# BAKERS' YEAST URIDINE NUCLEOSIDASE IS A REGULATORY COPPER CONTAINING PROTEIN

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SUMMARY: The enzymatic properties of homogeneous bakers' yeast uridine nucleosidase, prepared as previously described (G. Magni et al., J. Biol. Chem. (1975), 250, 9-13) have been further investigated, and in addition to glucose-6-phosphate and ribose the enzyme activity was inhibited by ribose-5-phosphate and ribulose-5-phosphate. The curves describing this inhibitions were sigmoidal and when the data were plotted according to Hill, n' values different from 1 were observed suggesting the existence of interactions among the inhibitory molecules binding sites. Furthermore the percentage of inhibition exerted by glucose-6-phosphate, ribose and ribose-5-phosphate on the enzyme activity varied at different pH values.The addition of various chelating agents to the activity assay mixture caused a strong inhibition of the enzyme activity and metal analysis by atomic absorption spectrophotometry, colorimetric methods and electronic paramagnetic resonance, indicated the presence of 1 copper atom per enzyme molecule. Finally the inhibition exerted by metal ions on the enzyme activity was described.

In our laboratory bakers' yeast uridine nucleosidase (EC 4.2.2.3) has been purified to homogeneity and its physical and enzymatic properties have been studied (1). Since the regulation of the "UMP Cycle", as we called the overall process which leads to a continuous recycling of UMP in yeast (1), is our major interest in the study of pyrimidine metabolism, we continued to examine the enzymatic properties of uridine nucleosidase. As we reported in our previous paper (1) uridine nucleosidase is inhibited, in addition to ribosylthymine, by other metabolites which do not seem directly implicated in the pyrimidine nucleosides metabolism. In the present study, however, we found that bakers' yeast uridine nucleosidase activity is inhibited by pentose phosphate pathway metabolites and of some metabolic significance appears to be the inhibition exerted by ribose-5-phosphate. In addition it is shown that the enzyme is sensitive to the action of various chelating agents and that uridine nucleosidase is a metalloprotein containing 1 copper atom per enzyme molecule.

## EXPERIMENTAL PROCEDURE

The enzyme was assayed by the spectrophotometric method with uridine as the substrate at 37° (1). A unit of enzymatic activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 nanomole of uridine per minute. The protein concentration was determined according to Schacterle and Pollack (2). The enzyme was purified as previously described (1), and all experiments have been performed using homogeneous enzyme preparations. Spectrophotometric measurements were performed using a Varian Model Cary 118 C spectrophotometer. Metal analysis have been performed with a Perkin Elmer 303 Atomic Absorption Spectrophotometer. Copper content and its valence state have been determined by colorimetric methods (3,4,5) and electron paramagnetic resonance measurements.

## RESULTS

Inhibition by ribulose-5-phosphate and ribose-5-phosphate. In addition to ribose, ribosylthymine and glucose-6-phosphate, bakers' yeast uridine nucleosidase is inhibited by ribulose-5-phosphate and ribose-6-phosphate: the end products of pentose phosphate pathway. When uridine nucleosidase is assayed in the presence of increasing concentrations of the inhibitor,highly sigmoidal inhibition curves are obtained. Fig. 1 shows the shape of the inhibition curves, and when the data were plotted according to Hill (see insets of Fig. 1 a and b) n' values markedly substrate concentration dependent were obtained, as summarized in Table I. Furthermore, for the inhibition exerted by ribose-5-phosphate at inhibitor concentrations higher than 0.025 mM, n' values considerably lower than 1 were observed, suggesting a negative cooperative effect between inhibitor molecules, as first described by Conway and Koshland (6) for the binding of NAD to 3'-phosphoglyceraldehyde dehydrogenase from rabbit muscle.

Effect of pH on the Enzyme Inhibition. Fig. 2 shows the effect of hydrogen ion concentration on the enzyme activity and on the inhibition exerted by ribose, glucose-6-phosphate and ribose-5-phosphate. The percent inhibition is referred to initial velocity observed in the absence of inhibitors at each pH value tested. It can be seen that the inhibition is markedly pH dependent in the case of glucose-6-phosphate and ribose-5-phosphate, whereas

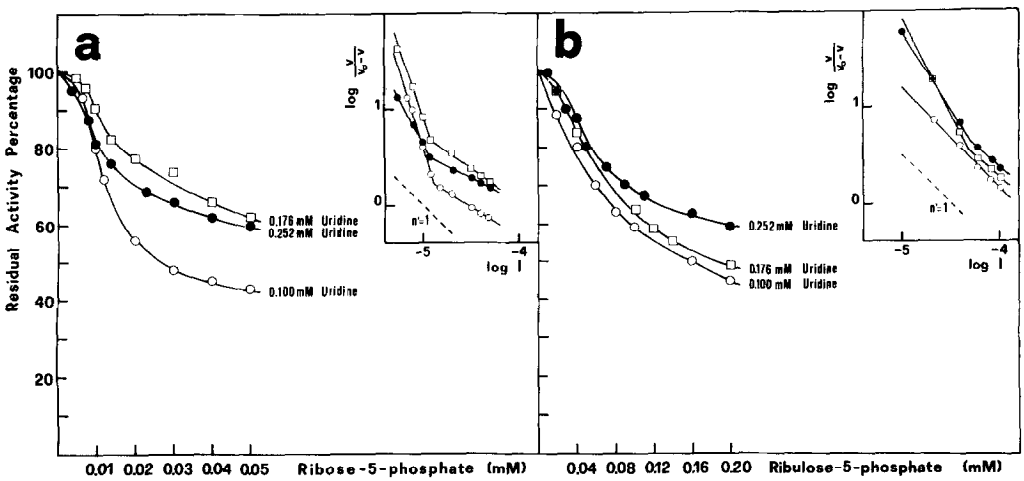


Fig. 1. Inhibition exerted on uridine nucleosidase activity by (a) ribose-5-phosphate and (b) ribulose-5-phosphate. Insets: inhibition values plotted according to Hill. Activity has been assayed as indicated in the text.

TABLE I. EFFECT OF RIBULOSE-5-PHOSPHATE AND RIBOSE-5-PHOSPHATE ON URIDINE NUCLEOSIDASE FROM BAKERS' YEAST[a].

| Inhibitor | Uridine Concentration (mM) | Concentration (mM) required for 50% inhibition | Hill coefficients (n') |
|---|---|---|---|
| Ribulose-5-phosphate | 0.100 | 0.145 | 1.1 |
| | 0.176 | 0.185 | 1.9 |
| | 0.252 | 0.268 | 1.6 |
| Ribose-5-phosphate | 0.100 | 0.027 | 3.8 |
| | 0.176 | 0.090 | 3.5 |
| | 0.252 | 0.120 | 2.1 |

[a]The enzyme activity was assayed as described under Experimental Procedure.

it remains almost constant in the case of ribose.

Effect of Metal Ions and Chelating Agents. The enzyme did not require the addition of any metal ion for full activity, and various metals were inhibitory when tested under the standard assay conditions. Among a variety of metal ions tested, $MnCl_2$, $NiCl_2$, $HgCl_2$, $CoCl_2$, $CdCl_2$, $ZnCl_2$ and $CuCl_2$ re-

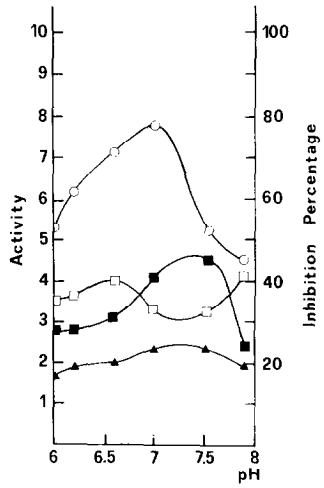


Fig. 2. Effect of pH on the enzyme inhibition. Uridine nucleosidase has been assayed in the absence O—O—O and in the presence of 0.04 mM ribose-5-phosphate □—□ 0.10 mM glucose-6-phosphate ■—■—■ and 3.00 mM ribose ▲—▲—▲
Activity is expressed as nanomoles of uridine hydrolyzed per minute.

TABLE II. INHIBITOR BINDING CONSTANTS FOR INHIBITION OF BAKERS' YEAST URIDINE NUCLEOSIDASE

| Compound | Ki | Type of inhibition |
|---|---|---|
| | M | |
| $ZnCl_2$ | $6.5 \times 10^{-6}$ | noncompetitive |
| $CdCl_2$ | $2.1 \times 10^{-5}$ | noncompetitive |
| $CoCl_2$ | $2.8 \times 10^{-4}$ | noncompetitive |
| $CuCl_2$ | $2.5 \times 10^{-4}$ | noncompetitive |
| $MnCl_2$ | $2.8 \times 10^{-4}$ | noncompetitive |
| $NiCl_2$ | $8.4 \times 10^{-4}$ | noncompetitive |
| o-phenanthroline | $3.4 \times 10^{-4}$ | competitive |
| Ethylenediaminetetracetate | $2.2 \times 10^{-4}$ | competitive |
| NaCN | $1.5 \times 10^{-2}$ | competitive |

Ki values were calculated from plots of 1/v versus 1/s. Each compound was included in the assay mixtures which were incubated for 5 minutes before uridine was added.

sulted inhibitory. Although the enzyme did not seem to require the addition of any metal for activity it is inhibited by various chelating agents as re

ported in Table II. In addition it has been found that $HgCl_2$ and 2-2' bypiridine are mixed inhibitors, being the concentrations required for 50% inhibition $0.65 \times 10^{-6}$ M and $2.4 \times 10^{-4}$ M respectively. 8-Hydroquinoline and neocuproine tested up to 2 mM concentration, and sodium azide tested up to 50 mM, did not affect the enzyme activity.

Metal Analysis. The inhibition exerted on the enzyme by metal-chelating agents strongly suggested that bakers' yeast uridine nucleosidase could be a metallo-enzyme. Metal analysis performed on different enzyme preparations by atomic absorption spectrophotometry revealed that the only metal present in significant quantity was copper, ranging its stoichiometry between 0.65 to 1.08 g. atom of copper per enzyme mol.. Whereas cobalt, zinc, manganese and iron were undetectable or present as traces. Copper content was also determined by the colorimetric cuprizone method (3) modified according to Nara et al. (4), and a stoichiometry approaching to 1.5 g. atom per enzyme mol. was observed. Furthermore in order to determine the valence state of copper, the method of Griffiths and Wharton (5) was used emploing biquinoline as copper reagent, and the results showed that all copper is in the cupric state. These results have been confirmed by electron paramagnetic resonance measurements which indicated the presence, in the enzyme, of an amount of paramagnetic copper corresponding to a value near 1 g. atom of metal per enzyme mol.

## DISCUSSION

Enzyme activities hydrolizing uridine have been identified in several organisms (7,8,9,10,11) but their molecular and enzymatic studies have received scant attention. In our laboratory we obtained from yeast homogeneous preparations of the enzyme (1) and studied its enzymatic and physical properties, because of the role which the enzyme might play in the "salvage" of pyrimidine nucleosides. The present study discloses for the first time that bakers' yeast uridine nucleosidase is allosterically inhibited by pentose phosphate pathway end products, and in particular by ribose-5-phosphate which is the common nucleotides precursor. The kinetics analysis of ribose-5-phosphate and ribulose-5-phosphate inhibitions indicated the existence of cooperativity between inhibitor molecules. As summarized in Table I n' values

vary with substrate concentration, ranging from 2.1 to 3.8 up to 0.023 mM ribose-5-phosphate and from 1 to 1.8 up to 0.100 mM ribulose-5-phosphate, whereas, at ribose-5-phosphate concentrations higher than 0.025 mM (Fig. 1 b) n' values lower than 1 are observed. Variation of enzyme inhibition as a function of pH has been observed with several regulated enzymes (6,7) and has been interpreted in terms of conformational alteration of the enzyme molecule, leading to a variation of accessibility of the binding sites to inhibitor molecules. This seems to be the case for uridine nucleosidase as shown by the data reported in Fig. 2.

The inhibition exerted on the enzyme activity by various metal-chelating agents induced us to analyze the protein for metal content and the data reported in this paper indicates that copper, as cupric ion, is the only metal present in significant amount.

Vallee (8) has critically discussed the experiments necessary to demonstrate that a specific protein is a metallo-protein and we think that the constant copper content of different enzyme preparations, the stoichiometry near to an integral number, the above mentioned inhibition by metal-chelating agents represent sufficient evidences, even though it would be desiderable to obtain an apoenzyme which is specifically reactivated by copper. All efforts to achieve this goal have been so far unsucessful.

The first question, in our opinion, which ought to be answered is whether bakers' yeast uridine nucleosidase is a new cuproprotein or a multifunctional enzyme (i.e. the protein is a known copper enzyme endowed with nucleosidase activity). For this purpose enzymatic optical and paramagnetic studies are in progress.

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