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OMP Decarboxylase: An Experimental Test of Electrostatic Destabilization of the Enzyme−Substrate Complex

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The first-order rate constant (k_{cat}) for the reaction catalyzed by OMP decarboxylase (ODCase; E.C. 4.1.1.23) (Scheme 1) exceeds the rate constant for the uncatalyzed reaction (k_{non}) in dilute aqueous solution at 25 °C by a factor of $\sim 10^{17}$.[1] More powerful catalysts are known,[2] but no other biological catalyst produces a larger rate enhancement without the assistance of metals or other cofactors. In the case of ODCase, catalysis appears to be achieved entirely through noncovalent interactions between the substrate and amino acid residues of the active site. Of the various mechanisms that have been proposed,[3] a particularly interesting possibility involves "electrostatic stress", in which the enzyme−substrate complex is destabilized by repulsive interactions between the scissile carboxylate group of substrate OMP and an aspartate residue at the active site. It has been suggested that relief from that stress, in the transition state for decarboxylation, might supply a majority (18 out of 33 kcal/mol) of the favorable free energy of activation that is needed to account for the observed rate enhancement.[4]

In an early discussion of ground-state destabilization mechanisms,[5] Jencks suggested that "a compound that does not have to overcome an energetically unfavorable desolvation or electrostatic destabilization upon binding will be able to bind more tightly, so that this tight binding can be used as a tool to help identify the destabilization mechanism." If repulsion were present between two anionic groups in the enzyme−substrate complex, one would expect that their unfavorable free energy of interaction would be *reversed* if one of the groups were made to carry a positive charge. To implement this approach, we prepared 6-methylaminouridine 5′-phosphate ($MAUMP^+$),[6] which bears a positively charged amino group near the position that would normally be occupied by the negatively charged scissile carboxylate group of OMP.

To obtain $MAUMP^+$, we first treated 5-bromouridine 5′-phosphate with potassium cyanide in DMSO, which gives a reasonable yield of 6-cyanouridine 5′-phosphate.[7] This intermediate was reduced selectively, at 25 °C, in methanolic HCl under 60 atm of H_2 using a Pd catalyst. By UV and ninhydrin analysis, hydrogenation appeared complete in <2 h. The product mixture from this step was reduced to a gum under vacuum and separated by anion-exchange chromatography (DEAE Sephacel), and the appropriate fractions were desalted by charcoal adsorption. [8]

In Figure 1, MAUMP inhibition is plotted as a function of changing pH.[9] The strength of inhibition is relatively invariant at high pH, decreasing as the pH is reduced until the point where the enzyme begins losing activity. The pH at which inhibition is half-maximal is in reasonable agreement with the pK_a value (6.6) of the inhibitor's exocyclic NH_3^+ group, determined separately by NMR experiments involving the chemical shift of the $^{13}C_7$ atom in buffered solutions of varying pH. Thus, ODCase exhibits modest affinity for MAUMP at pH values where its amino group is uncharged (apparent $K_i = 3.3 \times 10^{-6}$ M) but shows no apparent affinity for $MAUMP^+$.

The apparent pH dependence of ligand binding equilibria suggests the identity of the basic group that is undergoing titration

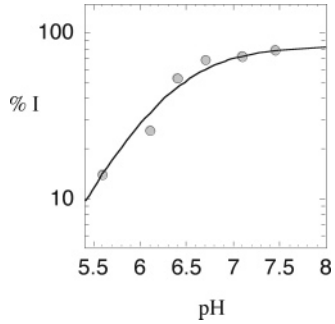


Figure 1. Inhibition of reaction velocity as a function of changing pH (with $E = 10^{-8}$ M, $S_o = 1.1 \times 10^{-6}$ M, $I = 1.3 \times 10^{-4}$ M). The solid line indicates the behavior expected for inhibition by the conjugate base of an acid with apparent p$K_a = 6.3$.

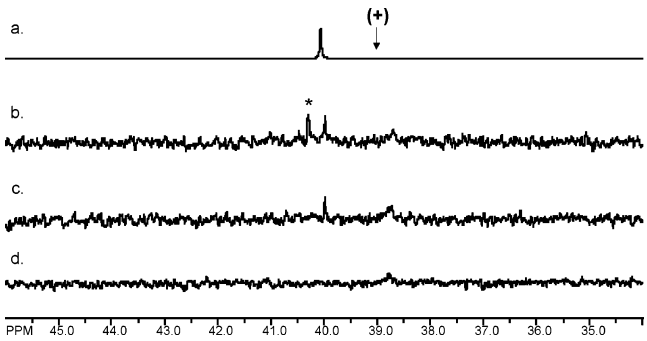
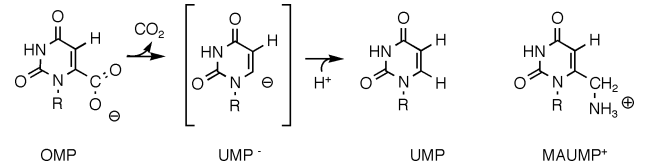


Figure 2. ^{13}C NMR spectra recorded at 25 °C of samples containing (a) [7-^{13}C]-MAUMP (1.2 mM) in potassium phosphate buffer (20 mM, pH 7.0) (b) components of spectrum a with 0.5 equiv of ODCase added, (c) components of spectrum b with 2 equiv of XMP added. Spectrum (d) is enzyme (0.6 mM) alone in phosphate buffer, pH 7.0. The (+) indicates the chemical shift of [7-^{13}C]-MAUMP free in solution with the amino group positively charged, and the (*) denotes the chemical shift of the inhibitor bound to ODCase.

Scheme 1



but does not rule out equivalent scenarios in which a proton may be taken up by a group on the protein. To evaluate the state of ionization of the amino group of enzyme-bound MAUMP, we used ^{13}C-NMR to examine the stable ODCase/MAUMP complex.[10] Figure 2 shows ^{13}C NMR spectra of [7-^{13}C]-MAUMP (1.2 mM) (a) free in potassium phosphate buffer (20 mM, pH 7.0), (b) with added ODCase (0.5 equiv), and (c) with added ODCase (0.5 equiv) plus the competitive inhibitor xanthosine 5′-monophosphate (XMP) (2 equiv, K_i 0.3 μM[11]). The bottom trace shows the ^{13}C NMR spectrum of ODCase alone in solution, pH 7.0. In spectrum b, the

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Table 1. Dissociation Constants (mol/L) of ODCase Ligands

ligand	10^6 K_d (25 °C, pH 7)
OMP[a]	0.7
UMP[a]	200[1]
MAUMP[b]	3.3
MAUMP$^{(+)}$[b]	≥15

[a] Reference 11. [b] This work.

resonance that arises at 40.3 ppm is attributed to enzyme-bound MAUMP, as judged by its absence from spectrum c in which XMP had been added at concentrations sufficient to displace MAUMP. Comparison of these spectra suggests that the active site binds MAUMP with its amino group uncharged.

These findings indicate that the affinity of ODCase for MAUMP, although easily measurable, is a little less than its affinity for substrate OMP (Table 1). Moreover, even that limited affinity appears to be lost when the amino group of MAUMP is converted to the ammonium form. That behavior is not easy to reconcile with the view that electrostatic repulsion, between the substrate's carboxylate group and the carboxylate groups of the active site (Asp-91 and Asp-96 in the yeast enzyme), plays an important role in destabilizing the ground-state E−S complex. Other evidence points to the same conclusion. Thus, the affinity of yeast ODCase for substrate OMP is 200-fold greater than its affinity for the product UMP, which lacks the substrate's scissile carboxylate group.[11] UMP appears to be bound in syn form,[12,13] which is relatively uncommon in free solution, amounting to roughly 1%.[14] It seems reasonable to infer that the actual affinities of UMP and OMP, in the syn forms in which they are bound, are similar and do not greatly favor UMP as would have been expected if the enzyme−OMP complex were destabilized, in a thermodynamic sense, by repulsion between negatively charged groups on the enzyme and substrate. Moreover, mutation of Asp-91 to alanine *weakens* the enzyme's affinity for OMP, rather than strengthening it as would have been expected if electrostatic repulsion between Asp-91 and the carboxylate group of OMP were to play an important role in destabilizing the native enzyme−substrate complex in its ground state.[11]

These observations appear to be consistent with the view that Lys-93 plays a critical role in catalysis by providing a favorable attractive electrostatic interaction with the C-6 carbanionic intermediate that is generated by elimination of CO_2, shown in Scheme 1. In several reported crystal structures, that lysine residue has been shown to form H-bonds to the enolate oxygen of 1-β-D-ribofuranosylbarbituric acid (BMP)[12,15,16] and to the lone pair of electrons of the ring nitrogen atom of 6-azauridine 5′-phosphate.[4] In the present experiments, the observed shielding of the C-7 resonance of enzyme-bound MAUMP, and the failure of ODCase to bind MAUMP$^+$, would seem understandable if the uncharged amino substituent of MAUMP were situated near this positively charged Lys-93.

If electrostatic repulsion is absent in the ground-state enzyme−substrate complex, how does ODCase avoid binding OMP with an affinity remotely approaching its affinity for the altered substrate in the transition state, with a nominal K_d value of $\sim 10^{-23}$ M?[1] It seems probable that this enzyme's unimpressive binding affinity for OMP, comparable in magnitude with that of the product UMP, may disguise repulsive or distortion effects that are relieved in the transition state, and are not revealed by the crystal structures of the enzyme with other ligands. The substrate phosphoryl group has recently been shown to contribute -12.3 kcal/mol to the free energy of stabilization of the transition state by yeast ODCase,[15] and other binding determinants on the enzyme and substrate have also been shown to make major contributions to substrate turnover.[3] It seems increasingly apparent that no single enzyme−substrate interaction will suffice to account for this enzyme's exceptional ability to discriminate between the forms of the substrate that are present in the transition state and in the ground state, and that multiple binding interactions must be working in concert.

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(8) MAUMP (λ_{max} 263, ϵ 12500, water) was characterized by ^{13}C decoupled ^{1}H NMR: (pyrazine standard = 8.7 ppm, δ): 3.94 (1H, d, J = 16 Hz, H7 [A]), 3.95 (2H, m, H4′/H5′), 4.25 (1H, d, J = 16 Hz, H7 [B], 4.5 (2H, m, H2′/H3′), 6.6 (1H, d, ^3J = 6.2 Hz, H1′), 6.5 (1H, d, ^3J = 4.3 Hz, H5), and by positive ion ES/MS, *m*/*z* 354.04 (calcd 354.07).
(9) Enzymatic decarboxylation was monitored on a Hewlett-Packard diode array spectrophotometer model 8452A, by the decreasing absorbance at 285 nM where $\Delta\epsilon_M$ is -1743 cm^{-1} (Lieberman, I.; Kornberg, A.; Simms, E. S. *J. Biol. Chem.* **1955**, *215*, 403). The K_i value of MAUMP was determined from the inhibited and uninhibited rates of decarboxylation using the standard expressions (Copeland, R. A. *Enzymes: A Practical Introduction to Structure Mechanism and Data Analysis*; John Wiley and Sons: New York, 2000; pp 310−313).
(10) Proton decoupled ^{13}C NMR spectra were recorded on a Varian spectrometer operating at 500 MHz and processed offline with SpinWorks (Marat, K. *2000 Spinworks*, v. 1.2; University of Manitoba: Winnipeg, Manitoba, Canada (ftp://davinci.chem.umanitoba.ca/pub/marat/SpinWorks**)**. A 5-mm broadband probe, 15° pulse width with a 0-s recycle time, and 4.1-s acquisition time were used throughout. Chemical shifts were internally referenced to added glycerol or methanol, which were assigned values of 62 and 49 ppm, respectively.
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