Properties of the C-terminal Domain of Enzyme I of the Escherichia coli Phosphotransferase System*

Received for publication, August 15, 2005, and in revised form, March 17, 2006 Published, JBC Papers in Press, March 19, 2006, DOI 10.1074/jbc.M508966200

Himatkumar V. Patel1,2, Kavita A. Vyasa3, Roshan L. Mattoo2, Maurice Southworth5, Francine B. Perlerv5, Donald Comb3, and Saul Roseman3

From the 4Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218 and 5New England Biolabs Inc., Ipswich, Massachusetts 01938

The bacterial phosphoenolpyruvate (PEP):glucose phosphotransferase system (PTS) mediates uptake/phosphorylation of sugars. The transport of all PTS sugars requires Enzyme I (EI) and a phosphocarrier histidine protein of the PTS (HPr). The PTS is stringently regulated, and a potential mechanism is the monomer/dimer transition of EI, because only the dimer accepts the phosphoryl group from PEP. EI monomer consists of two major domains, at the N and C termini (EI-N and EI-C, respectively). EI-N accepts the phosphoryl group from phospho-HPr but not PEP. However, it is phosphorylated by PEP(Mg2+2) when complemented with EI-C. Here we report that the phosphotransfer rate increases ~25-fold when HPr is added to a mixture of EI-N, EI-C, and PEP(Mg2+2). A model to explain this effect is offered. Sedimentation equilibrium results show that the association constant for dimerization of EI-C monomers is 260-fold greater than the $K_a$ for native EI. The ligands have no detectable effect on the secondary structure of the dimer (far UV CD) but have profound effects on the tertiary structure as determined by near UV CD spectroscopy, thermal denaturation, sedimentation equilibrium and velocity, and intrinsic fluorescence of the 2 Trp residues. The binding of PEP requires Mg2+2. For example, there is no effect of PEP on the $T_m$, an increase of 7 °C in the presence of Mg2+2, and ~14 °C when both are present. Interestingly, the dissociation constants for each of the ligands from EI-C are approximately the same as the kinetic ($K_a$) constants for the ligands in the complete PTS sugar phosphorylation assays.

The best characterized function of the phosphoenolpyruvate:glycose phosphotransferase system (PTS)4 is in the uptake of sugars by bacterial cells, which are phosphorylated during their translocation across the cytoplasmic membrane (for reviews see Refs. 1–3). There are many variants of the PTS, but all of them require the first two proteins in the phosphotransfer process, Enzyme I (EI) and the low molecular weight phosphocarrier system; PEP, phosphoenolpyruvate; EI, Enzyme I of the PTS; EI-N, Enzyme I N-terminal domain; EI-C, Enzyme I C-terminal domain; HPr, a phosphocarrier histidine protein of the PTS; LDH, lactate dehydrogenase; DTT, dithiothreitol.

PEP(Mg2+) + EI $\rightleftharpoons$ pyruvate + P-EI

REACTION 1

P-EI + HPr $\rightleftharpoons$ P-HPr + EI

REACTION 2

The phosphoryl group is then transferred from P-HPr to the sugar-specific proteins. The properties of EI have been reviewed previously (4, 5). As discussed in an earlier report (6), the phosphotransfer potential of PEP is so high that the PTS must be stringently regulated or cells would burst with accumulated sugar phosphate. One potential mechanism for this regulation is dependent on the properties of the monomer/dimer transition of EI. The dimer accepts the phosphoryl group from PEP, whereas the monomer does not, and association/dissociation is a very slow process relative to the phosphotransfer reactions. A report on the kinetics of EI phosphorylation and dimerization has recently been published (7), and the hydrodynamic properties of the monomer/dimer transition are described in the companion paper (36).

Early work on thermal unfolding of EI, using high sensitivity scanning calorimetry and partial proteolysis, showed that the monomer consisted of two major domains (8), EI-N, the N-terminal, and EI-C, the C-terminal domain. Each consists of about half the molecule, and a short linker polypeptide chain. EI-C is flexible and protease-sensitive, whereas EI-N is compact and protease-resistant. EI-N isolated after proteolysis of intact EI was not phosphorylated by PEP but did react with phospho-HPr, and the same result was obtained with molecularly cloned EI-N (9), which was identical in all respects to EI-N isolated by proteolysis (Reaction 3).

P-HPr + EI-N $\rightleftharpoons$ P-EI-N + HPr

REACTION 3

EI-N did not dimerize and yielded a binding or association constant (9), $K_a = 1.4 \times 10^5$ M⁻¹, for Reaction 4.

HPr + EI-N $\rightleftharpoons$ [HPr · EI-N]

REACTION 4

This value is similar to that obtained with intact EI and HPr (1.1 $\times 10^5$ M⁻¹). Finally, EI-N has been crystallized and its structure (10) resolved to 2.5 Å. Topographically, it resembles an ellipsoid 66 Å long, 16–26 Å wide, and contains two subdomains. One is superposable (topologically) over a subdomain of pyruvate phosphate dikinase, which is also phosphorylated by PEP on a His residue (11, 12). NMR experiments suggest that HPr binds to the cleft between the two subdomains of EI-N (13, 14).
EI-C is responsible for the dimerization of EI monomers (15) and was proposed to bind PEP and possibly Mg\(^{2+}\). The crystal structure of the EI-C domain of *Thermoanaerobacter tengcongensis* was recently reported (16) and comprises homodimers folded into a \(\beta\alpha_6\) barrel with three large helical insertions. The similarity to pyruvate phosphate dikinase is striking in that the active-site residues in the empty PEP binding domain of the EI-C adopt almost identical conformations as the liganded PEP-binding sites of pyruvate phosphate dikinase (11, 12, 16). The EI-C domain from *T. tengcongensis* exhibits 54% amino acid identity to that of *Escherichia coli*. Structural proposals can be made for the *E. coli* EI-C from the above crystal structure. However, as reported here and in earlier work, the crystal structure represents one of what must be many conformers of EI-C (see “Discussion” the companion paper (36)).

Some other important properties of *E. coli* EI-C can be summarized as follows: (a) EI-C was molecularly cloned (17) and shown to complement EI-N, both in vitro and in vivo. That is, EI-N was phosphorylated in the presence of EI-C, PEP, and Mg\(^{2+}\) (Reaction 5).

\[
\text{PEP(Mg}^{2+}\text{)} + \text{EI-N} + [\text{EI-C}] \rightarrow \text{pyruvate} + \text{P-EI-N} + [\text{EI-C}]
\]

**REACTION 5**

The kinetics of this system are presented here, with special emphasis on the effects of HPr. (b) EI-C is an acidic polypeptide (amino acids 256–575 of EI, 35,633 Da), containing 52 acidic and 37 basic residues. The calculated pI = 4.87, and it has a net charge of −15 at pH 7. (c) The 4 Cys and 2 Trp residues of EI are located in EI-C. The C terminus of EI is a Cys. The three internal SH groups are required for dimerization, whereas the C-terminal SH is not (18, 19). Dimerization does not involve formation of −S−S− bonds between the Cys residues. For the present purposes, the rates of reaction of the three internal Cys-SH groups with 5,5′-dithiobis-2-nitrobenzoic acid (18, 19) are of special interest. They change markedly in the presence of EI ligands (PEP, Mg\(^{2+}\), or HPr), thus illustrating, even in these early reports, that the EI-C domain adopts multiple configurations.

The present studies are concerned with determining the association constant of the EI-C monomers, its interactions with the two ligands Mg\(^{2+}\) and PEP, and their effects on some important properties of the polypeptide. In addition, we report the effect of HPr on the kinetics of phosphorylation of EI-N by mixtures of EI-C, PEP, and Mg\(^{2+}\).

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**—The following reagents were purchased from the indicated sources: DTT from Fisher Biotech, β-mercaptoethanol and PEP from Sigma, and EDTA from Fisher Scientific. Other chemicals and buffers from commercial sources were the highest purity available. The New England Biolabs IMPACT system, used for isolation of EI-C, is briefly described below.

Overproducing transformants were used to isolate and purify Enzyme I, HPr and EI-N as reported (9, 20–23). The buffers used were as follows: buffer A (20 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 0.1% Triton X-100, and 1 mM EDTA), buffer B (20 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 0.1% Triton X-100, 1 mM EDTA, and 50 mM DTT), buffer C (10 mM potassium phosphate, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.2 mM DTT), buffer D (10 mM potassium phosphate, pH 7.5, 50 mM KCl, 1 mM EDTA, and 0.2 mM DTT), and buffer F (20 mM potassium phosphate, pH 7.5, 50 mM NaCl, 5 mM MgCl\(_2\), 1 mM EDTA, 2 mM DTT).

**Molecular Cloning and Isolation of EI-C**—Unlike the other cytoplasmic PTS proteins (EI, HPr, and IIAGlc), and EI-N, which are relatively easy to molecularly clone, overexpress, and, therefore, to isolate in sufficient quantity, EI-C proved to be exceedingly difficult to isolate. We made several attempts to use commercial fusion protein systems in which the DNA encoding EI-C (amino acids 256–575 of EI) was fused with genes that specified marker proteins used for purification (e.g., maltose- and chitin-binding proteins). The marker protein was then cleaved from the desired product, such as EI-C, with a protease specific for the peptide linking the two fused proteins. We were unsuccessful in these attempts because EI-C is especially protease-sensitive, although it lacks the amino acid peptide sequences supposedly required by the specific proteases.

Success was finally achieved using the “IMPACT” system of New England Biolabs. This is an in situ system that is used to splice, posttranslationally, two fused proteins or, as in the present case, to isolate a protein in one step (see Ref. 24 and www.neb.com/neb/inteins.html).

The strategy is to start with a construct encoding the Saccharomyces cerevisiae vacuolar ATPase Subunit A intein with an embedded chitin-binding domain. This DNA sequence is then fused to the sequence that encodes EI-C at the C terminus of the intein. The fused protein binds to chitin beads and is thoroughly washed. In the presence of thiols such as DTT, the intein undergoes specific self-cleavage, which releases the target protein from the intein tag attached to the column of chitin beads.

Cells (BL21-DE3-DEI) harboring plasmid pTYB11-E1C were grown in 500 ml of Luria-Bertani medium containing ampicillin 75 μg/ml, at 37°C with shaking to an OD\(_{600}\) of 0.48 and then induced by adding isopropyl 1-thio-β-D-galactopyranoside (0.5 mM) for 15 h at 15°C. The cells were harvested, washed twice with and resuspended in 40 ml of column buffer A. Cells were lysed in the French Press, DNA was precipitated with streptomycin sulfate, and the suspension was centrifuged (200,000 × g) to remove debris. The supernatant was diluted to a final volume of 75 ml and transferred at 1 ml/min to a 12-ml chitin (fresh beads) column equilibrated with 100 ml of buffer A at 4°C. The column was washed with 20 column volumes of buffer A to remove nonspecific protein and flushed with 30 ml of buffer A containing 50 mM DTT (buffer B). The volume of buffer B above the resin was adjusted to 2 ml, and the column was maintained for 18 h at room temperature during which the conjugated protein is cleaved by the intein system and releases EI-C. The column was then allowed to equilibrate to 4°C, and the cleaved protein was eluted with buffer B at 1 ml/min. Fractions (4 ml) were collected. EI-C was found in the first four to five fractions. SDS-PAGE was performed to detect protein in the fractions, and the gels were stained with Coomassie Brilliant Blue.

**Analytical Ultracentrifugation: Sedimentation Equilibrium**—A Beckman Optima XL-I analytical ultracentrifuge with an AnTi60 rotor, equipped with absorption and interference optics, was used for sedimentation equilibrium experiments. These were performed at 5°C and 25°C using cells with carbon-filled six- or two-channel centerpieces (12 mm) and plane quartz windows. Samples of freshly dialyzed protein at 3–15 μM concentrations in 110-μl volumes were loaded into the right side of each cell, with the reference dialysate (115 μl) in the left channel, and centrifuged at different speeds, 17,000, 19,000, and 21,000 rpm for 18–24 h. Absorbance scans at 280 nm were acquired with a step size 0.002 cm at each speed. Likewise, for every speed, Raleigh interference fringes (as described in the Beckman manual) were acquired every 2 h. EI-C was in buffer C or buffer D. The effects of ligands such as Mg\(^{2+}\) and/or PEP at the indicated concentrations were studied under the same conditions.
Protein-specific volumes were calculated to be 0.7328 and 0.7413 ml/g at 5 °C and 25 °C, respectively, based on the amino acid composition of EI-C predicted by the corresponding DNA sequence (using the software sednterp, which is available at www.jphilo.mailway.com). Sedimentation equilibrium data were fitted and weighted globally to a model of reversible monomer dimer association using software “nonlin” (25) and/or XL-A/XL-1 data analysis software, by Beckman Instruments Inc., and “Sedimentation equilibrium” (26). Goodness of fit of data were determined by residuals (within less then ±0.01 for absorbance and to within less then ±0.0006 fringe units for interference). Apparent dimerization constants ($K_{d}$) were converted from either interference of 3.31 fringes/mg unit or of absorbance values at 280 nm to the true concentration-dependent association constants (expressed as molar concentrations of monomer) using a specific molar extinction coefficient at 280 nm of 19,540 cm$^{-1}$ mol$^{-1}$. Oligomers (higher than dimer) were present at < 0.8% of total protein. The predicted (from the DNA sequence) mass of the monomer is 35,633 Da.

Analytical Ultracentrifugation: Sedimentation Velocity—Analytical ultracentrifugation was performed as above. Both absorbance and interference optical detection systems were used to follow the movement of the boundary. All protein samples were dialyzed extensively against buffer C, and the dialysate (420 μl) was transferred to the reference sector. The protein solution (3–15 μM, 400 μl) was loaded into the sample chamber of a charcoal-filled Epon double-sector centriple. Following a 1-h temperature equilibration at 25 °C at rest, the rotor was accelerated to either 50,000 or 52,000 rpm, and refractive index profiles with the Rayleigh interference optical system and absorbance at 280 nm were recorded at regular intervals from initial partial depletion at the meniscus until the sedimentation boundary migrated outside the observable radial range. Data were modeled as a superposition of Lamm equation solutions, $c(s)$ with the software sedfit and also globally fitted with sedphat (27, 28) (both are available at www.analyticalultracentrifugation.com/default.htm). The sedimentation coefficient distribution $c(s)$ was calculated at a confidence level of $p = 0.68$ using maximum entropy regularization, and optimizing parameters and the meniscus position of the solution in the cell by nonlinear regression. Fits were obtained with root mean square deviations between 0.004 and 0.008 fringes or between 0.005 and 0.009 au for absorbance data. The sedimentation coefficients of the monomer and dimer were determined by integration of the main peak of $c(s)$. For monomer and dimer, the hydrodynamic frictional ratio ($f/f_0$) was determined by combining the measured s-value with the molar mass calculated from the amino acid sequence. The determined frictional ratios ($f/f_0$) are molecular constants that include the contributions from hydration. Using estimates of hydration based on the amino acid sequence this frictional ratio can be divided into a hydration and a shape factor, from which dimensions of elliptoid models were derived. These calculations were performed with sednterp.

Far UV CD Spectroscopy—CD spectroscopy was performed with a Jasco J-715 spectropolarimeter equipped with a Neslab water bath to maintain a constant temperature during analysis. Wave scans were acquired by sampling data at 0.2 nm intervals between 250 and 190 nm at a rate of 20 nm/min at 25 °C. Each spectrum represents an average of five scans. The solution contained the following components: EI-C (7 μM) and the specified ligand in buffer C, which was transferred to a 1-mm quartz cuvette.

For “melting” curves, the minima at 222 nm was selected for monitoring the loss of $\alpha$-helix as a function of temperature. The temperature was increased from 20 to 90 °C in steps of 1 °C per minute with 1-min intervals between steps for equilibration. Non-linear least squares fitting was used to determine the $T_m$ from the melting curves.

Near UV CD Spectroscopy—CD spectroscopy was performed on the same instrument as above. Wave scans were acquired by sampling data at 0.2 nm intervals between 350 and 250 nm at a rate of 20 nm/min at 25 °C. Protein in buffer C (36 μM, 1.2 ml) was loaded into a 10-mm quartz cuvette. The blank in all cases was buffer C containing the respective ligands, and the resulting spectra were subtracted from subsequent scans of the protein solutions. Each spectrum is an average of ten scans. The results are expressed as mean molar ellipticity (deg cm$^2$/dmol) at each wavelength.

Fluorescence Spectroscopy—Fluorescence spectroscopy was performed with an SLM Aminco spectrofluorometer. Spectra of EI-C (5 μM) in buffer E at 25 °C in the presence of the indicated ligand were acquired with polarizers positioned at 54.7° in the excitation light beam (4 nm bandwidth). Changes in the intrinsic tryptophan fluorescence intensity were monitored as a function of ligand concentration. The excitation wavelength was set at 291 nm (4 nm bandwidth), and emission was at 338 nm (4 nm bandwidth). Temperature was controlled by a programmable Neslab water bath using a water-jacketed turret containing the cuvettes (1 ml, with 1-cm path length). Samples in the cuvettes were stirred using a magnetic stirrer. Data were fit using non-linear least squares to the ADair equation to obtain $K_D$ values. Stoichiometry of binding of Mg$^{2+}$ to EI-C was estimated by fitting the data to a model of the dimer containing one, two, and four binding sites. Goodness of fit was determined using chi$^2$, residuals, and visual examination of curves. Of the models tested, that with two binding sites/monomer of unequal magnitude produced the best fit (see “Results”) for binding to Mg$^{2+}$. PEP titration was performed in the absence and presence of Mg$^{2+}$ (5 mM) by monitoring change in relative fluorescence intensity at 338 nm as the PEP concentration was varied.

Lactate Dehydrogenase Assays for PEP-dependent Protein Phosphorylation—When PEP phosphorylates a PTS protein, the kinetics of formation of one of the products, pyruvate, can be followed continuously using a coupled assay with lactate dehydrogenase and NADH (29, 30). Briefly, a solution of EI-C (5 μM), PEP (5 mM), with or without EI-N (7 or 11.7 μM), LDH (8 units), NADH (0.150 mM), in buffer F was placed in a cuvette (total volume, 200 μl) at 25 °C, and absorbance was monitored at 340 nm for ~20 min. Readings were taken every 0.1 s. HPt was added either at the start (14 μM final concentration) of the reaction or at the indicated time (8.1 μM).

Determination of Phosphoprotein Synthesis Using $[^{32}P]$PEP—$[^{32}P]$PEP was synthesized and stored as reported (31). EI-C (0.5–5 μM as indicated) and $[^{32}P]$PEP (2 nmol) in 50 μl of buffer F were first incubated at room temperature for 30 min. The reaction was started by adding EI-N (5.85–11.7 μM) and/or HPt (8.1 μM) at the indicated concentration; the total volume was 100 μl. Having determined in preliminary experiments that phosphorylation of EI-N with EI-C was slow (in minutes), the proteins were hand mixed rather than in a rapid quench apparatus (7, 31). Reactions were stopped by adding 50 μl of quench solution (5 mM urea plus 3 M KO H). Phosphoproteins were separated on a Superdex 75 column (Amersham Biosciences) by injecting 105 μl of the quenched reaction mixture at a flow rate of 1 ml/min in buffer containing 3 mM urea plus 35 mM Na$_2$PO$_4$, pH 11. Fractions of separated phosphoproteins (1.1 ml) were collected after 6 min, and the $[^{32}P]$P was determined by using a Liquid Scintillation Spectrometer Counter (Beckman).

RESULTS

Sedimentation Equilibrium Studies with EI-C—Sedimentation equilibrium experiments were conducted as described under "Experimental Pro-
Properties of the C-terminal Domain of Enzyme I

El-C, purified to apparent homogeneity, aggregated at low ionic strength, i.e. at NaCl concentrations below 50 mM, and was also sensitive to air oxidation of the SH groups. However, the protein was stable in solution under a variety of conditions when maintained in 10 mM potassium phosphate buffer, pH 7.5, 100 mM NaCl, 0.2 mM DTT, and 1 mM EDTA (buffer C). Unless otherwise specified, these were the conditions used for the experiments described below.

Sedimentation equilibrium experiments were conducted at three concentrations of the protein, ranging from 3 to 15 μM, at 5 and 25 °C, at three speeds (Fig. 1, see "Materials and Methods") and in the presence and absence of Mg²⁺ (5 mM) or of Mg²⁺ (5 mM) and PEP (10 mM). The following conclusions can be drawn from data in Table 1 and in the accompanying report (36) on intact EI. 1) The same trends were seen with EI-C as with intact EI (36). That is, increases in temperature, increased further in the presence of Mg²⁺, and increased yet again when both Mg²⁺ and PEP were present. 2) The most notable deviation between El and EI-C is the huge difference in the association constants. For instance, at 25 °C in the presence of Mg²⁺, the $K_a$ values are 0.65 μM⁻¹ and 170 μM⁻¹ for El and EI-C, respectively. This 262-fold increase is discussed below. 3) The $K_a$ for El-C was especially sensitive to high ionic strength. For example, as shown in Table 1, in the presence or absence of Mg²⁺, the $K_a$ at 25 °C drops 70- to 90-fold when the KCl concentration is increased from 0.1 to 0.5 M. These results suggest that ionic interactions are of primary importance in the dimerization process.

The results bear directly on all of the experiments that follow, where the concentration of El-C ranged from 3 to 15 μM. Under these conditions, El-C exists from 93 to 98% as the dimer.

**Sedimentation Velocity Studies**—These experiments were analyzed by continuous distribution as described under "Experimental Procedures." Typical results are shown in Fig. 2. Two distinct species are discernible, the presumptive monomer and dimer (exhibiting the larger s). The $s_{20,000}$ values for El-C monomers and dimers in the presence and absence of their ligands are listed in Table 1. There appears to be a consistent increase in the sedimentation coefficients, both for monomers and dimers, as the ligands were varied from none, to Mg²⁺, to Mg²⁺ plus PEP. This result correlates well with those obtained for intact El (36). The experimentally determined s-values yielded information (albeit speculative) about the shape and hydrodynamic volumes of El-C, suggesting that the presence of the ligands, especially both PEP and Mg²⁺, induce a compaction or change in shape of El-C, possibly both. El-C (monomer or dimer) may assume a prolate or an oblate shape. If the shapes of the molecules are assumed to be prolate, the calculations show that the diameter of monomers changes from 117 Å in the absence of any ligand to 93 Å in the presence of both ligands. For oblate ellipsoids, the diameter changes from 78 Å without ligands to 69 Å with both ligands. Similarly, for a dimer when the shapes are assumed to be prolate ellipsoids, the diameter changes from 151 Å in the absence of ligands to 130 Å in the presence of both ligands. For the oblate ellipsoid model, the diameter of dimer changes from 100 Å (no ligands) to 92 Å (with both ligands). Thus, the calculated diameters from either prolate or oblate ellipsoids, for both the dimers and the monomers, were consistently larger in the absence of both the ligands than in the presence of both the ligands. The ligands obviously induce changes in the hydrodynamic properties of the molecules, but defining the precise structural changes awaits further study (see "Discussion").

**CD Experiments with El-C:** Effects of Ligands—A solution of El-C (7 μM) in buffer C was analyzed by CD spectroscopy at 25 °C in the presence and absence of its ligands, and the data are shown in Fig. 3, which

### Table 1

| Conditions | 0.1 M NaCl¹ | 0.5 M NaCl² |
|------------|------------|------------|
|            | 5 °C       | 25 °C      | 5 °C       | 25 °C      |
|            | $s_{20,000}$ | $f/s_0$ | $s_{20,000}$ | $f/s_0$ |
| El         | 26.3       | 0.21       | 1.26       | 3.00 ± 0.03 | 1.32       | 4.65 ± 0.03 | 1.29       |
| El + MgCl₂ | 51         | 0.34       | 1.88       | 2.92 ± 0.02 | 1.30       | 4.53 ± 0.01 | 1.32       |
| El + MgCl₂ + PEP | >100⁴ | >10⁴ | >10⁴ | 3.00 ± 0.03 | 1.32       |

¹ Association constant determined in the presence of 0.1 M NaCl, i.e. in buffer C, and 0.5 M NaCl, i.e. in buffer D.
² If the shapes of the molecules are assumed to be prolate, the calculations give the following molecular diameters for the monomer: 117 Å when alone to 93 Å with both ligands.
³ For oblate ellipsoids, the diameter changes from 78 Å without ligands to 69 Å with both ligands.
⁴ If the shapes of the molecules are assumed to be prolate ellipsoids, the molecular diameters for the dimer changes from 151 Å in the absence of ligands to 130 Å in the presence of both ligands. For the oblate ellipsoid model, the diameter (2a) changes from 100 Å (no ligands) to 92 Å (with both ligands).
⁵ This was difficult to determine accurately due to high variance in fitting parameters and residuals in data fitting.

---

**FIGURE 1.** Sedimentation equilibrium analysis. Representative sedimentation equilibrium experiment with El-C (triangles, 3 μM; squares, 6 μM; and circles, 15 μM) in buffer C was performed at 25 °C. Both absorbance (B) and interference (number of fringes (D)) were measured. Data were fit (as described under "Experimental Procedures"), and the fitting curve (solid line) is shown for each set. The respective weighted residuals (A and C) are also shown for each fitting. For each of the three concentrations, sedimentation equilibrium was determined at three speeds (representative data for 17,000 rpm are shown here), four temperatures, and with different ligands (see text).

---

**TABLE 1**

| Conditions | 0.1 M NaCl¹ | 0.5 M NaCl² |
|------------|------------|------------|
|            | 5 °C       | 25 °C      | 5 °C       | 25 °C      |
|            | $s_{20,000}$ | $f/s_0$ | $s_{20,000}$ | $f/s_0$ |
| El         | 26.3       | 0.21       | 1.26       | 3.00 ± 0.03 | 1.32       | 4.65 ± 0.03 | 1.29       |
| El + MgCl₂ | 51         | 0.34       | 1.88       | 2.92 ± 0.02 | 1.30       | 4.53 ± 0.01 | 1.32       |
| El + MgCl₂ + PEP | >100⁴ | >10⁴ | >10⁴ | 3.00 ± 0.03 | 1.32       |

¹ Association constant determined in the presence of 0.1 M NaCl, i.e. in buffer C, and 0.5 M NaCl, i.e. in buffer D.
² If the shapes of the molecules are assumed to be prolate, the calculations give the following molecular diameters for the monomer: 117 Å when alone to 93 Å with both ligands.
³ For oblate ellipsoids, the diameter changes from 78 Å without ligands to 69 Å with both ligands.
⁴ If the shapes of the molecules are assumed to be prolate ellipsoids, the molecular diameters for the dimer changes from 151 Å in the absence of ligands to 130 Å in the presence of both ligands. For the oblate ellipsoid model, the diameter (2a) changes from 100 Å (no ligands) to 92 Å (with both ligands).
⁵ This was difficult to determine accurately due to high variance in fitting parameters and residuals in data fitting.
indicates significant secondary structure, such as α-helices. However, the important result is that there is no measurable change on adding the ligands PEP, or Mg\(^{2+}\), or PEP plus Mg\(^{2+}\). We concluded that at 25 °C these ligands, if they bind to EI-C, produce no gross change in secondary structure, such as a shift from β-sheet and random coil to α-helix, or vice versa.

On the other hand, the sedimentation equilibrium experiments indicated that there was an Mg\(^{2+}\) effect, an increase in the association constant for dimerization, implying that Mg\(^{2+}\) binds to EI-C under the conditions used for the CD studies. We therefore used other methods to study the interactions between EI-C and its potential ligands.

The first question was whether the ligands did or did not stabilize EI-C with respect to thermal unfolding. Ellipticity was determined at 222 nm as a function of temperature over the range 20 to 90 °C, and the data are shown in Fig. 4A. PEP alone has a minimal effect, an increase in the \(T_m\) of 0.6 °C. But Mg\(^{2+}\) alone increases the \(T_m\) by 7.5 °C, whereas the combination of Mg\(^{2+}\) plus PEP has the most dramatic effect, increasing the \(T_m\) by as much as 14.4 °C. The phenomena were further investigated by determining the changes in \(T_m\) as a function of PEP concentration (Fig. 4B), from which an approximate dissociation constant could be calculated, \(K_D = 0.28 \text{ mM}\), for the interaction of PEP with a mixture of EI-C and Mg\(^{2+}\), assuming 1 mol of PEP binds per mol of EI-C monomer (see below).

Because the thermal unfolding results clearly showed substantial stabilization of EI-C by the ligands, we re-examined CD spectroscopy as a method to detect changes in the structure of the protein. Far UV CD spectroscopy, at 200–250 nm, will show changes in secondary structure, whereas near UV CD spectroscopy, at 250–350 nm, will show changes in tertiary structure. Fig. 5 presents the results obtained by the latter method. There was no detectable effect with PEP alone, a slight effect with Mg\(^{2+}\) alone, and a substantial effect when both ligands were present. The regions of the spectrum that changed suggest that the aromatic amino acids are being affected. EI-C contains 13 Phe, 6 Tyr, and 2 Trp amino acids, so it would be difficult to ascribe the spectral changes to specific amino acids.

**Fluorescence Spectroscopy with EI-C**—Both Trp of EI are located in the EI-C domain, which exhibits the expected Trp excitation and emission spectra. The effects of the ligands on the spectra were tested as shown in Fig. 6, and both spectra exhibited significant changes with Mg\(^{2+}\) alone and with Mg\(^{2+}\) plus PEP (−25% increase at 338 nm). The fluorescence spectra show an increase in intensity but not a shift in the wavelength. This implies that there are no gross changes in conformation upon ligand binding. The spectral changes were sufficient to permit quantitative determinations of the binding constants for the ligands (Fig. 7). PEP alone gave no detectable effect (Fig. 7B), whereas Mg\(^{2+}\) alone produced significant changes (Fig. 7A). As in all the other methods that we tested, the maximum change was induced by the addition of
Properties of the C-terminal Domain of Enzyme I

Both ligands. A titration curve for PEP binding to EI-C/Mg$^{2+}$ is shown in Fig. 7B.

From this result, the dissociation constant of PEP to EI-C (Mg$^{2+}$) could be calculated, $K_D = 0.22$ mM. This value agrees surprisingly well with the $K_D$ (0.28 mM) determined by an entirely different method, the changes in $T_m$ described above. The calculations for estimating the $K_D$ of binding of Mg$^{2+}$ to EI-C are discussed below.

**Stoichiometry of Ligand Binding to EI-C**—The ligand: EI-C dissociation constants derived above are all based on one important assumption, i.e. the stoichiometry of binding is 1 mol of ligand/mol of EI-C monomer. To test this idea, the titration curves for Mg$^{2+}$ were simulated as shown in Fig. 7A using non-linear least squares to obtain binding curves to the Adair equation to obtain $K_D$ values. The stoichiometry of binding was estimated by fitting the data to models with either 0.5 Mg$^{2+}$ (i.e. one per dimer), or with one, two, or four binding sites per monomer. Of the models tested, that with two binding sites/monomer of unequal magnitude, $K_{D1} = 0.39$ mM and $K_{D2} = 4.8$ mM, produced the best fit.

**Kinetics of Phosphorylation of EI-N by EI-C and PEP(Mg$^{2+}$): Effect of HPr**—The EI-N domain of Enzyme I contains both the active site His (autophosphorylated by PEP) and the binding site for HPr. When EI-N was originally isolated by proteolysis of EI (8), it was shown that, unlike EI, it could not be phosphorylated by PEP (Mg$^{2+}$). However, it did accept the phosphoryl group from phospho-HPr. Subsequently, EI-N (9) and EI-C (17) were molecularly cloned, and EI-C was found to complement EI-N both in vitro and in vivo. That is, phospho-EI-N was formed when EI-C was added to a mixture of EI-N, PEP, and Mg$^{2+}$ (Reaction 5 in the introduction). Furthermore, when EI-N and EI-C were expressed in an EI mutant of E. coli, the cells regained the ability to utilize and grow on PTS sugars.

Here, we present studies on the kinetics of EI-N phosphorylation, and the surprising effect of HPr on the rate of this reaction. Two methods were used to study the kinetics, both at 25 °C. (a) A continuous kinetic technique (29, 30), where pyruvate formation was followed spectrophotometrically by coupling the phosphorylation reaction to the lactate dehydrogenase reaction (reduction of pyruvate by NADH). The continuous kinetic method gave the rate of total phosphoprotein formed, i.e. P-EI-N plus P-HPr when HPr was in the mixture. (b) In the second, a discontinuous method, the substrate was $[^{32}P]$PEP, and the products, $[^{32}P]$EI-N and $[^{32}P]$HPr, formed as a function of time, were separated chromatographically after quenching the reaction.

The pyruvate kinetic results shown in Fig. 8A present two experiments. The lower, straight line, which extends for 50 min, consisted of a mixture of EI-N (7 μM), EI-C (5 μM), PEP (5 mM), and Mg$^{2+}$ (5 mM). At the end of the experiment, only a small fraction (~30%) of the EI-N was phosphorylated. By sharp contrast, when HPr (14 μM) was present in the mixture, a rapid burst of phosphorylation was observed for ~10 min, at which point ~15 μM protein (EI-N and HPr) had been phosphorylated, or ~65% of the total phosphorylatable protein in the mixture. The reaction then proceeded more slowly as the substrate concentrations fell, until it was >95% complete in 50 min. Phospho-HPr is spontaneously hydrolyzed under these conditions (32) and explains the linear pyruvate formation late in the experiment.

An even more dramatic illustration of the HPr effect is shown in Fig. 8B. Here, the reaction was initiated with EI-N (11.7 μM), EI-C (5 μM), PEP (5 mM), and Mg$^{2+}$ (5 mM). As in Fig. 8A, there was a slow, linear phosphorylation. When HPr (7 μM) was added, however, there was a rapid burst of phosphorylation, so that >95% of the total HPr and EI-N were phosphorylated in ~30 min.

As noted above, $[^{32}P]$PEP was used in the second, discontinuous method to distinguish between the phosphorylatable proteins, and the
results are given in Fig. 9. Each of the mixtures in these experiments contained EI-C (which is not phosphorylated). Fig. 9A consists of two experiments; one incubation mixture also contained EI-N, whereas the second contained EI-N and HPr. The results are presented as total labeled phosphoprotein formed as a function of time and are similar to those obtained in Method 1 where pyruvate was measured. Panel B depicts the same data but shown only for 60 rather than 350 min. Also, panel B shows the results in the presence and absence of HPr.

Fig. 9C is the same experiment, but this time only with the complete mixture, i.e. containing HPr is shown. The total phosphoprotein formed as a function of time is the top line (filled circles), whereas the individual phosphoproteins are shown in the two lower curves. As can be seen, HPr is more rapidly phosphorylated than EI-N (for example, 4.2 μM P-HPr versus 1.8 μM P-EI-N in 10 min). A model is presented under “Discussion” to explain these results.

DISCUSSION

The schematic model shown in Fig. 10 will be used to discuss the results presented above. No corrections have been made for the spontaneous hydrolysis of phospho-HPr. At 25 °C, pH 7.5, the half-life of P-HPr is 35 min (32). For simplification, and to emphasize the flow of phosphate from free (uncomplexed) P-EI-N to HPr. See “Discussion” for more complete description.

Reaction I: EI-C Monomer/Dimer Transition—Sedimentation equilibrium results showed that EI-C is qualitatively similar to native EI in its associative properties, namely that it is cold-sensitive and affected positively by one of its ligands, Mg^{2+}. Quantitatively, however, the binding constants of the EI monomers are orders of magnitude less than those of EI-C monomers. At 25 °C, for instance, in the presence or absence of Mg^{2+}, the Kd for the self-association of the EI-C monomers was ~250-fold greater than the comparable values for EI monomers (36). The EI-N domain must decrease the tendency of the EI-C domain to self-associate when it is covalently linked in EI, perhaps by steric hindrance. In the concentration range 0.5–7 μM, used in all experiments described in this report, EI-C was at least 95% dimer.

Reaction II: Binding of Mg^{2+} and PEP to EI-C—Circular dichroism spectra (far UV) showed that EI-C contained substantial secondary structure, including α-helices, which agrees with the proposed secondary structure, based on sequence, and, more importantly, the crystal structure of EI-C from T. tengcongensis (16). However, when Mg^{2+} and/or PEP were added there were no significant changes in the spec-
Properties of the C-terminal Domain of Enzyme I

trum. If there are structural changes in the secondary structure on ligand binding, far UV CD spectroscopy was unable to detect them. Therefore, other methods were tested: (a) effects of the ligands on the thermal stability of EI-C and (b) fluorescence of the two Trp in the EI-C domain. Excellent agreement was obtained with these completely different procedures. Because rather large effects were observed, such as a 14.4 °C increase in the $T_m$ when EI-C was subjected to thermal denaturation in the presence of Mg$^{2+}$ and PEP, it was possible to titrate the polypeptide with the ligands and obtain binding constants.

Two important assumptions were made in interpreting these data. First, when there was no detectable change in $T_m$ or in fluorescence intensity, this meant that there was no binding of the ligand to EI-C at that concentration of ligand. Second, that at saturation of EI-C by the ligand, the ratio was 1 mol of ligand/mol EI-C monomer for the high affinity binding site as follows. 1) The binding curves did not indicate any cooperativity, suggesting that there was independent binding of a ligand to each of the EI-C monomers in the dimer. The curve fitting results showed that the best fit for the high affinity site was obtained with 2 mol of Mg$^{2+}$ per dimer. 2) There was no detectable binding of PEP in the absence of Mg$^{2+}$, indicating that binding of both ligands is ordered. In other words, Reaction II in the scheme in Fig. 10 is a two-step process, binding of Mg$^{2+}$ followed by binding of PEP. 3) Judging from the immediate changes in the fluorescence intensity of the Trp groups, the binding of the ligands is a very rapid process. 4) The dissociation constant of Mg$^{2+}$ and EI-C was determined. Curve fitting of the titration curve indicated that there was a high affinity site (per monomer), $K_D = 0.39$ mM, and a low affinity site, $K_D = 4.8$ mM. Interestingly, the apparent $K_D$ for Mg$^{2+}$ in enzymatic assays of Enzyme I (22) in the complete sugar phosphorylation system was 0.53 mM, close to that of the high affinity binding site. 5) Similarly, the $K_a$ for PEP and EI-C (Mg$^{2+}$) was 0.22–0.28 mM, which is surprisingly close to the $K_m$ for PEP in the sugar phosphorylation assay, 0.2–0.4 mM.

Sedimentation velocity was also performed to determine the sedimentation coefficient, which gives insight about the shape, rather than structure, of the protein; a successive increase in $s$-values was observed implying that a change in hydrodynamic properties of the protein, possibly compaction, accompanies binding of both ligands. Obviously more work is required to define the structural (and/or spatial) changes in EI-C, especially what happens when it binds to its ligands, but it is reasonable to conclude that the three-dimensional structure obtained from the crystals is but one of many conformers of EI-C. In the accompanying report (36), we propose a “swivel” model for the change in structure of intact EI when it binds Mg$^{2+}$ and PEP. Because we do not know the amino acid boundaries of the putative swivel, it could conceivably be part of what we have cloned as the EI-C domain. In fact, the near UV CD spectroscopic results (Fig. 5) indicate substantial changes in tertiary structure of EI-C in the presence of Mg$^{2+}$/PEP, reflected by an increase in the ellipticity over the range 250–270 nm. When the same method was applied to intact EI and to EI-H189Q, there were also substantial changes in the presence of the two ligands (36). However, in these cases, the ellipticity decreased, and over a broader range, 255–290 nm. Whether or not we have cloned the swivel as part of EI-C, a clear understanding of the structural changes accompanying binding of the ligands should provide important information on the structural changes that occur in intact EI.

Reaction III: Kinetics of EI-N Phosphorylation with EI-C, PEP, and Mg$^{2+}$—As reported (17), a mixture of EI-C, PEP, and Mg$^{2+}$ will phosphorylate EI-N. In the present studies, the kinetics was followed.

The process is quite slow. Over a period of 1–2 h, only a fraction (~30%) of the EI-N was phosphorylated, and the amount phosphorylated depended on the quantity of EI-C present (data not shown). In general, the quantity of EI-N phosphorylated never exceeded the quantity of EI-C in the mixture. We interpret these results in terms of several steps encompassed in what is designated Reaction III in Fig. 10: 1) EI-N binds to the dimeric EI-C-Mg$^{2+}$/PEP complex. Whether 1 or 2 mol of EI-N binds per mol of complex is not known with certainty, but it appears likely that 2 mol of EI-N bind per dimer of EI-C (see below). 2) The phosphoryl group is transferred to the EI-N moiety from the bound PEP, the pyruvate dissociates (it can be measured with LDH), and presumably it is rapidly replaced by another PEP. The major issue is whether phospho-EI-N dissociates at a significant rate from the EI-C complex. We think that this dissociation, if it occurs at all, must be very slow. This interpretation is based on the fact that the yield of phospho-EI-N did not exceed the amount of EI-C in the mixture. If EI-C acted as a catalyst, many EI-N molecules would have been phosphorylated. The results also suggest that dimeric EI-C binds two EI-N moieties and that both of these are phosphorylated in this mixture. 3) We suggest that the rate of the first step in Reaction III, the association of EI-N with the EI-C complex, is the major factor in determining the rate of EI-N phosphorylation.

Reaction IV: Effect of HPr—The addition of HPr results in a rapid increase in protein phosphorylation. Both species, HPr and EI-N, are phosphorylated. For instance, Fig. 8A shows that ~88% of these proteins (~19 μM) is phosphorylated in ~20 min, whereas in the absence of HPr, only ~15% of the EI-N is phosphorylated. The initial rates are (~32 nm/s) for the complete mixture, compared with (~1.3 nm/s) for the mixture lacking HPr, i.e. a 25-fold increase in the rate of protein phosphorylation.

The results can be explained as follows: 1) The rate constants for each of the phosphotransfer steps in Glc uptake by the E. coli Glc-specific system have been determined (7, 31, 33). The largest rate constants were found to be for the reversible transfer: P-EI plus HPr ⇄ EI plus P-HPr. In the forward direction, the rate constant is 200 × 10⁶ M⁻¹ s⁻¹, which places this transfer among the most rapid reported for enzyme reactions, i.e. in the class of diffusion rate limited reactions (34, 35). The rate constant for the reverse reaction, 3.9 × 10⁶ M⁻¹ s⁻¹, is also rapid, although 50-fold less than in the forward direction. 2) Preliminary experiments indicate that the transfer from P-EI-N to HPr (Reaction IV) has approximately the same rate constant as that for intact P-EI and HPr. Likewise, the reverse step (Reaction V) is very rapid. 3) The HPr effect is therefore explained as follows. A small quantity of complex is formed between EI-N and EI-C dimer containing Mg$^{2+}$ and PEP (Reaction III). The EI-N in the complex is phosphorylated. This, in turn, rapidly reacts with HPr (Reaction IV), part of which phosphorylates some of the uncomplexed EI-N (Reaction V). In this, we also propose that the [EI-C-EI-N-Mg$^{2+}$/PEP] complex does not dissociate, except for the pyruvate, which is replaced by PEP. The final result is that the process becomes repetitive until all available HPr and EI-N have been consumed.

Questions—The results presented here show that HPr can catalyze the formation of large quantities of EI-N. Similarly, native EI monomer, which cannot be phosphorylated by PEP, is rapidly phosphorylated by PEP when a small amount of dimer and HPr are present (7). What this may mean physiologically we can only surmise. Because there are many interactions between phospho-HPr and other PTS proteins, conceivably phospho-EI monomer may serve as a storage form of high energy phosphate independent of PEP. For instance, when the PEP concentration is low, P-EI monomer might be used to initiate some physiological process such as immediate uptake of a sugar, or phosphorylation
of IIA\textsuperscript{Gl}

to regulate adenylate cyclase or certain non-PTS sugar transporters.

Here, we focused on the two major domains of EI, EI-N and EI-C. Although some questions have been answered, many remain. For example, what are the binding constants and stoichiometry between EI-C and EI-N, or EI-C and P-EI-N? What are the effects of the ligands on these constants? Can we gain information on the binding constants of the domains in intact Enzyme I? Answers to these questions will be sought in future studies.

Acknowledgments—We thank Drs. Evangelos Moudrianakis (Johns Hopkins University) and Allen Minton (National Institutes of Health) for providing expert assistance and guidance in the analytical ultracentrifugation studies, Editor Robert Simoni and the referee who suggested that we conduct the near UV CD spectroscopic studies, and Dr. Doug Barrick who helped us with these experiments. Analytical high-performance liquid chromatography was performed with an 817 Bioscan system manufactured by Metrohm-Peak Ltd., Switzerland; Dr. Randy Benton kindly provided expert assistance with this equipment. Analytical high-performance liquid chromatography was performed with an 817 Bioscan system manufactured by Metrohm-Peak Ltd., Switzerland; Dr. Randy Benton kindly provided expert assistance with this equipment. We also thank Drs. Ludwig Brand and Dmitri Toptygin for valuable suggestions and use of fluorescence equipment, Dr. Ernesto Freire for valuable suggestions and use of fluorescence equipment, Dr. Doug Barrick who helped us with these experiments. Analytical high-performance liquid chromatography was performed with an 817 Bioscan system manufactured by Metrohm-Peak Ltd., Switzerland; Dr. Randy Benton kindly provided expert assistance with this equipment. We also thank Drs. Ludwig Brand and Dmitri Toptygin for valuable suggestions.

REFERENCES

1. Meadow, N. D., Fox, D. K., and Roseman, S. (1990) Annu. Rev. Biochem. 59, 497–542
2. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) Microbiol. Rev. 57, 543–594
3. Saier, M. H., Jr. (2001) J. Mol. Microbiol. Biotechnol. 3, 325–327
4. Chauvin, F., Brand, L., and Roseman, S. (1996) Rev. Microbiol. 47, 31–37
5. Ginsburg, A., and Peterkofsky, A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1332–1337
6. Weigel, N., Kukuruzinska, M. A., Nakazawa, A., Waygood, E. B., and Roseman, S. (1982) J. Biol. Chem. 257, 14477–14491
7. Meadow, N. D., Mattoe, R. L., Savtchenko, R. S., and Roseman, S. (2005) Biochemistry 44, 12790–12796
8. LiCalzi, C., Crocenzi, T. S., Freire, E., and Roseman, S. (1991) J. Biol. Chem. 266, 19519–19527
9. Chauvin, F., Fomenkov, A., Johnson, C. R., and Roseman, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7028–7031
10. Liao, D.-I., Silverton, E., Seok, Y.-J., Lee, B. R., Peterkofsky, A., and Davies, D. R. (1996) Structure 4, 861–872
11. Herzberg, O., Chen, C. C., Kapadia, G., McGuire, M., Carroll, L. J., Noh, S. J., and Dunaway-Mariano, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2652–2657
12. Herzberg, O., Chen, C. C., Liu, S., Tempczyk, A., Howard, A., Wei, M., Ye, D., and Dunaway-Mariano, D. (2002) Biochemistry 41, 780–787
13. Garrett, D. S., Seok, Y.-J., Peterkofsky, A., Gronenborn, A. M., and Clore, G. M. (1999) Nat. Struct. Biol. 6, 166–173
14. Peterkofsky, A., Wang, G., Garrett, D. S., Lee, B. R., Seok, Y. J., and Clore, G. M. (2001) J. Mol. Microbiol. Biotechnol. 3, 347–354
15. Brox, S. J., Talbot, J., Georges, F., and Waygood, E. B. (2000) Biochemistry 39, 3624–3635
16. Oberholzer, A. E., Bumann, M., Schneider, P., Bachler, C., Siebold, C., Baumann, U., and Erni, B. (2005) J. Mol. Biol. 346, 521–532
17. Fomenkov, A., Valiakhmetov, A., Brand, L., and Roseman, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8491–8495
18. Han, M. K., Walbridge, D. G., Knutson, J. R., Brand, L., and Roseman, S. (1987) Anal. Biochem. 161, 479–486
19. Han, M. K., Roseman, S., and Brand, L. (1990) J. Biol. Chem. 265, 1985–1995
20. Chauvin, F., Brand, L., and Roseman, S. (1994) J. Biol. Chem. 269, 20720–20724
21. Reddy, P., Fredd-Kuldell, N., Liberman, E., and Peterkofsky, A. (1991) Protein Expression Purif. 3, 179–187
22. Weigel, N., Waygood, E. B., Kukuruzinska, M. A., Nakazawa, A., and Roseman, S. (1982) J. Biol. Chem. 257, 14461–14469
23. Anderson, B., Weigel, N., Kundig, W., and Roseman, S. (1971) J. Biol. Chem. 246, 7023–7033
24. Perler, F. B. (2002) Nucleic Acids Res. 30, 383–384
25. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Halvorson, H. R. (1981) Biophys. J. 36, 575–588
26. Rivas, G., Stafford, W., and Minton, A. P. (1999) Methods Companion Methods Enzymol. 19, 194–212
27. Dam, J., and Schuck, P. (2004) Methods Enzymol. 384, 185–212
28. Schuck, P. (2000) Biophys. J. 78, 1606–1619
29. Waygood, E. B., Meadow, N. D., and Roseman, S. (1979) Anal. Biochem. 95, 293–304
30. Waygood, E. B., and Meadow, N. D. (1982) Methods Enzymol. 90, 423–431
31. Meadow, N. D., and Roseman, S. (1996) J. Biol. Chem. 271, 33440–33445
32. Waygood, E. B., Erickson, E., El-Kabbani, O. A. L., and Delbaere, L. T. J. (1985) Biochemistry 24, 6938–6945
33. Rohwer, J. M., Meadow, N. D., Roseman, S., Westerhoff, H. V., and Postma, P. W. (2000) J. Biol. Chem. 275, 34909–34921
34. Miller, B. G., and Wolfenden, R. (2002) Annu. Rev. Biochem. 71, 847–885
35. Fersht, A. R. (1985) Enzyme Structure and Mechanism, 2nd Ed., W.H. Freeman, New York
36. Patel, H. V., Vyas, K. A., Savtchenko, R. S., and Roseman, S. (2006) J. Biol. Chem. 281, 17570–17578