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Enzyme-catalyzed uridine phosphorolysis: S_N2 mechanism with phosphate activation by desolvation

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Abstract The rate of uridine phosphorolysis catalyzed by uridine phosphorylase from *Escherichia coli* decreases with increasing ionic strength. In contrast, the rate was increased about twofold after preincubation of uridine phosphorylase with 60% acetonitrile. These data correlate with known effects of polar and bipolar aprotic solvents on S_N2 nucleophilic substitution reactions. The enzyme modified with fluorescein-5′-isothiocyanate (fluorescein residue occupies an uridine-binding subsite [Komissarov et al., (1994) Biochim. Biophys. Acta 1205, 54–58]) was selectively modified with irreversible inhibitor SA-423, which reacts near the phosphate-binding subsite. The double-modified uridine phosphorylase is assumed to imitate the enzyme–substrate complex. Modification with SA-423 was accompanied with dramatic changes in the absorption spectrum of active site-linked fluorescein, which were identical to those for fluorescein in a hydrophobic medium, namely 80% acetonitrile. The data obtained suggest that an increase in active site hydrophobicity leads to phosphate desolvation and facilitates the enzymatic S_N2 uridine phosphorolysis reaction.

Key words: Uridine phosphorylase; *E. coli*; Mechanism; S_N2 substitution reaction; Active site desolvation; FITC

1. Introduction

Uridine phosphorylase (UPase) (EC 2.4.2.3) catalyzes phosphorolysis of uridine [1,2]. In general, uracil substitution with phosphate may proceed according to either an S_N1 or S_N2 mechanism (Fig. 1). Hydrated phosphate anion is a weak nucleophilic agent and usually takes part in S_N1 reactions only [3]. The aim of the present study is to get a further insight into the mechanism of UPase action. We have investigated the effects of ionic strength variation and of a bipolar aprotic solvent on the rate of enzyme-catalyzed uridine phosphorolysis. In an attempt to elucidate the mechanism of phosphate activation at the UPase active site, we have studied the spectral properties of enzyme-bound fluorescein (which occupies an uridine-binding subsite of the UPase [4]) and have selectively modified both uridine- and phosphate-binding subsites to demonstrate that active site hydrophobicity increases on enzyme–substrate complex formation.

2. Materials and methods

Tris, uridine and fluorescein-5′-isothiocyanate (FITC) were from Sigma Chemical Co. (USA). Acetonitrile was from Aldrich (USA). Sephadex G-50M was from Pharmacia (Sweden). All other chemicals were of reagent or analytical grade and used without further purification. SA-423 was synthesized in the laboratory of Dr. V.I. Fetisov (Institute of Physiologically Active Compounds, Russian Academy of Sciences, Chernogolovka, Russia). UPase with a specific activity of 100 U/mg (25°C) was isolated from the *E. coli* K-12 overproducing strain as described previously [5]. The assays of UPase activity [2] and spectral measurements were carried out on a Graficord UV-240 spectrofotometer (Shimadzu, Japan). Protein concentration was estimated according to Bradford [6] and from absorbance at 280 nm [1]. When indicated, salts were added to the reaction mixture (0.5 mM uridine, 50 mM phosphate, pH 7.0) before activity determination. UPase was incubated in the presence of salt or acetonitrile overnight at 4°C before use.

FTC-UPase was obtained as described earlier [4]. FTC-UPase (16 μM) was incubated at 20°C with 0.16 mM SA-423 in 50 mM Tris-HCl buffer, pH 7.1. A control sample of native UPase was incubated with SA-423 under the same conditions. All solutions contained 3% (v/v) of isopropanol. At various times (0–5 min), 10-μl aliquots were withdrawn from the control mixture for activity determination. After activity of the control sample had dropped to 10% of initial value, the modifying agent was separated on a Sephadex G-50M column. Thereafter the absorbance spectra of the double-modified UPase and FTC-UPase obtained were compared with that of fluorescein and between each other.

3. Results and discussion

It is known that the effect of solvent polarity on nucleophilic substitution reactions makes it possible to distinguish between S_N1 and S_N2 mechanisms [3]. The rate of enzymatic uridine phosphorolysis was found to be greatly influenced by the ionic strength of the solution. Increasing concentrations of different salts slows down the reaction rate. The fact that the uridine phosphorolysis rate depends on the ionic strength value (Fig. 2) testifies in the favour of an S_N2 mechanism. Alternatively, these results may reflect a decreased phosphate binding due to salt competition. To choose between these alternatives, the effect of solvent hydrophobicity on UPase activity was measured. When acetonitrile (> 50%; v/v) was added to UPase prior to activity determination, the rate of the enzymatic reaction under the standard conditions was increased about twofold (Fig. 3). The equilibration of the enzyme at high acetonitrile concentration is probably accompanied by an increase in aprotic solvent content at the UPase active site, thereby facilitating uridine phosphorolysis. Hence, the data obtained correlate well with known effects of polar and bipolar aprotic solvents on S_N2 nucleophilic reactions [3] and suggest that a single-displacement S_N2 mechanism is valid for enzymatic uridine phosphorolysis, in agreement with the proposal made for purine nucleoside phosphorylase [7,8].

Phosphate itself is a weak nucleophilic agent (a hard, low polarizable, highly-solvated base) and must be activated to

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Abbreviations: FITC, fluorescein-5′-isothiocyanate; UPase, uridine phosphorylase (EC 2.4.2.3) from *E. coli*; FTC-UPase, FITC-modified UPase.

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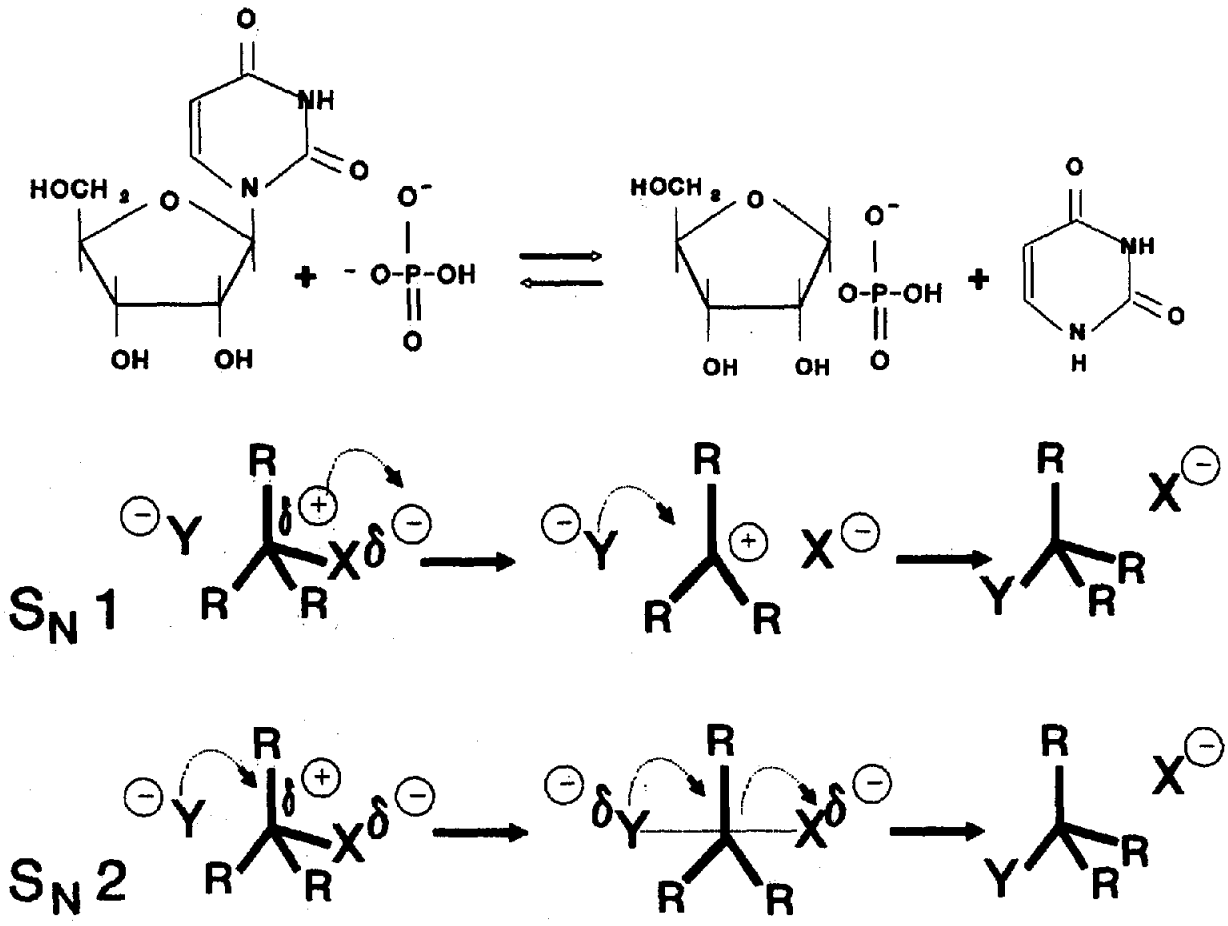

Fig. 1. The scheme of the enzymatic uridine phosphorolysis reaction and possible S_N1 and S_N2 mechanisms of nucleophilic substitution.

enter S_N2 reactions [3]. Phosphate binding at the UPase active site results in the structural rearrangements that are manifestated in absorbance spectra [4]. In UPase, selectively modified with FITC, the fluorescein residue is located at the putative uridine-binding subsite and does not affect the ability to bind the second substrate, phosphate [4]. To investigate possible structural rearrangements of active site in ternary enzyme–substrate complex, selective modification of FTC-UPase was carried out. Nearly stoichiometric amounts of a powerful inhibitor SA-423 irreversibly inactivate UPase by covalently interacting with the phosphate-binding subsite [9]. The modification with SA-423 was used to study the active site changes after modification of both uridine and phosphate subsites (mimicking enzyme–substrate complex formation).

The successive treatment of UPase with FITC and SA-423 was performed as described in section 2. The FTC-UPase and the double-modified enzyme were isolated by gel-filtration and their absorption spectra were compared with that of fluorescein (Fig. 4). The spectrum of FITC bound at the uridine subsite is red-shifted about 5 nm relative to that for the free fluorescein. The environment at this subsite appears to be similar to that observed for fluorescein in ethanol [10] and for FITC bound at the conformation-sensitive site of Na^+,K^+-ATPase from lamb kidney [11]. These data agree with FITC residing in a hydrophobic pocket of the UPase active site. The SA-423 modification was accompanied with an additional effect on the spectrum of the active-site-bound fluorescein (Fig. 4). The extinction coefficient of the double-modified UPase at 490–500 nm is about sixfold lower then that for FTC-UPase, which indicates differences in the fluorescein environment resulting from it specific interactions with nearby amino acids. The decrease in absorption for the double-modified enzyme is the

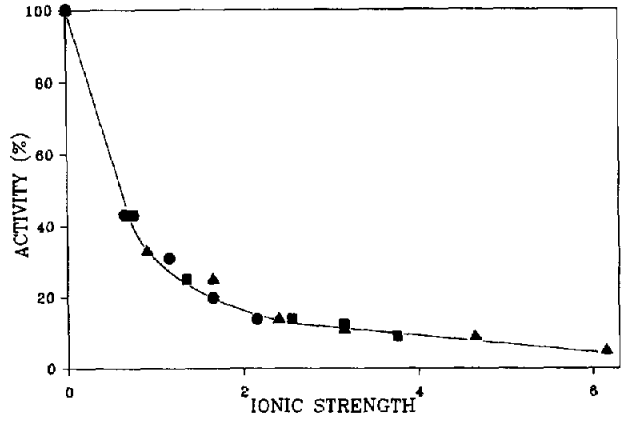

Fig. 2. Effect of the ionic strength value on the relative rate of the enzymatic uridine phosphorolysis. UPase was incubated with ammonium (▲) or sodium (■) sulfate or sodium chloride (●). Activity assays were performed at the same salt concentrations.

same as for the fluorescein in 80% acetonitrile (Fig. 4). After denaturation of the double-modified enzyme with 0.1% SDS (95°C, 2 min) both the red shift and absorption decrease were eliminated.

We have been able to demonstrate not only that the fluorescein environment at the putative uridine-binding subsite is apparently hydrophobic, but also that after the phosphate subsite was modified with SA-423 the active site hydrophobicity increases dramatically. It may reflect a less hydrated state of phosphate in the UPase active site in the enzyme–substrate complex. Finally, it is likely that the simultaneous filling of both uridine and phosphate subsites leads to structural rearrangements at the active site and phosphate desolvation results in its enhanced nucleophilicity.

These data are in agreement with results of kinetic investigation with molybdate dianion as a substrate for purine phosphorylase, glyceraldehyde-3-phosphate dehydrogenase and ATP-sulfurylase [12–14]. It was suggested that desolvation contributes to enzyme-enhanced nucleophilicity of phosphate and plays a role in the reactions of other phosphate-transfer enzymes [12,14]. On the other hand, it was shown that ATPases and adenylate kinase probably have mobile substrate-binding pockets which, undergo dramatic ligand-dependent conformational changes [10,15–17].

Altogether, the results of this study support the view that the enzymatic uridine phosphorolysis takes place according to S_N2 mechanism with phosphate activation due to its desolvation in the enzyme active site. The reaction may comprise the following steps: (i) uridine binds into hydrophobic pocket; (ii) a conformational change occurs on enzyme–substrate complex formation; (iii) active site hydrophobicity increases and phosphate anion nucleophilicity enhances through desolvation; (iv) uridine is attacked by the desolvated phosphate and the S_N2 substitution reaction takes place.

The mechanism of uridine phosphorolysis is obviously very complicated because of the complex nature of enzyme–substrate interaction and further studies are required for its elucidation. One cannot exclude the possibility that all the effects above are associated with conformational changes. Hopefully,

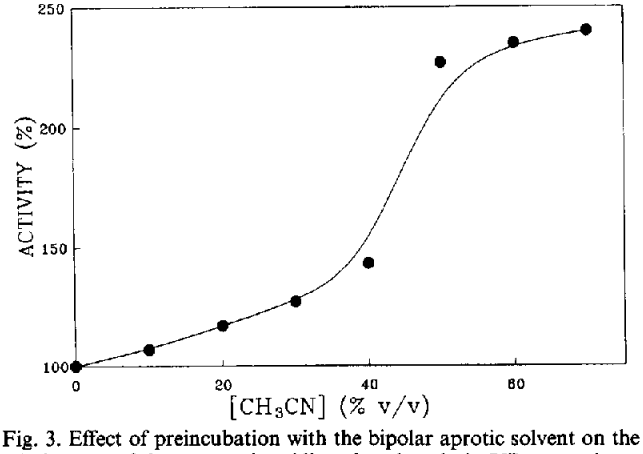

Fig. 3. Effect of preincubation with the bipolar aprotic solvent on the relative rate of the enzymatic uridine phosphorolysis. UPase was incubated overnight at indicated acetonitrile concentrations (v/v) and activity assays were performed under standard conditions.

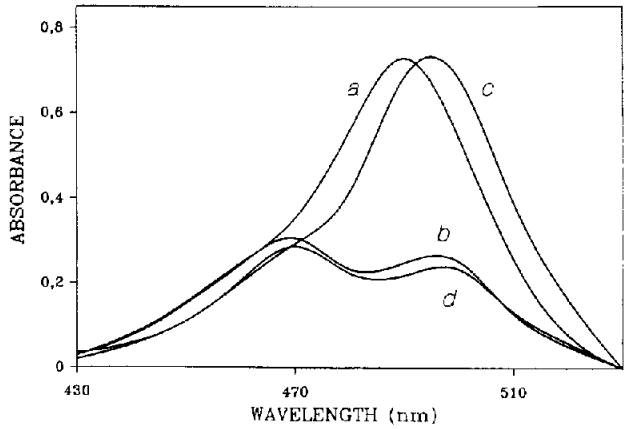

Fig. 4. Absorption spectra (50 mM Tris-HCl, pH 7.6) of fluorescein (a); fluorescein in the presence of 80% acetonitrile (v/v) (b); FTC-UPase (c) [4]; FTC-UPase modified with SA-423 (d).

the use of site-specific variants and X-ray analysis of native UPase and its various derivatives will yield substantial additional information about its molecular mechanism.

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