.5. Analytical ultracentrifugation

Beckman Optima Models XL-A and XL-I analytical ultracentrifuges equipped with absorption optics and four-place AN-Ti rotors were used for sedimentation equilibrium experiments at 20 and 4 °C as previously described [14]. Cells with carbon filled 6-channel centerpieces (12 mm) and plane quartz windows were used. Proteins were loaded into the right side of each channel: 0.080 mL of 0.33–0.40 (inner), 0.56–0.63 (middle), and 0.81–0.94 mg/mL (outer) versus 0.095 mL of reference dialysate buffer in left channels. At the beginning of each run, scans at 280 nm were made in the continuous mode (0.003 cm steps) with three averages at 3000 rpm in order to establish solvent and protein meniscii, plateau absorbances at 280 nm, and bottom radial positions for each channel. After rotors were accelerated to 10,000 rpm

at 20.0 or 4.0 °C, auto scans at 2 h intervals were made in 0.001 cm steps (step mode) with 7–13 averages for a total of 52 h before ending centrifuge runs. Generally, identical samples of freshly dialyzed or gel-filtered proteins were loaded into two cells and run at the same time in the two ultracentrifuges at 20.0 and 4.0 °C. Data obtained at 4 °C are not reported here since low temperature favors the monomer of EI or EI(H189A), but the data collected at 4 °C is useful for detecting any aggregation [13]. Densities of solvents were determined with the Anton-Paar Model DMA-58 densitometer at 20.00 ± 0.01 °C: buffer A, 1.0079 g/mL. Protein specific volumes were calculated to be 0.725 mL/g for EI and EI(H189A) from the amino acid compositions derived from DNA sequences and the values of Zamyatnin [23].

Global, weighted fits of sedimentation equilibrium data obtained at three concentrations of protein to a model of reversible monomer ⇄ dimer association (with fully competent species present) were made using software provided by Allen P. Minton (NIDDK/NIH). Baselines were held constant at zero absorbance at 280 nm (as pre-determined by buffer–buffer runs). Trimers and tetramers of enzyme I were not detectable. This analysis is extremely sensitive to cases in which chemical equilibrium of proteins with effectors was not achieved and in these cases the data could not be analyzed. For all accepted data, residuals from fits of data to a monomer–dimer equilibrium were randomly distributed around zero (0 ± 0.01 absorbance). For conversion of 280 nm absorbance values of apparent dimerization constants (K_{obs}) to the true concentration-dependent association constants, $K'_{1,2}$ values (expressed per molar concentrations of monomer), it is assumed that the extinction coefficient of the monomer does not change upon dimerization and that the specific absorbance coefficient at 280 nm (A) is 0.40 cm^2/mg [19]. Thus, $K_{obs} = A_2/A^2 = (2/\epsilon)(K_{1,2})$, where A, the specific absorbance of the monomer, and the molar extinction coefficient (ϵ) are for a 1.2 cm path length. A monomer M_r value of 63,500 for EI(H189A) is used to calculate, resulting in the equation: $\log K'_{1,2} = 4.183 + \log K_{obs}$.

3. Results and discussion

3.1. Thermal unfolding of EI(H189A) in the presence of substrates

Progress curves for intrinsic tryptophanyl residue fluorescence changes of EI(H189A) as a function of increasing temperature are shown in Fig. 1. Decreases in Trp fluorescence reflect C-terminal domain unfolding [17]. EI(H189A) with 2 mM $MgCl_2$ has been heated at a rate of 30 K h^{-1} in the presence of (Fig. 1, left to right) 5 mM pyruvate, 5 mM pyruvate and 0.2 mM PEP, 5 mM pyruvate and 1.0 mM PEP, or 1.0 mM PEP. There is a good fit of each data set to a two-state model of unfolding. Nevertheless, the thermal unfolding curve of EI(H189A) with 2 mM Mg^{2+} and 5 mM pyruvate indicates that aggregation occurs at >52 °C, which

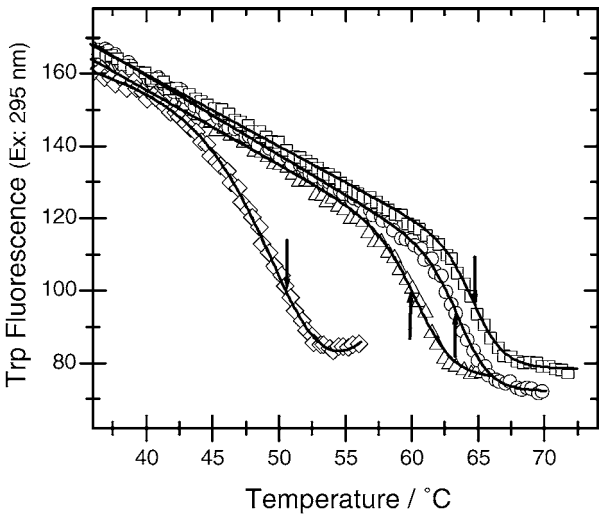

Fig. 1. Thermally induced changes in the Trp fluorescence of EI(H189A) in buffer A with 2 mM ME and added effectors at 30 K h^{-1} scan rate. Excitation was at 295 nm and emission was monitored at 342 nm. Added effectors in the presence of 2 mM Mg^{2+} are 5 mM pyruvate (◇), 5 mM pyruvate and 0.2 mM PEP (△), 5 mM pyruvate and 1 mM PEP (○), or 1 mM PEP (□). Transition temperatures are indicated by vertical arrows and the solid line for each data set shows the fit to a two-state unfolding model.

is not observed when PEP is present with pyruvate. The addition of 5 mM pyruvate to solutions containing 1.0 mM PEP and 2 mM Mg^{2+} counteracts the stabilizing effect of 1.0 mM PEP on the C-terminal domain, reducing the transition temperature (t_m) from ∼65 to 63 °C. When the concentration of PEP is decreased to 0.2 mM in the presence of 5 mM pyruvate and 2 mM Mg^{2+}, t_m is 60 °C for C-terminal domain unfolding. With 5 mM pyruvate and 2 mM Mg^{2+} alone, t_m is ∼50.5 °C, after adjusting the amplitude change.

The results shown in Fig. 2 indicate that the conformational stability of enzyme I is profoundly influenced by intracellular concentrations of pyruvate and PEP. When EI(H189A) is scanned by DSC in the presence of 1 mM PEP and 2 mM Mg^{2+} at pH 7.5, a single endotherm centered at ∼63 °C is observed. Both C- and N-terminal domains, which unfold at ∼63 °C, are considerably stabilized under these conditions [13]. The DSC profile in the presence of 5 mM pyruvate with 2 mM Mg^{2+} has a single, broad endotherm centered at ∼53 °C (Fig. 2). In this case, the C-terminal domain is stabilized by ∼4 °C, whereas the N-terminal domain is destabilized ∼1 °C, as determined by deconvolution assuming an independent two, two-state unfolding model [14]. The DSC profile of EI(H189A) with 5 mM pyruvate, 1 mM PEP, and 2 mM Mg^{2+} exhibits endotherms at T_{max} 54 and ∼63 °C, indicating that a partial uncoupling of C- and N-terminal domain unfolding occurs when 5 mM pyruvate is present [14]. When the concentration of PEP is decreased to 0.2 mM in the presence of 5 mM pyruvate and 2 mM Mg^{2+}, endotherms centered at 55 and 60 °C are observed. Since the binding of PEP is synergistic with that of Mg^{2+}, intracellular concentration of Mg^{2+} also

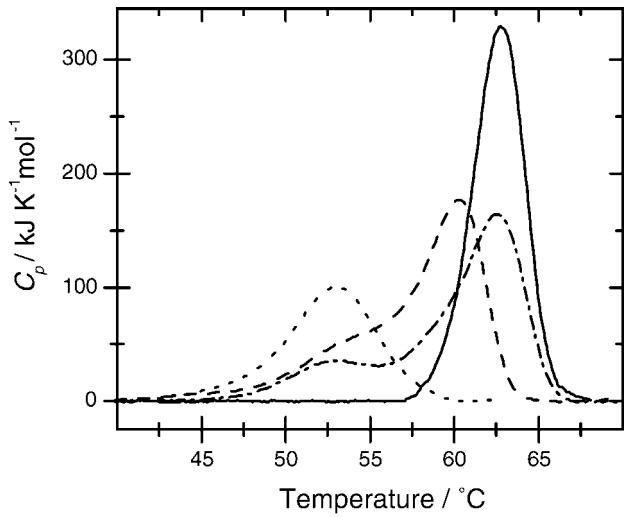

Fig. 2. DSC scans of EI(H189A) obtained in buffer A with 2 mM Mg^{2+} and the following effectors: 5 mM pyruvate (short dashes); 5 mM pyruvate and 0.2 mM PEP (long dashes), 5 mM pyruvate and 1 mM PEP (dash-dot-dash), or 1 mM PEP (solid line). DSC data are shown after instrument baseline subtraction and normalization for protein concentration and scan rate. Each scan is additionally corrected for a protein linear baseline.

influences the conformational stability of enzyme I in the presence of PEP (see below).

The midpoints of endotherms in Fig. 2 should be compared to the transition temperatures obtained for C-terminal domain unfolding of EI(H189A) shown in Fig. 1 under the same conditions. The shoulders centered at about 55 °C in mixtures of 5 mM pyruvate, 0.2 or 1 mM PEP, and 2 mM Mg^{2+} represent uncoupled N-terminal domain unfolding since these transitions are not observed when thermally induced changes in Trp fluorescence are monitored. The high-temperature endotherms of Fig. 2 observed for mixtures of PEP and pyruvate and PEP alone in the presence of 2 mM Mg^{2+} represent unfolding of coupled N- and C-terminal domains of EI(H189A). Deconvolutions (assuming a dependent, sequential model) of high-temperature endotherms observed for EI(H189A) with mixtures of 5 mM pyruvate, PEP, and Mg^{2+} give two transitions with one having a t_m value approximately corresponding to C-terminal domain unfolding [14]. Areas of endotherms in Fig. 2 (ΔH values) have little meaning and are not reported. In the absence of effectors, calorimetric and van't Hoff unfolding enthalpies have been reported for dephospho-EI [17] and EI(H189A) [13].

A protein concentration-dependence of dephospho-EI is not observed for DSC profiles in the absence of ligands even though a monomer–dimer equilibrium exists at 20 °C. Specifically, if dephospho-EI initially contains 30 or 60% dimer at 20 °C in DSC scans, the same thermodynamic parameters for reversible unfolding are measured (Table 3 of Dimitrova et al. [13]). In this case, the unfolding of C- and N-terminal domains are loosely coupled and the shape of the DSC endotherm and enthalpy for unfolding the N-terminal domain is similar to those of the isolated N-terminal domain, which is monomeric [17]. When ligand binding strongly promotes protein dimerization (see Table 2 below), the unfolding of C- and N-domains are tightly coupled with transition temperature increases of ~18 and 6–8 °C, respectively. These observations suggest that in the absence of ligands, dephospho-EI or EI(H189A) dissociates to monomers prior to or during thermally induced unfolding of the C-terminal domain. In contrast, ligands that preferentially bind to the dimer apparently maintain the dimeric form of dephospho-EI or EI(H189A) until thermal unfolding causes ligand dissociation. High-temperature endotherms in the latter case show no evidence of dimer dissociation.

Stabilizing effects of ligands on the C-terminal domain of enzyme I occur through the binding energy [24,25]. Thus, the effect of a ligand (L) on the transition temperature for a two-state unfolding of a macromolecular domain (with $T_c > T_0$) is directly related to the affinity constant for the ligand. Assuming that the ligand binds to a single site of each subunit and does not bind to unfolded C-terminal domains of EI(H189A), Eq. (17) of Brandts et al. [25] can be applied:

$$\ln(1 + K[\mathrm{L}]) = \left(\frac{\Delta H_c}{R}\right)\left(\frac{1}{T_0} - \frac{1}{T_c}\right) - \frac{\Delta C_p}{R}\left(\ln\left[\frac{T_0}{T_c}\right] + \frac{T_c}{T_0} - 1\right) \quad (1)$$

where T_0 and T_c in Kelvin are the transition midpoints in the absence and presence of ligand L, respectively; [L] the free concentration of ligand, R the gas constant, ΔC_p the heat capacity change for unfolding (assumed to be temperature-independent), which in this case is assumed to be 13.8 kJ K^{-1} mol^{-1} or the same as that measured for the amino terminal domain in buffer A [12] and ΔH_c (the enthalpy change at T_c) is calculated from

$$\Delta H_c = \Delta H_0 + \Delta C_p(T_c - T_0) \quad (2)$$

where ΔH_0 is the unfolding enthalpy in the absence of ligand. The free concentration of ligand is obtained from reiterative fitting taking into account the total ligand and protein concentrations. Eq. (7) of Brandts and Lin [26], in which the ΔH_0 value is used, yields the same K'_A value.

Table 1 summarizes calculated association constants at T_m from Eq. (1) using calculated ΔH_c values from Eq. (2) and the above assumptions. The unfolding enthalpy for EI(H189A) in the absence of ligands has been determined by DSC [13]. Generally, the observed ΔH values obtained from fitting data to a two-state unfolding model for the protein with ligands are less reliable due to variations in baseline fitting and tend to be somewhat higher than those calculated from Eq. (2). The K'_A value for pyruvate binding to EI(H189A) in the presence of 2 mM Mg^{2+} (Table 1) is in excellent agreement with the K_i value of 2 mM reported by Saier et al. [5]. In this case, K'_A is temperature-independent since the enthalpy for binding pyruvate is ~0 (see below). The binding of PEP stabilizes the C-terminal domain to

Table 1
Calculation of association constants at transition temperatures for EI(H189A) binding ligands from DSC and fluorescence measurements[a]

EI(H189A) μM subunit	Ligands (mM)	ΔH_{obs}[b] (kJ mol^{-1})	ΔH_c[c] (kJ mol^{-1})	T_m[b] (K)	K'_A at T_m (M^{-1})
3.15	–	188	–	318.6	–
4.09	1 PEP	293	251	323.1	2.2×10^3
3.31	5 Pyr/2 Mg^{2+}	347	255	323.4	4.9×10^2
3.46	1 PEP/2 Mg^{2+}	469	441	336.9	6.1×10^5

[a] Measurements are for EI(H189A) ± effectors in 20 mM K-phosphate and 100 mM KCl (pH 7.5) with 30 K h^{-1} scan rates. Results are from Dimitrova et al. [13,14].
[b] Enthalpy changes at T_m (ΔH_{obs}) and transition temperatures (T_m) are averages of 2–4 separate determinations from two, two-state analysis of DSC data in the absence of effectors when two endotherms are resolved and from two-state analyses of Trp fluorescence changes as a function of increasing temperature for C-terminal domain unfolding [17].
[c] Values of ΔH_c are calculated from Eq. (2).

Table 2
Substrate effects on dimerization constants for the active-site mutant EI(H189A): sedimentation equilibrium studies[a]

EI(H189A) (±mM effectors)	log $K'_{1,2}$ ((M monomer)$^{-1}$), 20 °C
No additions	5.49 ± 0.04
Hepes substituted for phosphate in buffer	5.76 ± 0.12
(+2 Mg^{2+})	5.30 ± 0.08
(+5 Pyr/2 Mg^{2+})	4.16 ± 0.11
(+5 Pyr/0.20 PEP/2 Mg^{2+})	5.67 ± 0.08
(+5 Pyr/1 PEP/2 Mg^{2+})	7.4 ± 0.5
(+1 PEP/2 Mg^{2+})	>8

[a] Buffer (±effectors) for equilibration of proteins prior to and during analytical ultracentrifugation is 20 mM potassium phosphate, 100 mM KCl, and 2 mM ME, pH 7.5, except when 20 mM Hepes/KOH is substituted for phosphate. Values of the dimerization constant expressed per M monomer (log K'_A) are averaged from global analysis of 6–9 data files (each with 3 concentrations of protein).

a much greater extent than does pyruvate due to the free energy difference between binding PEP and pyruvate to EI(H189A) in the presence of 2 mM Mg^{2+}; ($\Delta\Delta G' \sim -20.5$ kJ mol^{-1}).

Synergism between the binding of Mg^{2+} and PEP has been observed before [13]. In the absence of Mg^{2+}, the affinity constant of EI(H189A) for PEP is 2.2×10^3 M^{-1} at 49.9 °C (Table 1). The presence of 2 mM Mg^{2+} therefore increases the affinity constant of EI(H189A) for PEP more than 280-fold. From the T_m values of EI(H189A) in the presence of 0.2 mM PEP and 2 or 10 mM Mg^{2+} (63.3 or 64.4 °C), the calculated association constant for PEP is increased ~1.6-fold at ~64 °C by increasing Mg^{2+} concentrations from 2 to 10 mM [14]. These results indicate that 2 mM Mg^{2+} is nearly saturating.

3.2. Substrate effects on the dimerization of EI(H189A)

Table 2 gives dimerization constants (expressed as log $K'_{1,2}$ per M monomer) determined by sedimentation equilibrium at 20 °C and 10,000 rpm for EI(H189A) in the absence and presence of substrates. Values of log $K'_{1,2}$ are averages from global analyses of 6–9 data files (each having 3 concentrations of protein) after 26–44 h at 10,000 rpm when equilibrium has been reached. Representative sedimentation equilibrium data and fitting procedures for EI(H189A) in the absence of substrates are given in Dimitrova et al. [13].

For our studies on the effects of binding PEP, the availability of the inactive, active-site mutant EI(H189A) [12] has allowed a separation of binding effects from those of phosphorylation [13]. Moreover, the conformational stability of EI(H189A) is similar to that of nonphosphorylated enzyme I. Whereas the addition of Mg^{2+} to EI(H189A) has little effect on $K'_{1,2}$, the addition of 5 mM pyruvate with 2 mM Mg^{2+} decreases the dimerization constant by a factor of ~14 (Table 2). In contrast, essentially complete dimer formation is promoted by 1 mM PEP in the presence of 2 mM Mg^{2+} due to preferential binding of PEP to the dimer, and under these conditions both N- and C-domains are markedly stabilized (Fig. 2). The dimerization constant in this case is given in Table 2 as log $K'_{1,2} > 10^8$ M^{-1} which simply represents the limits in the sensitivity of absorbance measurements in sedimentation equilibrium scans at 280 nm. The addition of 5 mM pyruvate to EI(H189A) in the presence of ≤1 mM PEP and 2 mM Mg^{2+} substantially decreases the dimerization constant of EI(H189A). Under the latter conditions, destabilization and uncoupling of N- and C-terminal domain unfolding occurs (Fig. 2).

Overall, the results presented in Table 2 suggest that there is a strong correlation between the coupling of C- and N-terminal domain unfolding observed in DSC measurements (Fig. 2) and the dimerization constant of enzyme I. That is, the conformational stability of EI(H189A) is increased by 1 mM PEP and 2 mM Mg^{2+}, which strongly promote dimerization. The further addition of 5 mM pyruvate destabilizes N- and C-terminal domains of some of the protein population (Fig. 2), presumably by displacing PEP from some dimers, and decreases the average dimerization constant (Table 2).

3.3. Isothermal titration calorimetry

EI(H189A) in the presence of 2 mM Mg^{2+} has been titrated with PEP in buffer A with 2 mM Mg^{2+} at 20.0 °C in ITC experiments and a representative ITC experiment is shown in Fig. 3. From duplicate titrations, an average

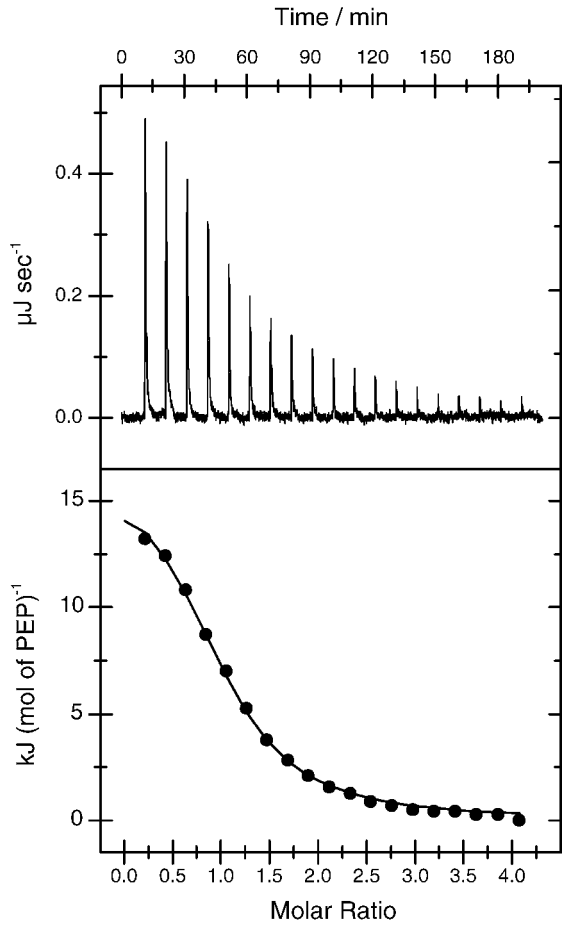

Fig. 3. A representative titration of EI(H189A) with PEP in buffer A + 2 mM Mg^{2+} at 20 °C. The concentration of the protein in the cell is 5.16 μM subunit (0.33 mg/mL) and the PEP concentration in the syringe is 0.3 mM. The upper panel shows the observed heats for the 2nd through 20th injection of 5 μL PEP at 10 min intervals after baseline correction. Heats of dilution of PEP are obtained from injections 19–20 and subtracted from the data for injections 2–18. The lower panel shows the corrected, normalized binding enthalpies vs. PEP/EI(H189A) molar ratio, which reflects the fact that heat is absorbed in this binding process. The data (●) are fitted to a one-site binding model (thin solid line) which give $K'_A = 9.3 \pm 1.1 \times 10^5\ M^{-1}$, $\Delta H = 17.2 \pm 0.8\ kJ\ mol^{-1}$, and $n = 0.97 \pm 0.03$ for the stoichiometry of PEP binding to the EI(H189A) subunit.

stoichiometry of 0.98 ± 0.03 for PEP binding to a subunit of EI(H189A) with an affinity constant of $1.0 \pm 0.1 \times 10^6\ M^{-1}$ ($\Delta G' = -33.7 \pm 0.2\ kJ\ mol^{-1}$) are obtained. Moreover, the average enthalpy for binding PEP to EI(H189A) in the presence of 2 mM Mg^{2+} at 20 °C is $+16.3 \pm 0.6\ kJ\ mol^{-1}$ giving $T\Delta S = 50.2 \pm 0.4\ kJ\ mol^{-1}$. ITC experiments under the same conditions as those conducted at 20 °C have been performed at 15 and 25 °C, yielding the ΔC_p plot shown in Fig. 4. The slope of the linear plot in Fig. 4 gives a value for $\Delta C_p = -1.4 \pm 0.1\ kJ\ K^{-1}\ mol^{-1}$ for EI(H189A) binding PEP in the presence of 2 mM Mg^{2+}. The value of ΔC_p for EI(H189A) binding PEP in the presence of Mg^{2+} is consistent with a PEP-promoted protein conformational change that favors dimer formation, which, in fact, occurs during ITC titrations.

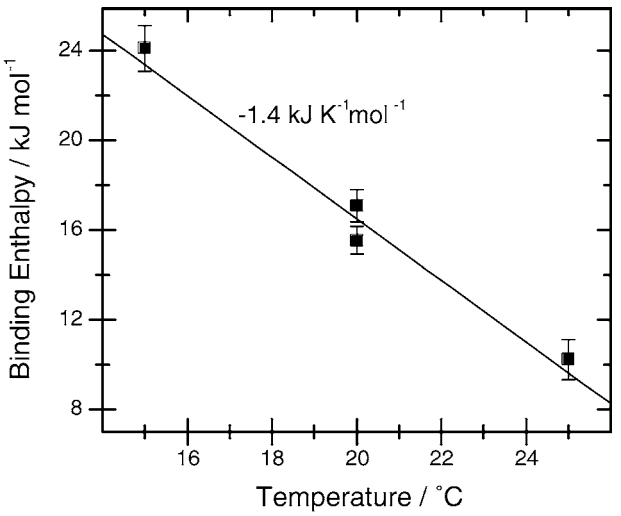

Fig. 4. Heat capacity plot of observed H values (■) for binding PEP to EI(H189A) in the presence of 2 mM Mg^{2+} and buffer A (pH 7.5) vs. temperature (15, 20, and 25 °C) from ITC measurements. The slope (ΔC_p) is shown ($-1.4 \pm 0.1\ kJ\ K^{-1}\ mol^{-1}$) with errors for each titration indicated.

The quantity $[\ln K_{20}/K_{63.7}] = 0.50$ using the values determined by ITC and DSC (Table 1). This indicates that ΔC_p is approximately $-0.16\ J\ K^{-1}\ mol^{-1}$ for extrapolation to T_m, which supports the view that the ΔC_p value for binding PEP with 2 mM Mg^{2+} primarily reflects the conformational change in EI(H189A) that strongly promotes dimer formation. In this case, it is incorrect to use the ΔC_p value from ITC to extrapolate a binding constant to T_m since in DSC measurements the protein has undergone conformational changes and dimerization before DSC scans.

ITC experiments at 20 °C with pyruvate added to EI in the presence of 2 mM Mg^{2+} show only the heat of dilution for pyruvate. This is consistent with an electrostatically driven reaction without a measurable, accompanying protein conformational change. The overall destabilizing effects of pyruvate observed in the present studies could arise from competition of PEP and pyruvate for a single binding site of the EI(H189A) subunit (Fig. 2). Pyruvate displacement of PEP will reverse the protein conformational changes and dimerization produced by PEP binding while decoupling the N-terminal domain unfolding.

4. Concluding remarks

Previous results have shown that phosphorylation of H189 [17] or substitution of Glu for His189 [12] in the N-terminal domain of EI destabilizes this domain and increases 10-fold the $K'_{1,2}$ for dimerization [13], which occurs through C-terminal domain interactions [10,11,27]. These results indicate that non-phosphorylated N-terminal domain inhibits the dimerization of enzyme I.

The present studies show a correlation between the coupling of N- and C-terminal domains during unfold-

ing/folding by substrates binding to the C-terminal domain and the dimerization constant for EI(H189A). In the presence of 2 mM Mg^{2+}, PEP binds with high-affinity ($10^6\ M^{-1}$ at pH 7.5, 20 °C), 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 [13,14]. Under the same conditions, the addition of 5 mM pyruvate ($K'_A = 4.9 \times 10^2\ M^{-1}$) decouples domain interactions and inhibits protein dimerization necessary for catalyzing the Mg^{2+}-dependent autophosphorylation of EI by PEP [14]. Thus, physiological concentrations of Mg^{2+}, PEP and pyruvate (and orthophosphate to a lesser extent [14]) 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 [13] and phospho-EI is poised for reversible phosphotransfer to HPr.

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