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URIDINE PHOSPHORYLASE FROM *ESCHERICHIA COLI*

KINETIC PROPERTIES AND MECHANISM

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Summary

Type I and Type II uridine phosphorylases (uridine: orthophosphate ribosyltransferase EC 2.4.2.3) are distinguished by their pH optima (Krenitsky et al. (1965) J. Biol. Chem. 240, 1281—1286).

A Type I enzyme was partially purified from *Escherichia coli*. The crossing pattern of the initial velocity analysis indicated that the catalytic mechanism involved the sequential addition of substrates to the enzyme. Product inhibition by uracil or by ribose 1-phosphate was linear competitive with uridine or with concentrations of phosphate below 3 mM. This indicated that the sequence of substrate addition was random rather than ordered. At concentrations of phosphate above 3 mM, product inhibition by uracil was complex.

The random mechanism of this Type I enzyme contrasts with the ordered mechanism of a Type II enzyme from rat liver (Kraut, A. and Yamada, E.W. (1971) J. Biol. Chem. 246, 2021—2030).

Introduction

Uridine phosphorylases from various sources fall into two groups by virtue of their pH optima for uridine phosphorolysis [1]. One group (Type I) is characterized by an optimum around pH 6.6. The other group (Type II) is characterized by an optimum around pH 8.

Although the Type I enzyme from guinea pig requires phosphate for ribosyl transfer from uridine to [^{14}C]uracil, high concentrations of phosphate are inhibitory [2]. It was suggested that the formation of a dead-end complex between enzyme and phosphate might cause this inhibition and that the productive order of binding of substrates to the enzyme might be nucleoside followed by phosphate. Later kinetic studies with a Type II enzyme from rat liver suggested an ordered sequential mechanism involving the initial addition of phos-

phate to the enzyme [3,4]. For the sake of comparison with the Type II enzyme, it was of interest to study the kinetics of a Type I enzyme.

This report describes the kinetic properties of a Type I uridine phosphorylase purified from *Escherichia coli*. The results indicated that its catalytic mechanism was random rather than ordered.

Experimental Procedure

Materials. Uracil and uridine were purchased from P-L Biochemicals; α-ribose 1-phosphate (dicyclohexylammonium salt) from Sigma; casamino acids (vitamin free) from Difco; DEAE-cellulose (DE-52) from Whatman.

Enzyme assays. All assays were performed at 25°C in 50 mM Tris · HCl at pH 7.4. The reactions were continuously monitored at 282 nm ($\Delta\epsilon = 1370$ $M^{-1} \cdot cm^{-1}$) with a Gilford recording spectrophotometer using a full scale recorder setting of 0.1 absorbance unit. All solutions were adjusted to pH 7.4 before addition to the reaction mixtures. In the kinetic experiments (Figs. 1—6), velocities were expressed as nmol of product formed per min per ml of reaction mixture. Protein [5] and phosphate [6] concentrations were determined by published methods.

Results

Enzyme purification

Table I summarizes the purification of uridine phosphorylase from *E. coli* B-96 (ATCC No. 13473). The cells were grown in aerated vessels at 36°C in a media containing 18.9 g $Na_2HPO_4 \cdot 7H_2O$, 6.3 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 g $(NH_4)_2SO_4$, 0.5 g adenosine, and 8 g casamino acids per l. Cells were harvested by centrifugation when the absorbance of the undiluted culture reached 2.8 at 600 nm. The cell paste was resuspended in two times its weight of 5 mM potassium phosphate buffer, pH 8.0. This suspension was stored in 5-ml aliquots at -73°C for up to 6 months without loss of enzyme activity.

Step 1: Supernatant of sonicate. Six of the thawed aliquots were separately sonicated twice for 12 s with a 3-min cooling interval in an ice bath. A Branson Model 5125 sonic oscillator was used at a power setting of six. The sonicates were pooled and centrifuged at 48 000 × *g* and 3°C for 20 min.

TABLE I

PURIFICATION OF URIDINE PHOSPHORYLASE FROM *E. COLI*

Step	Volume (ml)	Total activity (units) *	Specific activity (units */ mg protein)	Purification (-fold)	Recovery (%)
1. Supernatant of sonicate	16.5	100 000	377	1	100
2. Extract of calcium phosphate gel	73	82 800	2 040	5.4	83
3. DEAE-cellulose chromatography	21	31 900	8 940	24	32
4. ECTEOLA-cellulose chromatography	40	27 000	16 100	43	27

* Units = nmol/min; assay mixtures contained 0.77 mM uridine and 88 mM potassium phosphate at pH 7.4 and 25°C.

Step 2: Elution from calcium phosphate gel. A calcium phosphate gel suspension was prepared according to the method of Keilin and Hartree [7]. The suspension contained 36 mg of dry solid per ml and was aged for 5 months at 3° C before use. 10 ml of this suspension was slowly added with mechanical stirring at 3° C to the supernatant resulting from Step 1. After stirring for 20 min, the mixture was centrifuged at 9000 × *g* for 5 min and the supernatant fluid was discarded. The pellet was mixed with 25 ml of 20 mM potassium phosphate, pH 7.4, (Buffer A) for 10 min and then centrifuged as above. The supernatant fluid was discarded and the pellet was extracted with another 25 ml of Buffer A, but at 25° C instead of 3° C. This was followed by two extractions with 25-ml portions of 40 mM potassium phosphate, pH 7.4, at 25° C. The last three extracts were combined.

Step 3: DEAE-cellulose chromatography. The combined extracts from Step 2 were applied to a DEAE-cellulose column (2.5 cm in diameter and 7 cm in height) which had been equilibrated with Buffer A at 25° C. After a 40 ml wash with Buffer A, the enzyme was eluted with a 400 ml linear gradient of 20—400 mM potassium phosphate, pH 7.4. The phosphate concentration of the combined fractions having the highest enzyme activity was 170 mM.

Step 4: ECTEOLA-cellulose chromatography. The pooled fractions from the DEAE-cellulose column were dialyzed against 300 ml of water for 30 min at 25° C and then against 2 l of 40 mM potassium phosphate, pH 7.4, containing 15 mM 2-mercaptoethanol, for 17 h at 3° C. The dialyzate was applied to an ECTEOLA-cellulose column (2.5 cm in diameter × 6 cm in height) equilibrated with 40 mM potassium phosphate buffer, pH 8.0, containing 15 mM 2-mercaptoethanol. The enzyme was eluted from the column with this buffer. Fractions having the highest enzyme activity were combined. This activity was stable for at least 3 months at -73° C. Before use in the experiments described below, the preparation was desalted by passing aliquots (2 ml) through a Sephadex G-25 column (2 cm in diameter and 26 cm in height) which had been equilibrated with 20 mM Tris · HCl at pH 7.4 and 3° C.

Initial velocity analysis

Double reciprocal plots of initial velocities at various concentrations of uridine and at different fixed concentrations of inorganic phosphate resulted in a family of lines which intersected at a point above the abscissa to the left of the ordinate (Fig. 1A). A similar pattern resulted when phosphate was plotted as the variable substrate (Fig. 1B). In both cases, the reciprocals of the apparent maximal velocities (ordinate intercepts) were linear functions of the reciprocals of the fixed substrate concentrations (dashed lines in Figs. 1A and 1B). The K_m value for uridine extrapolated to infinite concentrations of phosphate was 0.091 mM. The corresponding value for phosphate was 2.9 mM.

Product inhibition

With uridine as the variable substrate, product inhibition by uracil (Fig. 2) and by ribose 1-phosphate (Fig. 3) were competitive. In both cases, the degree of inhibition was a linear function of the concentration of product (inserts of Figs. 2 and 3).

With phosphate as the variable substrate, product inhibition by ribose 1-

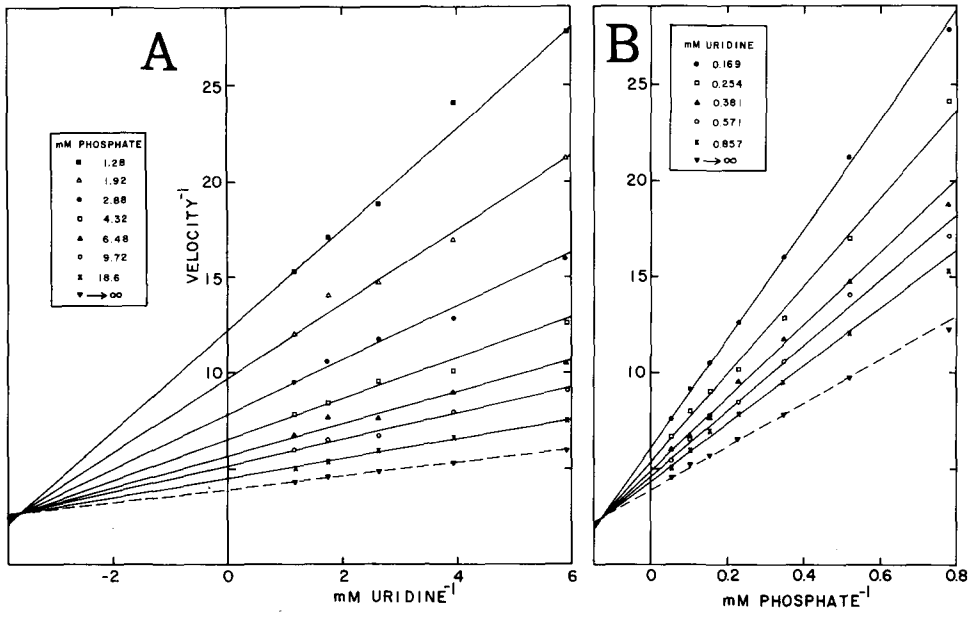


Fig. 1. Initial velocities with uridine (A) and phosphate (B) as the variable substrates. Apparent maximal velocities obtained from ordinate intercepts of one plot were used to generate the line (dashed) for concentrations extrapolated to infinity on the other plot. The K_m value for uridine at extrapolated infinite concentrations of phosphate determined from this line was 0.091 mM. The corresponding K_m value for phosphate was 2.9 mM. The K_i value for uridine determined from the point on the abscissa over which the lines intersect ($-1/K_i$) in Plot A was 0.28 mM. The K_i value for phosphate similarly determined from Plot B was 7.7 mM.

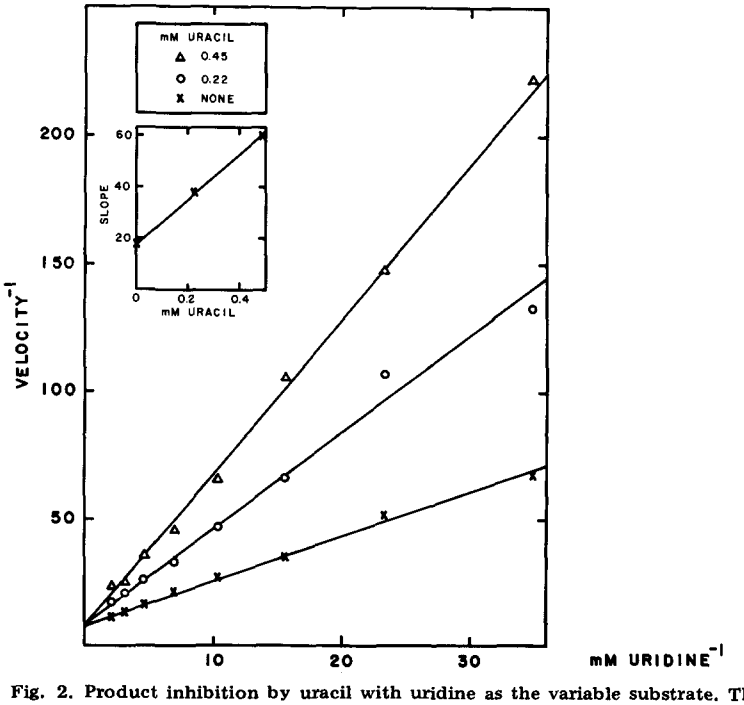
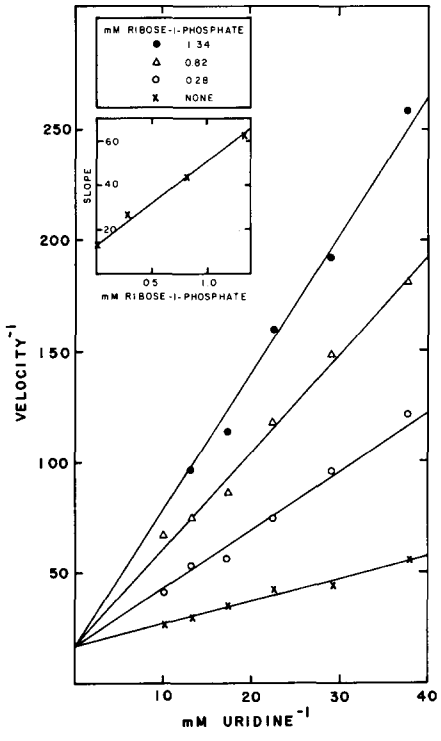


Fig. 2. Product inhibition by uracil with uridine as the variable substrate. The phosphate concentration was 0.64 mM. The K_i value for uracil was determined to be 0.2 mM.



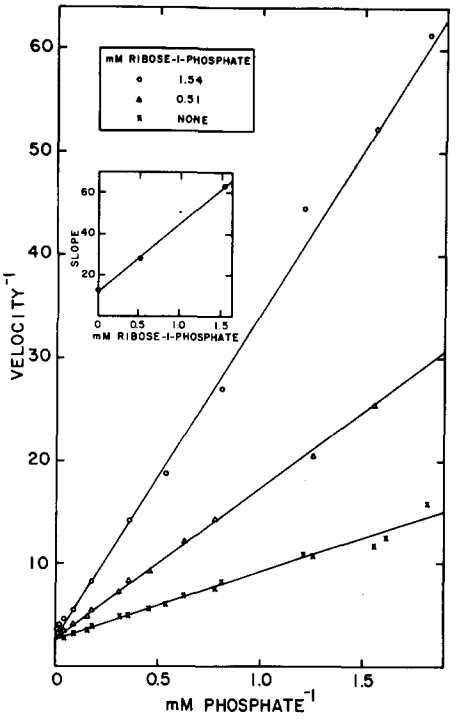


Fig. 3. Product inhibition by ribose 1-phosphate with uridine as the variable substrate. The concentration of phosphate was 10 mM. The K_i value of ribose 1-phosphate was determined to be 0.34 mM.

Fig. 4. Product inhibition by ribose 1-phosphate with phosphate as the variable substrate. The concentration of uridine was 0.25 mM. The K_i value for ribose 1-phosphate was determined to be 0.35 mM.

phosphate (Fig. 4) was linear competitive. Product inhibition by uracil with phosphate as the variable substrate was linear competitive at concentrations of phosphate below 3 mM (Fig. 5). However, concentrations of phosphate above 3 mM failed to completely overcome the inhibition by uracil (Fig. 6).

Discussion

The observed kinetics were subject to relatively simple interpretation by use of the rules compiled by Cleland [8]. The non-parallel lines of the initial velocity analysis (Fig. 1) clearly indicated that the catalytic mechanism was sequential rather than Ping Pong. The competitive nature of the product inhibition (Figs. 2—5) indicated that the sequence of substrate addition was random (rapid equilibrium) rather than ordered.

The only complexity in this otherwise simple mechanism was the departure from competitive inhibition by uracil at concentrations of phosphate above 3 mM (Fig. 6). One possible explanation for this anomaly is that the enzyme undergoes a conformational change at concentrations of phosphate above 3 mM which renders it more susceptible to inhibition by uracil. Another possibility is that a quaternary dead-end complex consisting of enzyme, uracil, and two

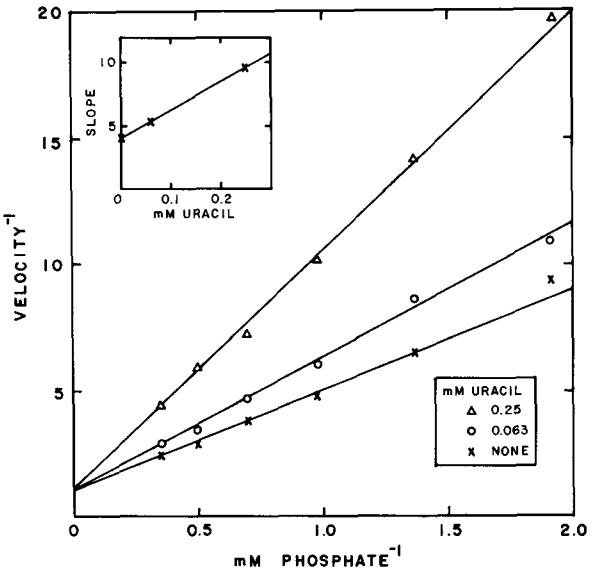


Fig. 5. Product inhibition by uracil with phosphate varied below 3 mM. The concentration of uridine was 0.18 mM. The K_i value for uracil was determined to be 0.18 mM.

molecules of phosphate is formed. Such possibilities would be difficult to distinguish experimentally.

As mentioned in the Introduction, uridine phosphorylases fall into two

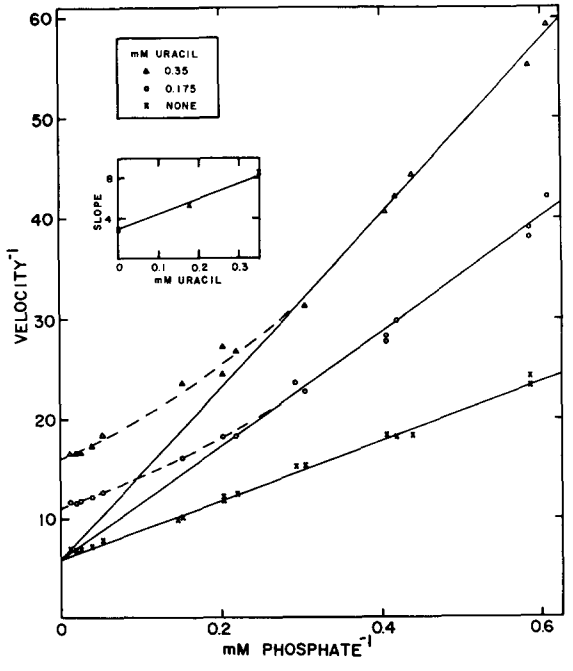


Fig. 6. Product inhibition by uracil with phosphate varied above and below 3 mM. The concentration of uridine was 0.22 mM. The K_i value for uracil was determined to be 0.2 mM.

groups by virtue of their pH optima [1]. The random mechanism proposed here for the Type I enzyme from *E. coli* contrasts with the ordered mechanism previously proposed for the Type II enzyme from rat liver [3,4]. Further studies on the mechanisms of Type I and Type II enzymes from other sources are required to determine if this mechanistic difference is yet another characteristic which distinguishes Type I from Type II uridine phosphorylases.

Acknowledgements

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