Escherichia coli Phosphoenolpyruvate-Dependent Phosphotransferase System. Evidence That the Dimer Is the Active Form of Enzyme I

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**ABSTRACT:** In vitro kinetic measurements have been performed by using purified HPr, Ei, and a membrane fraction of Eii from the *Escherichia coli* phosphoenolpyruvate-dependent sugar transport system. These measurements reveal very large lag times in the formation of methyl α-glucoside phosphate which are a function of the Ei and the Eii concentrations. The lag times decrease with increasing concentrations of Ei but they increase with increasing concentrations of Eii. When Eii, together with Mg²⁺ and phosphoenolpyruvate, is preincubated at 37°C before starting the kinetic measurements, the lag time can be decreased or eliminated. We have shown that the process responsible for the lag time is the activation of Ei by dimerization which is influenced by Mg²⁺ and phosphoenolpyruvate.

**Materials and Methods**

*Bacteria. E. coli* K 235 and *Salmonella typhimurium* SB 2950 were grown and harvested as stated previously (Dooijewaard et al., 1979a).

HPr was purified from *E. coli* K 235 according to the procedure of Dooijewaard et al. (1979a).

Ei was purified from *E. coli* K 235 by the method of Robillard et al. (1979). The ethylene glycol was removed by gel filtration over Sephadex G-75 after which the Ei was stored at 20°C in the lyophilized form.

Eii. The source of Eii was the cytoplasmic membrane fraction of *S. typhimurium* SB 2950. Frozen cells were resuspended in 25 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, and 1 mM NaCl (1 g wet weight cells/5 mL of buffer) and passed through a French pressure cell at 10000–15000 psi. The cell debris was removed by centrifugation for 30 min at 48000g. The supernatant was subjected to high-speed centrifugation (20000g for 2 h) to collect the membrane pellet. This pellet was washed once after resuspending in the same buffer to the original volume. The final pellet was resuspended in 20% of the original crude cell extract volume by using the same buffer and frozen at −20°C until use.

**Assay Procedure.** The phosphorylation reaction was carried out at 37°C in a final volume of 225 μL containing the following components: 5 μmol of potassium phosphate, pH 7.5; 2.5 μmol of KF; 0.25 μmol of DTT; 2 μmol of PEP (cylohexylammonium salt); 2 μmol of methyl α-glucopyranoside containing an amount of methyl α-D-[U-¹⁴C]glucopyranoside sufficient to produce 200000 cpm/μmol of α-MeGlc; 0.5 μmol of MgCl₂; 0.2 nmol of HPr; the specified amounts of Ei and Eii. After being incubated at 37°C, the phosphorylated sugar

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1 Abbreviations used: PEP, phosphoenolpyruvate; α-MeGlc, methyl α-glucopyranoside; PTS, phosphoenolpyruvate-dependent phosphotransferase system; DTT, dithiothreitol.
2 Ei purified by this procedure may be associated with ribonucleic acid (unpublished results).
**FIGURE 1:** Phosphorylation of α-MeGlc as a function of time and E₈ concentration. The E₈ concentrations are (µg/mL) (○) 0.25, (△) 0.38, (□) 0.50, (○) 0.63, and (◊) 1.25. E₉ (20 µL) was added as a membrane suspension, prepared as described under Materials and Methods.

**FIGURE 2:** Phosphorylation of α-MeGlc as a function of time and E₉ concentration. The E₉ concentrations are (µL) (○) 0.6, (△) 1.2, (□) 2.5, and (○) 5.0. The E₈ concentration was 0.63 µg/mL. The time curves have been measured and were linear for 80 min. Only the first 40 min of the time curve is presented to show the effect of E₉ on the lag time more clearly.

**FIGURE 3:** Rates of phosphorylation (A) and lag times (B) as a function of the E₉ concentration at several E₈ concentrations (µg/mL): (○) 0.25, (△) 0.38, (□) 0.50, (○) 0.63, (◊) 1.25. The determination of the rates and the lag times from the time curves, as presented in Figures 1 and 2, is described in the text.

was separated from the nonphosphorylated sugar via a column containing 1-mL bed volume Dowex Ag 1-X2 resin. The reaction mixture was loaded on the column and immediately washed with 20 mL of H₂O to remove nonphosphorylated sugar. Subsequently, the phosphorylated sugar was eluted with 12 mL of 1 M LiCl directly into scintillation vials. Emulsifier scintillator (Packard) (7.5 mL) was added and the radioactivity was counted in a Nuclear Chicago Mark I liquid scintillation counter. The counting efficiency was 60% and the background was 400-500 cpm. When the rate of phosphorylation was being measured, a single reaction vessel was used for each time curve. At stated time intervals 100 µL of the reaction mixture was pipetted directly onto a Dowex column and immediately rinsed with 20 mL of water.

**Preincubation studies** were executed in the following manner. Components were preincubated at 37 °C for 1 h in a total volume of 200 µL containing 25 mM Tris-HCl, pH 7.5, 1 mM DTT, and 1 mM NaN₃. After preincubation, the time curve was started by adding 25 µL of buffer containing the remaining components. This procedure avoids large changes of temperature and volume in between the preincubation step and the beginning of the time curve. Such changes in volume and temperature lead to large scattering in the measured rates and lag times for reasons which will be obvious at the end of Results.

**Phosphoenolpyruvate** (monocyclohexylammonium salt and potassium salt) and **dithiothreitol** were purchased from Sigma Chemical Co.

**Methyl α-[U-¹⁴C]glucopyranoside** (180 µCi/µmol) was purchased from the Radiochemical Centre, Amersham.

**Sephacryl S-200** was purchased from Pharmacia Fine Chemicals.

**Results**

**Lag Time Dependence on E₈ and E₉ Concentrations.** The time dependence of methyl α-glucoside phosphorylation as a function of E₈ concentration is presented in Figure 1. In these measurements E₉ is in excess so that the rate of phosphorylation is independent of the E₉ concentration but proportional to E₈ over a wide concentration range. All the curves in Figure 1 show an initial lag time before the steady-state rate of phosphorylation is attained. The value of the lag time is determined by extrapolating the linear portion of the time curve back to the time axis. As the E₈ concentration increases, the lag time decreases and approaches zero.

Figure 2 shows the time dependence of phosphorylation at a fixed E₈ concentration and varying concentrations of E₉. In this case the lag time increases with increasing E₉ concentrations.

The rates and lag times as a function of E₉ are summarized in Figure 3. Both the rate (Figure 3A) and the lag time (Figure 3B) increase with increasing E₉ concentrations until they reach a maximum value which is determined by the E₈ concentration. In keeping with the data in Figure 1, these rates increase with increasing E₈ concentrations; the lag times,
however, decrease. The dependence of the lag time and the rate on $E_I$ can be more clearly seen from the data replotted in Figure 4. When the data for 0.6 and 20 $\mu$L of $E_{II}$ from Figure 3 are plotted as a function of $E_I$, it is clear that there is an $E_I$ concentration dependence in the lag time even when the rate of phosphorylation is no longer $E_I$ concentration dependent. The relationship between the $E_I$ and $E_{II}$ concentrations, the rate, and the lag times will be treated under Discussion.

Preincubation Studies. In general, lag times arise from processes in which the levels of intermediates must be built up to their steady-state concentrations. These intermediates can be substrates or products in a chain of consecutive reactions or enzyme-substrate complexes or even protein–protein complexes as occur in multienzyme systems. A series of studies were executed in which various PTS proteins and substrates were preincubated in order to determine what kind of intermediates are involved in the present measurements. After preincubation the time dependence of the sugar phosphorylation activity was measured. The result presented in Figure 5 shows that the lag time decreases only if $E_I$ is preincubated with both $Mg^{2+}$ and PEP. Furthermore, preincubation influences only the lag time. The steady-state rate of phosphorylation remains unchanged as can be seen by comparing the linear portion of the control curve (O) and the curve obtained after preincubation of $E_I$ with $Mg^{2+}$ and PEP (¶). Preincubating $E_{II}$ and HPr separately or together with or without $E_I$ has no effect on the lag time. The minimum requirement to shorten the lag time is a preincubation of $E_I$ with, at least, $Mg^{2+}$ and PEP.

Figure 2 shows that the lag time increases with increasing $E_{II}$ concentrations. Since this is the reverse of the concentration dependence found for $E_I$ (Figure 1), it is necessary to consider whether the same lag time is involved for both enzymes or whether there is one process with a given lag time associated with $E_I$ and a second process with another lag time associated with $E_{II}$. These two possibilities can be distinguished in the following manner. If there is only one lag time which is sensitive to the levels of both $E_I$ and $E_{II}$, then a simple preincubation of $E_I$, $Mg^{2+}$, and PEP will always decrease the lag time, independent of the level of $E_{II}$ in the subsequent assay mixture. On the other hand, if there is one lag time associated with $E_I$ and a second associated with $E_{II}$, a preincubation of $E_I$, PEP, and $Mg^{2+}$ alone will only eliminate that portion of the lag time associated with the $E_I$-related process. If the rate of phosphorylation is measured under conditions where the lag time is proportional to the $E_{II}$ concentration, a portion of the lag time will still be present after preincubation. In Figure 6A, the rate of phosphorylation is dependent on $E_I$ (see lower plot). The corresponding lag time (upper plot) decreases with increasing $E_I$ concentration. The closed circles show that the lag time can be substantially decreased or eliminated over the entire $E_I$ concentration range by preincubating $E_I$ with $Mg^{2+}$ and PEP. The reciprocal experiment, measuring rates and lag times as a function of $E_{II}$ concentration, is presented in Figure 6B. In this series both the lag time and the rate are directly proportional to the $E_{II}$ concentration. Nevertheless, preincu-
obtained from the data in Figure 6. The data presented in Figure 6 suggest that there is only one process with one lag time sensitive to the levels of both EI and PEP.

**Time-Dependent Preincubation Studies.** The previous sections have demonstrated an inverse relationship between the EI concentration and the magnitude of the lag time. In addition, preincubation studies have correlated the lag time solely with an EI-associated process. In this section we wish to examine the connection between the EI concentration and the rate of decrease of the lag time during preincubations with Mg²⁺ and PEP.

Figure 7A shows the lag time remaining after preincubation as a function of the length of preincubation time and the EI concentration. These data are replotted in Figure 7B to show the percent decrease in the lag time as a function of the preincubation time and EI concentration. It can be clearly seen, from this figure, that the rate of decrease of the lag time during preincubation is dependent on the EI concentration in the preincubation mixture. The higher the EI concentration, the more rapid the decrease in the lag time.

**Kinetic Analysis.** The observations that (1) the lag time is inversely proportional to the EI concentration, (2) the rate of decrease of the lag time during preincubation is proportional to the EI concentration, (3) the rate of phosphorylation of α-MeGlc is not entirely linear with the EI concentration, and (4) preincubation of only EI with Mg²⁺ and PEP is sufficient to decrease the lag time suggest that EI must be activated by PEP and Mg²⁺ and that this activation process must include an aggregation of EI monomers either as a result of or as a requirement for phosphorylation by Mg²⁺ and PEP. A kinetic model will be presented in the Appendix which describes these observations; it allows us to derive expressions relating the lag time to the time required to activate EI by a dimerization process.

As shown in Scheme I, sugar phosphorylation can be written as two enzyme-catalyzed reactions, each operating according to a bi-bi ping-pong mechanism. For our present consid-

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3 The reason for taking ping-pong mechanisms is the existence of phosphorylated EI (our unpublished results; Hengstenberg, 1977) as well as the possibility to label membranes with [β³²P]PEP (Kundig, 1974) which indicates a phosphorylated EI intermediary.

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4 There is no information concerning the stoichiometry of PEP and HPr to EI dimer. In Scheme II we have presented the simplest case of one molecule of PEP and HPr reacting with one EI dimer. If the scheme proves to be different, it will not affect the qualitative behavior of the model.
is similar to that for a normal bi-bi ping-pong mechanism. Only the numerator is different from a normal linear enzyme concentration term because of the dimerization equilibrium. In this case the numerator represents the total dimer concentration which is a function of the enzyme concentration and of PEP and HPr concentrations (see also eq 9 and Discussion).

The expression for the lag time as a function of enzyme, PEP, and HPr concentrations (eq 15, Appendix)

\[ \text{lag time} = \frac{2}{k_a \left[ n + 1 - \frac{1}{A} \left( 1 + 2nA \right)^{1/2} \right]} \ln \left[ 1 + \frac{1 + nA - \left( 1 + 2nA \right)^{1/2}}{2(1 + 2nA)^{1/2}} \right] \]

demonstrates that increasing the EI concentration (n) decreases the lag time. This is in accordance with the experimentally found effect of EI on the lag time (Figure 4). The calculations also show that the lag time increases with increasing A and therefore that it should increase with increasing PEP and decrease with increasing HPr concentrations. The dependence of the lag time on PEP and HPr was measured to check the predictions of this model. Figure 8 shows that, in accordance with the model, the lag time indeed increases with PEP and decreases with HPr concentrations.

**Gel Filtration Studies.** The kinetic model presented in the Appendix and summarized above has been derived for a dimerization of EI monomers. In principle, however, association to higher molecular weight oligomers could also be involved. Since preincubation of EI with PEP and Mg²⁺ apparently shifts the equilibrium between monomers and oligomer, gel filtration studies in the presence or absence of PEP and Mg²⁺ were performed to determine the size of the oligomer.

The elution position of EI on a Sephacryl column run at room temperature in the absence of PEP and Mg²⁺ is equivalent to a molecular weight of approximately 70,000. However, when EI is preincubated with PEP and Mg²⁺ at 37 °C for 30 min and eluted with buffer containing PEP and Mg²⁺, it elutes as a single peak with a molecular weight of about 134,000. Leaving out either PEP or Mg²⁺ in the preincubation and subsequent column run results in a molecular weight 70,000. This agrees well with the observation from Figure 5 that preincubation of EI with either PEP or Mg²⁺ cannot decrease the lag time.

When the chromatographies were carried out at 4 °C, the following results were obtained. EI eluted as a single peak at the monomer position when buffers lacked Mg²⁺ and PEP and no preincubation step was employed. This is identical with the results obtained at room temperature with these conditions. However, even when EI was first preincubated at 37 °C with Mg²⁺ and PEP and then chromatographed in the presence of PEP and Mg²⁺ at 4 °C, it still eluted as a single peak in the monomer position. This implies that the dimer which is formed at 37 °C in the presence of Mg²⁺ and PEP is cold sensitive and dissociates at 4 °C. For this reason, large changes in volume or temperature while carrying out kinetic experiments can lead to substantial scatter in the data.

**Discussion**

EI is enzymatically active in the dimeric form. This has been shown by kinetic measurements and gel chromatography. When it is purified according to our method, it is a monomer of approximately 70,000 daltons lacking enzymatic activity. The activity reappears slowly in a sugar phosphorylation assay mixture, and eventually a constant rate of phosphorylation is achieved. The activation can also be achieved simply by preincubating EI with PEP and Mg²⁺. Gel filtration shows that under these circumstances the molecular weight is about 134,000. Therefore, the active form of EI has a molecular weight twice that of the inactive form.

**Effect of EI and Substrate Concentrations on the Lag Time.** The fraction of EI in the dimeric form in an assay is not solely determined by the dissociation constant (KD) and the EI concentration. As has been derived in the Appendix (eq 9), the total dimer fraction is also dependent on the PEP and HPr concentrations. Increasing these substrate concentrations leads to an increased total dimer concentration (Σ[EI]) in the case of PEP and a decreased total dimer concentration in the case of HPr. The PEP and HPr dependence of the lag time as described by eq 15 and experimentally confirmed (Figure 8) can be attributed to their effect on the total dimer concentration. EI in the assay mixture will be located in one of three pools: monomers, free dimers, and dimer complexes (see also eq 1). Adding PEP to a given EI concentration will cause a disturbance in the monomer–dimer equilibrium since PEP reacts with the free dimer to form phosphorylated dimers, thus raising the dimer complex concentration. The monomer–dimer equilibrium will be restored by refilling the free dimer pool from the monomer pool. This is the slow process, resulting in the lag time in sugar phosphorylation. The addition of HPr causes dephosphorylation of the dimer complex pool and an increase in the free dimer concentration. The monomer–dimer equilibrium will now be restored by dissociation of the dimers to monomers. Therefore, the total dimer concentration decreases upon addition of HPr, and less dimer has to be formed resulting in a shorter lag time. PEP and HPr affect not only the total dimer concentration but also the rate of dimer formation. This can be seen from eq 8 which describes the monomer concentration as a function of time and the concentrations of EI, PEP, and HPr. Differentiation of this equation (not shown) leads to the rate of total dimer formation which increases with increasing EI and PEP concentrations and decreases with increasing HPr concentrations.

Increasing the EI concentration leads to an increment not only in the total dimer formation rate but also in the total
dimer concentration. However, with increasing El concentration the lag time decreases, due to the concentration effect in the association reaction (eq 7), which is quadratic in the monomer concentration, 2k\_4[E\_m]^2.

**Influence of E\_II on the Lag Time.** Figure 3A,B shows that E\_II only affects the lag time when it is rate determining. When its concentration is in excess, E\_II has no effect on the lag time. When the E\_II concentration is lower, so that it is rate limiting, E\_II must be in excess; however, when E\_II first enters the reaction mixture, it is in an inactive form and activates slowly by association to a dimer. Suppose two experiments are carried out with the same E\_I concentration and different E\_II concentrations. In the first experiment, if we let the E\_I concentration be rate limiting, the lag time will reflect the time required to form the steady-state level of the total dimers. In the second experiment, we will let E\_II be rate limiting; now only the formation time for that amount of EI dimers sufficient to come into excess over E\_II will determine the lag time and that will be shorter than in the first experiment. Furthermore, the concentration of E\_II dimers which are in excess over E\_II is only determined by the E\_II concentration. Higher rate-limiting E\_II concentrations require higher total dimer concentrations if E\_I is to be in excess. Thus, at a fixed E\_I concentration, increasing E\_II concentrations lead to increasing lag times.

The fact that, at a fixed, rate-limiting E\_II concentration, higher E\_I concentrations decrease the lag time as found in Figure 4 (closed symbols) and eq 19 can also be explained. The concentration of E\_II dimers which are in excess over E\_II is only determined by the E\_II concentration. The total dimer formation rate, however, increases with increasing E\_I concentrations. Thus, the excess concentration of E\_I will be reached faster if the total E\_I concentration is higher. The result will be a decreasing lag time.

**Preincubation Studies.** The relative decrease of the lag time as presented in Figure 7 can be formulated by using eq 15. In a certain E\_I concentration range the relative decrease of the lag time reflects the fraction of the steady-state total dimer concentration formed during preincubation (F\_0). Therefore, the relative decrease of the lag time can be regarded as the total dimer formation rate. The model presented is also consistent with our observations that the steady-state rates of phosphorylation are independent of preincubation of E\_I, PEP, and Mg\^{2+}. Equation 14, which gives the steady-state rate as a function of enzyme and substrate concentrations, does not contain a term which represents the "start" concentration of dimers. Such a term is found, however, in the equation for the lag time (eq 15). The term F\_0 represents this start concentration and, as may be expected, the lag time decreases as F\_0 increases.

**Conclusion**

A kinetic model has been derived which explains the activation of E\_I through a dimerization process which is influenced by PEP and Mg\^{2+}. Physical evidence confirms this association state. The monomer/dimer ratio in the absence of PEP and Mg\^{2+} has not yet been determined. Nor is there any evidence that phosphorylation rather than a simple binding of PEP and Mg\^{2+} shifts the monomer–dimer equilibrium. Further study of these matters as well as a quantitative treatment of the kinetic parameters is now in progress.

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**Expression for the Dimer Concentration.** By use of the mechanism presented in Scheme II (Results), the total amount of enzyme I (E\_I) can be written as a summation of all forms:

\[ [E\_I] = [E\_m] + 2([E\_d]+[E\_d-PEP]+[E\_dP]+[E\_dP-HPr]) \] (1)

In this equation [E\_m] represents the free monomer concentration and the term in parentheses represents the total dimer concentration (sum of all dimers) consisting of the free dimer concentration [E\_d] and the dimer complex concentrations ([E\_d-PEP]+[E\_dP]+[E\_dP-HPr]). According to assumption b (see Scheme II, Results), the dimers are in equilibrium with each other and can be expressed in terms of the free dimer concentration [E\_d]

\[ [E\_d-PEP] = \frac{[PEP]}{K\_M-PEP}[E\_d] \] (2)

\[ [E\_dP-HPr] = \frac{k\_2[PEP]}{k\_4K\_M-PEP}[E\_d] \] (3)

\[ [E\_dP] = \frac{k\_2[PEP]K\_M-HPr}{k\_4K\_M-PEP}[E\_d] \] (4)

in which \( K\_M-PEP = (k\_4 + k\_2)/k\_4 \) and \( K\_M-HPr = (k\_4 + k\_4)/k\_3 \).

Substitution of eq 2-4 into eq 1 gives

\[ [E\_I] = [E\_m] + 2[E\_d]\left[ 1 + \frac{[PEP]}{K\_M-PEP} \left( 1 + \frac{k\_2}{k\_4} \left( 1 + \frac{K\_M-HPr}{[HP\_Pr]} \right) \right) \right] \] (5)

or

\[ [E\_I] = [E\_m] + 2[E\_d](1 + \frac{[K\_M-PEP]}{[PEP]} + \left( 1 + \frac{K\_M-HPr}{[HP\_Pr]} \right) \] (6)

The time dependence of the dimerization process is described by the differential equation

\[ 2d\sum[E\_d] \frac{dt}{dt} = 2k\_d[E\_m]^2 - 2k\_d[E\_d] \] (7)

Substitution of eq 5 into eq 7, followed by integration with the boundary condition that \([E\_m(t = 0)] = 0\) is equal to some constant, gives the expression for the monomer concentration as a function of time

\[ [E\_m] = \frac{K\_D}{4A}\left\{-1 + \left( 1 + 2nA \right)^{1/2} \right\} \{ 1 + n\_0A + (1 + 2nA)^{1/2} \}\} \left\{ 1 + n\_0A - (1 + 2nA)^{1/2} \right\} \exp \left[ \frac{-k\_d}{A} \left( 1 + 2nA \right)^{1/2} \right] \] (8)

where \( n\_0 = 4[E\_m(t = 0)]/K\_D, n = 4[E\_I]/K\_D, K\_H = k\_d/k\_4, \) and \( A = 1 + ([PEP]/K\_M-PEP)(1 + (k\_4/k\_4)(1 + K\_M-HPr/[HP\_Pr])) \).

The equilibrium (steady-state) value of the total dimer fraction is obtained from eq 8 by setting \( t \rightarrow \infty \):
Expression for the Reaction Velocity and the Lag Time.

The initial rate of phosphorylation can be obtained from eq 6:

\[
\frac{d[a\text{-MeGlc-6-P}]}{dt} = \frac{d[P\text{-HPr}]}{dt} = k_4[E,E_4p\text{-HPr}] = \frac{1}{2}(E_4E_i - [E_i^n(t)])
\]

Substitution of eq 8 into eq 10, followed by integration with the boundary condition \([a\text{-MeGlc-6-P}(t = 0)] = 0\), gives eq 11 for the concentration of \(a\text{-MeGlc-6-P}\) as a function of time as well as enzyme and substrate concentrations:

\[
[a\text{-MeGlc-6-P}(t)] = \left(1 + \frac{K_{s}\text{PEP}}{[\text{PEP}]} \right)\left(1 + \frac{K_{s}\text{HPr}}{[\text{HPr}]} \right) \left(\frac{K_D}{K_{s}E_4p}[n + \frac{1}{A} - \frac{1}{4A}\ln\left(1 - \frac{t}{1 + 2nA}\right)] - \frac{1}{2}\right)
\]

where \(F_0 = [[E_4] - [E_i^n(t = 0)]]/[[E_4] - [E_i^n(t = \infty)]]\) which is the fraction of the equilibrium total dimer concentration already present at \(t = 0\). Schematically, eq 11 can be represented by

\[
[a\text{-MeGlc-6-P}(t)] = at - b \ln \left[1 - c(1 - \exp(-dt))\right]
\]

or

\[
[a\text{-MeGlc-6-P}(t)] = at - e
\]

where \(e\) is another constant. The steady-state rate of phosphorylation is given by the coefficient \(a\) of the linear part of eq 12 and 13. The lag time is found by substituting \([a\text{-MeGlc-6-P}] = 0\) into eq 13. (This is in fact the extrapolation back to the time axis as done with the experimental time curves to determine the lag time.) The lag time from eq 13 is given by

\[
\text{lag time} = \frac{e}{a}
\]

Applying this to eq 11, we get the steady-state rate

\[
v = \frac{K_D}{8}\left(n + \frac{1}{A} - \frac{1}{A}(1 + 2nA)^{1/2}\right) + \frac{1}{k_4}\left(1 + \frac{K_{s}\text{HPr}}{[\text{HPr}]} \right)
\]

and the lag time

\[
\text{lag time } (\text{LT}) = \frac{2}{k_4\left(n + \frac{1}{A} - \frac{1}{A}(1 + 2nA)^{1/2}\right)} \ln \left[1 - \frac{1 - \frac{1}{4A}\ln\left(1 - \frac{t}{1 + 2nA}\right)}{1/(1 + 2nA)^{1/2}}\right]
\]

Combined Effect of \(E_4\) and \(E_4\text{p}\) on the Lag Time and the Rate of Phosphorylation.

The observation that an \(E_4\text{p}\) concentration dependence exists in the lag time (Figures 2 and 3) even though the lag time is solely an \(E_4\)-related process (Figures 5 and 6) can be understood if we examine the complete sequence of PTS reactions (Scheme I) and include the \(E_4\) dimerization process as the first step in this scheme. This is nothing more than lifting the earlier restriction on Scheme II that \(E_4\) be in excess.

Assuming that PEP and \(a\text{-MeGlc}\) are saturating and the rate of phosphorylation is linear with respect to \(p\) (total HPr concentration), the steady-state rate of phosphorylation is given by

\[
v = \frac{8K_{s}\text{HPr}}{K_{s}\text{HPr} + K_{s}\text{E_4p}[E_4\text{p}]}\times \frac{K_{s}\text{E_4p}}{K_{s}[E_4\text{p}][E_4]}\times \frac{1}{K_{s}[E_4\text{p}][E_4]}
\]

where \(K_{s}\) is the Michaelis constant for PEP.

Equation 16 explains the behavior of the two enzymes determining the rate of phosphorylation as found in Figure 3A.

At small \(E_4\text{p}\) concentrations the \(E_4\text{p}\)-containing term in the denominator is greater than the \(E_4\) \((n)\)-containing term. This latter term can be neglected, and the rate equation reduces to

\[
v = \frac{k_4K_D}{K_{s}\text{HPr}}\times \frac{[E_4\text{p}]}{[E_4]}\times \frac{K_{s}[E_4\text{p}]}{K_{s}[E_4\text{p}][E_4]}
\]

The corresponding lag time at any combination of \(E_4\) and \(E_4\text{p}\) concentrations is given by

\[
\text{LT} = \frac{1}{K_{s}E_4}\times \frac{[E_4\text{p}]^2}{[E_4]} + n + \frac{1}{A} - \frac{1}{A}(1 + 2nA)^{1/2}\times \frac{1}{K_{s}E_4}\times \frac{[E_4\text{p}]^2}{[E_4]} + n + \frac{1}{A} - \frac{1}{A}(1 + 2nA)^{1/2}\left(1 - F_0\right)
\]

where \(K_{s}' = k_4K_{s}\text{HPr}/(k_4K_{s}\text{E_4p}K_{D})\). The dependence of the lag time on \(E_4\text{p}\) as found in Figure 3B, is explained by eq 19. Calculations with eq 19 reveal that at rate-limiting amounts of \(E_4\text{p}\) the lag time increases when the \(E_4\text{p}\) concentration is raised. In this region, the lag time is also dependent upon \(E_4\); increasing the \(E_4\) concentration decreases the lag time. Higher \(E_4\text{p}\) concentrations will ultimately lead to neglecting the
Independent Activation of the Acetylcholine Receptor from *Torpedo californica* at Two Sites

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**ABSTRACT:** Membrane vesicles enriched in acetylcholine receptor were prepared from the electrophax tissue of *Torpedo californica*. The receptor was reduced with dithiothreitol to expose a sulfhydryl group near the ligand binding site and then treated in one of the following ways: (1) affinity alkylated with bromoacetylcholine, a receptor activator, (2) affinity alkylated with maleimidobenzyltrimethylammonium, a receptor inhibitor, or (3) reoxidized to the native state with dithiobis(2-nitrobenzoate). The affinity labels blocked half of the binding sites for α-bungarotoxin. The toxin sites not protected by the affinity labels were protected by carbamylcholine based on studies of toxin binding kinetics. The functional response of native and affinity-alkylated receptors was measured by a sodium ion flux procedure. In the absence of added cholinergic activators, only slow ion flux was observed. In the presence of carbamylcholine, a receptor activator, both native and modified membranes showed the increased sodium flux associated with functional receptors. The concentration of carbamylcholine required for a 50% maximal response was higher in the affinity-labeled membranes. Preincubation of the membranes with carbamylcholine blocked the increased ion flux, indicating that desensitization could be induced. The results provide evidence for the existence of two functional sites on the acetylcholine receptor. Each site corresponds to a bungarotoxin binding site and can be independently activated and desensitized.

The nicotinic acetylcholine receptor (AcChR) from electrophax tissue can be affinity alkylated by 4-maleimidobenzyltrimethylammonium (MBTA) and by bromoacetylcholine (BAC), following reduction of the receptor to expose a reactive sulfhydryl group near the active site (Karlin et al., 1975; Damle et al., 1978; Moore & Raftery, 1979a). Following alkylation, MBTA acts as an irreversible inhibitor of AcChR activation in intact electrophax cells from *Electrophorus electricus* (Karlin, 1969); in contrast, BAC acts as an irreversible activator, leading to prolonged depolarization (Silman & Karlin, 1969). Both affinity labels react specifically with the 40000 *M* subunit of AcChR from *E. electricus* and *Torpedo californica*, and this 40000 *M* subunit (the α chain) is presumed to contain the ligand binding site(s) (Karlin et al., 1975; Damle et al., 1978). Snake α-neurotoxins, which act as nearly irreversible inhibitors of AcChR activation, also bind to the α chain [for reviews, see Heidmann & Changeux (1978) and Barrantes (1979)]. However, there are two α-neurotoxin binding sites for each affinity-labeling site, and analysis of NaDodSO₄-polyacrylamide gel electrophoresis patterns indicates that there are at least two α subunits for each receptor monomer complex of *M*, 250000 (Karlin et al., 1975; Reynolds & Karlin, 1978). Since the binding of BAC and MBTA is mutually exclusive (Damle et al., 1978), it appears that one and only one of the two binding sites can be affinity labeled. Using a different affinity label, p-(tri-methylammonium)benzenediazonium fluoroborate, Weiland et al. (1979) recently found a 1:1 ratio of toxin to affinity-labeled sites.

For membrane-bound AcChR, kinetic and equilibrium binding studies using toxins have not yet revealed differences between the two toxin sites (Blanchard et al., 1979). In detergent solution, however, kinetic heterogeneity has been ob-

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1 Abbreviations used: AcChR, acetylcholine receptor; MBTA, 4-maleimidobenzyltrimethylammonium; BAC, bromoacetylcholine; AcCh, acetylcholine; Carb, carbamylcholine; VDB, vesicle dilution buffer; DTNB, 5,5'-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; [251]α-BgTx, iodinated α-bungarotoxin.