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Bacterial Phosphoenolpyruvate-Dependent Phosphotransferase System. Mechanism of the Transmembrane Sugar Translocation and Phosphorylation†

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ABSTRACT: The phosphoryl-group transfer from PHPr to glucose or α-methylglucose and from glucose 6-phosphate to these same sugars catalyzed by membrane-bound $E_{II}B^{Glc}$ of the bacterial phosphoenolpyruvate-dependent phosphotransferase system has been studied in vitro. Kinetic measurements revealed that both the phosphorylation reaction and the exchange reaction proceed according to a ping-pong mechanism in which a phosphorylated membrane-bound enzyme II acts as an obligatory intermediate. The occurrence of a phospho-IIB^{Glc}/III^{Glc} has been physically demonstrated by the production of a glucose 6-phosphate burst from membranes phosphorylated by phosphoenolpyruvate, HPr, and E_I. The observation of similar second-order rate constants for the production of sugar phosphate starting with different phosphoryl-group donors confirms the catalytic relevance of the phosphoenzyme IIB^{Glc} intermediate. The in vitro results, together with data published by other investigators, have led to a model describing sugar phosphorylation and transport in vivo.

The phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system catalyzes the concomitant transport and phosphorylation of PTS sugars across the cytoplasmic membrane of a great variety of bacteria [for recent reviews, see Hays (1978) and Robillard (1982)]. The transport process can be described by a minimum of two enzyme catalyzed reactions:[2]

$$\text{PEP} + \text{HPr} \underset{Mg^{2+}}{\overset{\text{enzyme I}}{\rightleftharpoons}} \text{PHPr} + \text{pyruvate} \quad (1)$$

$$\text{sugar}_{out} + \text{PHPr} \underset{Mg^{2+}}{\overset{\text{enzyme II complex}}{\rightleftharpoons}} \text{sugar–P}_{in} + \text{HPr} \quad (2)$$

Reaction 1 describes the phosphorylation of the phosphoryl-group carrier protein HPr, catalyzed by enzyme I. In this reaction, enzyme I, like HPr, undergoes a cycle of (de)-phosphorylation. In previous reports, we have studied the mechanisms of the phosphorylation of HPr and enzyme I (Dooijewaard et al., 1979b; Misset et al., 1980; Misset & Robillard, 1982; Hoving et al., 1981, 1982).

Sugar phosphorylation and translocation are mediated by several sugar-specific membrane-bound enzyme II complexes which all use PHPr as the phosphoryl group donating substrate

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[1] Abbreviations: PEP, phosphoenolpyruvate; PTS, PEP-dependent phosphotransferase system; DTT, dithiothreitol; Glc, glucose; Glc-6-P, glucose 6-phosphate; α-MeGlc, methyl α-glucopyranoside; BSA, bovine serum albumin; Gal-6-P, galactose 6-phosphate; TMG, methyl β-thiogalactoside.

[2] Throughout this paper, we will use the following nomenclature for the different reactions: phosphoryl-group transfer involves the overall, enzyme II catalyzed reaction PHPr + S ↔ S–P + HPr whereas phosphoryl-group exchange refers to the partial reaction in Scheme I (Ic). For reasons of simplicity, both the homologous and the heterologous systems are termed phosphoryl-group exchange. In the literature, other nomenclature is used as well: isotope exchange (homologous system), transphosphorylation, and exchange group translocation [both systems, cf. Saier et al. (1977a,b)].

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(reaction 2). Genetic and physicochemical studies have revealed that the various enzyme II complexes differ not only in substrate specificity but also in chemical composition. Jacobsen et al. (1979) have reported that a single membrane-bound protein specific for mannitol (E_{II}^{Mtl}) occurs in *Escherichia coli* and *Salmonella typhimurium*, and Kundig et al. (1971) have described a fully membrane-bound two-component complex (IIA/IIB) specific for mannose, glucose, and fructose. Sugar specificity resides with the IIA component, which can also be phosphorylated by PHPr (Kundig, 1974). The function of IIB has not been determined. *E. coli* and *S. typhimurium* also possess a second two-component system specific for glucose (IIB^{Glc}/III^{Glc}). III^{Glc} is a soluble and/or membrane-associated protein which serves as the phosphoryl-group donor to a membrane-bound IIB^{Glc}.

Kinetic investigations of the PTS-catalyzed phosphorylation and exchange reactions have been used to elucidate the catalytic mechanism of various enzymes II (Marquet et al., 1978; Perret & Gay, 1979; Simoni et al., 1973; Hüdig & Hengstenberg, 1980; Rose & Fox, 1971; Saier et al., 1977a,b; Raphaeli & Saier, 1978, 1980; Jacobson et al., 1979). A consistent picture, however, has yet to emerge. The kinetic data for about half of the systems studied suggest a ping-pong-type reaction mechanism involving a phosphoenzyme II intermediate. The data for the other systems including the two glucose systems just described are not consistent with a simple ping-pong mechanism and have been interpreted as indicating an ordered mechanism in which the phosphoryl group is passed from donor to acceptor without formation of a phosphoenzyme II intermediate. In one case, the mannitol-specific enzyme II, there is kinetic data suggesting both types of mechanisms, a ping-pong mechanism when P–HPr is the donor and an ordered mechanism when sugar phosphate is the donor (Raphaeli & Saier, 1980). The conclusions that no phosphoenzyme II intermediate exists in the case of the glucose-specific enzyme II are at variance with chemical modification studies which show that phosphorylation of enzyme II by phospho-HPr protects enzyme II from inactivation by *N*-ethylmaleimide and dephosphophosphorylation by addition of the sugar substrate removes this protection (Haguenauer-Tsapis & Kepes, 1977; Robillard & Konings, 1981).

Since phosphorylation and transport of PTS sugars are coupled processes, the correct definition of the physical mechanism of E_{II}-catalyzed transport will depend on the correct definition of the phosphorylated state of this enzyme.

In the following section, we present kinetic and, for the first time, physical data confirming the existence of a catalytically significant membrane-bound $P–E_{II}$ complex for the inducible glucose-specific enzyme II from *S. tryphimurium*.

Materials and Methods

Bacteria, Media, and Growth Conditions. Enzyme II preparations were obtained from *S. tryphimurium* PP1163 [*trpB223* Δ(*cysK-ptsHI*)*41 ptsM416 galP*, Tn*10* next to *ptsM*]. PP1163 was constructed by crossing PP801 with phage P22 grown on PP1133 on Luria plates containing tetracycline and looking for colonies unable to grow on galactose (Postma, 1981). The strain was grown to stationary phase at 37 °C in nutrient broth including 0.2% glucose, yielding 4 g of cells (wet weight) out of 2.5 L of medium. The cells were washed once in 0.9% cold NaCl and stored at −20 °C until use.

HPr was purified according to the method of Dooijewaard et al. (1979).

Enzyme I was purified according to Robillard et al. (1979) with the modification described by Brouwer et al. (1982) and Misset & Robillard (1982).

Enzyme II. The source of enzyme II was the washed cytoplasmic membrane fraction. Frozen cells were resuspended in 25 mM sodium phosphate buffer, pH 7.0 (1 g wet weight/5 mL of buffer), containing 1 mM DTT and 1 mM NaN_3. To rupture the cells, the suspension was passed through a French pressure cell at 0 °C, while maintaining the pressure at 10000–15000 psi. Cell debris was removed by centrifugation at 48000*g* for 30 min. The supernatant (crude cell extract) was subjected to centrifugation at 150000*g* for 3 h. The membrane pellet was resuspended to the original volume (wash suspension) by using a Potter tissue homogenizer. After a second high-speed centrifugation, the pellet was resuspended to one-fifth of the crude cell extract volume, frozen, and stored at −20 °C until use.

Urea/Butanol Treatment. The membranes were extracted with urea/butanol as described by Saier et al. (1977a) in order to remove Glc-6-P phosphatase activity. A quantitative recovery of the enzyme II activity was achieved (also see Results).

Glc-6-P was purified free from glucose by anion-exchange chromatography over a column of Dowex AG 1 × 2. After the column was loaded with the Glc-6-P solution, the resin was extensively washed with distilled water to remove glucose. Glc-6-P was eluted with 1 M ammonium bicarbonate and assayed with Glc-6-P dehydrogenase. Glc-6-P-containing fractions were pooled and lyophilized in order to remove the ammonium bicarbonate.

Kinetic Experiments. Phosphoryl-group transfer from PHPr to glucose (and analogues) was measured at 37 °C as a function of time (30 min). Due to a high ratio of enzyme I over enzyme II, no lag times were observed (Misset et al., 1980). Enzyme II concentrations were chosen such that conversion of the sugar did not exceed 10%. The reaction mixtures contained the following components: 25 mM sodium phosphate buffer, pH 7.0; 1 mM DTT; 1 mM NaN_3; 2.5 mM $MgCl_2$; 10 mM NaF; 10 mM PEP; 0.1 μM enzyme I; and the stated concentrations of sugar, HPr, and an E_{II}-containing protein fraction. In the experiments measuring the phosphoryl-group exchange between sugar and sugar phosphate, PEP, enzyme I, and HPr were omitted, but the reaction mixtures contained the stated concentrations of Glc-6-P. Sugar phosphate was separated from sugar by the ion-exchange method, and radioactivity was counted as described previously (Misset et al., 1980).

Concentrations of enzyme I and HPr were determined by active-site titration with ^{14}C-labeled PEP, measuring the initial burst of pyruvate formation (Brouwer et al., 1981). Molar concentrations of enzyme II were determined by the Glc-6-P burst procedure described under Results.

Protein was determined by using the biuret method with BSA as a standard.

Dithiothreitol and phosphoenolpyruvate (monopotassium salt) were purchased from Sigma.

^{14}C-Labeled Sugars. Glucose and methyl α-glucopyranoside were purchased from the Radiochemical Centre, Amersham. Sugar concentrations were calculated from the specific radioactivity as given by the manufacturer.

Results

The transfer of the phosphoryl group from PHPr to sugar depends on two enzymes, a soluble enzyme III^{Glc} and a membrane-bound enzyme IIB^{Glc}. The procedure described above for the preparation of the membranes to be used as the source of IIB^{Glc} involved a urea/butanol extraction which removes peripheral membrane proteins (Rephaeli & Saier, 1978). Since all soluble and loosely membrane-associated

III^{Glc} should have been removed by this treatment, it was assumed that α-MeGlc phosphorylation would not take place without added soluble III^{Glc}. We observed, however, that soluble III^{Glc} was not required. The extracted membrane fraction actively catalyzed phosphorylation with added PEP, E_I, and HPr alone. When the latter components were present in excess, the rates of phosphorylation were linearly dependent on the concentration of membrane protein. This result demonstrates that PHPr can react directly with an integrally membrane-bound component of the IIB^{Glc}/III^{Glc} system. It is consistent with the previous observation that crr mutants which completely lack soluble III^{Glc} (i.e., *S. typhimurium PP 780*; Scholte et al., 1982) transport and phosphorylate α-MeGlc to a certain extent (~5%) (Postma & Scholte, 1979). Furthermore, the authors discovered that crr mutants, which completely lack soluble III^{Glc}, as determined with III^{Glc} antibodies, contain a membrane-bound protein reacting with III^{Glc} antibodies. This III^{Glc}-type molecule presumably accounts for the phosphorylation activity measured in the absence of soluble III^{Glc} in the present study (Scholte et al., 1982).

Dependence of the Rate of Phosphorylation on the PHPr and Sugar Concentrations. In order to determine the reaction mechanism of the in vitro phosphoryl-group transfer from PHPr to glucose (and analogues), the steady-state rates of phosphorylation (V) were measured as a function of the concentrations of PHPr and the sugar. The assay mixtures contained enough enzyme I (see Materials and Methods) to maintain all the HPr as PHPr. As a result, the rates were linearly dependent upon the enzyme II concentration [for a theoretical treatment, see Misset et al. (1980) and Waygood et al. (1979)]. The substrate dependence of V is usually visualized in a Lineweaver–Burk plot which, in this case, can be recorded at a single fixed enzyme II concentration [cf. Misset & Robillard (1982)]. The observed patterns are indicative of the reaction mechanism. Figure 1 shows the Lineweaver–Burk plots for the PHPr and glucose concentration dependence of the reaction rates. Patterns of parallel lines are obtained not only with the PHPr/glucose combination shown in Figure 1 but also with PHPr/α-MeGlc (data not shown). These results indicate that the enzyme IIB^{Glc} catalyzes phosphoryl-group transfer from PHPr to sugar by a ping-pong mechanism. The full sequence of reactions expected to occur between PHPr and SP is listed in Scheme I. In this scheme,

Scheme I

$$\text{PHPr} + e_{III} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} e_{III}\text{–PHPr} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} e_{III}\text{P–HPr} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} e_{III}\text{P} + \text{HPr} \quad \text{(Ia)}$$

$$e_{III}\text{P} + e_{II} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} e_{III}\text{P–}e_{II} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} e_{III}\text{–}e_{II}\text{P} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} e_{III} + e_{II}\text{P} \quad \text{(Ib)}$$

$$e_{II}\text{P} + \text{S} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} e_{II}\text{P–S} \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} e_{II}\text{–SP} \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} e_{II} + \text{SP} \quad \text{(Ic)}$$

S represents the phosphoryl group accepting sugar (glucose or α-MeGlc) whereas the various enzyme II and enzyme III complexes are symbolized by e_{II} and e_{III}, respectively. The dependence of the steady-state rate of phosphorylation on the concentrations of total enzyme II ($[E_{II}]$), enzyme III, PHPr, and sugar, according to the reaction mechanism of Scheme I, is given by

$$\frac{[E_{II}]}{V} = \frac{1}{TN} + \frac{1}{k^{PHPr}}\frac{1}{[PHPr]} + \frac{1}{k^{S}}\frac{1}{[S]} + \frac{1}{k^{PEIII}}\frac{1}{[PEIII]} \quad (3)$$

in which $k^{PHPr} = k_1k_2k_3/(k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)$, $k^{PEIII} = k_4k_5k_6/(k_{-4}k_{-5} + k_{-4}k_6 + k_5k_6)$, and $k^{S} = k_7k_8k_9/(k_{-7}k_{-8} + k_{-7}k_9 + k_8k_9)$ and TN is the turnover number of the enzyme:

$$TN^{-1} = \frac{k_2 + k_{-2} + k_3}{k_2k_3} + \frac{k_5 + k_{-5} + k_6}{k_5k_6} + \frac{k_8 + k_{-8} + k_9}{k_8k_9}$$

k^{PHPr}, k^{PEIII}, and k^{S} are all second-order rate constants of the reactions

$$\text{PHPr} + E_{III} \xrightarrow{k^{PHPr}} E_{III}\text{P} + \text{HPr}$$

$$E_{III}\text{P} + E_{II} \xrightarrow{k^{PEII}} E_{II}\text{P} + E_{III}$$

$$E_{II}\text{P} + \text{S} \xrightarrow{k^{S}} \text{SP} + E_{II}$$

These rate constants are determined only by the corresponding partial reaction and, therefore, are independent of the other substrates involved. The turnover number, on the other hand, is a lumped value comprising rate constants from all partial reactions in scheme I and is dependent on all substrates involved. The apparent turnover number for IIB^{Glc} can be extrapolated from the y-axis intercepts in Figure 1. At subsaturating concentrations of III^{Glc}, the turnover number will be underestimated, but the conclusion concerning the ping-pong nature of the enzyme II catalyzed reaction remains unchanged. This is obvious from the form of eq 3. When $[E_{II}]/V$ is plotted vs. $[PHPr]^{-1}$ or $[S]^{-1}$, the E_{III} rate and concentration terms only affect the intercept (the turnover number) and not the slope of the curves.

The proposed ping-pong reaction mechanism implies that (i) reaction Ic in Scheme I should be measurable in the absence of nonparticipating substrates (and/or products) and (ii) the phosphorylated enzyme acts as an obligatory intermediate and, therefore, should be demonstrable. Both measurements will be described in the following section.

Phosphoryl-Group Exchange. Reaction Ic can be measured by phosphoryl-group exchange between sugar and sugar phosphate. Since both glucose and α-MeGlc act as substrates for enzyme IIB^{Glc}/III^{Glc}, phosphoryl-group exchange can be measured in either a homologous system (i.e., sugar with the corresponding sugar phosphate) or a heterologous system (i.e., sugar with different sugar phosphates).

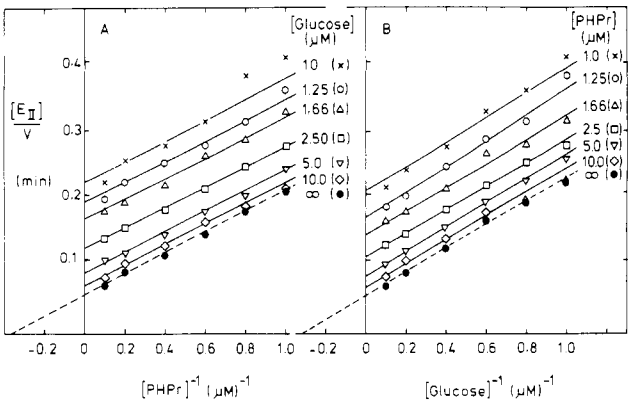


FIGURE 1: Steady-state rate of phosphorylation as a function of PHPr (A) and glucose (B) concentrations, plotted according to the method of Lineweaver–Burk. Experimental conditions are described under Materials and Methods. Enzyme IIB^{Glc}/III^{Glc} (membrane fraction) was used at a final concentration of 3.4 μg of protein/mL. The values of $[E_{II}]/V$ on the ordinate are expressed in the units of micromoles of E_{II} per minute per micromoles of Glc-6-P where the molar concentration of E_{II} was determined by the Glc-6-P burst method discussed in the text. The rates used to construct the curve for an infinite glucose concentration in panel A (filled symbols) were obtained from the y-axis intercepts in panel B. Similarly, the data for an infinite PHPr concentration in panel B were obtained from the y-axis intercepts in panel A.

Table I

phosphoryl-group		K_m^{app} (M) for the phosphoryl-group[a]		second-order rate constant[b] (M^{-1} min^{-1})		
donor	acceptor	donor	acceptor	k^{donor}	$k^{acceptor}$	TN (min^{-1})[c]
P-HPr	glucose	2.7×10^{-6}	3.2×10^{-6}	6.2×10^{6}	5.5×10^{6}	20
P-HPr	α-MeGlc	2.7×10^{-6}	7.1×10^{-6}	6.0×10^{6}	2.2×10^{6}	19
Glc-6-P	glucose	1.1×10^{-3}	0.6×10^{-6}	9.5×10^{3}	16.7×10^{6}	12
Glc-6-P	α-MeGlc	2.0×10^{-3}	5.9×10^{-6}	6.5×10^{3}	2.7×10^{6}	17

[a] The K_m values are determined from the Lineweaver-Burk plot of the infinite concentration of the second substrate. [b] The second-order rate constants for the various phosphoryl group donating and accepting substrates are determined from the slopes of the Lineweaver-Burk plots. The values determined from one experiment have a standard deviation of approximately 10%. [c] The turnover number (TN) is the y-axis intercept of the Lineweaver-Burk plot at an infinite concentration of the second substrate.

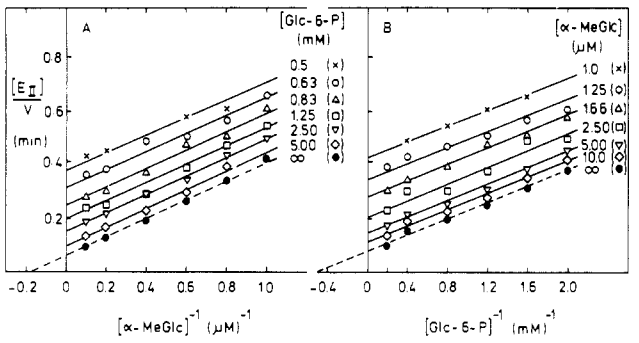


FIGURE 2: Steady-state rate of phosphoryl-group exchange between α-MeGlc and Glc-6-P, plotted according to Lineweaver–Burk. Enzyme IIB^{Glc}/III^{Glc} (membrane fraction) was used at a final concentration of 1.7 μg of protein/mL. The data at infinite Glc-6-P concentration, panel A, or infinite α-MeGlc concentration, panel B, were obtained as stated in the legend to Figure 1.

Steady-state equations can be derived for the phosphoryl-group exchange between sugar and sugar phosphate similar to those derived for the phosphoryl-group transfer from PHPr to sugar. The dependence of the steady-state rate[3] of phosphoryl-group exchange on the concentrations of total enzyme II (E_{II}), sugar (S), and sugar phosphate (S–P) is given by

$$\frac{[E_{II}]}{V} = \frac{1}{TN} + \frac{1}{k^S}\frac{1}{[S]} + \frac{1}{k^{S-P}}\frac{1}{[S-P]} \quad (4)$$

in which k^S and k^{S-P} are the second-order rate constants of the reactions $S + e_{II}P \xrightarrow{k^S} e_{II} + S-P$ and $S-P + e_{II} \xrightarrow{k^{S-P}} e_{II}P + S$, respectively. k^S in eq 4 is identical with k^S in eq 3. TN is again the turnover number of enzyme II and is a function of the particular sugar and sugar phosphate used. In contrast with the phosphorylation reaction, however, TN and $[E]/V$ are independent of $[E_{III}]$. As a result, the true TN can be extrapolated from the intercept of the Lineweaver–Burk plots at infinite substrate concentrations.

The reciprocal rates of the enzyme II catalyzed phosphoryl-group exchange between α-MeGlc and Glc-6-P are plotted in Figure 2 vs. the reciprocal acceptor concentration at varying concentrations of donor. Patterns of parallel lines are obtained, indicating that a ping-pong mechanism is operative. Exchange between glucose and Glc-6-P resulted in similar plots. Table I summarizes the second-order rate constants determined from the phosphoryl-group transfer and exchange experiments. From the fact that the values of k^{donor} are independent of the type of acceptor used and that the values of $k^{acceptor}$ are independent of the type of donor used, it may be concluded that the ping-pong mechanism in Scheme I is the proper mechanism describing the phosphoryl-group transfer from PHPr to sugar.

Michaelis Constants (K_m). K_m values for the substrates can be determined from kinetic measurements, such as described here. These constants represent an affinity of the enzyme for its substrate(s), although they are not the real dissociation constants of the enzyme–substrate complex. The value of K_m equals the substrate concentration which gives rise to half the maximal rate, obtained when the substrate is present at infinite concentration. Since in a two-substrate reaction the rate is determined by both substrate concentrations, the K_m value of one substrate will be a function of the concentration of the second substrate. For this reason, K_m values for one substrate are usually reported at infinite concentrations of the second substrate. The mathematical expression of this K_m can be derived from eq 3 and 4:

$$K_m^{substrate} = \frac{TN}{k^{substrate}} \text{ (M)} \quad (5)$$

in which $k^{substrate}$ is the second-order rate constant for the substrate involved. Since K_m is a function of the TN as well (eq 5), the absolute value will also depend upon the second substrate used. In general, no identical K_m values have to be obtained from experiments in which either phosphoryl-group transfer or exchange is measured. This is often overlooked in the literature. Instead, obtaining identical K_m values for both types of experiments is used as evidence that both reactions are part of the same reaction mechanism. This is correct only if the second-order rate constants determined for both types of experiments are the same. Table I summarizes the K_m^{app} values for the substrates used in our kinetic experiments as well as the turnover numbers obtained for each combination of substrates.

Demonstration of the Membrane-Bound Phosphorylated IIB^{Glc}/III^{Glc}. (*A*) *Pyruvate Burst.* We have previously shown that the concentrations of HPr and E_I could be determined by incubating these proteins with [^{14}C]PEP. An initial burst of [^{14}C]pyruvate occurs corresponding to the concentration of phosphorylated protein (Hoving et al., 1981; Brouwer et al., 1982; Misset & Robillard, 1982). This same procedure can be used to determine the concentration of membrane proteins capable of being phosphorylated from PHPr. We have added increasing amounts of membrane protein to a solution containing a fixed amount of HPr, E_I, and [^{14}C]PEP. An initial burst of pyruvate was formed followed by a linear increase in pyruvate concentration with time. The concentration of pyruvate at $t = 0$ was linearly dependent on the membrane protein concentration and yielded 8.6 nmol of phosphoprotein per mg of membrane protein. The rate constant for the hydrolysis of the phosphorylated membrane fraction, k_h, was estimated from the slow time-dependent formation of pyruvate

[3] In general, steady-state rates are forward reaction rates, obtained by measuring only small conversions of the substrates (≤10%). In the homologous system (glucose and Glc-6-P), chemical equilibrium exists, and therefore, the distribution of the ^{14}C label in the glucose and Glc-6-P pools can be followed until equilibrium. The forward (steady state) rate can be calculated from the resulting exponential plots (Hoving et al., 1981).

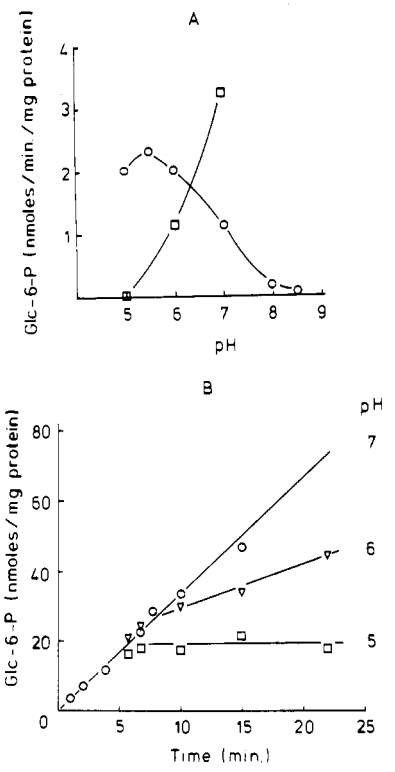


FIGURE 3: (A) Activity vs. pH profile of the enzyme IIB^{Glc} catalyzed phosphorylation (□) and exchange (○) reactions at 37 °C. The phosphorylation reaction mixture contained the following components at the start of the experiment: 25 mM sodium phosphate buffer, pH 7, 1 mM DTT, 1 mM NaN_3, 25 mM $MgCl_2$, 10 mM NaF, 20 μM PEP, 75 μM [^{14}C]glucose, 0.1 μM E_I, 0.7 μM HPr, and 0.2 mg/mL membrane protein. The exchange reaction mixture contained 0.25 mg/mL membrane protein. The phosphoryl-group donor was 5 mM Glc-6-P in place of PEP, E_I, and HPr. The [^{14}C]glucose concentration was 15 μM. The remaining components were identical with those in the phosphorylation reaction mixture. The reactions were allowed to proceed at pH 7 for 5 min during which time aliquots were removed and assayed for [^{14}C]Glc-6-P. The pH was then decreased to pH 5.0, 5.5, or 6.0 by addition of one part 250 mM sodium acetate buffer, pH 4.6, 4.85, or 5.05, respectively, to three parts reaction mixture. Alternatively, the pH was increased to pH 8.0 or 8.5 by a 1:3 addition of 250 mM tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 8.2 or 8.8, respectively. The reaction mixture maintained at pH 7 received a 1:3 addition of water. Aliquots were withdrawn at fixed intervals after the pH was changed and analyzed for [^{14}C]Glc-6-P. The rates of phosphorylation and exchange were calculated from these data. (B) Time dependence of the inhibition of the enzyme IIB^{Glc} catalyzed phosphorylation reaction after the pH was dropped to 7 (○), 6 (△), or 5 (□). Reaction conditions are the same as those described for (A).

which followed the initial burst; it was equal to 0.22 min^{-1}. The significance of this value with respect to the kinetic data presented here and by other authors will be treated under Discussion.

(B) Glc-6-P Burst. When the pyruvate burst is due, in part, to the formation of a phosphorylated IIB^{Glc}/III^{Glc} fraction in the membrane, subsequent addition of excess glucose should result in a burst of Glc-6-P equal in magnitude to the concentration of phosphoenzyme in the membrane. Since the entire complement of PTS proteins and substrates is present, however, there would be a continuous rapid production of Glc-6-P which could mask the initial burst. This complication can be eliminated by inhibiting, after the preincubation step, those reactions leading up to the production of phospho-IIB^{Glc}/III^{Glc} without inhibiting the reaction involving the transfer of the phosphoryl group to the sugar.

The data in Figure 3A show the pH dependence of the phosphorylation and exchange reactions after a sudden change

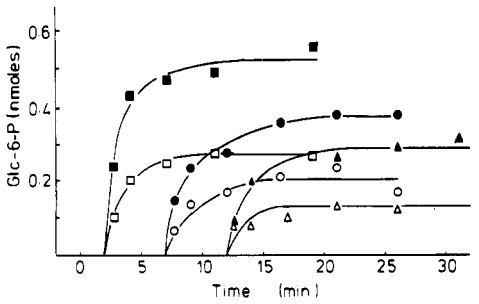
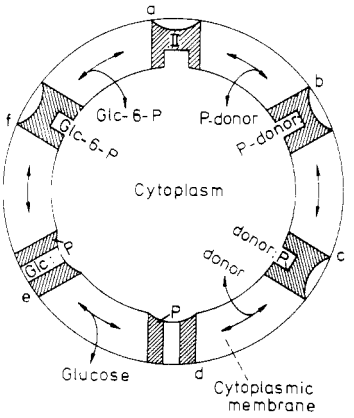


FIGURE 4: Glc-6-P burst determination. The amount of Glc-6-P present in the reaction mixture is plotted as a function of time after the addition of glucose. Reaction mixtures (300 μL final volume) containing 20 μM PEP, 0.7 μM HPr, 0.15 μM E_I, and either 0.25 mg/mL (open symbols) or 0.5 mg/mL (filled symbols) membrane protein were incubated for 3 min at 37 °C in 25 mM sodium phosphate buffer, pH 7, containing 1.0 mM DTT, 2.5 mM $MgCl_2$, and 7.5 mM NaF. A separate mixture was used for each time curve. At $t = 0$, the pH was lowered to 5 by adding concentrated sodium acetate buffer, pH 4.6, to a final concentration of 62.5 mM. [^{14}C]Glucose was added to a final concentration of 100 μM at $t = 2$, 7, or 12 min. Samples were drawn at the indicated times and analyzed for [^{14}C]Glc-6-P.

in pH. The reactions were started at pH 7 and allowed to proceed for 5 min during which time the rate of Glc-6-P production was monitored. The pH was then changed by addition of a concentrated buffer. The data show that the rates of phosphorylation decrease at lower pH. At pH 5, less than 1% of the pH 7 activity remains. In contrast to the phosphorylation reaction, the exchange reaction has a pH maximum of 5.5, and at pH 5, 75% of the maximum activity is retained. Since the last step of both the phosphorylation and exchange reactions is identical in the case of a ping-pong mechanims (E_{II}–P + Glc ⇌ E_{II} + G-6-P), the observed inhibition of the phosphorylation reaction at pH 5 must reflect a stronger pH dependence of one or more steps leading up to the production of phosphoenzyme II from PEP, E_I, and HPr. We conclude, therefore, that by employing a pH drop, the phosphorylation of E_{II} can be stopped while the dephosphorylation reaction can still proceed even if at a reduced rate. It should be noted that the concentrations of PTS components and substrates used in these experiments (see legend to Figure 3A) were selected with the Glc-6-P burst measurements in mind. Consequently, the rates of phosphorylation cannot be compared with the second-order rate constants listed in Table I which predict a much larger difference between the rates of phosphorylation and exchange at pH 7. The time dependence of the inhibition of the phosphorylation reaction after the pH drop is presented in Figure 3B. The new steady-state rate is achieved within 2–3 min after the pH is changed.

The data in Figure 3A,B indicate that the best way to demonstrate the existence of a membrane phospho-IIB^{Glc}/III^{Glc} intermediate is a pH-drop procedure involving the following steps: (i) preincubate membranes with PEP, E_I, and HPr at pH 7; (ii) drop the pH of the incubation mixture to 5 and incubate for 2 min to inhibit the reactions involved in the production of phospho-IIB^{Glc}/III^{Glc}; (iii) add excess glucose and measure the burst of Glc-6-P. The results of such an experiment are presented in Figure 4. The experimental details are given in the figure legend. The data show a definite burst in Glc-6-P production, the magnitude of which depends on the concentration of added membrane protein and the length of the pH 5 preincubation period. Since the HPr and E_I concentrations are constant in all the measurements, the differences in the amount of Glc-6-P formed reflects the difference in the amount of phospho-IIB^{Glc}/III^{Glc}. This is supported by the fact that a reaction mixture with twice the

Scheme II



amount of membrane protein yields twice the amount of Glc-6-P in the burst. These data support the occurrence of a membrane-bound phospho-IIB^{Glc}/III^{Glc} intermediate functional in the transfer of the phosphoryl group to glucose. The rate of hydrolysis of the phosphoenzyme at pH 5 can be determined by plotting the log of the concentration of Glc-6-P produced in the burst vs. the length of the pH 5 preincubation period. The first-order rate constant determined from such a plot is 0.05 min^{-1}. Extrapolation back to $t = 0$ yields the amount of Glc-6-P produced in the absence of hydrolysis.

Discussion

Mechanism of Sugar Phosphorylation and Transport. The in vitro phosphoryl-group transfer from PHPr to glucose (and analogues) proceeds via a ping-pong mechanism. This has been concluded from steady-state kinetics. The kinetic results (i.e., the sets of parallel lines) point to only an interaction between unphosphorylated enzyme II and P donor or phosphoenzyme II with the sugar at the substrate concentrations examined (see also the partial reactions in Scheme I). In the concentration ranges used, no other interactions of these substrates with the two enzyme forms occur, since this would have led to deviation of the parallel line patterns. The kinetic data were supported by the physical demonstration of phospho-IIB^{Glc}/III^{Glc} by using the Glc-6-P burst procedure. During the preparation of this paper, a report appeared (Begley et al., 1982) examining the stereochemical course of the enzyme IIB^{Glc} catalyzed phosphorylation of α-MeGlc. Each phosphoryl-group transfer step is expected to occur with inversion of the configuration at the phosphorus (Knowles, 1980). Starting with phosphoenolpyruvate, chiral at the phosphorus, they observed that the resulting α-MeGlc-P had a configuration at the phosphorus opposite to that of the phosphoenolpyruvate. This inversion of configuration implies five phosphoryl-group transfers or four phosphoenzyme intermediates, P–E_I, P–HPr, P–E_{III}^{Glc}, and P–E_{II}^{Glc}. Our kinetic and Glc-6-P burst data support their conclusions concerning the existence of a catalytically significant phosphorylated enzyme IIB^{Glc} species.

A model describing in vivo transport and phosphorylation of sugars is presented in Scheme II. Unphosphorylated enzyme II (a) has affinity primarily for P donor and not for the sugar. Furthermore, it does not allow penetration of the sugar through the membrane under normal circumstances (Postma et al., 1980). After having bound the P donor on the cytoplasmic side (b), enzyme II becomes phosphorylated (c) after which the donor diffuses off the enzyme (d). Phosphorylation of enzyme II is accompanied by creation of a sugar binding site(s) which must be available from the outside probably via a channel. After sugar binding (e), the phosphoryl group is transferred from the enzyme to the sugar (f) with a concomitant conformational change which prevents the sugar phosphate from leaking out.

Phosphoryl-Group Exchange. The phosphoryl-group transfer from PHPr to the sugar proceeds via a ping-pong mechanism (Scheme I). The results obtained with the sugar–sugar phosphate phosphoryl-group exchange substantiate this ping-pong mechanism, as indeed it should. Therefore, the actual translocation step in vivo is reaction Ic in Scheme I (see also Scheme II, pathways d–f and a). In vivo measurements of this partial reaction should result in phosphoryl-group exchange between sugar and sugar phosphate coupled to transport of both sugar moieties over the membrane in opposite directions. This partial reaction has been measured in vesicles by Saier et al. (1977b). In those experiments, right side out vesicles, loaded with Glc-6-P, catalyzed the uptake of α-MeGlc from the medium, concomitantly expelling glucose into the medium. During its uptake, α-MeGlc is phosphorylated. This experiment strongly favors the transport model given in Scheme II. In a subsequent paper, the authors studied Glc-6-P–α-MeGlc phosphoryl-group exchange in vitro (Raphaeli & Saier, 1978). In contrast with our results, their Lineweaver–Burk plot showed patterns of intersecting lines, from which the authors concluded that the mechanism of phosphoryl-group exchange was sequential (i.e., not involving phosphoenzyme II) rather than ping-pong. In order to explain their results, separate binding sites for sugar and sugar phosphate were suggested, enabling the formation of a ternary complex with the enzyme. Although it is difficult to give an explanation for the different results, some remarks can be made. Rough estimation of the rate constants and turnover numbers for the Glc-6-P–α-MeGlc phosphoryl-group exchange presented by Rephaeli & Saier (1978) reveals that our bacterial strain (PP 1163) catalyzes this reaction 10–50 times more efficiently. Therefore, hydrolysis of phospho-IIB^{Glc}/III^{Glc} (k_h = 0.22 min^{-1}) may play a role in their kinetics and be responsible for the patterns of intersecting lines. Second, contamination of Glc-6-P with glucose gives rise to nonparalles lines. Therefore, we have purified Glc-6-P free from glucose, a procedure not employed by Rephaeli & Saier (1978).

PHPr: Sugar Phosphoryl-Group Transfer Mechanisms in Other PTS. The first kinetic analysis concerning this reaction was published by Rose & Fox (1971) for the phosphorylation of β-glucosides by the inducible enzyme $II^{\beta Glc}$ from *E. coli*. The phosphoryl-group transfer from PHPr to the β-glucosides proceeded via a ping-pong mechanism.

In *E. coli*, inducible enzymes II are found for mannitol and sorbitol. Enzyme II^{Mtl} has recently been purified to homogeneity (Jacobson et al., 1979). Phosphoryl-group exchange between the sugars and their corresponding sugar phosphates has been demonstrated (Saier et al., 1977a; Jacobson et al., 1979). Furthermore, preliminary experiments revealed that PHPr–mannitol phosphoryl-group transfer proceeded via a ping-pong mechanism (Raphaeli & Saier, 1980).

In *Streptococcus faecalis*, a single membrane-bound enzyme II^{Glc} catalyzes the uptake and phosphorylation of glucose (Hügi & Hengstenberg, 1980). From kinetic experiments, the authors concluded that the PHPr–glucose phosphoryl-group transfer proceeded via a ping-pong mechanism. This was substantiated by the kinetics of phosphoryl-group exchange between Glc-6-P and glucose as well as galactose 6-phosphate and glucose.

Two inducible enzymes II exist in *Bacillus subtilis*: II^{Glc} and II^{Fru}. The former has been kinetically characterized by

Marquet et al. (1978). Phosphoryl-group transfer from PHPr to the glucose analogue α-MeGlc also proceeds via a ping-pong mechanism, as does the exchange between Glc-6-P and α-MeGlc. The inducible II^{Fru} has only been studied by means of phosphoryl-group exchange between fructose and fructose 1-phosphate (Perret & Gay, 1979). As expected for this partial reaction, phosphoryl-group exchange proceeds via a phosphoenzyme intermediate (ping-pong mechanism), which suggests that PHPr–fructose phosphoryl-group transfer will also proceed via a ping-pong mechanism. Unfortunately, in all cases mentioned, phosphoenzyme II has not been physically demonstrated, and, as a result, molar enzyme concentrations were not determined. Therefore, no comparison can be made of the turnover numbers and the second-order rate constants among the various experiments (transfer and exchange) or with those determined in the present paper (Table I). Despite this fact, our results, together with those described by other authors, clearly indicate that the ping-pong mechanism is general for many membrane-bound enzyme II species.

Controversial results have been obtained concerning the mechanism of phosphoryl-group transfer in the *Staphylococcus aureus* lactose PTS. Phosphoryl-group transfer proceeds from PEP via enzyme I, HPr, and soluble III^{Lac} to lactose. The latter transfer is catalyzed by the membrane-bound II^{Lac}. Kinetic analysis of the III^{Lac}–PTMG phosphoryl-group transfer indicated a sequential, rather than a ping-pong, mechanism (Simoni et al., 1973). No evidence for a phospho-II^{Lac} was obtained. This would imply that in the absence of (phospho) III^{Lac} no phosphoryl-group exchange between sugar and sugar phosphate does occur. Yet, phosphoryl-group exchange between Gal-6-P and TMG, in the absence of III^{Lac}, can be measured (Saier et al., 1977a). This observation suggests, in analogy with the cases described above, that phospho-II^{Lac} acts as an intermediate. However, no mechanism or kinetic constants were determined which allow comparison of the rates of exchange with the rates of transfer. Therefore, no reliable judgement can be made about the apparent discrepancy in these results. Careful analysis of phosphoryl-group transfer data together with exchange data is required to arrive at a proper reaction mechanism.

The kinetics of the *E. coli* E_{II}^{Mtl}-catalyzed exchange reaction and the constitutive *E. coli* E_{II}^{Man}-catalyzed phosphorylation and exchange reactions are also troublesome in this regard. Because Lineweaver–Burk plots revealing intersecting lines were obtained, Saier and co-workers concluded that the reaction mechanisms were ordered rather than ping-pong and that no phosphoenzyme II intermediate was involved. It is incorrect to conclude, on the basis of such data, that a ping-pong mechanism is not operational. Parallel line patterns in Lineweaver–Burk plots reflect only the most simple type of ping-pong mechanism. Nonparallel lines can occur even with a ping-pong mechanism if complications are involved such as hydrolysis of the phosphoenzyme intermediate, substrate- or product-induced rate alterations (regulation), formation of ternary or higher order complexes, etc. Both substrate and product inhibition occur in the exchange reactions catalyzed by E_{II}^{Mtl} and E_{II}^{Man} (unpublished data).

Quantitative Analysis. Scholte & Postma (1981) stated that, in vivo, the enzyme I catalyzed phosphorylation of HPr is the rate-limiting step in the overall PTS reaction. This conclusion seems contradictory to the kinetic data we have obtained for the enzyme I and enzyme II catalyzed reactions. The second-order rate constants for the phosphorylation and dephosphorylation of enzyme I and enzyme II are 10^8–10^9 and 10^6–10^7 $M^{-1}\cdot min^{-1}$, respectively (Hoving et al., 1982; Misset & Robillard, 1982; Table I). From these data, it would appear that the enzyme II catalyzed reaction should be rate limiting in the overall PTS reaction. In order to correlate the results of Scholte & Postma (1981) with our data, it must be assumed that the in vivo enzyme II activity is much higher or that the in vivo E_I activity is much lower that that determined in vitro (Hoving et al., 1982; Misset & Robillard, 1980).

Acknowledgments

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Registry No. PTS, 56941-29-8; Glc-6-P, 56-73-5; Glc, 50-99-7; α-MeGlc, 97-30-3; enzyme II, 37278-09-4.

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Purification and Characterization of the Androgen Receptor from Rat Ventral Prostate†

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ABSTRACT: The androgen receptor has been purified from rat ventral prostate cytosol by a combination of differential DNA–Sepharose 4B chromatography and testosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine–Sepharose 4B affinity chromatography. Approximately 8 μg of protein was obtained from 38 g of rat ventral prostate, with a yield of 24%. The receptor was purified approximately 120 000-fold. Silver nitrate staining of a sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel revealed a major polypeptide band migrating at 86 000 daltons. Affinity labeling of a partially purified receptor preparation with either 17-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one or 17β-hydroxy-[1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one 17-(2-bromoacetate) produced a major band of radioactivity migrating at 86 000 daltons on a $NaDodSO_4$ gel. Under nondenaturing conditions, a M_r of 85 000 was determined by gel filtration (42 Å) and sucrose gradient sedimentation analysis (4.5 S). The purified receptor had an isoelectric point of 6.3. [^{3}H]-4,5α-Dihydrotestosterone, bound to the purified receptor, was displaced with 4,5α-dihydrotestosterone > testosterone ≫ progesterone > 5α-androstane-3α,17β-diol > 17β-estradiol > cortisol. A number of physicochemical properties of the purified receptor were similar to those of the receptor in crude cytosol.

Receptor proteins are essential for the biological function of androgens in male accessory sex organs [review articles by Liao et al. (1975), Mainwaring (1978), and Chan & Tindall (1981)]. By defining the molecular properties of these proteins, we will further our understanding of the mechanism by which receptors mediate biological events within target tissues. Recently, we developed a procedure to purify an androgen receptor from steer seminal vesicle to apparent homogeneity as determined by $NaDodSO_4$[1] gel electrophoresis (Chang et al., 1982). This procedure combined two techniques, differential DNA chromatography and steroid affinity chromatography, which had been used for purifying other steroid hormone receptors (Coty et al., 1979; Wrange et al., 1979; Westphal & Beato, 1980; Kuhn et al., 1975; Govindan & Sekeris, 1978; Sica & Bresciani, 1979). Because the rat prostate has been one of the most widely studied male accessory glands (Baulieu & Jung, 1970; Fang & Liao, 1971; Mainwaring & Irving, 1973; Tindall et al., 1975; Wilson & French, 1976, 1979), the purification of the prostate receptor would represent an important step in our efforts to understand the mechanism of androgen action in this target tissue. This paper describes the purification of the androgen receptor from rat ventral prostate cytosol and the characterization of a number of its physicochemical and steroid-binding properties.

Experimental Procedures

Materials. [1,2,4,5,6,7-$^3H_6$]Dihydrotestosterone, 143 Ci/mmol, was purchased from Amersham. 17-Hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]R1881, 87 Ci/mmol), nonradioactive R1881, and Enhance were obtained from New England Nuclear (all other steroids were from Steraloids). Leupeptin was a gift from the United States–Japan Cooperative Cancer Research Program. 17β-Hydroxy-[1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one 17-(2-bromoacetate) (147 Ci/mmol) and 17β-hydroxy-5α-androstan-3-one 17-(2-bromoacetate) were synthesized and provided by Dr. Thomas J. Lobl of the Upjohn Co. All other reagents were of analytical grade.

Buffers. The following buffers were used: TED buffer, 50 mM Tris-HCl buffer containing 1.5 mM EDTA and 1.5 mM dithiothreitol (pH 7.4 at 22 °C); TEDG buffer, TED buffer containing 20% glycerol; TEDK buffer, TED buffer containing 0.5 M NaCl; sodium borate buffer, 25 mM sodium borate (pH 8.0 at 22 °C); imidazole buffer, 25 mM imidazole hydrochloride (pH 7.4 at 22 °C).

Preparation of Cytosol. Male Sprague-Dawley rats (3–4 months old) were sacrificed 24 h after orchiectomy. Ventral prostate lobes were removed and frozen quickly in liquid ni-

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[1] Abbreviations: dihydrotestosterone, 4,5α-dihydrotestosterone; [^{3}H]R1881, 17-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one; R1881, 17-hydroxy-17α-methyl-4,9,11-estratrien-3-one; [^{3}H]dihydrotestosterone 17β-bromoacetate, 17β-hydroxy[1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one 17-(2-bromoacetate); leupeptin, a mixture of *N*-acetyl- and *N*-propionyl-L-leucyl-L-leucyl-DL-arginine aldehyde hydrochlorides; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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