THE β-GLUCOSIDE SYSTEM OF *ESCHERICHIA COLI*

II. KINETIC EVIDENCE FOR A PHOSPHORYL-ENZYME II INTERMEDIATE*

Steven P. Rose‡ and C. Fred Fox†

Department of Biochemistry, University of Chicago, Chicago, Illinois, and Department of Bacteriology and the Molecular Biology Institute, University of California, Los Angeles, California

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SUMMARY. The β-glucoside specific enzyme II (enzyme II^{bgl}) of the phosphoenolpyruvate dependent phosphotransferase system of *Escherichia coli* exhibits properties indicative of a "Ping-Pong" kinetic mechanism.

In *Escherichia coli* the first step in β-glucoside catabolism is transport and phosphorylation mediated by a β-glucoside specific enzyme II (1) of the phosphotransferase system initially described by Kundig, Ghosh and Roseman (2). The phosphorylation process is described in the following scheme.

$$\text{P-enolpyruvate} + \text{HPr} \underset{}{\overset{\text{enzyme I}}{\rightleftharpoons}} \text{P}\sim\text{HPr} + \text{pyruvate}$$

$$\text{P}\sim\text{HPr} + \beta\text{-glucoside} \xrightarrow{\text{enzyme } II^{bgl}} \beta\text{-glucoside-6-P} + \text{HPr}$$

We noted apparent inconsistencies in the kinetic values obtained where substrate concentration was varied at constant HPr or *vice versa*. A systematic study shows that the kinetic properties of the enzyme II mediated reaction are those expected of a two substrate reaction sequence involving a substituted enzyme intermediate.

METHODS. In membranes derived from strain W1895D1-bgl^+c, the single activity

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* The initial paper in this series is reference 1.

† Address reprint requests to C. F. Fox, Department of Bacteriology, University of California, Los Angeles, California 90024.

‡ Current address: Department of Biological Chemistry, University of California, Los Angeles, California 90024.

catalyzing TEG[1] phosphorylation is enzyme II^{bgl}. The procedure for growth of this strain and for the preparation of twice washed membranes has been described (1). The purification procedures for enzyme I (40-fold purified), phosphoglucosidase A, and HPr (150-fold purified) will be described in detail elsewhere (3-4). The 0.4 ml assay system for TEG phosphorylation by enzyme II^{bgl} contained 12.5 mM P-enolpyruvate, 10 mM KF, 50 μM $MgCl_2$, 2.5 mg/ml of bovine serum albumin, 50 mM Tris·HCl of pH 7.6, 5 mM 2-mercaptoethanol, 174 μg of enzyme I, 11 μg of twice washed membranes as the source of enzyme II^{bgl}, and HPr and TEG at the indicated concentrations. Enzyme I and P-enolpyruvate are present in sufficient excess so that essentially all of the HPr is in the form of P∿HPr. The reactions were initiated by the addition of washed membranes to an otherwise complete system and incubated at 28° C for 20 minutes. The formation of phosphoryl TEG was determined by a published procedure (1), except that the entire Dowex-1 eluate of 5 ml was mixed with 6.5 ml of Patterson-Green solution (5) containing 6:7 v/v of Triton X-100 (Rohm and Haas) and toluene for scintillation counting.

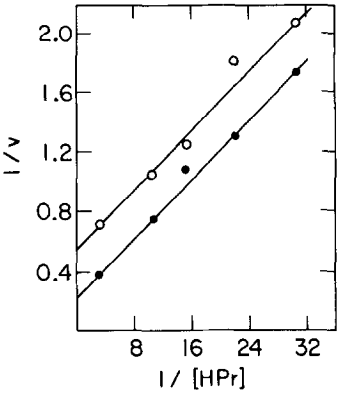

FIGURE 1. Double reciprocal plots for the enzyme II^{bgl} mediated reaction where HPr is varied at constant TEG concentrations. Units are 1/[HPr], mg^{-1} ml and 1/v, $nmoles^{-1}$ phosphoryl TEG formed in 20 minutes. Concentrations of TEG are 250 μM (●) and 50 μM (○).

[1] Abbreviations used are: TEG, ethyl-1-thio-β-D-glucopyranoside; TPG, phenyl-1-thio-β-D-glucopyranoside; TPG-6-P, TPG-6-phosphate; PNPG, *p*-nitrophenyl-β-D-glucopyranoside; PNPG-6-P, PNPG-6-phosphate.

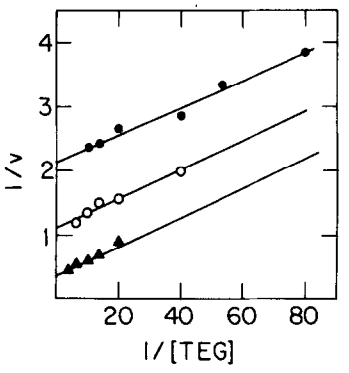

FIGURE 2. Double reciprocal plots for the enzyme II^{bgl} mediated reaction where TEG is varied at constant HPr concentrations. Units are 1/[TEG], mM^{-1} and 1/v, $nmoles^{-1}$ of phosphoryl TEG formed in 20 minutes. Concentrations of HPr are 32.5 µg/ml (●), 65 µg/ml (○) and 325 µg/ml (▲).

RESULTS. Double reciprocal plots of 1/v vs. 1/[HPr] or 1/v vs. 1/[TEG] for the enzyme II^{bgl} reaction mediated by washed membranes are presented in Figures 1 and 2. The parallel straight lines obtained when one of the substrates is varied at constant concentrations of the other substrate are indicative of a double displacement or "Ping-Pong" kinetic mechanism. The steady state rate equation for this kinetic mechanism, using Cleland's formulation (6), is

$$1/v = \frac{K_a}{V_1 a} + \frac{1}{V_1}\left(1 + \frac{K_b}{b}\right)$$

where v, a, b, K_a, K_b, and V_1 represent initial velocity, concentration of the varied substrate, concentration of the unvaried substrate, limiting Michaelis constant of a, limiting Michaelis constant of b, and the maximal velocity repsectively. By plotting 1/a vs. 1/v, an apparent maximal velocity (v') is obtained for each concentration of b. From secondary plots of 1/b vs. 1/v', V_1 and K_b can be calculated. Using this value of V_1, K_a can be calculated from the primary plot of 1/a vs. 1/v. From the separate experiments depicted in Figures 1 and 2, values of 0.35 and 0.46 mg per ml respectively were calculated for K_{HPr} at saturating TEG (limiting $K_{P\sim HPr}$), and values of 0.14 and 0.17 mM respectively for K_{TEG} at saturating P~HPr (limiting K_{TEG}).

TABLE I. Irreversibility of β-glucoside phosphorylation mediated by enzyme II^{bgl}

	System	Incubation time (minutes)	Change in absorbance at 420 nm
1.	Complete	120	< 0.005
2.	Add HPr	120	< 0.005
3.	Add HPr, enzyme I and P-enolpyruvate	5	2.86

The complete system (0.5 ml) contained 1.4 mg of enzyme II^{bgl}, 193 µg of phospho-β-glucosidase A, 1 mM PNPG, 1 mM TPG-6-P (3), 50 µM $MgCl_2$, 10 mM KF, 75 mM Tris·HCl of pH 7.6, and 7.5 mM 2-mercaptoethanol. Enzyme II mediated transfer of phosphate from TPG-6-P to PNPG would be detected by the increase in absorbance at 420 nm resulting from the phospho-β-glucosidase catalyzed release of *p*-nitrophenol from PNPG-6-P. Where indicated, the reaction vessels also contained 7.5 mM P-enolpyruvate, 64 µg of HPr, and 44 µg of enzyme I. Reactions were incubated at 28° C and terminated by the addition of 0.05 ml of 50% (w/v) trichloroacetic acid. After a 10 minute centrifugation at 10,000 x g, 0.3 ml of the supernatant solution was added to 0.6 ml of 2 M KOH, and the absorbance compared with that of a similarly treated sample identical to the complete system above, but for the omission of TPG-6-P.

The inability of enzyme II^{bgl} to catalyze a phosphoryl group exchange between TPG-6-P and PNPG (Table I) shows that the enzyme II^{bgl} mediated reaction is essentially irreversible in extracts, the rate of the forward reaction exceeding that of the back reaction by at least 4 orders of magnitude.

DISCUSSION. A sequence of steps consistent with the kinetic findings reported here is given in Figure 3. The evidence for a "Ping-Pong" kinetic mechanism is given in Figures 1 and 2. The data in Table I indicate that the transfer of phosphate from phosphoryl enzyme II to the glycoside substrate is essentially irreversible in extracts.

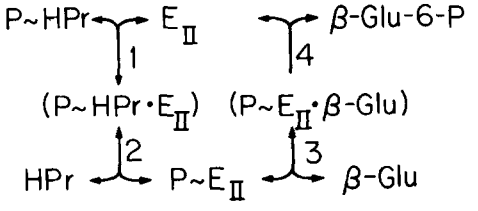

FIGURE 3. Proposed scheme for β-glucoside phosphorylation by enzyme II^{bgl}. See text for details. Symbols used are P~HPr, phosphoryl-HPr; E_{II}, enzyme II; β-Glu, β-glucoside; β-Glu-6-P, β-glucoside-6-P.

Our kinetic results may be contrasted with those of Nakazawa, Simoni, Hays and Roseman which indicate a more complex scheme for P-enolpyruvate linked transport (and phosphorylation) of lactose in *Staphlococcus aureus* (7). Data obtained with the *S. aureus* system indicate ternary complex formation between a specifically induced protein (enzyme II^{lac}) which presumably determines sugar binding, a second specifically induced protein (factor III^{lac}) which is phosphorylated by P~HPr, and the glycoside substrate. In *S. aureus* extracts, factor III^{lac} is a soluble protein. For β-glucoside phosphorylation, on the other hand, no inducible protein corresponding to factor III^{lac} can be detected in the soluble fraction of sonic extracts. Our kinetic data, however, do not preclude the possibility that the β-glucoside phosphorylating system could be composed of more than one membrane bound protein.

The principal interest in the enzymes II stems from their apparent role in the transport of their substrates for phosphorylation. Kaback (8) and Roseman (9) have proposed that transport mediated by an enzyme II is intimately coupled to phosphorylation. If the β-glucoside binding site for phosphorylatio depicted in step 3 of Figure 3 is also responsible for binding β-glucosides for transport, our data indicate that enzyme II^{bgl} linked transport is likely to be mediated by a phosphoryl-enzyme II intermediate.

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