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A Raman-active competitive inhibitor of OMP decarboxylase

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Abstract

6-Cyanouridine 5′-phosphate was shown to act as a competitive inhibitor of yeast OMP decarboxylase, with a K_i value of 1.1×10^{-5} M. Upon binding by the active site of yeast OMP decarboxylase (EC 4.1.1.23), the Raman stretching frequency of the nitrile group of 6-cyanouridine 5′-phosphate decreases from 2240 to 2225 cm^{-1}. Based on the behavior of a model compound, 6-cyano-1,3-dimethyluracil, and on vibrational calculations, the observed change in stretching frequency is attributed to desolvation of the ligand, and distortion of the ligand in which the nitrile group moves out of the plane of the pyrimidine ring. Similar distortions may play a role in substrate activation by OMP decarboxylase, contributing to the catalytic process.

Keywords: OMP decarboxylase; Ground state destabilization; Raman spectroscopy; Catalysis; Decarboxylation; Nitrile group; ODCase

1. Introduction

In the enzymatic conversion of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP) (Scheme 1), the altered substrate in the transition state ($S^{\ddagger}$) is bound by yeast OMP decarboxylase (ODCase) with unusually high affinity. Dividing the observed

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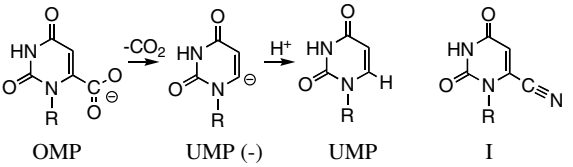

Scheme 1.

value of k_{cat}/K_m ($6.3 \times 10^7\,M^{-1}\,s^{-1}$) by the extrapolated rate constant for uncatalyzed decarboxylation of the model compound 1-methylorotate (k_{non}, $10^{-16}\,s^{-1}$), yields a nominal dissociation constant for E–S‡ of 10^{-24} M [1]. The ground state enzyme–substrate (E–S) and enzyme–product (E–P) complexes, on the other hand, dissociate with apparent K_d values of 0.7×10^{-6} and 2.0×10^{-4} M, respectively [2]. Unlike most enzymes that catalyze decarboxylation, ODCase does not employ a metal ion or other cofactor, and isotope effects argue against the intervention of a covalent intermediate [3]. The absence of those features has left the mechanism of action of this enzyme very much in question, although there seems to be general agreement that the major burden in activating the carboxyl group of OMP is borne by non-covalent interactions.

Indications of the binding forces at work, in the specific region of the active site of ODCase that would ordinarily be occupied by the scissile carboxylate group of OMP, have been obtained by studying the inhibitory activity of UMP derivatives with varying substituents at the 6 position (Table 1), including 6-hydroxyUMP (BMP) [4], 6-methylamino-UMP [5], and the 6-carboxamidoUMP analogs [6,7].

Here, we report that 6-cyanouridine 5′-phosphate (**I**), hitherto described only as an intermediate in the chemical synthesis of OMP, is a competitive inhibitor that can be utilized for Raman spectroscopy experiments to probe the environment of the enzyme's active site. Although the nitrile group is not a perfect isostere for a carboxyl group, competitive inhibition experiments indicate that ODCase's binding affinity for **I** (1.1×10^{-5} M) is comparable with that of OMP. The possibility of using the –CN vibrational frequency as a reporter of enzyme–ligand interactions was suggested recently [8] by model studies in which a correlation was established between the stretching frequency of acetonitrile's –CN group and the properties of the solvent in which it was dissolved [9].

As shown in Fig. 1, the presence of an equimolar concentration of ODCase decreases the –CN stretching frequency of **I** by $15\,cm^{-1}$ (from 2240 to $2225\,cm^{-1}$). In a separate experiment, the intensity of the band at $2225\,cm^{-1}$ arising from the bound ligand population was shown to be reduced to background levels by the addition of a saturating

Table 1
Observed binding affinities for 6-substituted UMP derivatives with yeast ODCase

6-Substituent	Observed binding affinity ($\times 10^{-6}$ M)
–C(=O)NH_2	620 [6]
–H	200 [2]
–$CH_2NH_3^+$	$\geqslant$15 [5]
–C≡N	11 [this work]
–CH_2NH_2	3 [5]
–CO_2^-	0.7 [2]
–C(=S)NH_2, C(=Se)NH_2	0.0015 [7]
–O^-	0.000009 (4 °C) [4]

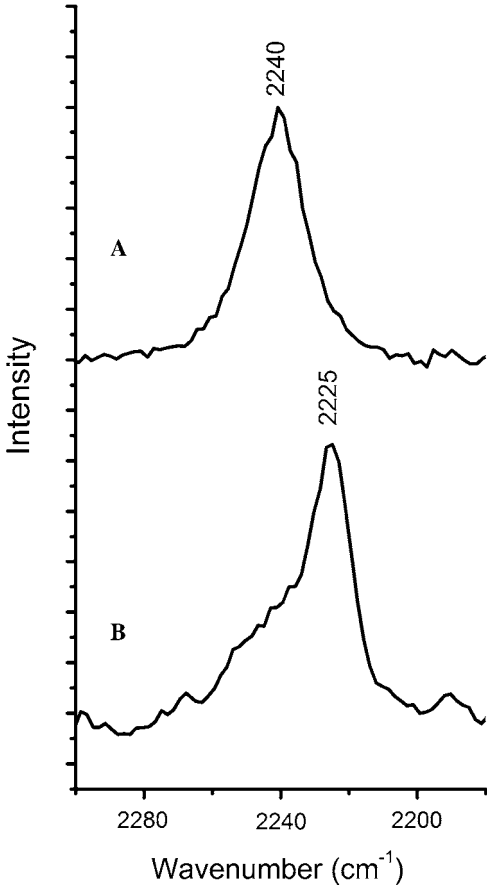

Fig. 1. Raman stretching frequency of the nitrile group of **I** in absence (A) and presence of ODCase (B).

concentration of the competitive inhibitor xanthosine 5′-monophosphate (XMP) (K_i 10^{-7} M [10]), again indicating that **I** is bound at the enzyme's active site [7].

This is not the first example in which protein binding has been shown to perturb the stretching frequency of a –CN group from its value in aqueous solution. In a previous study, the –CN frequency of a nitrile-derivatized tyrosine residue was shown to decrease by 8.8 cm^{-1} when the modified peptide ligand, that it was part of, was bound by calmodulin [6]. That shift was attributed to hydrophobic interactions between the tyrosyl –CN group and non-polar residues in the binding pocket, based on the observation that THF produced a similar decrease in the –CN frequency of a model compound compared with the value observed in aqueous solution.

Adopting a similar approach, we determined the effect of polar and non-polar solvents on the –CN stretching frequency of the model compound 6-cyano-1,3-dimethyluracil (**II**). Over a broad range of organic solvents, there was little variation in the stretching frequency of the –CN group (Table 2). Thus, comparable stretching frequencies were observed in formamide (2229 cm^{-1}) and isopropanol (2226 cm^{-1}), despite the >90 unit difference in dielectric constants between the two solvents. In contrast, the –CN stretching

Table 2
Observed –CN frequencies of 6-cyano-1,3-dimethyluracil (**II**) dissolved in the indicated solvents

Solvent	cm^{-1}
Water	2235
DMSO	2226
Formamide	2229
Isopropanol	2226
Methanol	2227
Acetic acid	2227

frequency of **II** in water (2235 cm^{-1}) differs from the value observed in other solvents by between 6 and 9 cm^{-1}. This apparent dichotomy suggests that the –CN stretching frequency of **I**, although limited in its ability to indicate solvent polarity, is sensitive to the presence of bulk water. The bound form of BMP, a powerful inhibitor of ODCase, has been shown by crystallographic analysis to be almost completely buried by interactions within the active site of yeast ODCase and stripped of solvating water molecules [11]. The present results are consistent with the view that binding removes **I** from contact with solvent water, but the observed difference in –CN stretching frequencies between **I** (aq) and E–**I** is twice that which could be accounted for by a desolvation effect (Fig. 2).

Inhibitor distortion may offer a plausible explanation for the additional shift in the –CN frequency of E–**I**. An earlier indication that native ODCase may distort its ligands was provided by a crystallographic analysis showing that the C6–C7 bond of OMP was twisted from planarity with the pyrimidine ring, in its complex with a catalytically inactive double mutant (D70A, K72A) of the enzyme from *Methanobacterium thermoautotrophicum* [12]. In view of the strong similarity in structure and reactivity between the enzymes from *M. thermoautotrophicum* and yeast [13], it seemed reasonable to suppose that distortion might also arise in the E–**I** complex. Shown in Table S1 are –CN stretching frequencies of benzonitrile, calculated for structures in which its –CN group is moved out of the plane of the benzene ring to an increasing extent. These values indicate that deviations from planarity would be expected to cause the stretching frequency to decrease. A 10° distortion was calculated to lead to a 2.4 cm^{-1} decrease, whereas a 20° distortion led to a 9.9 cm^{-1} decrease. These data suggest that a significant fraction of the "additional shift" in the –CN stretching frequency of **I** upon binding may be attributable to ~20° distortion of the –CN group from the plane of the pyrimidine ring. The strength of inhibition observed with 5,6-dihydroorotidine 5′-phosphate, (R + S, K_i 2.5×10^{-7} M [14]) a non-reactive form of the substrate in which the carboxylate group projects at an angle from the edge of the heterocyclic ring, further indicates that the active site is in fact optimized to bind such non-planar substrate analogs.

The action of ODCase is conspicuously dependent on interactions remote from the site of chemical transformation of the substrate. For example, comparison of the k_{cat}/K_m values

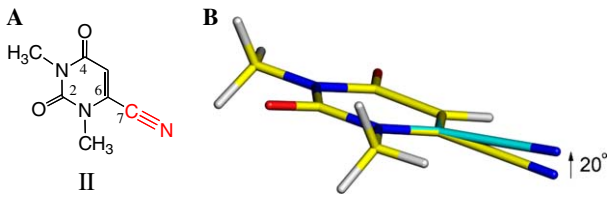

Fig. 2. 6-Cyano-1,3-dimethyluracil. (A) Chemical structure, (B) cartoon showing proposed distortion.

of OMP and 2′-deoxyOMP shows that the 2′-hydroxyl group contributes 4.6 kcal/mol to the rate enhancement produced by the yeast enzyme [15]; and the contribution of the 5′-phosphoryl group appears to be even greater ($\geqslant$12.5 kcal/mol) [16]. In addition to stabilizing the transition state, the rate-enhancing effects of these non-reacting groups can be interpreted as evidence favoring ground state destabilization (GSD), by reducing the burden that this enzyme faces in distinguishing $S^{\ddagger}$ from the substrate in the ground state [17]. Recently, it was suggested that GSD involving charge repulsion might play a significant role in the mechanism of ODCase, although computational [18] and experimental [5] tests have furnished little support for its involvement in catalysis.

The present results suggest another possibility: that GSD may occur—at least in part—by bending the C6–C7 bond of OMP out of the pyrimidine plane before it is cleaved. In the absence of solvent, this distortion would allow active site residue(s) to move closer to the vinylic C-6 atom at which negative charge develops in the transition state [19]. Based on mutagenesis studies [10] and analysis of the crystal structure of the yeast enzyme in complex with BMP [11], one of these residues is likely to be Lys-93 (yeast numbering). Juxtaposition of this cationic residue and the C-6 atom would by expected to stabilize the UMP(−) intermediate [20] by attractive electrostatic interactions. Even in bulk water, the proximity of a cationic group in the chemical transition state can exert a profound effect on reactivity, as indicated by the fact that glycine (H_3N^+-CH_2-CO_2^-), for example, undergoes decarboxylation many orders of magnitude more rapidly than does acetate (H_3C-CO_2^-) [19].

In summary, we have characterized a previously undescribed competitive inhibitor of yeast ODCase, and used Raman spectroscopy to study its interaction with this enzyme. Experimental and computational results indicate that the stretching frequency of a nitrile group typically decreases if it is desolvated or distorted, *or both*, as seems to occur in the E–**I** complex. We propose that the enzyme–ligand interactions involved in perturbing the structure of **I** may also play a role in activating substrate OMP for decarboxylation. It should be emphasized, however, that this activation pathway would only complement, not substitute for, the role played by transition state stabilization in the activity of ODCase. That view is supported by the fact that OMP is distorted, yet unreactive, when bound to a mutant form of ODCase from *M. thermoautotrophicum*.

2. Experimental

2.1. Synthesis

Compounds **I** (λ_{max}, 280 nm) and **II** (λ_{max}, 291 nm) were prepared from the appropriate 5-bromo derivative and KCN (2 equiv) in DMSO using 18-crown-6 ether as catalyst [6]. Purification of **I** was carried out by chromatography using a charcoal column, and **II** was recrystallized from water. Both products exhibited ^{1}H NMR shifts consistent with their assigned structures.

2.2. Spectroscopy

Raman spectra were obtained at 25 °C, using 500 mW of 752 nm excitation as described [21]. A Raman spectrum of ODCase (80 μL of 4×10^{-4} M) was acquired for 5 min in a 2 mm by 2 mm rectangular quartz cell. One equivalent of **I** was then added to the ODCase

solution, and the spectrum re-recorded without changing the optical alignment or the cell position. This step did cause some of the soluble enzyme present in the cuvette to precipitate, reducing the true E:**I** ratio to <1. Difference spectra were calculated by subtraction (Win-IR, Princeton Instrument Corp.), choosing an appropriate scaling factor to remove any residual protein signals. For the solvent study, spectra were obtained from samples containing **II** ($\sim 10^{-3}$ M) dissolved in the indicated solvents, using the same instrument settings. Wavenumbers ($\pm 2\,cm^{-1}$) were assigned by comparison with an external chloroform-d1 standard.

3. Computational

DFT calculations were performed in Gaussian98 using a 6-311G** basis set [22]. The nitrile group was constrained in the desired geometry and the vibrational spectrum was calculated for the minimum energy structure.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2005.12.001.

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