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Conformational stability changes of the amino terminal domain of enzyme I of the *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase system produced by substituting alanine or glutamate for the active-site histidine 189: Implications for phosphorylation effects

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Abstract

The amino terminal domain of enzyme I (residues 1–258 + Arg; EIN) and full length enzyme I (575 residues; EI) harboring active-site mutations (H189E, expected to have properties of phosphorylated forms, and H189A) have been produced by protein bioengineering. Differential scanning calorimetry (DSC) and temperature-induced changes in ellipticity at 222 nm for monomeric wild-type and mutant EIN proteins indicate two-state unfolding. For EIN proteins in 10 mM K-phosphate (and 100 mM KCl) at pH 7.5, $\Delta H \cong 140 \pm 10$ (160) kcal mol^{-1} and $\Delta C_p \cong 2.7$ (3.3) kcal K^{-1} mol^{-1}. Transition temperatures (T_m) are 57 (59), 55 (58), and 53 (56) °C for wild-type, H189A, and H189E forms of EIN, respectively. The order of conformational stability for dephospho-His189, phospho-His189, and H189 substitutions of EIN at pH 7.5 is: His > Ala > Glu > His-PO_3^{2-} due to differences in conformational entropy. Although H189E mutants have decreased T_m values for overall unfolding the amino terminal domain, a small segment of structure (3 to 12%) is stabilized ($T_m \sim 66$–68 °C). This possibly arises from an ion pair interaction between the γ-carboxyl of Glu189 and the ϵ-amino group of Lys69 in the docking region for the histidine-containing phosphocarrier protein HPr. However, the binding of HPr to wild-type and active-site mutants of EIN and EI is temperature-independent (entropically controlled) with about the same affinity constant at pH 7.5: $K'_A = 3 \pm 1 \times 10^5$ M^{-1} for EIN and $\sim 1.2 \times 10^5$ M^{-1} for EI.

Keywords: active-site mutants H189E and H189A; amino terminal domain; differential scanning calorimetry; enzyme I of the *E. coli* phosphoenolpyruvate:sugar phosphotransferase system; phosphorylation; secondary structure; thermal stability

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Abbreviations: PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate:sugar phosphotransferase system; EI, enzyme I (575 amino acid residues) of the PTS; EIN, amino terminal domain (residues 1–258 + Arg, containing an introduced C-terminal Arg residue) of enzyme I of the PTS; EIN(H189E) and EI(H189E), EIN and enzyme I, respectively, in which the active site His189 is replaced by Glu; EIN(H189A), EIN in which His189 is replaced by Ala; HPr, histidine-containing phosphocarrier protein of the PTS; *pts*$^+$, wild-type strain of *E. coli*; Δ*pts*, mutant strain of *Escherichia coli* with a deletion of the operon encoding genes expressing the PTS proteins HPr, enzyme I, and enzyme IIA^{glc}; DSC, differential scanning calorimetry; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid.

The bacterial phosphoenolpyruvate (PEP):sugar phosphotransferase system couples the phosphorylation and translocation of sugars. The PTS is composed of two cytosolic proteins (enzyme I and HPr) and sugar-specific components (enzymes II). PEP is the phosphoryl group donor in a Mg^{2+}-dependent autophosphorylation of enzyme I on the N3 atom of His189. Phospho-enzyme I reversibly transfers its phosphoryl group to the N1 atom of histidine 15 of HPr (Meadow et al., 1990). 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., 1997a) structures of EIN show that the nonphosphorylated, active-site His189 is buried near the interface between two subdomains: an α/β-domain (residues 1–20 and 148–230) and an α-domain (residues 33–143) consisting of four helices arranged in two hairpins. Upon phosphorylation, His189 rotates toward the surface with only small structural changes detected (Garrett et al., 1998).

A solution structure of the 37,460 M_r complex between dephospho-EIN and HPr, solved by NMR spectroscopy (Garrett et al., 1999), shows a surface complementation between H2, H2′, H3, and H4 α-helices in the α-subdomain of EIN and helices 1 and 2 of HPr with little disturbance of either protein structure. Recent isothermal titration calorimetry experiments demonstrate that HPr binds to the isolated α-helical domain of enzyme I but not to a deletion mutant of enzyme I lacking that domain (Zhu et al., 1999).

Chauvin et al. (1996) have cloned and purified a slightly longer EIN construct (residues 1–268 with a C-terminal Cys added) and reported similar binding parameters for the interactions of HPr with this EIN construct and intact enzyme I. Thermodynamic parameters determined for reversible, two-state thermal unfolding of nonphosphorylated EIN (either 1–258+Arg or 1–268+Cys) agree (Chauvin et al., 1996; Nosworthy et al., 1998).

Phosphorylation of the active-site His189 destabilizes both EIN (1–258+Arg) and the amino terminal domain of intact enzyme I (Nosworthy et al., 1998). This finding together with that of Van Nuland et al. (1995) that phosphorylation destabilizes HPr suggests that a unidirectional transfer of the phosphoryl group of PEP to enzyme I, HPr, enzymes II, and finally, irreversible transfer to sugars is favored.

Since the phosphoryl group on His189 of EIN or enzyme I hydrolyzes during thermal unfolding studies, protein engineering has been used to make H189E mutants of EIN and enzyme I that are expected to have properties of phosphorylated forms. The H189A mutant of EIN also has been produced and studied. This is an approach suggested by Huffine and Scholtz (1996) who substituted negatively charged Asp for serine 46 and Glu for His15 of HPr from *Bacillus subtilis* to mimic phosphorylated forms. This communication reports the hydrodynamic properties, thermal stability, and interactions with HPr of wild-type EIN, EIN(H189A), EIN(H189E), EI, and EI(H189E). Although the transition temperature for overall unfolding the H189E mutant of EIN is lower than that for H189A and wild-type EIN, a small segment of structure is stabilized in H189E mutants of both EIN and enzyme I. The implications of these findings on phosphotransfer to HPr are discussed.

Results

Homogeneity, size, and shape of EIN, and active-site mutants

Analyses of sedimentation equilibrium data obtained as before (Nosworthy et al., 1998) have shown that wild-type EIN and the active-site mutant proteins EIN(H189A) and EIN(H189E) are monomeric with molecular weights within 3% of those calculated from amino acid compositions (~28,300 M_r). In sedimentation velocity experiments (using a capillary synthetic boundary centerpiece at 40,000 rpm and 20.0 °C), the proteins sedimented as homogeneous species with corrected sedimentation coefficients, $s_{20,w}$ = 2.6 ± 0.1 S, corresponding to calculated Stokes radii of 27 ± 1 Å. The latter value is consistent with the dimensions of 32 Å diameter × 78 Å length for the EIN ellipsoid found by X-ray crystallography (Liao et al., 1996), assuming random tumbling of EIN in the centrifugal field.

Thermal unfolding of wild-type and mutant EIN proteins

In DSC studies at scan rates of 30 and 60 °C/h, the unfolding reactions of wild-type and active-site mutants of EIN are reversible. DSC profiles for first scans and corresponding fits of each data set to a two-state model of unfolding (with ΔC_p = 2.7 kcal K^{-1} mol^{-1}) for dephospho-EIN, EIN(H189A), and EIN(H189E) in 10 mM K-phosphate, pH 7.5 buffer are shown in Figure 1. Substitution of Ala or Glu for His189 decreases the thermal stability as demonstrated by decreases of maxima in the excess heat capacity plots of the main thermal transitions. The order of overall

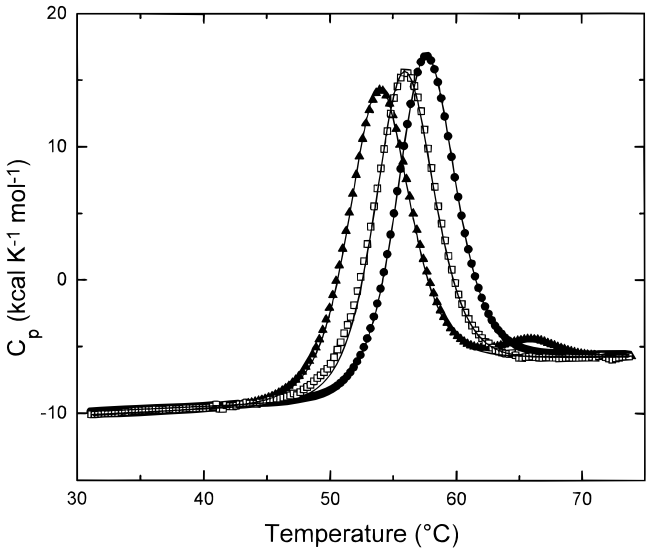

Fig. 1. Representative DSC scans for wild-type and active-site mutants of EIN (1–258+Arg) made from 10–80 °C at 60 °C/h scan rate and 1 mg/mL protein in 10 mM K-phosphate, pH 7.5 buffer. Truncated DSC profiles for wild-type EIN (●), EIN(H189A) (□), and EIN(H189E) (▲) together with a fit of each data set to a two-state model of unfolding with ΔC_p = 2.7 kcal K^{-1} mol^{-1} (——).

conformational stability of EIN proteins was H189E < H189A < H189, with T_m values of 53, 55, and 57 °C, respectively.

An additional small endotherm with a T_m value of ~66 °C is always present in DSC scans of EIN(H189E), both before and after analytical gel filtration through Superose-12. Further evidence that the small endotherm at 66 °C observed with EIN(H189E) is not due to an impurity is the fact that it also is present in DSC scans of the full length EI(H189E) mutant protein (see below). The small endotherm with $T_m \sim 66$ °C of EIN(H189E) is 3% of the area of the main transition. Possibly, an ion pair interaction between the γ-carboxyl of the glutamyl residue and a basic side-chain group in its vicinity stabilizes a small segment of the EIN structure (see below).

To assess possible shielding effects at higher ionic strengths, DSC experiments have been repeated for dephospho-EIN, EIN(H189A), and EIN(H189E) in 20 mm K-phosphate buffer (pH 7.5) in the presence of 100 mM KCl. The conformational stability of all three proteins is increased under these conditions and the differences between the T_m values of wild-type and mutant EIN proteins are decreased (Table 1). In all cases, first scans in the presence of 100 mM KCl give excellent fits to a two-state model of unfolding with $\Delta C_p = 3.3 \pm 0.3$ kcal K^{-1} mol^{-1} and little scan rate dependence. The parameters obtained from fitting DSC data for wild-type and mutant EIN proteins to a two-state unfolding model are given in parentheses in Table 1. The small, high-temperature endotherm (~4 kcal mol^{-1}) for EIN(H189E) also manifests a T_m increase to ~68 °C in the presence of 100 mM KCl.

The reversible thermal unfolding of wild-type and active-site mutant EIN proteins in 10 mM K-phosphate, pH 7.5 buffer has been monitored by equilibrium CD measurements at 222 nm (Fig. 2). Ellipticity changes are given after 15 min at each temperature, well after CD changes were complete. Progress curves of ellipticity changes at 222 nm as a function of increasing temperature are shown by the solid symbols in Figure 2A–C. Ellipticity changes have also been measured during cooling from ~70 °C, and the open symbols in Figure 2A–C show CD measurements at 222 nm after completion of time-dependent CD changes at each temperature. Some hysteresis occurs during cooling, which is not surprising when taking into account the time that each protein sample is exposed to high temperatures. The fit of each equilibrium CD data set obtained during temperature increases to a two-state model of unfolding is shown by the solid line in Figure 2A, 2B, or 2C. The parameters of the fit in each case are given in Table 1. The dashed line in Figure 2 is drawn through T_m values. The equilibrium CD data indicate that the secondary structure of EIN(H189E) is ~2 °C less stable than that of EIN(H189A), which itself has a T_m value ~2 °C lower than that of wild-type EIN. Note that the post-transitional high-temperature CD measurements for EIN(H189E)

Table 1. *Summary of thermodynamic parameters for two-state unfolding of EIN at pH 7.5*[a]

EIN	T_m (°C)	ΔH (kcal mol^{-1})	Method
Dephospho-EIN	56.9 (59.4)	152 (158)	DSC (60°C/h)
	56.9 (59.0)	149 (159)	DSC (30°C/h)
	55.9	127	Equil. CD[b]
Phospho-EIN	50.2 (53.5)	130 (136)	DSC (60°C/h)[c]
EIN(H189A)	55.8 (57.9)	143 (153)	DSC (60°C/h)
	55.4 (57.6)	144 (156)	DSC (30°C/h)
	54.0	118	Equil. CD[b]
EIN(H189E)	53.7 (56.7)	135 (153)	DSC (60°C/h)
	53.3 (56.4)	138 (160)	DSC (30°C/h)
	52.1	128	Equil. CD[b]

[a] EIN, amino terminal domain of enzyme I (residues 1–258+Arg) with His189 being the site of phosphorylation or mutation by substitution of Ala (H189A) or Glu (H189E). Protein concentrations were ~1 mg/mL in 10 mM K-phosphate, pH 7.5 for DSC and CD measurements (nonparenthetical values). An average value for $\Delta C_p = 2.7 \pm 0.3$ kcal K^{-1} mol^{-1} was estimated from DSC profiles (obtained at scan rates of 60 °C/h) and was used as a constant value for fits of equilibrium CD data as a function of increasing temperature to a two-state model of unfolding. Average values of T_m (±0.2 °C) and ΔH (±5 kcal mol^{-1}) are from 2–3 DSC experiments at 60 °C/h scan rate or 1–2 DSC at 30 °C/h; values in parentheses were obtained in the presence of 20 mM K-phosphate, pH 7.5, 100 mM KCl with and without the addition of 1 mM EDTA, under which conditions a value of $\Delta C_p = 3.3 \pm 0.3$ kcal K^{-1} mol^{-1} was obtained. (Results were the same with or without 1 mM EDTA in buffers.)

[b] CD monitored at 222 nm until time-dependent changes were complete (see Fig. 2).

[c] From Nosworthy et al. (1998) and the present study with 90–94% phosphorylated EIN.

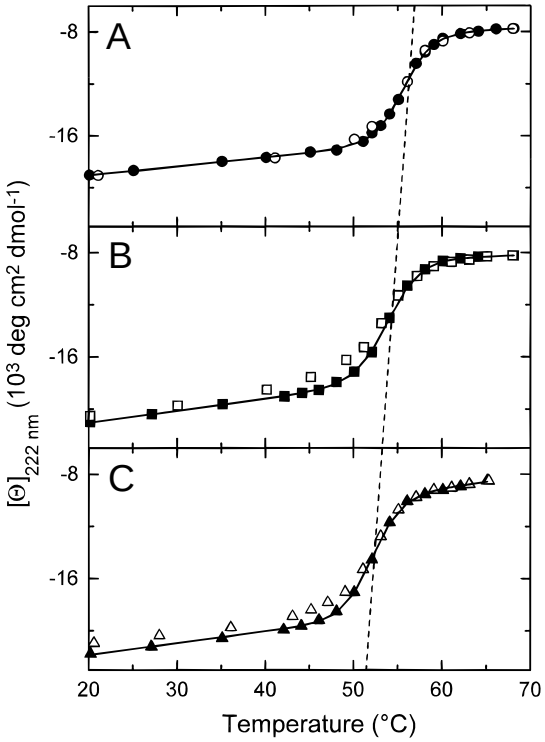

Fig. 2. Equilibrium CD measurements at 222 nm (after time-dependent changes) as a function of increasing (closed symbols) or decreasing temperature (open symbols) with 1 mg/mL EIN protein in 10 mM K-phosphate, pH 7.5 buffer. CD data for (**A**) wild-type EIN, (**B**) EIN(H189A), and (**C**) EIN(H189E), respectively, are measured during heating from 20 to ~70 °C and during subsequent cooling from ~70 to 20 °C. The solid line in each case is the fit of the CD data during temperature increases to a two-state model of unfolding [with $\Delta C_p = 2.7$ kcal K^{-1} mol^{-1}]. The dashed line is drawn through T_m values for the three proteins.

compared to those for wild-type and EIN(H189A) suggest small additional secondary structural changes.

All T_m values are lower in equilibrium CD measurements (at zero scan rate) than in DSC measurements at a scan rate of 30 °C/h (Table 1), suggesting that the latter scan rate is still too fast for complete equilibrium to be reached at each temperature. For CD measurements in a continuous mode as a function of increasing temperature at either 30 or 60 °C/h, T_m and ΔH values are the same as obtained from DSC at the same scan rate within experimental error (data not shown), which indicates that temperature calibrations for both instruments agree.

Thermodynamic parameters for the two-state unfolding of the wild-type and H189A and H189E EIN proteins are summarized in Table 1. EIN(H189E) has a lower overall stability than has wild-type, nonphosphorylated EIN even though a small segment of structure is stabilized in this mutant protein (see above). The T_m value of 50 °C for phosphorylated EIN in 10 mM K-phosphate, pH 7.5 reported previously (Nosworthy et al., 1998) and confirmed in the present study is lower than that measured for EIN(H189E). Increasing ionic strength increases the conformational stabilities of wild-type (dephospho- and phospho-forms) and mutant EIN proteins. Also, the value of ΔC_p is increased from 2.7 to 3.3 kcal K^{-1} mol^{-1} by the addition of 100 mM KCl. The T_m values of phospho-EIN and EIN(H189E) are increased ~3 °C, as compared to ~2 °C for wild-type and EIN(H189A), by the presence of 100 mM KCl, which suggests that neutral salt has an increased shielding effect in cases in which negative charges are introduced into the active site. Unfolding enthalpies are ~5 cal/g, which is in the range of 5–9 cal/g at 50 °C measured for the unfolding of small globular proteins (Makhatadze & Privalov, 1994).

The thermal unfolding of EIN (wild-type or active-site mutants) is completely reversible. However, repeatability of DSC scans is dependent on the time of equilibration at low temperatures (10 or 15 °C) between scans. When 15 min at 10 °C is allowed between DSC scans of EIN in 10 mM K-phosphate (pH 7.5), repetitive scans show an increasing broadening of the endotherm below the transition temperature with isotemperatures of 54.3 and 64.4 °C, and a decreasing amplitude of the main endotherm (Fig. 3). Pre- and post-transitional base lines and total areas are unchanged from those obtained in the first scan. The changes observed in repetitive scans shown in Figure 3 are more pronounced when 100 mM KCl is present (data not shown). Together, the results obtained with 15 min equilibration at 10 °C between scans suggest that an equilibrium refolding intermediate accumulates. When equilibrium times at 10 or 15 °C between repetitive DSC scans is increased to ≥1 h, DSC profiles are completely reproducible. Thus, the complete refolding (and possible annealing) of thermally unfolded EIN requires longer times than does equilibration of the VP-DSC instrument.

Thermal unfolding of phospho-EIN, phospho-enzyme I, and EI(H189E)

DSC scans of phosphorylated forms of EIN and EI are compared to those of EI(H189E) at low and high ionic strength, pH 7.5 in Figure 4. The phosphoryl group on His189 is stabilized by the presence of 100 mM KCl[6] and the DSC profiles in Figure 4 show that more than 90% of EIN and EI are phosphorylated with the small, high-temperature shoulder in each case representing the dephospho-amino terminal domain. Whereas ~90% phosphorylated EIN could be isolated by rapid gel filtration in the absence of KCl, much lower yields of phospho-EI are obtained by the same treatment (data not shown). The unfolding of the phosphorylated N-terminal domain of EI ($T_m \cong 50.2$ °C) masks that of the C-terminal domain ($T_m \cong 46.8$ °C). This is illustrated by showing the DSC profile of dephospho-EI under the same conditions (Fig. 4; EI & EI-P).

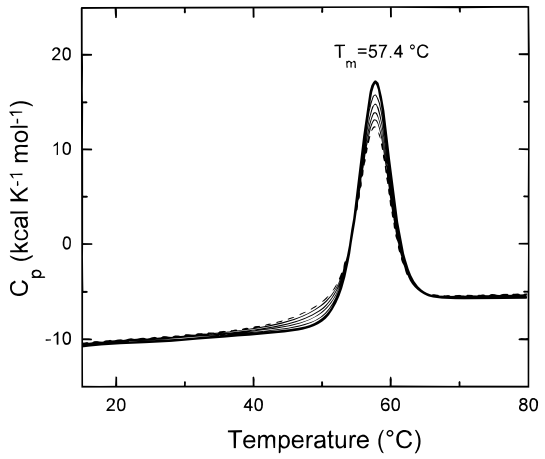

Fig. 3. Six consecutive DSC scans of wild-type EIN (1 mg/mL in 10 mM K-phosphate, pH 7.5 buffer) from 10 to 80 °C at a rate of 60 °C/h with 15 min equilibration at 10 °C between scans. The first, second through fifth, and sixth scans are shown by the heavy solid, thin solid, and dashed lines, respectively.

The EI(H189E) mutant shows unfolding transitions at 43 (46), 52.8 (53.8), and 65 (67) °C in the absence (and presence of 100 mM KCl) at pH 7.5. Note that the post-transition endotherm for EI(H189E) with T_m of 65–67 °C is considerably larger than that observed for EIN(H189E), representing ~20 and 3% of the areas for N-terminal domain unfolding, respectively (Figs. 1, 4). Thus, the C-terminal domain in EI(H189E) appears to augment the stabilization of a structural segment that unfolds at ~60–70 °C. The area of the endotherm centered at ~53–54 °C for N-terminal domain unfolding in EI(H189E) [$\Delta H \cong 130$ kcal mol^{-1}] is decreased proportionately to the increased area of the post-transition endotherm (T_m = 65–67 °C). This is consistent with the segment of structure stabilized in EI(H189E) being a part of the N-terminal domain.

The post-transition endotherms at 60–72 °C of EIN(H189E) and EI(H189E) are not observed with phosphorylated forms of EIN and EI. Yet, it seems likely that a small population of phosphorylated EIN or EI (that is undetected in DSC) have the same segment of structure stabilized as exhibited by EIN(H189E) and EI(H189E). If so, this has important implications for phosphotransfer to HPr (see below).

Interactions of wild-type and active-site mutants of EIN with HPr

The binding of HPr to EIN has been studied by titration calorimetry at 16 and 30 °C at pH 7.5. The binding parameters (K'_A and ΔH) for titrations of EIN proteins with HPr at 303 K are given in

[6] The phosphoryl group on His189 of EIN or enzyme I, in particular, was stabilized by the presence of 100 mM KCl in pH 7.5 buffers at ≤20 °C.

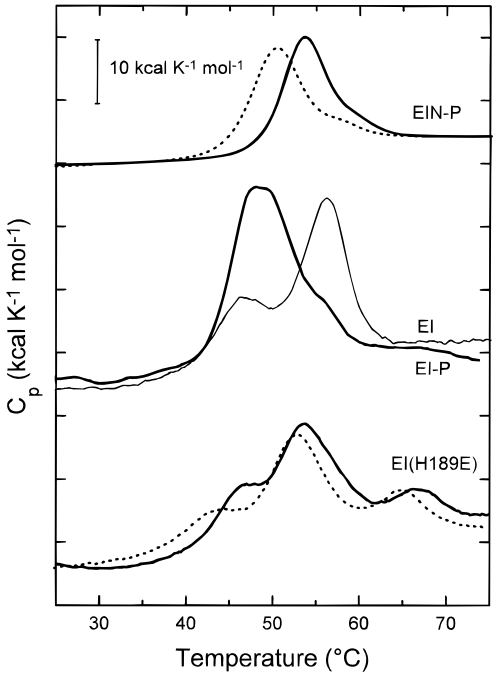

Fig. 4. DSC scans obtained at a rate of 60 °C/h for 94% phosphorylated EIN [EIN-P, 1.0 mg/mL], 90% phosphorylated enzyme I [EI-P, 0.48 mg/mL, solid, thick line], dephospho-enzyme I [EI, 0.22 mg/mL, thin line], and full length EI(H189E) [0.18–0.22 mg/mL] shown after subtraction of the instrument base line and normalization for concentration and scan rate. Heavy and thin solid lines are for proteins at pH 7.5 in 20 mM K-phosphate and 100 mM KCl; EI and EI(H189E) dialysate buffer had 1 mM EDTA added; dotted lines are for gel-filtered EIN-P and dialyzed EI(H189E) in 10 mM K-phosphate, pH 7.5. EI-P also was rapidly collected by gel filtration. Deconvolution of DSC data for phosphorylated EI and EI(H189E) in the presence of 100 mM KCl (data not shown) give: $T_m \cong 46$ °C for C-terminal domains; $T_m = 50$ °C for phosphorylated N-terminal domain; and $T_m = 53$ °C ($\Delta H \cong 130$ kcal mol^{-1}) for N-terminal domain with Glu substituted for His189. In addition, EI(H189E) has a small endotherm (~23 kcal mol^{-1}) with $T_m = 67$ °C.

Table 2A. Within experimental errors, dephospho-EIN and active-site mutants of EIN had the same affinity constant ($K'_A = 3 \pm 1 \times 10^5$ M^{-1}) and enthalpy value ($\Delta H = +6 \pm 1$ kcal mol^{-1}) for binding HPr at 303 K. Enthalpic changes on binding HPr to EIN proteins are similar at 289 and 303 K (data not shown). Thus, the binding of HPr to EIN is entropically controlled with $\Delta S \cong +45$ cal K^{-1} mol^{-1} at 303 K. The apparent association constant of 3×10^5 M^{-1} for HPr binding to wild-type EIN at 303 K is about twofold higher than K'_A values at 298 and 313 K reported previously (Chauvin et al., 1996; Garrett et al., 1997b).

After adding approximately a two- to threefold molar excess of HPr to EIN in ITC experiments, recovered EIN and HPr mixtures have been scanned in DSC at a rate of 60 °C/h. Values of T_m for EIN proteins in the absence (T_0) and presence (T_c) of the indicated free concentration of HPr are given in Table 2A. In the absence of EIN under the conditions used for obtaining the data in Table 2, HPr has $T_m = 63.3$ °C and an unfolding enthalpy of 75 kcal/mol at pH 7.5. However, at the low free concentrations of HPr (<25 μM) present in titration mixtures of EIN and HPr, an endotherm for free HPr is not observed (Fig. 5). At ~20 μM free HPr, the EIN proteins are stabilized ~3 °C during thermally induced unfolding.

The effect of ligand (L) or protein binding (with $T_c \geq T_0$ of EIN) on the transition temperature for a two-state unfolding of a macromolecule is directly related to the affinity constant for the ligand (Becktel & Schellman, 1987; Brandts et al., 1989). Assuming that ligand (HPr) cannot bind to EIN or the amino terminal domain of enzyme I when this domain is unfolded, Equation 17 of Brandts et al. (1989) can be applied:

$$\ln(1 + K_L[\mathrm{L}]) = (\Delta H_0/R)(1/T_0 - 1/T_c) - (\Delta C_p/R)(\ln[T_0/T_c] + T_c/T_0 - 1) \quad (1)$$

where T_0 and T_c are transition midpoints in the absence and presence of ligand L, respectively; [L] is the free concentration of ligand (HPr); ΔH_0 is the experimental enthalpy change for thermal unfolding EIN at midpoint T_0 in the absence of HPr; ΔC_p is

Table 2. *Binding of HPr to wild-type, dephospho-EIN, active-site EIN mutants, dephospho-enzyme I, and EI(H189E) in 10 mM K-phosphate (pH 7.5), 100 mM KCl, and 1 mM EDTA*

Protein	$[\mathrm{HPr}]_{free}$ (10^{-6} M)[b]	T_0 (K) (− HPr)	T_c (K) (+ HPr)	K'_A (10^5 M^{-1}) 303 K	K'_A (10^5 M^{-1}) Calculated at T_0[b]	ΔH (kcal mol^{-1}) Binding HPr 303 K	ΔH_0[c] at T_0
A: DSC following ITC titrations of EIN proteins with HPr at 303 K[a]							
EIN wt	17.4	332.6	335.0	3.2 ± 1.3	2.5	6 ± 1	158
EIN(H189A)	23.8	331.0	334.1	2.6 ± 0.4	3.0	5 ± 1	153
EIN(H189E)	21.8	330.0	333.3	3.2 ± 0.5	3.8	7 ± 1	153
B: DSC of dephospho-enzyme I and EI(H189E) ± HPr							
EI wt	21.0	329.6[d]	331.6[d]	—	1.2	—	150[d]
EI(H189E)	21.0	326.6[d]	328.6[d]	—	1.2	—	136[d]

[a] The binding of HPr was 1.0 equiv/EIN in ITC at ≤30 °C; the errors in the fits for K'_A and ΔH values from duplicate ITC experiments at 30 °C are indicated.

[b] Values of K'_A at T_0 from DSC data were calculated from Equation 1 (see text and Fig. 5).

[c] Values are from Table 1 at a scan rate of 60 °C/h.

[d] T_0 and T_c are the transition midpoints in the absence and presence of HPr, respectively, for the N-terminal domain of EI; ΔH_0 is the unfolding enthalpy of the N-terminal domain at T_0 in the absence of HPr.

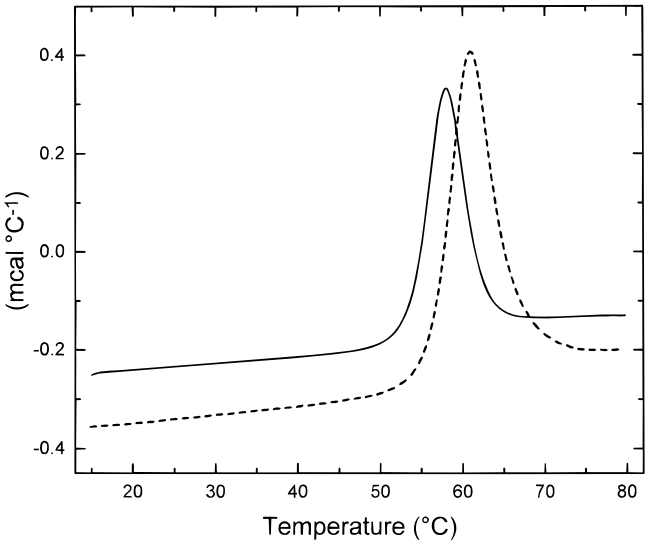

Fig. 5. DSC scans at 60 °C/h of EIN(H189A) in the absence (solid line) and presence of HPr (dashed line) from 10 to 80 °C in 10 mM K-phosphate, 100 mM KCl, and 1 mM EDTA, pH 7.5 buffer. The concentrations of EIN(H189A) in the absence and presence of HPr were 0.0368 and 0.0282 mM, respectively, and that of HPr was 0.0465 mM when present. The DSC data were corrected for the instrument baseline and normalized for scan rate but not for protein concentrations.

the heat capacity change for the thermal unfolding of EIN protein (3.3 kcal K^{-1} mol^{-1}); and R is the gas constant. Reiterative fitting has been used for final adjustments of $[HPr]_{free}$ and the values of K_A', the association constants calculated at T_0 from Equation 1 for HPr binding to the wild-type and active-site mutant EIN proteins (see Table 2A, which also gives other parameters needed for these calculations). The values of K_A' calculated at T_0 for HPr binding to EIN approximately agree with those measured at 303 K. This corroborates the entropic nature of EIN-HPr protein–protein interaction.

The stabilization of the amino terminal domain of enzyme I by HPr binding also has been measured, and the results calculated from Equation 1 are given in Table 2B. In these experiments, dephospho-EI and EI(H189E) were scanned at 60 °C/h at 0.22 mg/mL concentration in the absence and presence of 21 μM free HPr. Under these conditions, the endotherm for HPr (T_m = 63 °C), when present, is clearly visible (molar ratio of HPr/EI $\cong$ 6.8). Deconvolution of DSC data for both dephospho-EI and EI(189E) in the absence and presence of HPr yield T_0 and T_c values for the unfolding of the amino terminal domain in each case. The calculated K_A' value of 1.2×10^5 M^{-1} at ~53–56 °C and pH 7.5 is the same for dephospho-EI and EI(H189E) binding to HPr, which is a somewhat lower affinity constant than that measured for either dephospho-EIN or EIN(H189E).

Discussion

The appreciable hydrolysis of the phosphoryl group bonded to His 189 of EIN or enzyme I during repetitive DSC scans led us to study EIN(H189E) and EI(H189E) as stable analogues of phosphorylated forms. The overall conformational stability of the amino terminal domain of enzyme I is decreased by substituting Glu or Ala for the active-site His189. These results, together with our observations on the destabilization of the amino terminal domain of enzyme I (in either EIN or EI) by phosphorylation of His189 (Nosworthy et al., 1998; present studies), puts the order of conformational stability of the amino terminal domain with respect to position 189 substitutions at pH 7.5 as His > Ala > Glu > His-PO_3^{2-}. The observed differences in stability can be attributed to differences in conformational entropy since ΔC_p and ΔH at the same temperature for unfolding wild-type and active-site mutants of EIN are the same (D'Aquino et al., 1996).

In the nonphosphorylated form, His189 of EIN is buried in a cleft between the α and α/β subdomains and the solvent inaccessible N3 atom of His189 accepts a hydrogen bond from the hydroxyl of Thr168 (Liao et al., 1996; Garrett et al., 1997a; Fig. 6A). For phosphorylation to occur, His189 (in the g^+ conformation) rotates toward the surface and the N3 atom of the imidazole ring (g^- conformation) becomes bonded directly to phosphate and the negative charge on the phosphate oxygen is stabilized by an ion pair interaction with the side-chain of Lys69 (Garrett et al., 1998).

Since the hydrophobic cleft between subdomains of EIN containing nonphosphorylated His189 will not accommodate a negative charge (Liao et al., 1996), the substituted glutamate residue at position 189 is expected to be in the "out" conformation (Fig. 6B). Indeed, the shielding effect produced by 100 mM KCl suggests that the γ-carboxyl group of Glu189 in EIN(H189E) is solvent accessible as is phospho-His189 in phosphorylated EIN.

In the Glu189 conformation shown in Figure 6B, the ϵ-amino group of Lys69 is ~2.5 Å from the γ-carboxyl oxygen of Glu189, favorable for an ion pair interaction. A small segment of structure is apparently stabilized by this ion pair interaction, as shown by a small endotherm at ~66 °C (~4 kcal/mol) in DSC of EIN(H189E) at pH 7.5 and low ionic strength (Fig. 1). EI(H189E) exhibits a larger post-transition endotherm at 66–68 °C (~23 kcal mol^{-1}), and a proportionally lower enthalpy for N-terminal domain unfolding, than does EIN(H189E) (Figs. 1, 4). Thus, the presence of the C-terminal domain in full length EI(H189E), which is involved in dimerization, appears to augment the stabilization of a segment of the N-terminal domain structure that unfolds at ~60–70 °C.

Although the phosphoryl group on His189 also is ~2.5 Å from the ϵ-amino group of Lys69 (Garrett et al., 1998), DSC provides no evidence of a stabilized segment of N-terminal amino domain structure in phosphorylated EIN or EI. Nevertheless, it is entirely possible that this occurs but such conformations are not sufficiently populated to be detected by DSC. The modeled phosphoryl group on His189 of EIN is within 6 Å of acidic residues Glu125 and Asp129 (Garrett et al., 1999) and the γ-carboxyl group of the modeled Glu189 of EIN is within 6 Å of Glu125 (Fig. 6B), which will have overall destabilizing effects on protein conformations.

The substitution of Ala for His189 in EIN gives the same van der Waals radii as for the buried His189 without the imidazole ring. Thus, EIN(H189A) should have an identical conformation to that shown in Figure 6A without the stabilizing hydrogen bond to the hydroxyl group of Thr168. Also, a cavity will be formed that is normally occupied by the imidazole ring, and water molecules are expected to fill this cavity to satisfy hydrogen bonding to Thr168. Both of these factors could explain the decreased conformational stability observed for EIN(H189A) relative to that of wild-type EIN. Note also that EIN(H189A), like the wild-type, dephospho-EIN, exhibits only one two-state unfolding transition.

The solution structure for the nonphosphorylated EIN:HPr complex shows that HPr interacts with H2, H2′, H3, and H4 of the α-subdomain of EIN (Garrett et al., 1997b, 1999). The N-terminal end of H2 contains Lys69, which is stabilized by substitution of

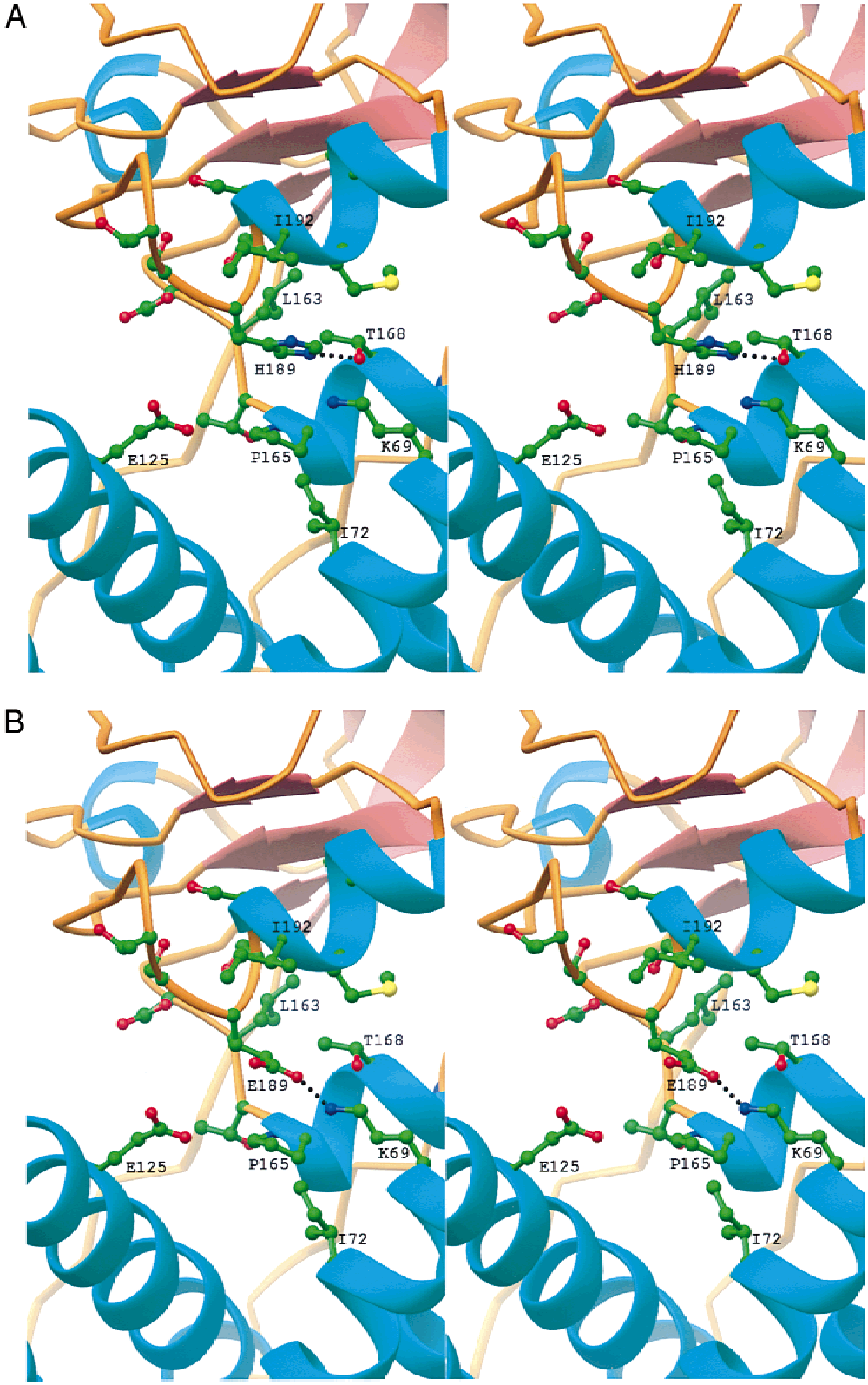

Fig. 6. Close-up stereo views of the EIN active site centered on (**A**) His189 or (**B**) Glu189 using the coordinates from the X-ray structure of Liao et al. (1996). Selected side chains that have any atom of the residue within 6 Å of any atom of Glu modeled at postion 189 are displayed. Color scheme for side chains: green, carbon; red, oxygen; blue, nitrogen; yellow, sulfur. **A:** Active-site view of wild-type EIN with the N3 atom of His (in the buried g^+ conformation) at a distance of 2.68 Å from the oxygen of the hydroxyl group of Thr168; dotted line shows this contact. **B:** Active-site view with glutamate modeled into the structure in place of His189 in the solvent accessible g^- conformation (Garrett et al., 1998) without energy minimization. Distance between the γ-carboxyl oxygen of Glu189 and the ϵ-amino group nitrogen is 2.5 Å; a dotted line shows this modeled contact.

Glu for His189 (Fig. 6B) or possibly by phosphorylation of His189. Thus, the segment of structure stabilized by EIN(H189E) probably involves the end of H2 (residues 68–75) in EIN. The EIN-HPr interactions in the complex reported by Garrett et al. (1999) involve electrostatic interactions between 67E, 68E, and 74E of EIN and residues 17R and 24K of HPr and hydrophobic contacts in the active-site region of HPr (15H, 16T, 17R, 20A, 21Q, 22F, 23V, and 24K) are made by 71A, 72I, 74E, 75G, 76H, and 78M of EIN. Hydrophobic interactions between the H4 helix of EIN and helix 1 (H15-A20) of HPr also occur but only 125E (Fig. 6B) is within 6 Å of the modeled Glu189 residue. Thus, the stabilization of a segment of H2 of EIN by phosphorylation would be expected to facilitate the docking of HPr. Garrett et al. (1999) observed that the binding of HPr to dephospho-EIN involves a helix-to-helix interface with essentially no changes in conformation relative to the uncomplexed proteins. In fact, the apparent association constants and enthalpic changes for wild-type EIN, EIN(H189A), and EIN(H189E) binding HPr at pH 7.5 and 30 °C are essentially the same (Table 2A). The binding of EIN to HPr is entropically controlled and temperature-independent with $K_A' \cong 3 \times 10^5\ M^{-1}$, $\Delta H \cong 6 \pm 1$ kcal mol^{-1}, and $\Delta S \cong +45$ cal K^{-1} mol^{-1}. These thermodynamic parameters reflect mainly solvent displacement, rather than ordering effects, on complex formation (Hilser et al., 1996). Also, the apparent association constants of dephospho-EI and EI(H189E) for HPr are the same at 53–56 °C, although the measured K_A' of $1.2 \times 10^5\ M^{-1}$ is slightly lower than that for EIN proteins binding HPr (Table 2).

The amino terminal domain of enzyme I reversibly transfers the phosphoryl group bonded to the N3 atom of His189 to the N1 atom of His15 of HPr (Seok et al., 1996). Replacement of the active-site His15 of *B. subtilis* HPr by the negatively charged glutamate residue has been found by Huffine and Scholtz (1996) to significantly decrease the overall conformational stability of this protein. The substitution of Ala for His15 actually stabilized HPr against urea-induced unfolding (Huffine & Scholtz, 1996). However, the NMR studies of Rajagopal et al. (1994) have detected only local changes in HPr structure upon phosphorylation of His15. Subsequently, Van Nuland et al. (1995) found that phosphorylation of His15 of HPr indeed introduces a torsion angle strain in the His15 backbone that is released upon phosphoryl group transfer by using both NMR and molecular dynamics refinement. This result is consistent with the results of Huffine and Scholtz (1996).

A destabilization of the overall conformations of the amino terminal domain of enzyme I and HPr by phosphorylation promotes a unidirectional transfer of the phosphoryl group from PEP to enzymes II and finally, irreversibly to the specific sugars being translocated across the membrane (Van Nuland et al., 1995; Nosworthy et al., 1998). Moreover, phosphorylation of His189 in the amino terminal domain of enzyme I is proposed here to stabilize a local segment of structure, thereby facilitating the docking of the small HPr acceptor protein for phosphoryl group transfer to His15 of HPr. Such a mechanism must involve faster on and off rates rather than changes in affinity.

Materials and methods

DNA methods: Mutagenesis of the active site of EI and EIN

pR6, an expression vector encoding *Escherichia coli* EI (Reddy et al., 1991), was used as a template for polymerase chain reactions (PCRs) to mutagenize the active-site His189 to Ala or Glu. Reverse mutagenic primers (69-mers) encompassing the sequence from bases 1139–1071 of the Genbank sequence (ecoptshi.genbank) were synthesized on a Model 394 DNA/RNA Synthesizer (Applied Biosystems, Foster City, California). The GTG sequence at bases 1082–1080 was changed to GGC (corresponding to Ala) or CTC (corresponding to Glu) in the mutagenic primers. PCR amplification of the EI sequence used one of the mutagenic primers and a forward PCR primer starting at 70 bases upstream of the EI start site. The purified PCR products (about 700 bp) were digested with *Nde*I (located at the EI start site) and *Pin*AI (located at bases 1128–1133). The fragments were ligated into pR6 similarly digested with the two restriction endonucleases. The ligated plasmids were introduced into *E. coli* GI698 as well as a *pts* deletion derivative of that strain by electroporation as described previously (Nosworthy et al., 1998). All the constructs were verified by DNA sequencing by the dideoxy method of Sanger et al. (1977) using an Applied Biosystems automated sequencer. The clones harboring the mutated EI sequences were used to construct clones encoding similarly mutated EIN proteins. pLP2 is an expression vector encoding *E. coli* EIN (Seok et al., 1996). The *Nde*I–*Pin*AI fragments from the mutated pR6 plasmids described above were ligated into pLP2 digested with the same restriction endonucleases. The clones were introduced into the two forms of *E. coli* GI698 as described above.

Proteins

Wild-type, nonphosphorylated EIN and H189A and H189E mutants of EIN and wild-type EI and EI(H189E) were expressed and purified from a 2-L culture of the Δ*pts* strain GI698 as previously described (Nosworthy et al., 1998). Final yields after concentrating proteins to 2 mL were 60 to 90 mg. Proteins were homogeneous in SDS-PAGE (Laemmli, 1970) and were stored at −80 °C in 10 mM Tris·HCl/100 mM NaCl, pH 7.5 buffer used for purification. Before DSC or other measurements, proteins were dialyzed at 4 °C overnight against several changes of 10 or 20 mM K-PO_4 ± 100 mM KCl ± 1 mM EDTA, pH 7.5 buffer using Slide-A-Lyzer Cassettes (3,500 and 10,000 MW cutoff for EIN and EI, respectively, Pierce, Rockford, Illinois). Specific absorption coefficients used for spectrophotometric determinations of protein concentration were $A_{280nm,1cm} = 0.190$ cm^2/mg for EIN (28,346 M_r; Nosworthy et al., 1998) and $A_{280nm,1cm} = 0.40$ cm^2/mg for EI (Waygood, 1986) with a monomeric M_r of 63,600. HPr (9119 M_r) was purified as previously described (Reddy et al., 1991) and the concentration of HPr in a stock solution (5.2 mg/mL) was determined in a Beckman Model XL-I analytical ultracentrifuge by the method described by Nosworthy et al. (1998) and found to be 105% of that determined by the spectrophotometric method of Waddell (1956). All chemicals were of the highest purity available, and all solutions were prepared with deionized and filtered water from a Milli-Q Plus system.

Phosphorylated, wild-type enzyme I was prepared by incubating the dephospho protein (15 mg/mL) with 25 mM Tris/HCl, 100 mM NaCl, 5 mM PEP, 2 mM $MgCl_2$, and 2 mM 2-mercaptoethanol, pH 7.5 for 30 min at 25 °C, followed by rapid gel filtration of ~0.25 mL through a PD-10 (G25M sephadex) column (Pharmacia, Uppsala, Sweden), equilibrated with the desired buffer or overnight dialysis at 4 °C against degassed buffer containing 20 mM K-phosphate, pH 7.5 and 100 mM KCl[6] for DSC.

Phosphorylated wild-type EIN (40 mg/mL) was prepared by a 2 h incubation at 25 °C in 25 mM Tris, 100 mM NaCl, 20 mM

PEP, 2 mM $MgCl_2$, 0.7 μM enzyme I, and 1.1 μM HPr at pH 7.5 (Garrett et al., 1998) followed by gel filtration in 10 mM K-phosphate, pH 7.5 or dialysis at 4 °C as above for phospho-EI.

Circular dichroism

CD measurements were performed with a Jasco J-710 spectropolarimeter using water-jacketed (0.2 mm path length) cylindrical cells and an external programmable water bath (Neslab RTE-110) as previously described (Nosworthy et al., 1998). Equilibrium CD measurements at 222 nm for both temperature increases and decreases were made by equilibration at each indicated temperature for 20 min (about 5 min of which was required for instrument equilibration at the set temperature) while monitoring the CD signal vs. time until no further changes in CD were recorded. Equilibrium CD data obtained during the heating cycle were analyzed for fits to a two-state model of unfolding with the program of Kirchhoff (1993).

Differential scanning calorimetry

DSC measurements were performed using the VP-DSC (Plotnikov et al., 1997) ultra-sensitive calorimeter (MicroCal, Inc., Northampton, Massachusetts) and the Nano-DSC (Privalov et al., 1995) calorimeter (Calorimetry Sciences Corp., Provo, Utah). Calibrations were as previously described (Ginsburg & Zolkiewski, 1991). The VP-DSC was run without feedback and 15–30 min equilibration times at 10 °C was used before or between scans. DSC data were corrected for instrument base lines (determined by running the dialysate buffer just prior to placing protein in the sample cell) and normalized for scan rate and protein concentration. Data obtained for EIN proteins were analyzed with the EXAM program of Kirchhoff (1993), which uses linear least-squares fitting of pre- and post-transitional baselines (with a sigmoidal connection centered at T_m). For final fitting of DSC data for nonphosphorylated EIN or EIN mutants to a two-state model of unfolding, N (the number of moles undergoing unfolding) was set to unity (assuming no error in concentration determinations of EIN samples), and the value of ΔC_p was held constant at the value determined from 5–7 data files in the EXAM program. Deconvolutions of DSC data for thermal unfolding EI and EI(H189E) in the absence and presence of HPr were performed using ORIGIN software from MicroCal, Inc. Excess heat capacity (C_p) is expressed as kilocalories per Kelvin per mole, where 1.000 cal = 4.184 J.

Isothermal titration microcalorimetry

All experiments utilized the OMEGA and MCS titration calorimeters from Microcal, Inc. The reference cell was filled with water and acted as a thermal reference to the sample cell. Instruments were calibrated by determining heats of dilution of sucrose at 30 °C, using the equations of Gucker et al. (1939), and were within 2% of calculated values. The sample cell was filled with ~35 μM of E1N solution (effective volume of the cell ~1.4 mL). The 250 μL injection syringe was filled with 330–375 μM HPr. Both HPr and EIN protein solutions were dialyzed in separate Slide-A-Lyzer Cassettes against the same buffer for chemical equilibration. The titration was initiated by the preliminary injection of 2 μL of the HPr solution, followed by 12–14 injections of 20 μL each every 5 min at 30 or 16 °C. During each titration experiment, cell contents were stirred at a constant rate of 395 rpm. The heats produced after saturation of E1N proteins with HPr were used to correct reaction heats for the heat of dilution of HPr into buffer. The final ratios of HPr/E1N in titration experiments were 1.7 with wild-type E1N, 2.6 with EIN(H189E), and 2.7 with EIN(H189A), respectively. A nonlinear least-squares minimization was performed using Microcal ORIGIN software to fit for stoichiometry (n), apparent association constant (K'_A), and standard enthalpy change for the binding of HPr to EIN.

After ITC titrations, samples were removed from the cell and reservoir (~2.0 mL) and directly loaded into the VP-DSC. The final ratios of HPr/E1N were 1.3 to 1.7 in DSC experiments.

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