Transient State Kinetics of Enzyme IICB\textsubscript{Glc}, a Glucose Transporter of the Phosphoenolpyruvate Phosphotransferase System of \textit{Escherichia coli}

**EQUILIBRIUM AND SECOND ORDER RATE CONSTANTS FOR THE GLUCOSE BINDING AND PHOSPHOTRANSFER REACTIONS**

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During translocation across the cytoplasmic membrane of \textit{Escherichia coli}, glucose is phosphorylated by phospho-IIAG\textsubscript{Glc} and Enzyme IICB\textsubscript{Glc}, the last two proteins in the phosphotransfer sequence of the phosphoenolpyruvate:glucose phosphotransferase system. Transient state (rapid quench) methods were used to determine the second order rate constants that describe the phosphotransfer reactions (phospho-IIAG\textsubscript{Glc} to IICB\textsubscript{Glc} to Glc) and also the second order rate constants for the transfer from phospho-IIAG\textsubscript{Glc} to molecularly cloned IIBG\textsubscript{Glc}, the soluble, cytoplasmic domain of IICB\textsubscript{Glc}. The rate constants for the forward and reverse phosphotransfer reactions between IIAG\textsubscript{Glc} and IICB\textsubscript{Glc} were 3.9 \times 10^{10} and 3.1 \times 10^{10} M\textsuperscript{-1} s\textsuperscript{-1}, respectively, and the rate constant for the physiologically irreversible reaction between [P]IIICB\textsubscript{Glc} and Glc was 3.2 \times 10^{10} M\textsuperscript{-1} s\textsuperscript{-1}. From the rate constants, the equilibrium constants for the transfer of the phospho-group from His\textsubscript{Cys} to the phosphorylation site Cys of IICB\textsubscript{Glc} or IICB\textsubscript{Glu} were found to be 3.5 and 12, respectively. These equilibrium constants signify that the thio phospho-group in these proteins has a high phosphotransfer potential, similar to that of the phospho-histidinyl phosphotransferase system proteins. In these studies, preparations of IICB\textsubscript{Glc} were invariably found to contain endogenous, firmly bound Glc (estimated K\textsubscript{b} \approx 10^{7} M). The bound Glc was kinetically competent and was rapidly phosphorylated, indicating that IICB\textsubscript{Glc} has a random order, Bi Bi, substituted enzyme mechanism. The equilibrium constant for the binding of Glc was deduced from differences in the statistical goodness of fit for the phosphotransfer data to the kinetic model.

The bacterial phosphoenolpyruvate:glucose phosphotransferase system (PTS) comprises dozens of cytoplasmic and membrane proteins, most of which are the sugar-specific components of the system (for reviews, see Refs. 1–4). The PTS has several important functions in enteric bacteria, shown in Fig. 1.

The bacterial phosphoenolpyruvate:glycose phosphotransferase system; IIAG\textsubscript{Glc}, the phosphocarrier protein component of the glucose-specific PTS in \textit{E. coli} (this protein was called IIIG\textsubscript{Glc} in the older literature); IICB\textsubscript{Glc}, the glucose-specific, integral membrane transporter of the PTS in \textit{E. coli}; IIAG\textsubscript{Glu}, the phospho-IIAG\textsubscript{Glc} component of the glucose-specific PTS in \textit{E. coli} (this protein was called IIIG\textsubscript{Glu} in the older literature); IICB\textsubscript{Glu}, the glucose-specific, integral membrane transporter of the PTS in \textit{E. coli}; PEP, phosphoenolpyruvate; Glc, glucose; [\textsuperscript{32}P]Glc, [\textsuperscript{32}P]-glucose; [\textsuperscript{3}H]Glc, [\textsuperscript{3}H]-glucose; M, millimolar; BisTris, 2-(bis(hydroxyethyl)-amino)-2-(hydroxyethyl)methylpropane-1,3-diol.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials used for the assay of Glc are given in supplemental data. 1-\textit{O Dioleoylphosphatidyl-DL-glycerol (P-9664; Sigma); N-lauroylsarcosine (L-5777; Sigma); adenosine 5\textsuperscript{-}[\textgamma-\textsuperscript{32}P]triphasphate.
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The concentration of endogenous Glc after treating the membrane preparations as described above was variable but was always in the same range as the concentration of IICB\textsuperscript{Glc}. Modeling the kinetics of Reactions V, VI, and VII (Fig. 2, Scheme I) required an understanding of the kinetic properties of this pool of endogenous Glc as well as decisions about how to model the kinetics of Reactions V(\(i\)), VI(\(i\)), and VII(\(i\)) when \([\text{HI}]\text{Glc}\) was added to the syringe containing the solution of [\(^{32}\text{P}\)]IIA\textsuperscript{Glc}. These methods are described in supplemental data on kinetics.

General Methods—Assays for the PTS proteins by measuring the rate of sugar phosphorylation were performed as described (9, 12). The method of Bradford (13) was used for soluble proteins; the reagent was from Bio-Rad. The modification of the method of Lowry by Markwell et al. (14), calibrated as previously described (6), was used for IIA\textsuperscript{Glc} and for membrane proteins.

The concentration of HPr or IIA\textsuperscript{Glc} was measured by using the lactate dehydrogenase coupled assay (12) (using homogenous auxiliary proteins), which measures the quantity of protein that can accept a phospho-group from PEP. The concentrations of HPr or IIA\textsuperscript{Glc} estimated from the specific activity of the [\(^{32}\text{P}\)]PEP agreed with the results of the lactate dehydrogenase assay within 5%.

IIIB\textsuperscript{Glc} was quantified by three methods: (a) by solutions of the protein, purified to apparent homogeneity, were thoroughly dialyzed and analyzed for nitrogen by the method of Jaenicke (15); (b) quantitative phosphorylation of the protein using an excess of [\(^{32}\text{P}\)]PEP of accurately known specific activity and catalytically quantitative of Enzyme I, HPr, and IIA\textsuperscript{Glc}; (c) by the lactate dehydrogenase assay (12). When all three methods were applied to the same sample, the results agreed to within 10%.

IICB\textsuperscript{Glc}, both purified and in membranes, was quantified by two methods. (a) The sample was assayed for its activity in PEP driven sugar phosphorylation, using a range of concentrations of IIA\textsuperscript{Glc}. These data were used to calculate the \(V_{\text{max}}\), which was converted to the concentration of the IICB\textsuperscript{Glc} protein by using the specific activity of the homogenous enzyme (97 \(\mu\text{mol of sugar phosphate/min/mg of IICB}\textsuperscript{Glc}\) (9). (b) IICB\textsuperscript{Glc} was also quantified by quantitatively labeling the protein in membrane suspensions with [\(^{32}\text{P}\)]PEP of accurately known specific activity (1–3 TBq/mol) (6) as follows. A mixture of 50 mM potassium phosphate buffer (pH 7.5), 5 mM MgCl\(_2\), 25 nmol of [\(^{32}\text{P}\)]PEP, 10 pmol of enzyme I, 7 pmol of HPr, 15 pmol of IIA\textsuperscript{Glc}, and ~150 pmol of IICB\textsuperscript{Glc} in a volume of 100 \(\mu\text{l}\) was incubated at room temperature for 15 min. It was then quenched with 50 \(\mu\text{l}\) of 0.6 \(\text{m} \cdot \text{KOH}\) and analyzed by gel filtration chromatography by the same methods used for rapid quench samples (see below). When applied to three membrane preparations, the two methods agreed to within 15%. Membranes prepared from ZSC112\textsuperscript{ΔG} exhibited insignificant activity in the PEP-driven sugar phosphorylation assay as well as insignificant labeling with [\(^{32}\text{P}\)]PEP.

Purification of Proteins—Enzyme I, HPr, and IIA\textsuperscript{Glc} were separately overproduced in cells carrying the relevant plasmids. The proteins were purified by the methods used previously (6). IIB\textsuperscript{Glc}-His\(_{\text{a}}\), was purified by the method of Buhr et al. (7), except that a Superose 12 HR 10/30 column (Amersham Biosciences) was substituted for Sephadex G75. The final preparations were apparently homogeneous as judged by SDS-PAGE. IICB\textsuperscript{Glc}-His\(_{\text{a}}\), was purified using modifications of the method of Waebel et al. (11); a Superose 12 HR 10/30 column was used, but neither Glc nor methyl \(\alpha\)-d-glucopyranoside was added to solutions. Before being employed in either rapid quench experiments or in the PTS sugar phosphorylation assay, the IICB\textsuperscript{Glc} was activated, except as noted, by mixing with an equal volume of a solution of lipid/detergent.
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mixed micelles (5 mg/ml dioleyl phosphatidyl glycerol, 1 mg/ml sodium lauroyl sarcosinate), as described by Bouma et al. (9).

Synthesis of \[^{32}\text{P}\]PEP—The enzymatic synthesis (16) was performed with the modifications described previously (6). The purification by anion exchange chromatography was further modified by the substitution of KCl for triethylamine/H\textsubscript{2}CO\textsubscript{3} as the eluant. We suspect that triethylamine or a contaminant in it occasionally interferes with the phosphotransfer reactions (data not shown). To stop the enzymatic reaction, 25 \(\mu\)l of 20% \((v/v)\) Norite A suspension was added to the reaction mixture. The Bio-Rad AG1-x8 column was equilibrated with 10 mM BisTris/Cl, pH 6.0, which was also a component of all of the eluant solutions. The reaction mixture (including the Norite A) was placed onto the column, which was then washed with 5 ml of the buffer, and the PEP was eluted with 5 ml of 0.1 M KCl. The PEP was eluted with 0.3 M KCl, 0.5–ml fractions were collected in tubes containing 40 \(\mu\)l of 0.2 M Na\textsubscript{3}PO\textsubscript{4}, 0.2 M K,PO\textsubscript{4} and counted by liquid scintillation counting. The peak concentration of \[^{32}\text{P}\]IIAGlc was prepared under the conditions used for the preparation of \[^{32}\text{P}\]IIAGlc—using a molar ratio of HPr/IIAGlc/IIBGlc = 1:24:24. A Superdex 75 HR 10/30 column (Amersham Biosciences) was employed to fractionate the proteins.

\[^{32}\text{P}\]IICB\textsubscript{Glc} was prepared from purified protein, which was sonicated for 30 s in a bath type sonicator in the presence of a 10-fold molar excess of \[^{32}\text{P}\]llAGlc and incubated for 15 min (with no added phospholipid). The proteins were separated on a Superdex 200 HR 10/30 column.

The columns used to fractionate the phosphorylation mixtures were equilibrated with 20 mM carbonate/bicarbonate (pH 9.5) buffer containing 1 mg/ml bovine serum albumin. The specific activity of all four phosphoproteins ranged from 10 to 40 TBq/mol, with emphasis on the stability of \[^{32}\text{P}\]IICB\textsubscript{Glc} under quench conditions.

Stability of \[^{32}\text{P}\]IIAGlc and \[^{32}\text{P}\]IICB\textsubscript{Glc} under quench conditions—\[^{32}\text{P}\]IIAGlc and \[^{32}\text{P}\]IICB\textsubscript{Glc} were isolated as described and stored at \(-80^\circ\text{C}\) in 20 mM carbonate/bicarbonate buffer (pH 9.5). Tests of the effect of pH on the rate of hydrolysis of the phospho-group were made by diluting 10 ml of 0.5 \(\mu\)M \[^{32}\text{P}\]IIAGlc solution into 3.5 ml of buffer or diluting 100 \(\mu\)l of 0.33 \(\mu\)M \[^{32}\text{P}\]IICB\textsubscript{Glc} into 4.0 ml of buffer. All equipment and vessels were pretreated with bovine serum albumin to minimize adsorption of protein. The buffers were as follows: pH 2, 0.1 M HCl/KCl; pH 3.8, 50 mM citric acid/sodium citrate; pH 6.0 and pH 8.1, 50 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4}; pH 10.1, 50 mM Na\textsubscript{2}CO\textsubscript{3}/NaHCO\textsubscript{3}; pH 12, 25 mM Na\textsubscript{2}PO\textsubscript{4}; pH 13, 0.1 M NaOH; pH 14, 1 M NaOH; and pH 14.3, 2 M NaOH (the last three solutions were prepared from fresh, commercial 2 M NaOH standard solution). The mixtures were incubated at 23 °C for 5 min to 4 h and filtered through 23-mm diameter polyvinylidene difluoride transfer membranes (Millipore Corp.) in a vacuum apparatus that allowed collection of the filtrate; both the filter and the filtrate were counted to ensure quantitative recovery of the radioactivity. Tests of the membrane with \[^{32}\text{P}\]P, showed that the background was negligible and that washing of the filter was not required. Other controls showed that protein adsorption to the membranes was quantitative.

The high rate of hydrolysis above pH 13 (see "Results") made use of a quench solution containing 1 M KOH unsuitable for the phosphotransfer measurements involving IICB\textsubscript{Glc}, especially since heating of the quenched reaction is required to fully denature \[^{32}\text{P}\]IIAGlc (6). The conditions for quenching that were developed for maintaining the phospho-S-Cys bond intact were as follows: 0.1 M KOH, 3 \(\mu\)l urea, and heating for 5 min at 55 °C. These conditions yield a level of hydrolysis sufficiently low (less than 1% per min) to allow preparation (with careful timing) of the quenched samples for separation by gel filtration (where the rate of hydrolysis is negligible).

We unexpectedly found that IICB\textsubscript{Glc} is rapidly fragmented when heated under the conditions developed for the chromatography of \[^{32}\text{P}\]IIAGlc. When heated to 55 °C for 5 min in 0.1 M KOH, 3 \(\mu\)l urea quench solution, as much as 65% of the radioactivity in \[^{32}\text{P}\]IIAGlc or \[^{32}\text{P}\]IICB\textsubscript{Glc}. His\textsubscript{g} appears in fractions containing 10–20 kDa molecules. These fractions also contain the six histidine residues from IICB\textsubscript{Glc}. His\textsubscript{g} as shown by using dot blots treated with anti-His antibodies (data not shown); corresponding fractions from control membranes do not bind the anti-His antibodies. Urea (3 \(\mu\)l) enhances the rate of fragmentation by about 30%. These results suggest that a peptide bond somewhere in the linker region between the B and C domains is very labile at high pH. Optimal conditions for attaining rapid quenching while minimizing protein cleavage and hydrolysis of the phospho-group were found to be 0.2 M KOH (final concentration in the quenched reaction) with no heating before injection onto the gel filtration column. Careful timing between thawing the quenched samples and injection produced a reproducible 13 ± 3% fragmentation, with acceptable speed of quenching of the reaction. The raw data for the concentration of the phosphoproteins in quenched reactions was therefore corrected by 13%.

Rapid Quench Assays—The present study employed the rapid quench apparatus used previously, and all of the details for its set-up were the same (6). Stock solutions of \(^{32}\text{P}\)-labeled proteins were diluted with the same solution used to fractionate the phosphorylation mixture at the time of its preparation (see above). Stock solutions of IIBGlc or membrane suspensions were diluted with 100 mM phosphate buffer (pH 7.5), 0.5 mM EDTA, 0.5 mM dithiothreitol, and 1 mg/ml bovine serum albumin. The phosphate buffer was pH 7.5 (rather than pH 6.5 (6)) to correlate with work on the kinetics of Enzymes II published by the time the present work was started (e.g. see Refs. 17 and 18). The rate of phosphotransfer between HPr and IIAGlc is not significantly affected by a change in pH from 6.5 to 7.5 (6)3 Another significant modification (described above) was of the conditions used for quenching the reaction. Preparation of the solutions for rapid quench experiments required large dilutions from stock solutions and a change from the frozen state to ambient temperature (\(-23^\circ\text{C}\), at which all experiments were performed. The diluted solutions were therefore preincubated for 1 h at ambient temperature before the experiment was started.

When the phosphotransfer between IIAGlc and IIBGlc was studied, a Superdex 75 HR 10/30 column (6) was used to separate the proteins in the quenched reactions. When the phosphotransfer reactions between IIAGlc and IICB\textsubscript{Glc} were studied, the column was a Superose 12 HR 10/30 (Amersham Biosciences). This column cannot resolve Glc-6-P from inorganic phosphate, which is always present because of hydrolysis of the phosphodonor protein during storage following its preparation. For this purpose, a separate aliquot of each quenched reaction mixture was chromatographed on a Superdex Peptide HR 10/30 column (Amersham Biosciences) that was equilibrated with 35 mM Na\textsubscript{3}PO\textsubscript{4}, 0.1 M Na\textsubscript{2}SO\textsubscript{4}.

When \[^{31}\text{H}\]Glcp was used in rapid quench experiments, the \[^{31}\text{H}\]Glcp-6-P was isolated by anion exchange chromatography using a modification of the method used for PTS sugar phosphorylation assays (12). Aliquots (100 \(\mu\)l) of the quenched solution were diluted with 900 \(\mu\)l of water, and the pH was reduced to between 9.5 and 10 by the addition of

\[^{3}\text{N. D. Meadow, R. S. Savtchenko, S. J. Remington, and S. Roseman, unpublished data.}\]
FIGURE 1. A diagram of the Glc-specific PTS from E. coli. The phosphorylated amino acid in each of the four proteins is indicated. There are five phosphotransfer reactions, each designated by the Roman numeral used throughout this work. The glucose permease, IIBC^{Glc}, is shown separated into its two domains, the phosphorylation domain IIB^{Glc}, which extends into the cytoplasm, and the sugar recognition and binding domain IIC^{Glc}, which is an integral membrane domain. The reactions of IIBC^{Glc} are drawn as conventionally represented and do not illustrate the random order mechanism, presented under "Results," in which Glc binds either to unphosphorylated or to phosphorylated IIBC^{Glc}.

FIGURE 2. Phosphotransfer from [P]IIA^{Glc} to Glc and the random order of addition mechanism for IIBC^{Glc}. The upper panel shows the proposed catalytic mechanism of IIBC^{Glc}; it is a Bi Bi, random order of addition, substituted enzyme mechanism. The lower panel shows the scheme of balanced first and second order equations for the reactions of the Bi Bi, random order mechanism, except Reaction IVa, which applies only to the IIB^{Glc} domain. The rate constants of the reactions (k_{XXX}) are determined (using the kinetic simulator, KinSim) by numerical integration of the differential equations defined by the chemical reactions. The signs of the rate constants (k_{XXX}) are positive for reactions proceeding left to right. Reactions pertaining to [^{1}H]Glc are identified by a 1. These data are treated as separate reactions in the model, because the appearance of [^{1}H]Glc-6-[^32P] was a separate data set, independent of the data on the appearance of total Glc-6-[^32P]. In modeling with the simulator, these constants were held equal to the corresponding constants for unlabeled Glc; therefore, they are not given in the tables or figures. Rate constants that are omitted were assigned values of 0 in the simulator because of their low thermodynamic reversibility. In this work, Reactions IV and V are referred to as the "upper pathway," and Reactions VI and VII are referred to as the "lower pathway."

**RESULTS**

Introduction—In what follows, reactions are identified by the Roman numerals assigned in Scheme I (Fig. 2). Representative progress curves are shown in Figs. 3 and 5–7. It is important to emphasize that in these figures, each panel represents one experiment. The rate constants of the reactions were estimated from the experimental progress curves by numerical integration (see "Experimental Procedures"). The data from studying the phosphotransfer reaction between IIAGlc and IIBC^{Glc} (with experiments using either [^{32P}]IIA^{Glc} or [^{32P}]IIBC^{Glc}) were fitted to Reaction IVa. The data from the phosphotransfer reactions from [P]IIA^{Glc} to Glc via IIBC^{Glc} were fitted to Reactions IV, V, VI, and VII. When [^{1}H]Glc was added to an experiment, Reactions Vt, VI, and VIIt were included in the model. TABLE ONE presents a summary of all of the rate constants that we report in this work; some of the rows in TABLE ONE present results from replicate experiments, not the data from single experiments.

Rates of Phosphotransfer between IIAGlc and IIBC^{Glc}-His_{6}—In the E. coli Glc-specific PTS, the last protein-protein phosphotransfer step in the upper pathway (Fig. 2) is from His_{6}^{60} in IIAGlc to Cys_{421} in IIBC^{Glc} (Reaction IV in Figs. 1 and 2). The subsequent and final reaction (V) is the phosphotransfer to Glc. As described below, kinetic measurements with both purified and membrane-bound IIBC^{Glc} were complicated by the presence of endogenous Glc, a problem that could be avoided by using IIBC^{Glc} as the phosphoacceptor. IIBC^{Glc} is the cytoplasmic domain of the integral membrane protein; the cloned fragment (10,739 Da) comprises residues 1–4 of the amino terminus of IIBC^{Glc}, followed by residues 391–476, and is terminated by a His_{6} cartridge (7). Molecularly cloned IIBC^{Glc}-His_{6} is a soluble and readily purified protein containing the phosphorylation site (Cys_{421} in IIBC^{Glc}) but not the Glc binding site.
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Its phosphotransfer reaction is designated Reaction IVa in Scheme I (Fig. 2).

A typical progress curve and the estimated rate constants for the reversible transfer of a phospho-group from \([^{32}P]IIA\)Glc to IIBGlc-His6 are shown in Fig. 3. The rate constants obtained from a global analysis of the data from four experiments (three using \([^{32}P]IIA\)Glc and one using \([^{32}P]IIAGlc\)) are given in TABLE ONE, row 1. There was good agreement between the constants obtained by starting the reaction from either direction. This implies that there are no significant concentrations of intermediate complexes between the two reacting proteins prior to the last step: transfer of the phosphoryl group to the acceptor and separation of the proteins to yield the products. These rate constants yield an equilibrium constant of 3.5 for Reaction IVa, indicating that the thiophosphate linkage has a very high phosphate transfer potential, close to that of phospho-IIA\(\text{Glc}\).

Stability of \([^{32}P]IIBGlc\) and \([^{32}P]IIICBGlc\) under Quench Conditions—

The phosphoesters \([^{32}P]IIBGlc\) and \([^{32}P]IIICBGlc\) showed unexpected instability of the phospho-group at pH 14, the pH of the quench solution developed for the phospho-His proteins (6). The stability of both phosphoproteins was therefore studied as a function of pH; the results, from pH 2 to 14, are shown in Fig. 4. Between pH 2 and 12, the rate constants for the hydrolysis of the phospho-group are similar in magnitude to those published for the hydrolysis of butyliophosphate (23), cysteamine-3-phosphoric acid (24), and the thiophosphopeptides derived from IICBGlc (25) and from IIICBGlc (26). Although the rate constants for hydrolysis of butyliophosphate and cysteamine-3-phosphoric acid exhibit bell-shaped curves in the pH range 1–6, this was not observed with the thiophosphoesters of any of the PTS proteins.

There is, however, a more important difference in the properties of the thiophosphoproteins above pH 12. Butyliophosphate is very stable in the pH range 10–14, whereas the thiophospho-PTS proteins are not. Above pH 12, the behavior of the two thiophospho-PTS proteins

![FIGURE 3. Transfer of \([^{32}P]\)phospho-group from \([^{32}P]IIA\)Glc to IIBGlc-His6 (data modeled by Reaction IVa). Experimental and theoretical progress curves are shown for the transfer reaction. The rapid quench experiment was conducted as described under “Experimental Procedures” using the following initial concentrations (after mixing): \([^{32}P]IIA\)Glc = 54 nM; IIBGlc-His6 = 21 nM produced by hydrolysis of \([^{32}P]IIA\)Glc during storage; IIBGlc-His6, \(K_D = 45\) nM. \([^{32}P]IIA\)Glc-His6, IIBGlc-His6. The solid and dashed lines are the theoretical progress curves fitted by nonlinear least squares (using Fitsim) to the differential equation defined by Reaction IVa, A, the first second of the progress curves on a linear time scale. B, the full time course of the reaction (30 s) on a logarithmic time scale; the ordinate is to the same scale as A, and the same symbols are used. Time points longer than 10 s were obtained after hand mixing. The rate constants from the model as follows: \(k_{IIA} = 10.0 \pm 0.3 \times 10^{-6} \text{ M}^{-1} \text{s}^{-1}\) and \(k_{IVA} = 5.7 \pm 0.2 \times 10^{-6} \text{ M}^{-1} \text{s}^{-1}\). The calculated apparent equilibrium constant for Reaction IV is therefore \(K_{eq} = 2.7\). The data from this experiment are included in the global analysis shown in TABLE ONE, row 1.

### TABLE ONE

| Rate constants of the phosphotransfer reactions from \([^{32}P]IIA\)Glc to IIBGlc-His6 or through IICBGlc to glucose |
|---|
| The constants were estimated either by manual fitting of the data from individual experiments using \(K_{eq}\) (19, 20), or by nonlinear least squares fitting of the data from groups of experiments using Fitsim (21). The analyses were performed by first choosing a \(K_{eq}\) for the sugar binding reaction; the table shows the results obtained for \(K_{eq} = 10^{-6}, 10^{-5}, \text{ or } 10^{-4} \text{ M}\). The \(K_{eq}\) was used to calculate, from the total concentration of Glc and IICBGlc before mixing, the concentrations of free Glc, free IICBGlc, and IICBGlc-Glc present in the syringe. For each value of the \(K_{eq}\), at least seven pairs of values for \(k_{IIA}\) and \(k_{IVA}\) were chosen (the results from only three or four of these pairs are shown in the table). Finally, the simulation was performed keeping \(k_{IIA}\) and \(k_{IVA}\) fixed while fitting the rate constants for the phosphor transfer reactions. |
| **Fixed constants**<sup>a</sup> &nbsp; &nbsp; &nbsp; | **Fitted constants**<sup>b</sup> | **S.D. × 10<sup>-6</sup>** | **Row** |
|---|---|---|---|
| \(K_{IIA}\) | \(k_{IIA}\) | \(k_{IVA}(k_{IVA})\) | \(k_{IVA}(k_{IVA})\) | \(k_{IVA}\) | \(k_{IVA}\) | |
| &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| 10<sup>-6</sup> | 1 × 10<sup>6</sup> | 1 | 3 ± 2 | 0.4 ± 1 | 2 ± 1 | 2 ± 1 | 20 ± 30 | 30 | 2 |
| 10<sup>-6</sup> | 1 × 10<sup>6</sup> | 0.01 | 3 ± 2 | 0.4 ± 2 | 2 ± 1 | 20 ± 30 | 30 | 3 |
| 10<sup>-6</sup> | 1 × 10<sup>6</sup> | 0.0001 | 3 ± 2 | 0.4 ± 2 | 2 ± 1 | 20 ± 30 | 30 | 4 |
| 10<sup>-7</sup> | 1 × 10<sup>6</sup> | 100 | 3.0 ± 0.3 | 0.24 ± 0.1 | 2.3 ± 0.3 | 1.7 ± 0.3 | 5.4 | 5 |
| 10<sup>-7</sup> | 1 × 10<sup>6</sup> | 0.01 | 3.2 ± 0.4 | 0.27 ± 0.1 | 2.4 ± 0.3 | 1.6 ± 0.3 | 5.4 | 6 |
| 10<sup>-7</sup> | 1 × 10<sup>6</sup> | 0.0001 | 3.5 ± 0.4 | 0.29 ± 0.2 | 2.5 ± 0.3 | 1.4 ± 0.2 | 5.1 | 7 |
| 10<sup>-8</sup> | 1 × 10<sup>6</sup> | 100 | 3.9 ± 0.4 | 0.31 ± 0.1 | 3.2 ± 0.4 | 1.0 ± 0.2 | 5.0 | 8 |
| 10<sup>-8</sup> | 1 × 10<sup>6</sup> | 0.01 | 10 ± 20 | 0.5 ± 0.9 | 0.0 ± 10 | 2 ± 2 | 32 | 9 |
| 10<sup>-8</sup> | 1 × 10<sup>6</sup> | 0.01 | 40 ± 80 | 9 ± 20 | 0.5 ± 0.5 | 2 ± 1 | 32 | 10 |
| 10<sup>-8</sup> | 1 × 10<sup>6</sup> | 0.001 | 40 ± 90 | 10 ± 40 | 0.7 ± 0.5 | 2 ± 1 | 32 | 11 |
| Manual fitting of three experiments using wild type IICBGlc in membranes<sup>c</sup> | | | | | | | |
| 10<sup>-7</sup> | 1 × 10<sup>5</sup> | 0.01 | 1.5 | 0.25 | 1.5 | 1.5 | 12 |
| 10<sup>-7</sup> | 1 × 10<sup>5</sup> | 0.01 | 2.0 | 0.50 | 1.5 | 2.0 | 13 |
| 10<sup>-7</sup> | 1 × 10<sup>5</sup> | 0.01 | 3.5 | 0.33 | 2.7 | 2.5 | 14 |

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*<sup>a</sup> Constants associated with glucose binding.*

*<sup>b</sup> Constants involving unlabeled glucose and tritiated glucose were forced to be equal, so only one is given here.*

*<sup>c</sup> S.D. of all of the data points from the theoretical values.*

*The significance of the S.E. of the individual rate constants is limited to whether it is less than one-fourth the magnitude of the constant itself (21, 37), and in the fits with \( K_{eq} = 10^{-4} \text{ M}\), the S.E. values range from about one-half to about one-tenth the magnitude of the rate constant with which they are associated.*

*In these experiments, \( [^{3}H] \)Glc was added to the \([^{32}P]IIA\)Glc solution. Two additional time courses were obtained, one that had no Glc added and the other that had Glc added to the IICBGlc solution. The rate constants derived from the additional eight data sets agreed well with those presented in the table.*

*Varying the \( K_{eq} \) for Glc binding to IICBGlc from \( 10^{-4} \) to \( 10^{-3} \text{ M} \) had essentially the same effect as shown for IICBGlc-His6. The best fit was obtained when the \( K_{eq} \) was set at \( 10^{-4} \text{ M} \).*
The rapid quench experiment shown in Fig. 5 was designed to determine this. The experiment measured the rate of the phosphotransfer reactions from $^{32}\text{P}\text{IIA}^{\text{Glc}}$ to Glc via IICB$^{\text{Glc}}$. A preparation of wild type membranes was used that was washed only once and neither incubated nor dialyzed, so that the molar ratio of endogenous Glc to IICB$^{\text{Glc}}$ was higher (22:1) than that in the other experiments reported here (2:1 to 4:1). The figure shows only the data on the production of $^{32}\text{P}\text{IIA}^{\text{Glc}}$ and Glc-6-$^{32}\text{P}$, the utilization of $^{32}\text{P}\text{IIA}^{\text{Glc}}$ is not shown.

For the analysis shown in Fig. 5A it was assumed that the Glc in IICB$^{\text{Glc}}$-Glc was not kinetically competent (i.e. the rate constant, $k_{\text{VII}}$, was forced to 0). The result was a poor fit between the theoretical curve and the data points, but this was the best fit that we could obtain. If the bound Glc is not kinetically competent, then $^{32}\text{P}\text{IIA}^{\text{Glc}}$ should accumulate before any sugar phosphate is formed. It is clear, however, that Glc-6-$^{32}\text{P}$ appeared more rapidly than $^{32}\text{P}\text{IIA}^{\text{Glc}}$. Thus, as seen in Fig. 5B, when Reaction VII is assumed to be active and is assigned a non-zero value in the simulation, very good agreement is obtained between the theoretical curves and the data points. In all of our experiments, substantially better theoretical fits to the data were obtained when kinetically active IICB$^{\text{Glc}}$-Glc was included in the model.

Kinetics of Phosphorylation of Endogenous and Exogenous Glc—At the instant of mixing of IICB$^{\text{Glc}}$ with exogenous Glc added to the $^{32}\text{P}\text{IIA}^{\text{Glc}}$, there are three pools of the sugar: exogenous Glc, pool 3; free endogenous Glc, pool 2; and bound endogenous Glc (IICB$^{\text{Glc}}$-Glc), pool 1. The foregoing assumes that the endogenous Glc is all accessible to the IICB$^{\text{Glc}}$ and that it participates in a binding equilibrium with the enzyme. This is the case as shown in supplemental data on Glc. Further, as shown above, the endogenous Glc is kinetically competent, but what is its rate of phosphorylation relative to the exogenous Glc? In other words, how rapidly do the exogenous and endogenous Glc pools equilibrate relative to the phosphotransfer reactions starting with phospho-IIA$^{\text{Glc}}$?

The experiment shown in Fig. 6 clearly shows that the bound endogenous Glc is phosphorylated more rapidly (Reaction VII) than it equilibrates with the exogenous pool of Glc (Reaction VI). In this experiment, the rate of phosphotransfer from $^{32}\text{P}\text{IIA}^{\text{Glc}}$ to Glc via IICB$^{\text{Glc}}$ was measured, but only the data on the production of $^{3}\text{H}\text{Glc-6-$^{32}\text{P}$}$ and total Glc-6-$^{32}\text{P}$ are shown. The experiment was conducted in two parts. In the first part, $^{3}\text{H}\text{Glc}$ was added to the syringe containing the IICB$^{\text{Glc}}$ and endogenous Glc. In other words, the exogenous labeled pool was permitted to mix and equilibrate for more than 30 min with the endogenous Glc before the measurements were begun. The data points and the fitted curve for total Glc-6-$^{32}\text{P}$ and $^{3}\text{H}$-labeled Glc-6-$^{32}\text{P}$ were coincident, showing complete equilibration. In the second part of the experiment, the $^{3}\text{H}$-Glc was added to the syringe containing the $^{32}\text{P}\text{IIA}^{\text{Glc}}$ and came into contact with the endogenous unlabeled Glc and IICB$^{\text{Glc}}$ only when mixed. There was a clear difference between the rates of phosphorylation of the endogenous Glc and the exogenous $^{3}\text{H}$-Glc for about the first 10 s of the progress curve (Fig. 6 shows only the first 1.5 s). Complete equilibration of the two pools took about 10 s under the conditions used for the experiment shown in Fig. 6, whereas measurable phosphorylation of Glc from IICB$^{\text{Glc}}$-Glc is seen at the first time point (25 ms).

Phosphorylation of IICB$^{\text{Glc}}$: Kinetics of the Complete System—the results described above establish Reactions VI and VII, the lower pathway in Fig. 2, as an active pathway for phosphorylating Glc. The transient state kinetics of the upper pathway in Fig. 2 (Reactions IV and V), the pathway most often used to describe the PTS enzymes II, will now be characterized. The rate constants of all of the reactions involving enzyme. This is the case as shown in supplemental data on Glc. Further, as shown above, the endogenous Glc is kinetically competent, but what is its rate of phosphorylation relative to the exogenous Glc? In other words, how rapidly do the exogenous and endogenous Glc pools equilibrate relative to the phosphotransfer reactions starting with phospho-IIA$^{\text{Glc}}$?
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Fig. 6. Comparison of the rate of phosphorylation of exogenous [3H]Glc when added to different reaction solutions. Data from two parts of a rapid quench experiment were used to measure the rate of transfer of the 32P-labeled phospho-group from [32P]IIA-Glc to Glc via IICBGlc. The data from the progress curves were modeled by the differential rate equations defined by Reactions IV, V and VI, VII and VIII. Only the data for the two radioactive forms of Glc are shown; the data for the phosphoproteins are not shown. A, experiment 1; [3H]Glc was added to the IICBGlc-His6 solution (○ •) and allowed to equilibrate with the endogenous Glc for 30 min. After mixing with the labeled phospho-IIA-Glc, the initial concentrations were as follows: [32P]IIA-Glc = 138 nM; IIAGlc = 25 nM (produced by hydrolysis of [32P]IIA-Glc during storage); total IICBGlc-His6 = 97 nM (IICBGlc-Glc = 47 nM calculated from a Kd of 10 μM; total Glc = 95 nM (95 nM endogenous and 10 nM [3H]Glc). Experiment 2; [3H]Glc was added to the [32P]IIA-Glc solution (○ •) and mixed with IICBGlc containing endogenous Glc. After mixing, the initial concentrations were as follows: [32P]IIA-Glc = 128 nM; IIAGlc = 35 nM (produced by hydrolysis of [32P]IIA-Glc during storage); total IICBGlc-His6 = 87 nM (IICBGlc-Glc = 43 nM calculated from a Kd of 10 μM; total Glc = 110 nM (85 nM endogenous, 25 nM [3H]Glc (from the [32P]IIA-Glc solution). The data were fitted manually; the solid line is a theoretical curve. Experiment 1 shows total Glc-6-[3H]Glc (○) and [3H]Glc-6-[32P]Glc (•). Experiment 2 shows total Glc-Glc (○) and [3H]Glc-6-[32P]Glc (•). In experiment 2, the specific activity of the [3H]Glc changes continuously for the first several seconds (see supplemental data on kinetics). The rate constants for the experiment had been determined, Kinsim was used to simulate the time course of the change in the specific activity. This time course was then used to calculate the exact concentration of [3H]Glc-6-[32P]Glc at the specific time points. Therefore, the dashed line is drawn through the data points (i.e. it is not a theoretical fit). These data are included in the global analyses shown in TABLE ONE, rows 2-11.

Rows 2–11 of TABLE ONE show the rate constants obtained from a series of global analyses of the data from four experiments that measured the rate of phospho-group transfer from [32P]IIA-Glc to Glc via IICBGlc-His6, in which exogenous [3H]Glc was added to the [32P]IIA-Glc. Each row shows the effects of varying the Kd and/or the rate constants associated with Reactions VI and VII. In this experiment, a large proportion of the phospho-group reacts with the enzyme to form the phosphoenzyme. The second important question was whether the His tag attached to IICBGlc affected the kinetic behavior of the proteins. Fig. 7 shows that IICBGlc-His6 is as catalytically efficient as the wild type protein, as does the more comprehensive summary in TABLE ONE (cf. rows 5–8, IICBGlc-His6, with rows 12–14, IICBGlc).

Keq and Rate Constants for the Binding of Glc to IICBGlc and Their Effects on Determination of the Rate Constants of the Phosphotransfer Reactions—It is evident that the kinetic characteristics of the sugar binding reaction (Reaction VI) will affect the analysis of the phosphotransfer reactions. The binding reaction determines the relative concentrations of free and bound reactants present at the initiation of the reaction as well as their behavior as phosphorylation proceeds. The rate constants of the sugar binding reaction cannot be determined by any known method. Moreover, even the apparent binding constant (Kapp) could not be determined by flow dialysis for reasons presented in supplemental data on Glc. Semiquantitative values for Kapp, ranging from 1.8 × 10^7 to 9 × 10^8, were obtained from the centrifugation experiments (supplemental data on Glc). We were, however, able to deduce likely values for Kapp from the statistics of fitting the phosphotransfer data. Analysis of four data sets by the nonlinear least squares method (TABLE ONE) showed the following. (a) The values chosen for Kd were influenced by the effect of the rate constants of the phosphotransfer steps and, importantly, on the statistical goodness of the fit. As shown in TABLE ONE, the smallest S.E. value of the rate constants of the four phosphotransfer reactions was obtained when a Kd of 10 μM was used. There was a very high degree of uncertainty in the phosphotransfer rate constants when the Kd was set at 10^-6 or 10^-7 μM; the S.E. values were often larger than the constants themselves. (b) In sharp contrast, the rate constants for binding of Glc to IICBGlc and association of the complex (k+V and k-V) could be varied as much as 6 orders of magnitude without a large effect on the rate constants for the phosphotransfer reactions. This small effect is consistent with the progress curves in Fig. 7, which show that the bulk of the reaction was completed in about 100 s, whereas the experiments from the gel filtration columns (supplemental data on Glc) suggest that the t50 of the binding reaction is about 12 min.

The experiment shown in Fig. 7B permitted independent estimates of the rate constants associated with Reactions VI and VII. In this experiment, a large proportion of the IICBGlc was complexed even when Kd was designated at 10^-6 μM. The experiment involved a mixing with the labeled phospho-IIA-Glc (see supplemental data on Glc) which was estimated by manual fitting as 2.5 × 10^6 M^-1 s^-1 (TABLE ONE, row 14), in agreement with the other estimates in rows 5–8. The rate constants for Reactions VI and VII were also estimated by the nonlinear least squares method (see supplemental data on kinetics) and corroborate those shown in TABLE ONE.
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DISCUSSION

The transient state kinetic experiments reported here were intended to determine the rate constants for the last two steps in the phosphotransfer reaction from phospho-IA\(^{\text{Glc}}\) to IICB\(^{\text{Glc}}\) to Glc by E. coli cells, namely the phosphotransfer reactions from phospho-IA\(^{\text{Glc}}\) to IICB\(^{\text{Glc}}\) to Glc (Reactions IV and V in Figs. 1 and 2). Initially, we conducted these studies with highly purified preparations of IICB\(^{\text{Glc}}\) in lipid/detergent mixtures, but the results were variable, whereas natural membranes containing active IICB\(^{\text{Glc}}\) gave reproducible results (TABLE ONE).

Confirmation of the results obtained with membranes was obtained with IIB\(^{\text{Glc}}\), the soluble, homogeneous domain of the intact protein. IIB\(^{\text{Glc}}\) contains the phosphorylation site Cys of IICB\(^{\text{Glc}}\). The cloned IIB\(^{\text{Glc}}\) domain has kinetically properties that are very similar to those of the whole protein. Both the forward and backward rate constants of phosphotransfer between IIA\(^{\text{Glc}}\) and IIB\(^{\text{Glc}}\) are somewhat larger than those involving the intact membrane protein, IICB\(^{\text{Glc}}\), perhaps expected from the smaller mass of IIB\(^{\text{Glc}}\) and the complexity of the membrane preparations.

Steady-state measurements of IICB\(^{\text{Glc}}\) activity (9, 22, 28, 29) also corroborate the rate constants reported here for Reactions IV and V. The rate constants \(k_{\text{cat}}\) and \(k_{\text{cat}}\) are equivalent to the two respective specificity constants of IICB\(^{\text{Glc}}\) for [P]IIA\(^{\text{Glc}}\) and Glc (i.e. \(k_{\text{cat}} = k_{\text{cat}}(\text{IIA}^{\text{Glc}})\) and \(k_{\text{cat}} = k_{\text{cat}}(\text{Glc})\)). Assuming that the mechanism of the enzyme is Ping Pong (30), the agreement between our results and the calculated specificity constants is good. The latter cluster around \(4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) for \(k_{\text{cat}}(\text{IIA}^{\text{Glc}})\) compared with \(3.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) for \(k_{\text{cat}}\) and around \(3.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) for \(k_{\text{cat}}(\text{Glc})\) compared with \(2.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) for \(k_{\text{cat}}\). The advantage of the rate over the specificity constants is that the rate constants are affected by fewer experimental errors.

A computer model has been developed that can predict the kinetic behavior of the Glc PTS under a variety of conditions, both in vivo and in vitro (22). The model was based, in part, on preliminary results from our kinetic experiments. What we consider to be the definitive experimental rate constants are presented here. The effects of the new constants on the predictions of the model will be presented in a separate report.

From the rate constants, we can calculate the corresponding equilibrium constants for the reactions, phospho-IIA\(^{\text{Glc}}\) to IIB\(^{\text{Glc}}\) or IICB\(^{\text{Glc}}\) (3.5 and 12, respectively). These equilibrium constants appear to be the first data that permit comparison of the standard free energies of hydrolysis of two phosphocysteinyl PTS proteins with those of the phosphohistidinyl PTS proteins. Briefly, the phosphotransfer potential of [P]IIA\(^{\text{Glc}}\) is somewhat less than that of [P]IIA\(^{\text{Glc}}\). But it is, like the other phosphoproteins of the PTS, a “high energy” phosphocompound. The implications of this observation will be elaborated in a future publication on the kinetics and thermodynamics of the complete pathway of the Glc-specific PTS in E. coli. Whether these phosphotransfer potentials are important for the catalytic action of another class of phosphoS-Cys proteins, the protein-tyrosine phosphatases of eukaryotic cells (8) remain to be determined. One could speculate, however, that these enzymes may transfer the phospho-group to substances in addition to water (i.e. they may act as phosphotransferases as well as phosphatases).

At the outset of this work, both the highly purified enzyme and the membranes containing IICB\(^{\text{Glc}}\) were unexpectedly found to contain a “contaminant” that was phosphorylated by the enzyme when it was supplemented with [\(^{32}\text{P}\)]IIA\(^{\text{Glc}}\). The “contaminant” was identified (supplemental data) as Glc that is in equilibrium with IICB\(^{\text{Glc}}\) with an estimated \(K_D\) of \(10^{-7} \text{ M}\); the Glc is kinetically competent. Our data suggest that the sources were very low levels of contamination of laboratory water and reagents and a cellular source, possibly glycogen.

Erni and co-workers (31) purified IICB\(^{\text{Glc}}\) to apparent homogeneity from Salmonella typhimurium and E. coli and were the first to characterize this transporter, finding, for instance, that it contained a phos-
phosphorylation site Cys in the B domain similar to that found in II\text{V\text{th}} (26). They found that isolated [\text{32P}]IICBGlc could transfer the phospho-group to Glc, but the rate of the reaction was exceedingly slow relative to the rate constants reported here (32). There are several possible explanations for this difference (e.g. the enzyme was perhaps partially denatured during its isolation (32), or perhaps there are differences between the physical state of the purified enzyme in the lipid/detergent mixtures compared with its state in the natural membranes).

Garcia-Alles et al. (18, 33) reported that IICBGlc from \textit{E. coli} exhibits steady-state kinetics that are biphasic when a large range of sugar concentrations (50 \text{mM} to 5 \text{mM}) is tested. The authors attribute this to the presence of at least three (and perhaps four) catalytic sites that fall into two classes, one with higher affinity for Glc (K\text{a} \sim 10 \text{mM}) but lower phosphorylation activity and the other with low affinity for Glc (K\text{a} \sim 300 \text{mM}) but about 6 times the phosphorylation activity of the high affinity class. II\text{V\text{th}} also has high and low affinity sites that are delimited at 5 \text{mM} Mtl, and the low affinity site has the higher capacity (17). Our measurements were made at Glc concentrations between 0.08 and 0.2 \text{mM} (with one instance of 5 \text{mM}), which are all well within the high affinity region. We have no information about the kinetics of IICBGlc in the low affinity region. The presence of multiple reactive sites and their kinetic properties will bear on interpreting the physiological significance of the lower branch of the mechanism of IICBGlc (Fig. 2).

The unphosphorylated forms of several Enzymes II bind their sugar substrates (34); bound Mtl is phosphorylated (35), and the enzyme was postulated to have a random order of addition mechanism by analysis of steady-state kinetic data (17, 36). We find that Glc bound to unphosphorylated IICBGlc is kinetically competent; therefore, IICBGlc also has a random order of addition mechanism. It is obvious that the relative flux through the two branches will be very dependent on the rate of binding of Glc to IICBGlc, but K\text{vI} is one of the least certain of the rate constants presented here. The lower branch of the pathway may be of physiological significance under conditions that deplete IICBGlc of phosphogroups (i.e. low cellular concentrations of PEP and/or the presence of other PTS sugar substrates) in the presence of Glc.

In summary, we have analyzed an Enzyme II of the PTS with transient-state kinetic methods and have found that IICBGlc has a random order of addition, Bi Bi, substituted enzyme mechanism with the following properties. (a) The lower branch has a small effect on the flux through the enzyme under the conditions used in our experiments. Since the magnitude of the effect is dependent on the magnitude of k\text{vI} (the rate constant for Glc binding to IICBGlc) and the sugar concentration, under other conditions, the lower branch of the kinetic mechanism could become physiologically significant. (b) We have been able to estimate the rate constants for the binding of Glc to IICBGlc although they are not directly measurable. (c) Although IICBGlc is the fourth protein in the PTS pathway to which the phospho-group from PEP is transferred, the phosphoenzyme retains a phosphotransfer potential much higher than that of ATP. An overview of the kinetics and thermodynamics of the glucose-specific PTS will be presented elsewhere. (d) Finally, our results confirm the data used to build a kinetic model that showed that control of flux through the Glc-specific PTS of \textit{E. coli} is exerted at the last steps of the pathway, the phosphotransfer reactions of IICBGlc, in cells grown on glucose to minoxidirectional phase (22). The model successfully replicated the flux both \textit{in vivo} and \textit{in vitro}, which suggests that its extension to other sugar-specific Enzymes II will enhance our ability to predict cellular responses to a variety of physiological conditions.

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REFERENCES
1. Meadow, N. D., Fox, D. K., and Roseman, S. (1990) \textit{Annu. Rev. Biochem.} 59, 497–542
2. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) \textit{Microbiol. Rev.} 57, 543–594
3. Robillard, G. T., and Broos, J. (1999) \textit{Biochim. Biophys. Acta} 1422, 73–104
4. Siebold, C., Flükiger, K., Beutler, R., and Erni, B. (2003) \textit{FEBS Lett.} 504, 104–111
5. Hu, K. Y., and Saier, M. H., Jr. (2002) \textit{Res. Microbiol.} 153, 405–415
6. Meadow, N. D., and Roseman, S. (1996) \textit{J. Biol. Chem.} 271, 33440–33445
7. Buh, A., Flükiger, K., and Erni, B. (1994) \textit{J. Biol. Chem.} 269, 23437–23443
8. Guan, K. L., and Dixon, J. E. (1991) \textit{J. Biol. Chem.} 266, 17026–17030
9. Bouma, C. L., Meadow, N. D., Stover, E. W., and Roseman, S. (1987) \textit{Proc. Natl. Acad. Sci. U. S. A.} 84, 930–934
10. Pelton, J. G., Torchia, D. A., Meadow, N. D., Wong, C.-Y., and Roseman, S. (1991) \textit{Biochemistry} 30, 10043–10057
11. Waebber, U., Buh, A., Schunk, T., and Erni, B. (1993) \textit{FEBS Lett.} 324, 109–112
12. Waygood, E. B., and Meadow, N. D. (1982) \textit{Methods Enzymol.} 90, 423–431
13. Bradford, M. (1976) \textit{Anal. Biochem.} 27, 10–13
14. Markwell, A. M. K., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) \textit{Anal. Biochem.} 87, 206–210
15. Iversen, L. (1975) \textit{Sci. Biochem.} 1, 623–627
16. Roosien, F. F., Brink, J., and Robillard, G. T. (1983) \textit{Biochim. Biophys. Acta} 760, 185–187
17. Lolkema, J. S., ten Hoeve-Duurkens, R. H., and Robillard, G. T. (1993) \textit{J. Biol. Chem.} 268, 17944–17949
18. Garcia-Alles, L. F., Zahn, A., and Erni, B. (2002) \textit{Biochemistry} 41, 10086
19. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1989) \textit{Annu. Rev. Microbiol.} 43, 305–312
20. Anderson, K. S., Sikorski, J. A., and Johnson, K. A. (1988) \textit{Biochemistry} 27, 7795–7806
21. Zimmerle, C. T., and Frieden, C. (1989) \textit{Biochim. J.} 258, 381–387
22. Rohwer, J. M., Meadow, N. D., Roseman, S., Westerhoff, H. V., and Postma, P. W. (2000) \textit{J. Biol. Chem.} 275, 34099–34121
23. Herr, E. B., and Koshland, D. E. J. (1957) \textit{Biochim. Biophys. Acta} 25, 219–220
24. Akerfeldt, S. (1960) \textit{Annu. Rev. Physiol.} 22, 103–138