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Effects of Substrate Binding Determinants in the Transition State for Orotidine 5′-Monophosphate Decarboxylase

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To evaluate the effects of individual binding determinants on transition state stabilization, the binding properties of substrates and competitive inhibitors of the OMP decarboxylase activity of human UMP synthase were compared with those of fragments obtained by cutting these ligands at various positions. The ribofuranosyl group generates little binding affinity (as indicated by comparison of the k_{cat}/K_m values of orotidine with that of orotic acid, and of the K_i value of 6-hydroxyuridine with that of 6-hydroxyuracil), but seems to constrain the relative mobilities of the pyrimidine ring and the phosphoryl group in such a way as to optimize their contributions to transition state stabilization. The phosphoryl group of OMP appears to contribute approximately 10 kcal/mol of binding free energy to transition state stabilization, as indicated by comparison of the k_{cat}/K_m value of OMP with that of orotidine, and of the K_i value of the transition state analogue inhibitor 6-hydroxy-UMP with that of the corresponding ribonucleoside. This substituent effect, one of the largest that has been recorded for an enzyme reaction, is of special interest in view of the phosphoryl group's considerable distance from the site of substrate transformation.

INTRODUCTION

In the final step in pyrimidine biosynthesis, orotidine 5′-phosphate (OMP) is decarboxylated to form uridine 5′-phosphate (UMP) (Fig. 1). In mammals, orotidine 5′-monophosphate decarboxylase (ODCase, EC 4.1.1.23) is joined to orotate phosphoribosyltransferase (EC 2.4.2.10) in the bifunctional complex UMP synthase (*1*), but ODCase exists as a distinct protein in lower organisms (*2*). In the case of the yeast enzyme, recent evidence suggests the presence of one or more tightly bound zinc atoms, which may be involved in promoting substrate decarboxylation (*3*). Comparison of k_{cat} for the enzymatic decarboxylation of OMP with k_{non} for the spontaneous reaction in neutral solution, in the absence of catalysts, shows that ODCase is an exceptionally proficient enzyme, enhancing the rate of reaction by more than 17 orders of magnitude (*4*).

Several lines of evidence suggest that enzymatic decarboxylation of OMP proceeds by a mechanism that does not involve formation of covalent bonds between the enzyme and the substrate (*5*, *6*), implying that the generation of this remarkable affinity depends on the action of noncovalent binding forces alone. In the present work, we examine the contributions made by substrate binding determinants to the

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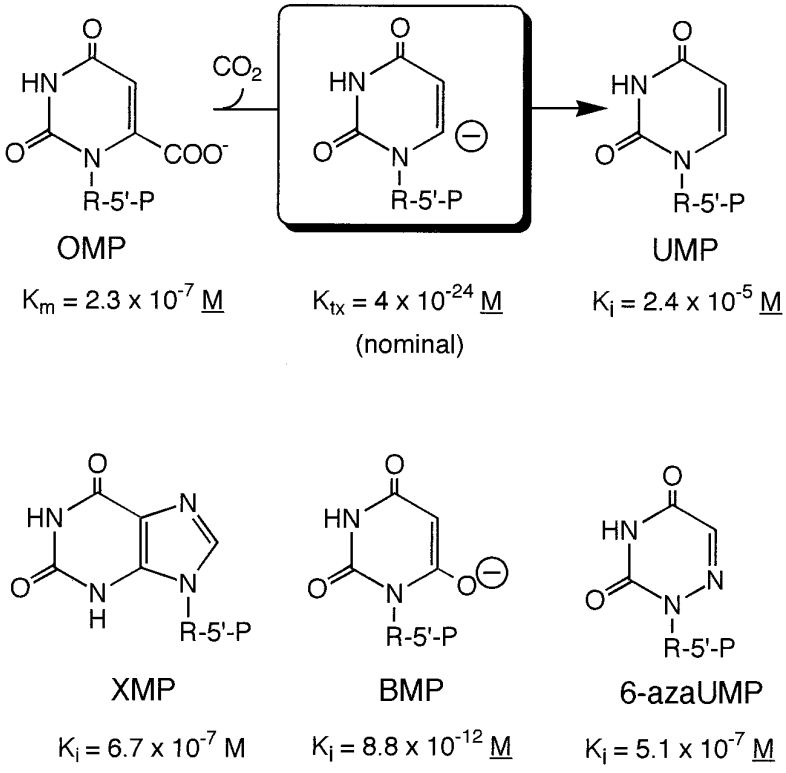

FIG. 1. The reaction catalyzed by OMP decarboxylase, and the structures and apparent binding affinities of several competitive inhibitors.

free energy of binding, using compounds that contain various structural features of substrates, products, and the stable transition state analogue inhibitor 6-hydroxyuridine 5′-phosphate (1-(5′-phospho-β-D-ribofuranosyl)barbituric acid, or BMP) (*7*). The results identify some structural features of the substrate that are recognized by ODCase during the initial binding event, and several additional features that appear to be recognized in the transition state. Of particular interest are the contributions of the ribofuranosyl group and the 5′-phosphoryl substituent of OMP, which contribute to transition state stabilization in very different ways.

EXPERIMENTAL

Materials

Orotidine 5′-monophosphate (OMP), orotidine, ribose 5′-phosphate, uridine 5′-phosphate (UMP), uridine, 6-hydroxyuridine 5′-phosphate (BMP), 6-hydroxyuridine, barbituric acid, xanthosine 5′-phosphate (XMP), and 6-azauridine were pur-

chased from Sigma Chemical Co. Human UMP synthase, purified and stored as described by Livingstone and Jones (*8*), was used as a source of decarboxylase activity. Enzyme concentrations were calculated from absorbance readings at 280 nm using a molar extinction coefficient of 23,380 M^{-1} cm^{-1}, estimated from the amino acid composition (*9*).

Methods

Enzymatic decarboxylation of OMP to UMP was followed by monitoring the decrease in absorbance at 285 nm, where $\Delta\varepsilon = -1650$ M^{-1} cm^{-1} (*8*). ODCase activity was usually observed at 37°C in Mops buffer (1.5×10^{-2} M, pH 7.2), containing 1,4-dithiothreitol (10^{-3} M), EDTA (10^{-4} M), in the presence of substrate OMP (10^{-4} M). Rates of enzymatic decarboxylation of orotate and orotidine were determined by monitoring the decrease in absorbance at 300 nm ($\Delta\varepsilon = -2725$ M^{-1} cm^{-1}) and 296 nm ($\Delta\varepsilon = -856$ M^{-1} cm^{-1}), respectively. Assays were carried out at room temperature in Mops buffer (1.5×10^{-2} M, pH 7.2), containing 1,4-dithiothreitol (10^{-3} M). These reactions approached completion after 26 h for orotic acid and 60 h for orotidine, in the presence of very high concentrations of UMP synthase (3.1×10^{-6} M in subunits), yielding uracil and uridine, respectively.

K_i values were determined by comparing the rate of decarboxylation in the presence and absence of various concentrations of inhibitor, using double reciprocal plots. The K_m value of OMP (2.3×10^{-7} M) is much lower than the substrate concentrations ($\sim 10^{-4}$ M) that are normally used in the spectrophotometric assay. Under those conditions, with OMP at a concentration well in excess of K_m, a few of the present inhibitors inhibited ODCase only slightly. To increase the sensitivity of detection in those cases, K_i values were determined using a radioactive assay, monitoring the release of $^{14}CO_2$ from the carboxyl substituent of 7-^{14}C-labeled OMP (*10*). Reaction mixtures were incubated at 37°C for 20 min in Tris-HCl buffer (1.5×10^{-2} M, pH 7.2), containing dithiothreitol (10^{-3} M), and OMP (1.2×10^{-6} M). In every case, inhibition appeared to be competitive.

RESULTS

Table 1 shows kinetic parameters for the action of the OMP decarboxylase activity of human UMP synthase on orotic acid, orotidine, and orotidine 5′-phosphate. In each case, the apparent dissociation constant of the substrate in the transition state, K_{tx} (final column), was calculated by dividing the rate constant for spontaneous decarboxylation of 1-methylorotic acid ($k_{non} = 2.8 \times 10^{-16}$ s^{-1}) (*4*) by k_{cat}/K_m observed for the substrate in question, in the present experiments. Values of k_{non} could not be determined directly for the nucleoside and nucleotide, because their N-glycosidic bonds are not sufficiently stable to withstand the conditions that are needed to observe spontaneous decarboxylation (140–200°C, pH 7) (*4*). However, recent observations indicate that orotic acid and 1-methylorotic acid are similar in their rates of decarboxylation (B. G. Miller and R. Wolfenden, unpublished), so that this assumption seems unlikely to introduce substantial error. Table 2 shows K_i