The Monomer/Dimer Transition of Enzyme I of the Escherichia coli Phosphotransferase System*

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Enzyme I (EI) is the first protein in the phosphotransfer sequence of the bacterial phosphoenolpyruvate:glycose phosphotransferase system. This system catalyzes sugar phosphorylation/transport and is stringently regulated. Since EI homodimer accepts the phosphoryl group from phosphoenolpyruvate (PEP), whereas the monomer does not, EI may be a major factor in controlling sugar uptake. Previous work from this and other laboratories (e.g. Dimitrova, M. N., Szczepanowski, R. H., Ruvinov, S. B., Peterkofsky, A., and Ginsburg A. (2002) Biochem. 41, 906–913), indicate that $K_a$ is sensitive to several parameters. We report here a systematic study of $K_a$ determined by sedimentation equilibrium, which showed that it varied by 1000-fold, responding to virtually every parameter tested, including temperature, phosphorylation, pH (6.5 versus 7.5), ionic strength, and especially the ligands Mg$^{2+}$ and PEP. This variability may be required for a regulatory protein. Further insight was gained by analyzing EI by sedimentation velocity, by near UV CD spectroscopy, and with a nonphosphorylatable active site mutant, EI-H189Q, which behaved virtually identically to EI. The singular properties of EI are explained by a model consistent with the results reported here and in the accompanying paper (Patel, H. V., Vyas, K. A., Mattoo, R. L., Southworth, M., Perler, F. B., Comb, D., and Roseman, S. (2006) J. Biol. Chem. 281, 17579–17587). We suggest that EI and EI-H189Q each comprise a multiplicity of conformers and progressively fewer conformers as they dimerize and bind Mg$^{2+}$ and finally PEP. Mg$^{2+}$ alone induces small or no detectable changes in structure, but large conformational changes ensue with Mg$^{2+}$/PEP. This effect is explained by a “swiveling mechanism” (similar to that suggested for pyruvate phosphate dikinase (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)), which brings the C-terminal domain with the two bound ligands close to the active site His$^189$.

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3 The abbreviations used are: PTS, phosphoenolpyruvate:glycose phosphotransferase system; PEP, phosphoenolpyruvate; EI, Enzyme I of PTS; EI-N, Enzyme I N-terminal domain; EI-C, Enzyme I C-terminal domain; HPr, a phosphocarrier histidine protein of the PTS; M/D, monomer/dimer; DTT, dithiothreitol.

The phosphoenolpyruvate:glycose phosphotransferase system (PTS), is widely distributed in bacteria and has several important roles in these cells, the most general being PTS sugar uptake where these substrates are translocated across the cytoplasmic membrane concomitant with their phosphorylation. Indeed, the PTS was discovered as a sugar phosphorylation/transport system. This system catalyzes sugar phosphorylation/transport and is stringently regulated. Since EI homodimer accepts the phosphoryl group from phosphoenolpyruvate (PEP), whereas the monomer does not, EI may be a major factor in controlling sugar uptake. Previous work from this and other laboratories (e.g. Dimitrova, M. N., Szczepanowski, R. H., Ruvinov, S. B., Peterkofsky, A., and Ginsburg A. (2002) Biochem. 41, 906–913), indicate that $K_a$ is sensitive to several parameters. We report here a systematic study of $K_a$ determined by sedimentation equilibrium, which showed that it varied by 1000-fold, responding to virtually every parameter tested, including temperature, phosphorylation, pH (6.5 versus 7.5), ionic strength, and especially the ligands Mg$^{2+}$ and PEP. This variability may be required for a regulatory protein. Further insight was gained by analyzing EI by sedimentation velocity, by near UV CD spectroscopy, and with a nonphosphorylatable active site mutant, EI-H189Q, which behaved virtually identically to EI. The singular properties of EI are explained by a model consistent with the results reported here and in the accompanying paper (Patel, H. V., Vyas, K. A., Mattoo, R. L., Southworth, M., Perler, F. B., Comb, D., and Roseman, S. (2006) J. Biol. Chem. 281, 17579–17587). We suggest that EI and EI-H189Q each comprise a multiplicity of conformers and progressively fewer conformers as they dimerize and bind Mg$^{2+}$ and finally PEP. Mg$^{2+}$ alone induces small or no detectable changes in structure, but large conformational changes ensue with Mg$^{2+}$/PEP. This effect is explained by a “swiveling mechanism” (similar to that suggested for pyruvate phosphate dikinase (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)), which brings the C-terminal domain with the two bound ligands close to the active site His$^189$.

The system has been extensively studied and reviewed (4, 5). Although variations of the basic motif are known, the most general phosphotransfer sequence is as follows.

$$\text{Sugars} \rightarrow \text{Enzymes II} \rightarrow \text{HPr} \rightarrow \text{Enzyme I} \rightarrow \text{PEP}$$

### SCHEME 1

Each step is physiologically reversible except for the last, phosphotransfer to the sugar. The phosphotransfer potential of PEP is 14.7 kcal/mol, about twice that of ATP and greater than any other naturally occurring phosphate derivative. Since the phosphotransfer potentials of the PTS proteins are close to that of PEP, the energetics of the system strongly favor sugar uptake (6). From these considerations alone, it is apparent that the PTS must be stringently regulated, and indeed it is. Even the earliest results on the glucose permease by Kepes (7), before the PTS was discovered (2), showed that when a noncatabolizable Glc analogue, methyl α-D-glucopyranoside, is taken up by intact cells, the rate of uptake declined virtually immediately. Thus, the progress curves for uptake of PTS sugars resemble hyperbolas. These results are observed not only with intact cells but also with membrane vesicles supplied with unlimited quantities of PEP (8). We originally suggested Enzyme I as a potential candidate for governing the system (6). This idea is based on the facts that EI monomer forms a homodimer (9, 10), that the dimer but not the monomer is phosphorylated by PEP in the presence of Mg$^{2+}$, and that the rate of association/dissociation is surprisingly slow, much slower than sugar uptake (11–13). This difference in rates suggests that regulation of sugar transport could be affected by factors or ligands (e.g. metabolites or other proteins) that temporarily shift the M/D transition away from its normal equilibrium value. The extreme case would be conversion of all dimer to monomer, which would stop sugar uptake until the system slowly recovered as the monomer spontaneously dimerized.

As indicated in the references cited above, the EI M/D transition has been studied by sedimentation equilibrium, analytical gel filtration chromatography, fluorescence spectroscopy, and more recently by Ginsburg and co-workers (1, 14, 15) by sedimentation equilibrium and microcalorimetry. These reports give the $K_a$ of the monomer to dimer under various conditions and, in some cases, the $K_a$ of the phosphoprotein. However, the need for additional information on the M/D transition is further emphasized by recent findings (16) that EI can be seques-

The accompanying paper (37) describes the behavior of the C-terminal domain of EI. Based on the results described here and with the...
C-terminal domain, a model is offered to explain the apparent anomalous association/dissociation behavior of Enzyme I.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were purchased from the indicated sources: DTT from Fisher, β-mercaptoethanol and PEP from Sigma, EDTA from Fisher, S-300 Sepharose beads from Amersham Biosciences, and Ni2+-nitrilotriacetic acid–agarose beads from Qiagen Inc. Other chemicals and buffers from commercial sources were of the highest purity available. Overproducing transformants were used to isolate and purify Enzyme I and HPr as reported (11, 18–21).

Molecular Cloning and Purification of EI-H189Q—DNA preparation and analysis, restriction enzyme digests, ligation, and transformations were performed according to standard techniques (22). A QuickChange site-directed mutagenesis kit (Stratagene) was used to mutate the active site His189 of EI to Gln. BL21-DE3-ΔEI cells carrying the plasmid pET 21a EI-H189Q were grown to an OD600 = 0.5, induced with isopropyl β-D-thiogalactopyranoside (1 mM), and further allowed to grow at 37 °C for 2 h. The cells were harvested, and the protein was purified by the method used for wild type EI, which yielded a partially purified protein. This preparation was then purified to apparent homogeneity by passing the protein over a 450-ml S-300 Sepharose (Amersham Biosciences) gel filtration column connected to a fast protein liquid chromatography (Amersham Biosciences) system. The column was equilibrated with 50 mM potassium phosphate buffer, pH 6.5, containing 5 mM EDTA, 40 mM L-aminocaproic acid, 0.2 mM DTT, and 5 mM MgCl2. The sample was applied, and the column was eluted in the above buffer at a flow rate of 1 ml/min. Fractions of 2 ml were collected, and SDS-PAGE was performed. The gels were stained with Coomassie Brilliant Blue, and fractions containing apparently homogeneous proteins were combined and concentrated. The protein migrated at the expected molecular weight.

Analytical Ultracentrifugation: Sedimentation Equilibrium—Analytical ultracentrifugation was performed as follows. A Beckman XL-I analytical ultracentrifuge, with an AnTi60 rotor, equipped with absorption and interference optics, was used for sedimentation equilibrium experiments. These were performed at the indicated temperatures, usually 5, 15, 25, and 35 °C using cells with carbon-filled six-channel or two-channel centerpieces (12 mm) and plane quartz windows. Samples of freshly dialyzed protein at three concentrations, ranging from 3 to 15 μM, in 110-μl volumes were transferred to the right side of each cell, with the reference dialysate (115 μl) in the left channel, and centrifuged at three different speeds, ranging from 17,000 to 21,000 rpm for 18–24 h. Absorbance scans at 280 nm were acquired every 2 h with a step size of 0.002 cm at each speed. Likewise, for every speed, Raleigh interference fringes (as described in the Beckman manual) were acquired every 2 h. The following buffers were employed: each contained 0.2 mM DTT to protect the sensitive SH groups in EI: buffer B, 100 mM KCl, 10 mM potassium phosphate, 1 mM EDTA, 0.2 mM DTT at pH 6.5; buffer C, 100 mM KCl, 10 mM potassium phosphate, 1 mM EDTA, 0.2 mM DTT at pH 7.5; buffer D, 50 mM potassium phosphate, 1 mM EDTA, 0.2 mM DTT at pH 6.5; buffer E, 50 mM potassium phosphate, 1 mM EDTA, 0.2 mM DTT at pH 7.5; buffer F, 100 mM KCl, 10 mM Hepes, 1 mM EDTA, 0.2 mM DTT at pH 7.5; buffer G, 75 mM KCl, 10 mM potassium phosphate, 10 mM Hepes, 0.2 mM EDTA, 0.2 mM DTT at pH 7.5.

The pH values given above were measured on the mixtures at room temperature. Protein partial specific volumes for EI were calculated to be 0.7359, 0.7402, 0.7443, and 0.7487 ml/g at 5, 15, 20, 25, and 35 °C, respectively. These calculations were based on the amino acid compositions of the respective proteins predicted by the corresponding amino acid sequence (using the software sednterp, which is available on the World Wide Web at www.jphilo.mailway.com). Sedimentation equilibrium data were fitted, weighted globally to a model of reversible monomer dimer association using “nonlin” software (23) and/or XL-A/ XL-I data analysis software (Beckman Instruments) and sedimentation equilibrium (24). Goodness of fit of data was determined by residuals (within ± 0.01 for absorbance and to within ± 0.0006 fringe units for interference). Apparent dimerization constants (Kobs) 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 24,410 cm−1 mol−1 for EI. Oligomers (higher than dimer) were present at <0.8% of total protein. The predicted (from the DNA sequence) mass of the monomer is Mr = 63,562 daltons for EI.

Sedimentation experiments with known and potential ligands were conducted similarly. The ligands were Mg2+ and/or PEP and/or HPr. Potential ligands included glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-diphosphate in the presence of Mg2+ and PEP.

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 μg, 400 μl) was loaded into the sample chamber of a charcoal-filled Epon double-sector centerpiece. Following a 1-h temperature equilibration at 25 °C at rest, the rotor was accelerated to either 45,000 or 50,000 rpm, and refractive index profiles...
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**Table 1**

| Experiment | Temperature°C | Ligands | Absorbance | Interference | Average | Reported values |
|------------|---------------|---------|-------------|--------------|---------|----------------|
| 1          | 5             | None    | 0.086       | 0.065        | 0.076   | A               |
|            | 15            |         | 0.22        | 0.25         | 0.23    |                 |
|            | 20            |         | 0.39        | 0.21         | 0.3     |                 |
|            | 25            |         | 0.33        | 0.42         | 0.38    |                 |
|            | 35            |         | 0.19        | 0.23         | 0.21    |                 |
| 2          | 5             | None    | 0.05        | 0.033        | 0.042   | B               |
|            | 15            | 50 mM   | 0.128       | 0.122        | 0.125   |                 |
|            | 25            | KP      | 0.208       | 0.22         | 0.214   |                 |
|            | 35            |         | 0.181       | 0.125        | 0.153   |                 |
| 3          | 5             | Mg²⁺   | 0.081       | 0.057        | 0.069   | C               |
|            | 15            |         | 0.312       | 0.315        | 0.313   |                 |
|            | 25            |         | 0.668       | 0.634        | 0.651   |                 |
|            | 35            |         | 0.29        | 0.296        | 0.293   |                 |
| 4          | 5             | HPr     | 0.018       | ND           | 0.018   | D               |
|            | 15            |         | 0.085       |              | 0.085   |                 |
|            | 25            |         | 0.315       |              | 0.315   |                 |
|            | 35            |         | 0.095       |              | 0.095   |                 |
| 5          | 5             | Mg²⁺   | 0.073       | ND           | 0.073   |                 |
|            | 15            |         | 0.425       |              | 0.425   |                 |
|            | 25            | HPr     | 0.952       |              | 0.952   |                 |
|            | 35            |         | 0.893       |              | 0.893   |                 |
| 6          | 5             | Mg²⁺   | 1.23        | 1.24         | 1.23    | E               |
|            | 15            |         | 4.1         | 3.42         | 3.76    |                 |
|            | 25            | PEP     | 5.58        | 5.44         | 5.51    |                 |
|            | 35            |         | 6.15        | 7.89         | 7.02    |                 |
| 7          | 5             | Mg²⁺   | 0.073       | ND           | 0.074   |                 |
|            | 15            | PEP     | 0.425       |              | 1.29    |                 |
|            | 25            | HPr     | 0.952       |              | 2.04    |                 |
|            | 35            |         | 0.893       |              | 1.39    |                 |
| 8          | 5             | Mg²⁺   | 0.71        | 0.85         | 0.78    |                 |
|            | 15            | PEP     | 1.76        | 1.34         | 1.55    |                 |
|            | 25            | Pyr     | 1.97        | 1.84         | 1.94    |                 |
|            | 35            |         | 2.12        | 2.71         | 2.41    |                 |

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 sedsoft and also globally fitted with sedphat (25, 26); these are available on the World Wide Web 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 $Fw$ and the meniscus position of the solution in the cell by nonlinear regression. Fits were obtained with root mean square deviations between 0.002 and 0.008 fringes or between 0.004 and 0.009 absorbance units for absorbance data. The sedimentation coefficients of the monomer and dimer was 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 ellipsoid models were derived. These calculations were performed with sednterp. We emphasize that the $f/f_0$ ratios are approximations because of the assumptions required for the calculations and that discussions of "shape" (e.g. oblate and prolate ellipsoids) are only raw estimates. Whatever the shapes may be, the changes observed when the ligands are present are clearly significant.

**Near and 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. For near UV CD, 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. En in buffer C (17 μM, 1.2 ml) was placed in a 10-mm quartz cuvette. The blank in all cases was buffer C with the respective ligands and was subtracted from subsequent scans of the protein solutions. The final spectrum is an average of 10 scans. The results are expressed as mean molar ellipticity (degrees · cm²/dmol) at each wavelength. For far UV CD spectroscopy, the concentrations of protein were only 7 μM, and the path length of the cuvette was 1 mm.

**RESULTS**

**Sedimentation Equilibrium Studies with Enzyme I**

Sedimentation equilibrium experiments with En purified to apparent homogeneity were conducted as described under “Experimental Procedures.” Data were collected by following protein concentrations in the sedimentation cells both by UV absorbance and by interference spectroscopy, and typical results are shown in Fig. 1. The effects of the following parameters on the M/D transition were tested: temperature,
TABLE 2
Effects of temperature, ligands, and phosphorylation on the EI monomer/dimer transition at pH 6.5

| Experiment | Temperature | Ligands | $K_a$ (Absorbance) | $K_a$ (Interference) | $K_a$ (Average) | Reported values |
|------------|-------------|---------|--------------------|---------------------|-----------------|----------------|
| 1          | 5           | None    | 0.074              | 0.068               | 0.071           | A              |
|            | 15          |         | 0.25               | 0.43                | 0.34            |
|            | 20          |         | 1.96               | 0.99                | 1.47            |
|            | 25          |         | 2.26               | 1.06                | 1.66            |
|            | 35          |         | 1.1                | 0.83                | 0.96            |
| 2          | 5           | None    | 0.028              | 0.031               | 0.03            |
|            | 15          | 50 mM KPi| 0.176              | 0.2                 | 0.188           |
|            | 25          |         | 0.305              | 0.472               | 0.388           |
|            | 35          |         | 0.261              | 0.392               | 0.327           |
| 3          | 5           |         | 0.76               | 0.82                | 0.79            |
|            | 15          | Mg$^{2+}$| 1.86               | 1.77                | 1.82            |
|            | 25          |         | 4.57               | 4.97                | 4.77            |
|            | 35          |         | 3.94               | 4.11                | 4.03            |
| 4          | 5           |         | 3.12               | 3.26                | 3.19            |
|            | 15          | Mg$^{2+}$| 13.5               | 15.5                | 14.5            |
|            | 25          | PEP     | 41.2               | 43                  | 42.1            |
|            | 35          |         | 47.3               | 49.2                | 48.3            |
| 5          | 5           |         | 0.986              | 1.18                | 1.08            |
|            | 15          | PEP     | 4.46               | 4.96                | 4.71            |
|            | 25          | Pyr     | 13.9               | 15.2                | 14.6            |
|            | 35          |         | 14.8               | 15.9                | 15.3            |

At pH 6.5—The maximum $K_a$, 48 $\mu M^{-1}$, was obtained with phospho-EI in the presence of its ligands, Mg$^{2+}$ and PEP, at 35 °C. The minimum value was 0.03 $\mu M^{-1}$ at 5 °C in the absence of ligands and in 50 mM phosphate. The marked pH effect is further discussed below.

Temperature

Since it was first purified, EI has been recognized to be a cold-sensitive protein. At the four temperatures studied, 5, 15, 25, and 35 °C, the results were fairly consistent. $K_a$ increased with temperature, the optimum generally being at 25 °C, but in one important case, the phosphoprotein in the presence of its ligands, the largest $K_a$ was at 35 °C. The effect of varying the temperature while keeping all other parameters constant was generally about 10 for the ratio $K_a$ at optimum temperature/$K_a$ at 5 °C.

pH and Ionic Strength

Enzyme I has usually been assayed for activity at pH 6.5 or 7.5. The M/D transition was surprisingly resistant to this change or remarkably sensitive, depending on the temperature and ionic strength. The results are shown in Fig. 3. Under one set of conditions (25 °C, 10 mM potassium phosphate, 100 mM KCl), the $K_a$ was 4-fold greater at pH 6.5 than at pH 7.5. However, this effect was suppressed by increasing the phosphate concentration to 50 mM or by decreasing the temperature.

The pH effects at 25 °C, illustrated in Fig. 4 show that $K_a$ is 4–8-fold greater at pH 6.5 than at pH 7.5. The remaining results discussed here were obtained at pH 7.5, the pH most often employed to assay sugar phosphorylation by the PTS, and closer to the intracellular pH.

Ligands

Interestingly, in light of the data we report in the accompanying paper (37) for the EI carboxyl-terminal domain, EI-C, the effects of the ligands Mg$^{2+}$ alone, HPr alone, and a combination of the two had relatively little effect on $K_a$. PEP alone was not studied because of the time required and the possibility of a low rate of phosphate transfer to EI over this period of time in the absence of Mg$^{2+}$.
The presence of Mg2+ phosphorylated, and for this reason the experiments were conducted in the presence of Mg2+ ions over a period of many hours, it was essential to keep the protein fully phosphorylated. For the sedimentation studies, which were performed at concentrations 3–15 μM. The following concentrations were used: Mg2+ 5 mM; PEP, 20 mM except at 1 mM in the presence of 5 mM pyruvate. The other ligands were as follows: glucose 6-phosphate (G6P, 5 mM), fructose 6-phosphate (F6P, 5 mM), and fructose 1,6-diphosphate (F1,6P, 5 mM).

Phosphorylation

The phosphoprotein is known to have a higher association constant than the dephosphoprotein. For the sedimentation studies, which were performed over a period of many hours, it was essential to keep the protein fully phosphorylated, and for this reason the experiments were conducted in the presence of Mg2+ and PEP, meaning that two parameters were being varied. As will be emphasized below, the effect of the two ligands on the association constants was clear.

Conclusion

Dimerization of EI monomer is sensitive to virtually every parameter tested, which means that the dimerization domain (EI-C) must be highly flexible and capable of responding to these changes in the microenvironment. This property is further considered in the accompanying report (37).

Sedimentation Equilibrium Studies with EI-H189Q

Table 3 summarizes the $K_a$ values obtained with the active site mutant EI-H189Q at pH 7.5. This mutant cannot be phosphorylated. Again, unexpected results were obtained. In the absence of ligands or in the presence of Mg2+, at the four temperatures used for these studies, the association constants were close to but somewhat less than those obtained with native EI. However, in the presence of both PEP and Mg2+, the $K_a$ values for the mutant were 2–3-fold greater than for native phospho-EI and 40–100-fold greater than dephospho-EI in the absence of the ligands. The results are further discussed below and compared with those obtained with other nonphosphorylatable EI mutants, EI-H189A and EI-H189E (1, 14).

Sedimentation Velocity Studies

These experiments were conducted, and the data were analyzed as described under “Experimental Procedures.” Typical results are shown in Fig. 5 for native EI in the absence of ligands. Two distinct species are apparent, the presumptive monomer and dimer, the dimer exhibiting the larger sedimentation value. The $c(s)$ distributions for EI and EI-H189Q monomers and dimers in the presence and absence of their ligands are listed in Table 4. Three conclusions can be drawn from these data. (a) As expected, each dimer exhibited a larger value of the sedimentation coefficient than the corresponding monomer. (b) There is a consistent increase in the sedimentation coefficients, both for monomers and dimers, as the ligands were varied from none, to Mg2+, to Mg2+ + PEP.

### Table 3

| Protein     | Ligands    | Average $K_a$ (μM) |
|-------------|------------|-------------------|
| EI          | None       | 0.076, 0.23, 0.38, 0.21 |
| EI-H189Q    | None       | 0.048, 0.17, 0.34, 0.27 |
| EI          | Mg2+      | 0.069, 0.31, 0.65, 0.29 |
| EI-H189Q    | Mg2+      | 0.039, 0.22, 0.43, 0.22 |
| EI          | Mg2+ + PEP| 1.2, 3.8, 5.5, 7 |
| EI-H189Q    | Mg2+ + PEP| 2.8, 8.8, 15, 22 |

FIGURE 3. Dimerization of EI depends on ionic strength, pH, and temperature. Shown is $K_a$ of EI in 10 mM KPi, 100 mM KCl (triangles), or 50 mM KP, (squares) at the indicated pH. The highest association constant was at pH 6.5.

FIGURE 4. Effects of ligands on dimerization of EI at pH 6.5 and 7.5. The data were selected from Tables 1 and 2 and are shown here for clarity to emphasize the large pH effect. EI was used at concentrations 3–15 μM. The following concentrations were used: Mg2+, 5 mM; PEP, 20 mM except at 1 mM in the presence of 5 mM pyruvate. The other ligands were as follows: glucose 6-phosphate (G6P, 5 mM), fructose 6-phosphate (F6P, 5 mM), and fructose 1,6-diphosphate (F1,6P, 5 mM).

FIGURE 5. Sedimentation coefficient distribution (c(s)) from sedimentation velocity analyses. EI (3–15 μM) and EI-H189Q (3–15 μM) in buffer C were analyzed at either 45,000 or 50,000 rpm at 25 °C. Sedimentation scans were collected at 3-min intervals and monitored by both interference and UV absorbance methods. Sedimentation velocity data were analyzed using a continuous c(s) distribution model implemented in the program sedfit. Representative sedimentation data (about half of the data for the sake of clarity), with c(s) fits to each data set, are shown for EI (6 μM) in A, and resulting residuals are given in B. All data were included in the calculations of c(s) distributions. The sedimentation coefficient distribution, c(s), was calculated using maximum entropy regularization. The sedimentation coefficients obtained from these c(s) fitting are shown in C.
(c) Perhaps the most interesting result was that the \( s_{20, w}^0 \) values for native EI and EI-H189Q agreed within the experimental errors of each of the values. This significant finding indicates that phosphorylation of His\(^{189} \) in native EI has relatively little effect on the hydrodynamic properties of the protein compared with the large effects that result from binding of the ligands.

The sedimentation results characterize definitive hydrodynamic properties of the protein. Conversion of these values to estimates of the shape and hydrodynamic volumes of EI can be done, but is virtually pure speculation. Nevertheless, we have performed the calculations, and the results suggest that the presence of the ligands, especially both PEP and Mg\(^{2+} \), induce a compaction or change in shape of EI, possibly both (see Table 4).

**CD Spectroscopy**

Initially, CD spectroscopy was performed over the range 200–250 nm. There were no significant differences in the spectra in the absence and presence of the ligands (data not shown), implying that the ligands did not affect the secondary structure of the protein or cause partial unfolding of the polypeptide chain.

We also extended the CD spectral studies to the near UV range, 250–350 nm, and the results are shown in Fig. 6 with native EI and the mutant EI-H189Q. With both proteins, there were small changes with Mg\(^{2+} \) alone but marked changes when both Mg\(^{2+} \) and PEP were present. Since all of the spectra were acquired at 25 °C, well below the \( T_m \) of EI, and since there were no significant changes in the secondary structure, we conclude that binding of the ligands results in substantial changes in the tertiary structure of EI.

The changes in spectra shown in Fig. 6 are usually ascribed to the aromatic amino acids in the protein. However, EI contains the following aromatic amino acids: 18 Phe, 9 Tyr, and 2 Trp. Thus, it would be difficult, from these data, to determine which of these residues are involved in the change.

**DISCUSSION**

**Native Enzyme I—**As indicated earlier, the kinetics of uptake of Glc and its nonmetabolizable analogue, methyl \( \alpha \)-D-glucopyranoside, by *Escherichia coli* are similar. Rapid initial uptake is followed by an immediate and continuous decline in the rate, so that in the case of methyl \( \alpha \)-D-glucopyranoside, for example, the progress curve resembles a hyperbola. By determining each of the 10 rate constants in the sequence of phosphotransfer reactions in the Glc PTS system, it was possible to construct a model for predicting the uptake rate by intact cells (27). But this model could only predict the initial rate and does not explain why it continuously declines thereafter.

In our continuing studies on this problem, it became apparent that the published information on the EI \( K_m \) was insufficient, and a systematic study was required that could ultimately be applied at least to in vitro assay conditions, to uptake experiments with membrane vesicles (8), and conceivably to in vivo conditions.

The results are shown in Tables 1 and 2 for accuracy, and some of the values are shown in Figs. 2–4 for clarity. The substance of the sedimentation equilibrium data is that an association constant cannot be assigned to EI without specifying the conditions. This constant can vary by as much as 350-fold at pH 7.5 and as much as 685-fold at pH 6.5 at constant ionic strength. Remarkably, the range at all conditions tested is more than 3 orders of magnitude. \( K_m \) is sensitive to almost every parameter tested, including temperature, the presence of the ligands Mg\(^{2+} \) and PEP, pH, and ionic strength (and possibly the concentration of inorganic phosphate). The major effects were obtained with temperature, pH, and especially the two ligands, Mg\(^{2+} \) and PEP. Potential ligands, such as sugar phosphates, had no significant effect on the \( K_m \). The pH effect is further considered below.

We originally showed (28) that the EI monomer comprises three domains, EI-N at the N terminus, EI-C at the C terminus, and a linker region. EI-N is relatively rigid, stable to proteolysis, and contains both the active site (His\(^{189} \)) and the HP binding site. The three-dimensional crystal structure of EI-N has been determined (29). The crystal structure of the EI-C domain of *Thermotoga maritima* has also recently been reported (30). EI-C is flexible, very sensitive to proteases, and contains the dimerization domain. Little is known concerning the linker domain, except that it is presumed to be short, whereas EI-N and EI-C each consist of about half of the molecule. Some properties of the EI-C domain, including the effects of the ligands Mg\(^{2+} \) and PEP, are the subject of the accompanying report (37).

Ginsburg and co-workers (1, 14) have published an extensive and important series of studies on the effects of the ligands on the conformational stability and dimerization of EI using both the native protein and two active site nonphosphorylatable mutants, EI-H189A and EI-H189E. Their work relevant to native EI is cited in Table 1. They

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**TABLE 4**

| Conditions       | Monomer | Dimer   | $s_{20, w}^0$ | $f_{20, w}$ | $s_{20, w}^0$ | $f_{20, w}$ |
|------------------|---------|---------|--------------|-------------|--------------|-------------|
| EI               | 3.73 ± 0.07 | 1.46 | 5.82 ± 0.05 | 1.48 |
| EI + MgCl₂      | 3.95 ± 0.04 | 1.38 | 5.92 ± 0.04 | 1.46 |
| EI + MgCl₂ + PEP| 4.07 ± 0.09 | 1.34 | 6.11 ± 0.03 | 1.42 |
| EI-H189Q        | 3.78 ± 0.08 | 1.44 | 5.89 ± 0.04 | 1.47 |
| EI-H189Q + MgCl₂| 3.97 ± 0.05 | 1.37 | 5.96 ± 0.03 | 1.45 |
| EI-H189Q + MgCl₂+ PEP| 4.14 ± 0.08 | 1.32 | 6.19 ± 0.03 | 1.4 |

* * If the shapes of the molecules are assumed to be prolate, the calculations give the following molecular diameters for the monomer: 183 Å when alone to 143 Å with both ligands; for oblate ellipsoids, diameter changes from 110 Å when alone to 96 Å (with both ligands), respectively.

**FIGURE 6.** Near UV CD spectra of EI and EI-H189Q. Shown are near UV CD spectra of EI (A) (17 μm) and EI-H189Q (B) (17 μm), alone or in the presence of MgCl₂ (4 mM) alone or with MgCl₂ and PEP (5 mM) as indicated. The buffer mixture was 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 0.5 mM EDTA, and 0.5 mM DTT at 25 °C.
observed large increases in $K_a$ when EI is exposed to the ligands Mg$^{2+}$ + PEP, but these are not the result of phosphorylation but rather the result of a change in conformation of EI. We confirm and extend this conclusion here and in the accompanying paper (37).

The Nonphosphorylatable Mutants, H189E, H189A, and H189Q—The three mutants show both similarities and marked differences in their $K_a$ values (all values are at pH 7.5). No correction has been made for the difference in temperatures used in these studies, 20 °C for H189E and H189A (1, 14) and 25 °C for H189Q in our work. For instance, the $K_a$ for the Gln mutant is about 7–10-fold greater than the values obtained with EI or with H159A or H159Q. Both H159A and H159Q behave similarly to each other and to native EI in the absence of ligand or in the presence of Mg$^{2+}$. However, when both PEP and Mg$^{2+}$ are present, there is no detectable dissociation of H189A; the $K_a$ is at least 7–20-fold greater than the values for native EI and EI-H159Q. We therefore conclude the following. (a) The ligands, not phosphorylation, are the major determinants in the dimerization process. They also cause conformational changes. (b) Because of substantial differences in the behavior of the mutants, it appears that the active site in the N-terminal domain also plays a significant role in the dimerization process.

We had originally selected a Gln substitution for His$^{189}$ because both NMR and x-ray crystallographic studies showed that it caused minimal perturbation of the structure in the PTS protein IIAGlc$^{32}$ (31). His and Gln are approximately isosteric and can make approximately the same hydrogen bonds (i.e. each has two locations for hydrogen bonding). Depending on orientation, either can be a donor or acceptor, and the hydrogen-bonding atoms can be in approximately the same spatial location in each case. Table 3 compares the $K_a$ values for native EI with the H189Q mutant at four temperatures, with and without the ligands. EI-H189Q virtually mimics native EI, except when both Mg$^{2+}$ and PEP are present, which results in a very large jump in $K_a$ for both proteins. The value for the mutant is about 3-fold greater than that for the native phospho-EI.

A Model to Explain the Behavior of Enzyme I—In addition to uptake of its sugar substrates, the bacterial phosphotransferase system plays key roles in various cellular phenomena. The PTS regulates diauxic growth, certain non-PTS sugar transporters, adenylate cyclase, glycerol kinase, chemotaxis to PTS-sugars, and more (4, 5).

The known mechanisms for these regulatory phenomena are illustrated by IIAGlc$^{32}$, one of the sugar-specific pair of proteins required for Glc transport by the enteric bacteria. IIAGlc$^{32}$ interacts with at least eight other proteins (32). In some cases, regulation is effected by phosphate transfer, and in others it is affected by the binding of IIAGlc or phospho-IIAGlc, but not both, to the target protein. All of the phosphate transfer reactions of the PTS except the last (phosphorylation of the sugar acceptor) are readily reversible. This means that the ratio phosphate-IIAGlc/IIAGlc depends on the state of phosphorylation of the other PTS proteins and ultimately of the first protein in the cascade, Enzyme I. Perhaps this explains why the $K_a$ of EI is so sensitive to so many parameters and varies about 1000-fold.

We attempt to explain the curious behavior of this protein by the model shown in Fig. 7. In the model, we focus on the effects of the two ligands, Mg$^{2+}$ and PEP on EI, but also on the important active site nonphosphorylatable mutant, H189Q. The model is not only consistent with all of the data presented in this paper on the numerous $K_a$ values for EI and EI-H189Q but also with the sedimentation constants and the results obtained by far UV CD spectroscopy. Additionally, the model in Fig. 7 explains the results obtained with the C-terminal domain (EI-C) of Enzyme I (37). The effects of the ligands on EI-C were studied by equilibrium and velocity sedimentation, near and far UV CD spectroscopy, thermal denaturation, and fluorescence spectroscopy.

The scheme shows the two major structural domains of EI, EI-N and EI-C, in one of the monomers but does not specify the length of the linker region, which plays an important role in what follows.

(a) In the absence of ligands, EI monomer comprises a collection of conformers (designated $W$) because of the flexibility of the EI-C domain. This flexibility was shown in early studies on the rates of reaction of the four -SH groups in the EI-C domain with the 5,5'-dithiobis-2-nitrobenzoic acid reagent (33). The C-terminal Cys-SH group reacted very rapidly under all conditions. The three internal -SH groups reacted more slowly and at different rates, depending on which ligands were present. For instance, in the presence of HPr, the rate increased, although there is no apparent connection between the HPr binding site in EI-N and the three internal -SH groups in EI-C. In the presence of Mg$^{2+}$, the rate decreased, and when both PEP and Mg$^{2+}$ were added, the rate was slowest by far, suggesting perhaps that the three -SH groups are shielded in the presence of the two ligands.

(b) Similar results are reported in the accompanying paper (37). PEP alone has little to no effect on the thermal denaturation of EI-C or the intensity of fluorescence of the two Trp residues in EI-C (the only Trp in EI). Mg$^{2+}$ alone increases the $T_m$ by about 7 °C and significantly increases Trp fluorescence. However, the combination of the ligands, PEP + Mg$^{2+}$, increases the $T_m$ by 14.4 °C, accompanied by a large increase in Trp fluorescence.

(c) Reaction 1 in Fig. 7 suggests that some of the conformers of the monomers, designated relaxed, can and do dimerize but that the dimer also consists of a group of relatively relaxed, and possibly fewer, conformers (labeled $X$).

(d) In Reaction 2 in Fig. 7, Mg$^{2+}$ is added and binds to the EI-C domain (37), and the conformations become more restricted, designated $Y$ (increased $K_a$ and sedimentation constants).

(e) In Reactions 3–5 in Fig. 7, PEP is added, and the combination of the two ligands results in a major change in conformation and/or shape of the dimer to what is designated compact (Z), consisting of a few conformers or perhaps only one conformer. Two possibilities are presented.

(i) The binding of the PEP and the swivel (conformational change) occur in two steps, binding (Reaction 3) to give a potential transitory intermediate, followed by the conformational change or “swivel” (Reaction 4). (ii) A concerted reaction takes place when the PEP is added (Reaction 5).

(f) The experimentally determined $K_a$ values and sedimentation coefficients are also given in Fig. 7. The successive increase in $s_{20,0}$ (sedimentation coefficient) upon binding of ligands (Table 4) suggests a change in the hydrodynamic properties of EI and that Mg$^{2+}$ and especially both Mg$^{2+}$ and PEP induce compaction or a change in shape or both. These quantitative values correlate surprisingly well with the speculations. Additionally, the results of the UV CD spectroscopic analyses of EI and EI-H189Q (Fig. 6) lead to the same conclusion (i.e. there is no significant change in secondary structure (far UV) but a major change in tertiary structure (near UV) when Mg$^{2+}$ and PEP are both present). Conceivably, this change in tertiary structure reflects the “swivel.” Mg$^{2+}$ alone has only a minor effect on the near UV CD spectrum, although Mg$^{2+}$ binds to the EI-C domain (PEP does not in the absence of Mg$^{2+}$) (37).

(g) The marked pH effect, a 4–8-fold increase in $K_a$ at pH 6.5 versus pH 7.5, is interpreted to mean that one or more protonated His residues (not the active site) are involved in dimerization. His is the only amino acid with an ionizable side chain close to the pH 6.5–7.5 range (pK$_a$ —
There are eight His residues in Enzyme I, four each in the N- and C-terminal domains, respectively. A His is located approximately in the linker domain in the protein.

**Pyrurate Phosphate Dikinase**—A novel swiveling domain mechanism for the enzyme pyruvate phosphate dikinase was suggested in the elegant studies by Herzberg et al. (34). This enzyme catalyzes the following reaction:

\[
\text{ATP} + \text{Pi} + \text{pyruvate} = \text{AMP} + \text{PPi} + \text{PEP}
\]

Crystallographic results (34) showed that the enzyme undergoes a major change in structure during the reaction. The active site His domain, in contact with the nucleotide binding domain, is phosphorylated by ATP (actually two reactions involving pyrophosphate). The phospho-His domain then swivels so that it comes into contact with the PEP-pyruvate domain. The phosphoryl group is subsequently transferred to the pyruvate to give PEP.

Given the similarity to pyruvate phosphate dikinase, here we suggest that it is the EI-C domain with the bound ligands, PEP + Mg\(^{2+}\), that swivels to bring the ligands close to His\(^{189}\), expediting a phosphotransfer from the PEP to the His. After the phosphate is transferred, the pyruvate dissociates from the catalytically active conformer and is replaced by another PEP, maintaining the compact or constricted configuration. In the mutants H189Q and H189A, there is no phosphate transfer, and the compact conformation is maintained as long as the ligands are present, which would explain the higher \(K_a\) values for the mutants.

**Conclusion**—Crystallographic studies have established the structures of *E. coli* EI-N (29), *T. tengcongensis* EI-C (30), and intact EI from *Staphylococcus carnosus*.\(^4\)

Clearly, these structures are “snapshots” of what must be multiple conformers. We therefore suggest that the schematic diagram in Fig. 7 is a more correct representation of EI.

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\(4\) W. Hengstenberg, personal communication.
Monomer/Dimer Transition of Enzyme I

At least some of the speculations in Fig. 7 can be experimentally tested. For instance, what are the precise limits of the putative “swiveling domain” in the linker between EI-N and EI-C? What will happen if this domain is made relatively rigid and/or more flexible, perhaps by deleting it or by substituting proline or polyalanine, or perhaps by substituting Gln for one or more His residues?

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REFERENCES

1. Dimitrova, M. N., Szczepanowski, R. H., Ruvinov, S. B., Peterkofsky, A., and Ginsburg A. (2002) Biochem. 41, 906–913
2. Kundig, W., Ghosh, S., and Roseman, S. (1964) Proc. Natl. Acad. Sci. U. S. A. 52, 1067–1074
3. Simoni, R., Levinthal, M., Kundig, F., Kundig, W., Anderson, B., Hartman, J., and Roseman, S. (1967) Proc. Natl. Acad. Sci. U. S. A. 58, 1963–1970
4. Meadow, N. D., Fox, D. K., and Roseman, S. (1990) Annu. Rev. Biochem. 59, 547–560
5. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1963–1967
6. Weigel, N., Kukuruzinska, M. A., Nakazawa, A., Waygood, E. B., and Roseman, S. (1982) J. Biol. Chem. 257, 10787–10793
7. Kepes, A., and Cohen, G. N. (1962) in The Bacteria (Gunsalus, I. C., and Stanier, R. Y., eds) Vol. 1, pp. 179–221, Academic Press, Inc., New York
8. Liu, K. D. F., and Roseman, S. (1983) J. Biol. Chem. 258, 1067–1074
9. Kukuruzinska, M. A., Harrington, W. F., and Roseman, S. (1982) J. Biol. Chem. 257, 14479–14484
10. Kukuruzinska, M. A., Turner, B. W., Ackers, G. K., and Roseman, S. (1984) J. Biol. Chem. 259, 11679–11681
11. Chauvin, F., Brand, L., and Roseman, S. (1994) J. Biol. Chem. 269, 20270–20274
12. Chauvin, F., Brand, L., and Roseman, S. (1996) Res. Microbiol. 147, 471–479
13. Meadow, N. D., Mattoo, R. L., Savtchenko, R. S., and Roseman, S. (2005) Biochemistry 44, 12790–12796
14. Dimitrova, M. N., Peterkofsky, A., and Ginsburg, A. (2003) Protein Sci. 12, 2047–2056
15. Ginsburg, A., and Peterkofsky, A. (2002) Arch. Biochem. Biophys. 397, 273–278
16. Patel, H. V., Vyas, K. A., Li, X., Savtchenko, R. S., and Roseman, S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7486–7491
17. Tsien, R. Y. (1998) Annu. Rev. Biochem. 67, 509–544
18. Chauvin, F., Fomenkov, A., Johnson, C. R., and Roseman, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7028–7031
19. Reddy, P., Fred-Kuldeck, N., Liberman, E., and Peterkofsky, A. (1991) Protein Expression Purif. 2, 179–187
20. Weigel, N., Waygood, E. B., Kukuruzinska, M. A., Nakazawa, A., and Roseman, S. (1982) J. Biol. Chem. 257, 14461–14469
21. Anderson, B., Weigel, N., Kundig, W., and Roseman, S. (1971) J. Biol. Chem. 246, 7023–7033
22. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1996) Current Protocols in Molecular Biology, Greene Publishing Associates, New York
23. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Halvorson, H. R. (1981) Biochim. Biophys. Acta 679, 57–58
24. Rivas, G., Stafford, W., and Minton, A. P. (1999) Methods Companion Methods Enzymol. 19, 194–212
25. Dam, J., and Schuck, P. (2004) Methods Enzymol. 384, 185–212
26. Schuck, P. (2000) Biophys. J. 78, 1606–1619
27. Rohwer, J. M., Meadow, N. D., Roseman, S., Westerhoff, H. V., and Postma, P. W. (2000) J. Biol. Chem. 275, 34909–34921
28. LiCalzi, C., Croczeni, T. S., Freire, E., and Roseman, S. (1991) J. Biol. Chem. 266, 19519–19526
29. Liao, D.-I., Silverton, E., Seok, Y.-J., Lee, B. R., Peterkofsky, A., and Davies, D. R. (1996) Structure 4, 861–872
30. Oberholzer, A. E., Bumann, M., Schneider, P., Bachler, C., Siebold, C., Baumann, U., and Erni, B. (2005) J. Mol. Biol. 346, 521–532
31. Pelton, J. G., Torchia, D. A., Remington, S. J., Murphy, K. P., Meadow, N. D., and Roseman, S. (1996) J. Biol. Chem. 271, 33446–33456
32. Roseman, S., and Meadow, N. D. (1990) J. Biol. Chem. 265, 2993–2996
33. Han, M. K., Roseman, S., and Brand, L. (1990) J. Biol. Chem. 265, 1985–1995
34. 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
35. Zhu, P. P., Szczepanowski, R. H., Nosworthy, N. J., Ginsburg, A., and Peterkofsky, A. (1999) Biochemistry 38, 15470–15479
36. Chauvin, F., Brand, L., and Roseman, S. (1994) J. Biol. Chem. 269, 20263–20269
37. Patel, H. V., Vyas, K. A., Mattoo, R. L., Southworth, M., Perler, F. B., Comb, D., and Roseman, S. (2000) J. Biol. Chem. 281, 17579–17587