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The effective molarity of the substrate phosphoryl group in the transition state for yeast OMP decarboxylase

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Abstract

The second order rate constant (k_{cat}/K_m) for decarboxylation of orotidine by yeast OMP decarboxylase (ODCase), measured by trapping $^{14}CO_2$ released during the reaction, is $2 \times 10^{-4}\ M^{-1}\ s^{-1}$. This very low activity may be compared with a value of $3 \times 10^{7}\ M^{-1}\ s^{-1}$ for the action of yeast OMP decarboxylase on the normal substrate OMP. Both activities are strongly inhibited by 6-hydroxy UMP (BMP), and abrogated by mutation of Asp-96 to alanine. These results, in conjunction with the binding affinity of inorganic phosphate as a competitive inhibitor ($K_i = 7 \times 10^{-4}$ M), imply an effective concentration of 1.1×10^{9} M for the substrate phosphoryl group in stabilizing the transition state for enzymatic decarboxylation of OMP. The observed difference in rate (1.5×10^{11}-fold) is the largest effect of a simple substituent that appears to have been reported for an enzyme reaction.

Keywords: OMP; OMP decarboxylase; ODCase; Orotidine; Connectivity effect; Effective molarity; Transition state; Binding discrimination

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1. Introduction

Comparison of an enzyme's activity on its usual substrate, with its activity on a truncated version of the substrate that is presumably capable of entering the active site without steric hindrance, has shown that the binding of groups remote from the site of substrate transformation sometimes plays a prominent role in enzyme action [1]. Hexokinase, for example, is virtually inactive in catalyzing the hydrolysis of ATP, despite the ability of water to enter any cavity that can accommodate glucose, the normal phosphoryl acceptor [2]. A rate loss upon truncation of the substrate can be explained by utilization of binding forces to overcome the loss of translational, rotational, and internal entropy [3]. Thus the free energy of binding of a substrate A–B by an enzyme, compared with the sum of free energies of binding of its component parts A and B, would be expected differ by the rotational, translational, and internal entropies of the molecules. Comparisons of this kind are of theoretical and practical interest, in view of the potential magnitude of connectivity effects in determining the binding affinities of substrates in the ground state and transition state, and of protein–ligand interactions in general.

With a half-time of 78 million years in neutral solution at 25 °C, decarboxylation of OMP (Fig. 1) is one of the more difficult reactions in biology. Yeast OMP decarboxylase (ODCase) enhances the rate of this reaction by a factor of 10^{17} [4]. The crystal structures of OMP decarboxylase from yeast and several species of bacteria reveal numerous interactions between the active site and the ribose and phosphoryl groups of nucleotide inhibitors, but few enzyme interactions with the pyrimidine ring where decarboxylation occurs [5–8]. The complex network of interactions between the active site and the phosphoryl group accords with the finding that the binding affinities of inhibitory nucleosides fall 3–6 orders of magnitude short of the binding affinities of the corresponding nucleotides [9].

In earlier work on yeast ODCase no decarboxylation of orotidine or orotic acid could be detected using a UV assay [9]. In the present work, we used a more sensitive radioassay to assess the contribution that the substrate's 5′-phosphoryl group makes to k_{cat}/K_m for yeast ODCase, and to this enzyme's corresponding affinity for the altered substrate in the transition state. Here we report the reduction in rate that results from removal of the 5′-phosphoryl group of OMP, and the effective molarity of this group at the active site in the transition state for decarboxylation (see Table 1).

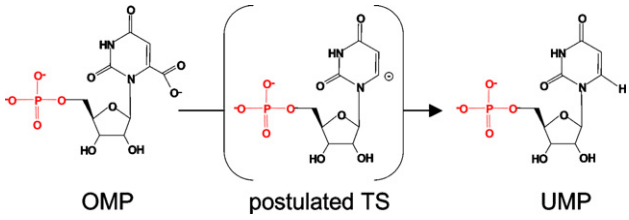

Fig. 1. OMP decarboxylase catalyzes the removal of the 6-carboxylate group from the pyrimidine ring of orotidine 5′-monophosphate. The effect of the phosphoryl group (red) was studied in this work.

Table 1
Comparison of orotidine and OMP decarboxylation by yeast OMP decarboxylase

	k_{cat} (s^{-1})	K_m (M^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
OMP	22	7×10^{-7}	3×10^{7}
Orotidine	$\geqslant 7 \times 10^{-6}$	$\geqslant 3 \times 10^{-2}$	2×10^{-4}

2. Materials and methods

2.1. Synthesis of [^{14}C]orotidine from 5-Br uridine

Adopting a procedure similar to that used earlier for the synthesis of OMP [10], 5-bromouridine (20 mg) and $Na^{14}CN$ (0.9 mg) were dissolved in DMSO (3 mL) and allowed to react at room temperature for 48 h. After removal of DMSO by rotary evaporation, KOH (1 mL, 3.4 M) was added to the crude 6-CN uridine and the mixture was heated to 60 °C for 4 h. After neutralization, the sample was purified over a Dowex 2-Cl^- anion exchange column and eluted with HCl (0.1 M). The product obtained in an overall yield of 56% was identical with authentic orotidine in its UV absorption ($\lambda_{max} = 267$) [11] and H NMR spectrum (δ 5.55 ppm (1′ H), δ 5.75 ppm (5 H), and δ 4.32 (3′ H)) [12].

2.2. Source of enzyme

All experiments were performed with the C155S mutant of OMP decarboxylase. Mutation of residue Cys-155 to serine results in a more stable protein than WT OD-Case, but does not affect ligand binding, substrate turnover, or the overall structure of the enzyme (unpublished data). Both C155S and D96A/C155S yeast OMP decarboxylase were expressed in *Escherichia coli* and purified by anion exchange chromatography over a Poros IIQ (Perspective Biosystems) and HR 10/10 MonoQ (Amersham Pharmacia Biotech) column as previously described [13].

2.3. Assay of orotidine decarboxylation

The decarboxylation of orotidine was measured by trapping the product $^{14}CO_2$ on KOH-soaked filter paper in a scintillation vial sealed by a rubber septum [14]. A typical reaction mixture (0.5 mL), containing OMP decarboxylase (1–5 mg/mL), [^{14}C]orotidine (1–9 mM), and Mops (100 mM, pH 7.2), was incubated at 23 C for 4–48 h. 10% Glycerol was added to the reaction mixture to stabilize the enzyme during incubations lasting longer than 16 h. Reactions were terminated by injection of $HClO_4$ (0.2 mL 4 M) through the septum to denature the protein and drive the $^{14}CO_2$ out of solution. To reduce chemiluminescence, the filter paper was incubated overnight in ScintiVerse E scintillation fluid (Fisher Scientific) before scintillation counting. In some experiments, the OMP decarboxylase inhibitor barbituric acid nucleoside 5′-phosphate (BMP, $K_i = 8.8 \times 10^{-12}$ M) [16] was used as a control.

Catalytic rescue was attempted with $NaNO_3$, $NaNO_2$, Na_2HPO_3, and NaCl at a concentration of 100 mM.

2.4. Assay of OMP decarboxylation

In control experiments, the decrease in activity of the enzyme during long incubations at room temperature was analyzed spectrophotometrically by monitoring the decrease in absorbance at 279 nm ($\Delta\varepsilon = 2.4\ mM^{-1}\ cm^{-1}$) [15] due to conversion of OMP (Sigma) to UMP. The decarboxylation of ^{14}C-OMP was measured at a substrate concentration of 2.7×10^{-7} M, in Mops buffer (0.05 M, pH 7.2) containing NaCl (0.1 M) and 10% glycerol.

3. Results and discussion

A single phosphoryl group, 9 Å away from the site of catalysis, distinguishes orotidine from the normal substrate OMP. Since no enzymatic decarboxylation of orotidine could be detected by monitoring the UV absorbance change at 279 nm, a more sensitive assay was needed to determine whether orotidine is a slow substrate. Fig. 2 shows that the amount of radioactivity released from [^{14}C]orotidine increases linearly with time over 48 h in the presence of OMP decarboxylase at 21 °C. The prolonged period of incubation required for these observations, even in the presence of highly concentrated enzyme, rendered it desirable to test the stability of the enzyme. Enzyme activity was found to decrease approximately 25% over the course of a 48 h incubation (Fig. 2), and a modest correction for this loss of activity was made in estimating the enzyme's true activity on orotidine.

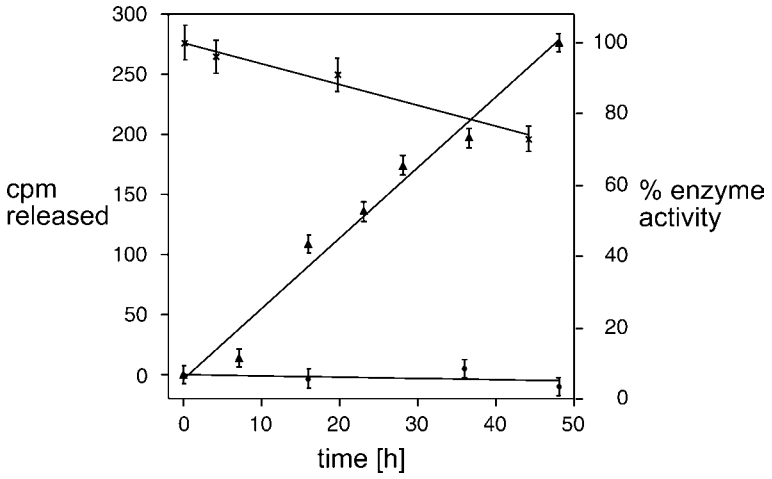

Fig. 2. The $^{14}CO_2$ released from [^{14}C]orotidine increases linearly with time in the presence of ODCase (▲). No $^{14}CO_2$ release is observed with the catalytically inactive ODCase mutant D96A (●). The enzymatic activity decreases by ~25% over 48 h (×).

The rate of [^{14}C]orotidine decarboxylation was found to increase linearly with increasing substrate concentration (Fig. 3). From the dependence of the rate on substrate concentration, a value of $k_{cat}/K_m = 2 \times 10^{-4}\ M^{-1}\ s^{-1}$ was calculated for the decarboxylation of orotidine (Fig. 3). Compared with the decarboxylation of the normal substrate OMP ($k_{cat}/K_m = 3 \times 10^{-7}\ M^{-1}\ s^{-1}$), the absence of the phosphoryl group decreases the second order rate constant for decarboxylation by a factor of 1.5×10^{11}. No significant rate enhancement was observed when nitrate, nitrite, phosphite or phosphate was added to the orotidine decarboxylation assay in an attempt to "rescue" enzyme activity on the truncated substrate. The enzyme's activity on orotidine was inhibited by the tight binding inhibitor BMP ($K_i = 8.8 \times 10^{-12}$ M) [16], indicating that the enzyme's low activity on orotidine is inherent in yeast OMP decarboxylase, and does not arise from contamination with an unknown enzyme. Mutation of Asp-96 to alanine results in a protein that binds OMP with an affinity comparable with the wild-type enzyme, but is devoid of catalytic activity [17]. When this inactive mutant enzyme was incubated with [^{14}C]orotidine, no detectable $^{14}CO_2$ was released over a period of 48 h (Fig. 2).

The very weak binding of orotidine, as indicated by the absence of any indication of substrate saturation at concentrations up to 9×10^{-3} M (Fig. 3), is confirmed by the absence of any inhibition of the decarboxylation of OMP by orotidine, with OMP present at a concentration (2×10^{-7} M) somewhat lower than its K_m value (7×10^{-7} M). These latter observations, conducted at very low concentrations of enzyme and OMP, were subject to substantial experimental error because of the instability of yeast OMP decarboxylase at very high dilutions. These inhibition experiments suggest a lower limit of $K_i = 3 \times 10^{-2}$ M for orotidine, consistent with the lower limit suggested by the experiments using orotidine as a substrate (Fig. 3).

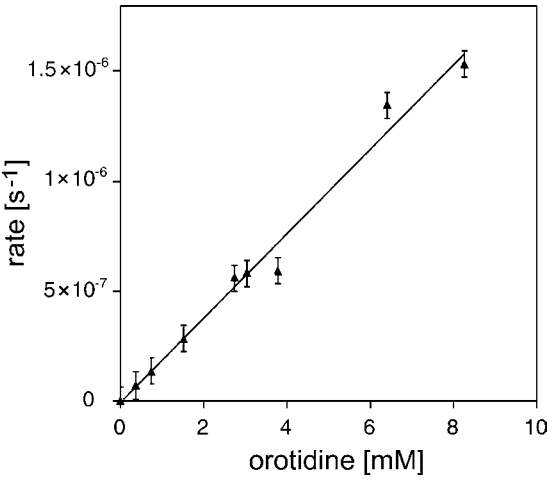

Fig. 3. The rate of $^{14}CO_2$ release over 16 h increases linearly with the concentration of orotidine. No saturation kinetics are observed up to a orotidine concentration of 9 mM. The rate of enzymatic activity is corrected for the decrease in enzymatic activity over the time of incubation.

The substantial error of this assay may account, at least in part, for the discrepancy between the present findings and a K_i value of 1.5×10^{-3} M reported earlier for orotidine [18]. Because of solubility limitations, it was not possible to extend these observations to concentrations of orotidine higher than 2×10^{-2} M. Even if such experiments had been feasible, it would have been difficult to interpret in view of the possibility that very high concentrations of this nucleoside might affect activity by changing the monomer–dimer equilibrium or substrate affinity [15].

In earlier work on triosephosphate isomerase, the presence of a single phosphoryl group on the substrate has been shown to contribute 8 orders of magnitude to the rate enhancement produced by triosephosphate isomerase, enough to account for almost all the rate enhancement produced by that enzyme [19]. In the present case, we find that a single substrate phosphoryl group, by its presence, contributes 11 orders of magnitude to the rate enhancement produced by ODCase. This contribution, although it is the largest that appears to have been recorded for a simple substituent in an enzyme reaction, does not fully account for the 10^{17}-fold rate enhancement produced by OMP decarboxylase. Comparing the K_m values of orotidine ($>10^{-2}$ M) and OMP (10^{-6} M), it appears that at least 4 orders of magnitude of the rate enhancement arise from binding interactions that do not discriminate between the substrate in the ground state and the transition state.

The maximal dissociation constant K_{TX} of an altered substrate in the transition state can be calculated by dividing the rate constant for the uncatalyzed reaction, k_{non}, by the second order rate constant k_{cat}/K_m. For OMP, the value of K_{TX} is 4.4×10^{-24} M [4], which may be compared with a value of 7×10^{-13} M for orotidine obtained in the present experiments. The dissociation constant of inorganic phosphate from yeast OMP decarboxylase is 7×10^{-3} M [9]. The sum of the negative free energies of binding of the truncated substrate orotidine and the missing phosphate falls 12.3 kcal short of the negative free energy of binding of the altered substrate OMP in the transition state. This connectivity effect can be expressed as an effective concentration of 1.1×10^{9} for the phosphoryl group in the transition state. Fig. 4 compares the connectivity effect in yeast OMP decarboxylase with connectivity effects that have been reported for other enzymatic reactions.

The structural means by which these very large connectivity effects are expressed in catalysis remains somewhat uncertain. The prospects of obtaining structural information concerning the ability of yeast ODCase to discriminate between OMP and orotidine appear to be clouded by this enzyme's weak affinity for orotidine. In the reported structures of ODCase complexes with nucleotides, however, the phosphoryl group exhibits multiple interactions with the enzyme (Gly-234, Arg-235, and Tyr-217). It seems reasonable to speculate that the pronounced difference in transition state binding affinity between these two similar substrates may be related to the ordering and closing of loop 207–217 upon nucleotide binding [6]. In the ordered form of this loop, there is a hydrogen bond between the hydroxyl group of Tyr-217 and the phosphoryl group, and mutation of that residue to alanine reduces the catalytic efficiency of yeast ODCase by 4.7 kcal/mol in free energy [9]. For such a movement to lower the free energy of activation, the benefit of increased contact would need to outweigh the cost of distorting the enzyme from its native, or open,

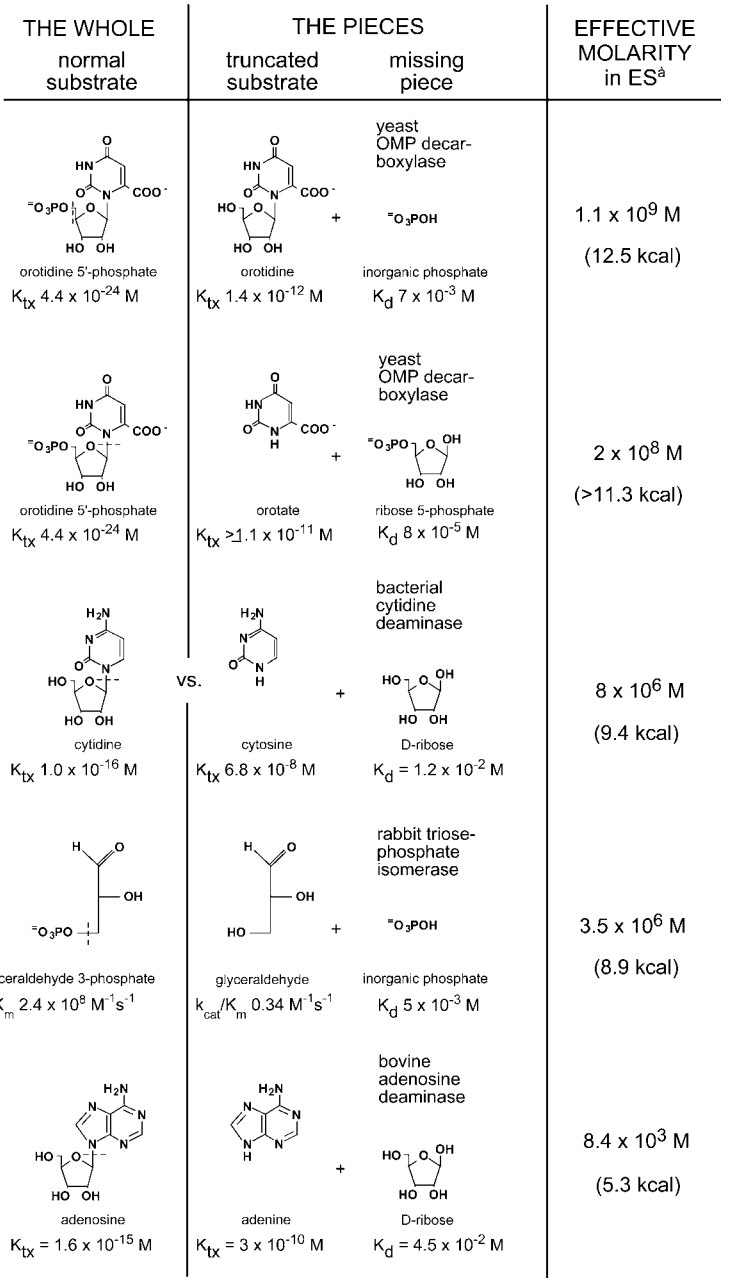

Fig. 4. Connectivity effect in enzyme reactions. Many of the values in this figure [9,20] are dissociation constants (K_{TX} or K_d), but the tabulated values for the two aldehydic substrates of triosephosphate isomerase [19] describe k_{cat}/K_m, which is inversely related to the value of the dissociation constant K_{TX}.

structure. The cost of distorting the enzyme from its native open structure, which must be paid as part of this process, would be expected to be minor if it involved a hinge-like movement of two parts that were otherwise well ordered [21]. The constraints imposed by formation of one ''weak'' bond can enhance the probability of forming a second, and such effects may in principle raise the apparent concentration of the reaction partner to high levels (3). In earlier work, effective concentrations of 10^5–10^8 M have been determined experimentally by making ''cuts'' in enzymes or substrates, then comparing the binding affinities of these ''pieces'' with those of the native enzyme or substrate in the transition state [22]. Combined with the large binding contributions that have been observed for individual groups [23], these levels of synergism appear to bring the transition state affinities of enzymes within reach of ordinary forces of attraction.

Acknowledgments

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References

[1] D.E. Koshland Jr., Proc. Natl. Acad. Sci. USA 44 (1958) 98–104.
[2] A. Kaji, S.P. Colowick, J. Biol. Chem. 240 (1965) 4454–4462.
[3] W.P. Jencks, Proc. Natl. Acad. Sci. USA 78 (1981) 4046–4050.
[4] A. Radzicka, R. Wolfenden, Science 267 (1995) 90–93.
[5] T.C. Appleby, C. Kinsland, T.P. Begley, S.E. Ealick, Proc. Natl. Acad. Sci. USA 97 (2000) 2005–2010.
[6] B.G. Miller, A.M. Hassell, R. Wolfenden, M.V. Milburn, S.A. Short, Proc. Natl. Acad. Sci. USA 97 (2000) 2011–2016.
[7] N. Wu, Y. Mo, J. Gao, E.F. Pai, Proc. Natl. Acad. Sci. USA 97 (2000) 2017–2022.
[8] P. Harris, J.-C. Navarro Poulsen, K.F. Jensen, S. Larsen, Biochemistry 39 (2000) 4217–4224.
[9] B.G. Miller, M.J. Snider, S.A. Short, R. Wolfenden, Biochemistry 39 (2000) 8113–8118.
[10] P.W. Landesman, Design, synthesis and evaluation of potential inhibitors of pyrimidine biosynthesis: a mechanistic approach, Ph.D. thesis, S.U.N.Y at Buffalo, 1982.
[11] A.M. Michelson, W. Drell, H.K. Mitchell, Proc. Natl. Am. Chem. Soc. USA 37 (1951) 396–399.
[12] F.E. Hruska, J. Am. Chem. Soc. 93 (1971) 1795–1797.
[13] B.G. Miller, J.A. Smiley, S.A. Short, R. Wolfenden, J. Biol. Chem. 274 (1999) 23841–23843.
[14] M.E. Jones, P.R. Kavipurapu, T.W. Traut, Methods Enzymol. 51 (1978) 155–157.
[15] D.J.T. Porter, S.A. Short, Biochemistry 39 (2000) 11788–11800.
[16] H.L. Levine, R.S. Brody, F.H. Westheimer, Biochemistry 19 (1980) 365–369.
[17] B.G. Miller, M.J. Snider, R. Wolfenden, S.A. Short, J. Biol. Chem. 276 (2001) 15174–15176.
[18] B.G. Miller, G.L. Butterfoss, S.A. Short, R. Wolfenden, Biochemistry 40 (2001) 6227–6232.
[19] T.L. Amyes, A.C. O'Donnoghue, J.P. Richard, J. Am. Chem. Soc. 123 (2001) 11325–11326.
[20] W.M. Kati, S.A. Acheson, R. Wolfenden, Biochemistry 31 (1992) 7356–7366.
[21] R. Wolfenden, Mol. Cell. Biochem. 3 (1973) 207–211.
[22] B.G. Miller, R. Wolfenden, Annu. Rev. Biochem. 71 (2002) 847–885.
[23] W.M. Kati, R. Wolfenden, Acc. Chem. Res. 23 (1991) 209–215.