Chemical models and their mechanistic implications for the transformation of 6-cyanouridine 5′-monophosphate catalyzed by orotidine 5′-monophosphate decarboxylase†

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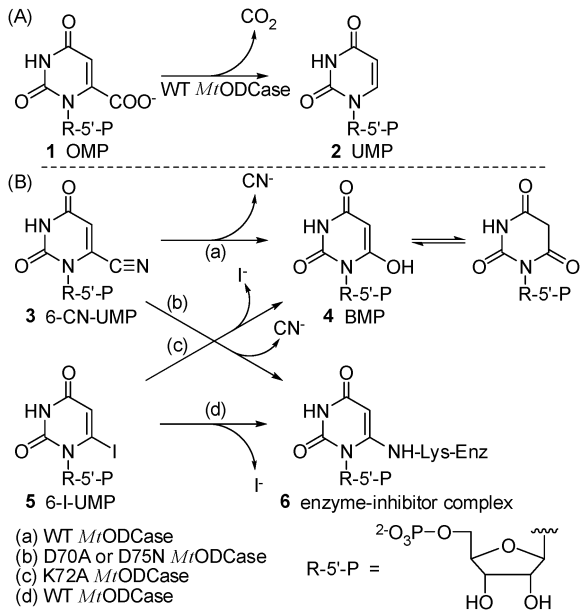
The reactions of 6-cyano-1,3-dimethyluracil have been studied as chemical models to illustrate the mechanism for the transformation of 6-cyanouridine 5′-monophosphate (6-CN-UMP) to barbiturate ribonucleoside 5′-monophosphate (BMP) catalyzed by orotidine 5′-monophosphate decarboxylase (ODCase). The results suggest that the Asp residue in the ODCase active site plays the role of a general base in the transformation.

ODCase (EC 4.1.1.23) catalyzes the decarboxylation of OMP (**1**) to uridine 5′-monophosphate (UMP, **2**) in the final step of the *de novo* pyrimidine nucleotide biosynthesis (Scheme 1(A)). ODCase is one of the most proficient enzymes known.[1,2] Unlike most of the other decarboxylases, ODCase contains no metal ions or small molecule cofactors, and there is no evidence for the existence of a covalent intermediate during the catalysis. These facts suggest that the proficient catalyst operates by a novel chemical mechanism.[3–6]

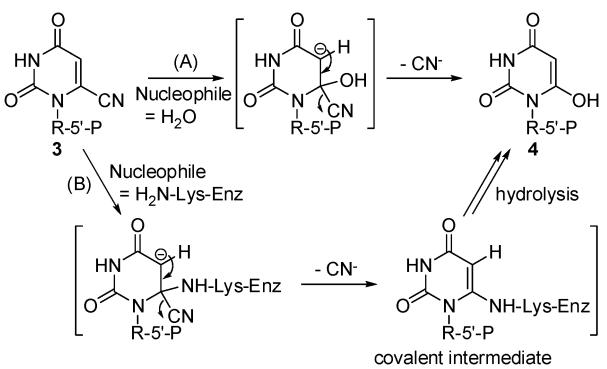


Scheme 1

In 2005, Kotra *et al.* discovered that 6-CN-UMP (**3**) underwent hydrolysis to form BMP (**4**) in the active site of ODCase from *Methanobacterium thermoautotrophicum* (*Mt*ODCase) (Scheme 1(B)(a)).[7] It was the first example that ODCase catalyzes an alternative substrate to undergo a reaction other than decarboxylation. We speculated that both the transformation of 6-CN-UMP (**3**) and the decarboxylation of OMP (**1**) (Scheme 1(A) & (B)(a)) would involve the same or similar chemical actions. Understanding the chemical reactions of 6-cyanouracil derivatives could open up an opportunity for studying the enzymatic mechanism of ODCase.

The reactions of 6-cyanouracil derivatives reported by Senda *et al.* in the 1970s[8–10] have received our attention. When 6-cyano-1,3-dimethyluracil (6-CN-1,3-DMU, **7**) was treated with sodium methoxide, *n*-butylamine or hydrazine, the 6-cyano group was replaced by the incoming nucleophiles to give the corresponding 6-substituted 1,3-dimethyluracils (6-MeO-1,3-DMU (**8**), 6-*n*-BuNH-1,3-DMU (**9**) and 6-H_2NNH-1,3-DMU (**10**), respectively). We envisioned that the nucleophilic substitutions of 6-CN-1,3-DMU (**7**) could potentially be a chemical model to account for the enzymatic transformation of 6-CN-UMP (**3**).

We rationalized that the *Mt*ODCase-catalyzed transformation of 6-CN-UMP (**3**) to BMP (**4**) could occur in two possible pathways:[11] (A) *Direct hydrolysis*: A water molecule undergoes the nucleophilic substitution of the 6-cyano group to form BMP (**4**) (Scheme 2(A)); (B) *Covalent intermediate*: A nucleophilic residue undergoes the nucleophilic substitution at the 6-position of 6-CN-UMP (**3**) to form a substrate-enzyme covalent complex. Further hydrolysis of the covalent intermediate accomplishes the transformation to form BMP (**4**)



Scheme 2

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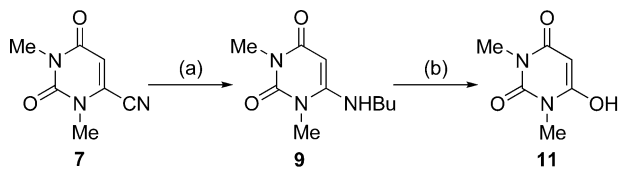
(Scheme 2(B)) (also proposed by Rudolph *et al.* in their recent paper[12,13]). X-Ray crystallography and sequence alignments have identified conserved amino acid residues in the active site consisting of two lysines and two aspartates.[14,15] The function of the Asp–Lys–Asp–Lys tetrad remains unclear except for the charge interactions and the formation of a hydrogen-bonding network. Herein, we postulate that the Lys and/or the Asp will participate in the catalytic reaction.

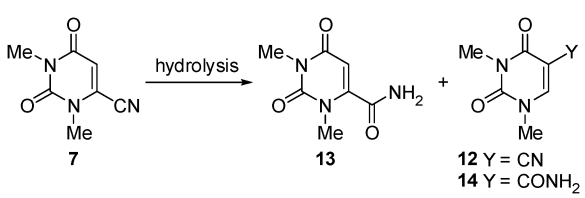
In an effort to examine the covalent mechanism (Scheme 2(B)) with the participation of the Lys residue, 6-CN-1,3-DMU (**7**) was used as the chemical model and was reacted with *n*-butylamine to afford 6-*n*-Bu-NH-1,3-DMU (**9**) in analogy to the formation of the substrate-enzyme covalent intermediate. Hydrolysis of 6-*n*-Bu-NH-1,3-DMU (**9**) in an aqueous HCl ethanol solution furnished the formation of 6-OH-1,3-DMU (**11**) (Scheme 3). This chemical model suggests that the proposed covalent mechanism could be feasible.[12,13] However, Kotra *et al.* reported that 6-iodouridine 5′-monophosphate (6-I-UMP, **5**) irreversibly inhibited the catalytic activities of *Mt*ODCase.[16] The inhibitor was covalently bound to the wild-type *Mt*ODCase between the 6-position of the uridine base and the Lys residue in the active site (Scheme 1(B)(d)). Hydrolysis of the Lys-UMP complex to BMP and free enzyme did not occur. In contrast, the covalent adduct was not observed when 6-CN-UMP (**3**) was incubated with the wild-type *Mt*ODCase, and the enzyme activity was inhibited solely by BMP (**4**). Thus, based upon their results, we have concluded that the transformation of 6-CN-UMP (**3**) to BMP (**4**) does not go through the Lys-UMP complex (Scheme 2(B)).

In an attempt to learn if the enzymatic transformation of 6-CN-UMP (**3**) was due to the direct displacement of the cyano group with H_2O, 6-CN-1,3-DMU (**7**) was subjected to the reactions with several oxygen-nucleophiles in protic and aprotic solvents. These reactions gave a variety of products including the migration of the cyano group from the 6-position to the 5-position,[8–10] and the hydrolysis of the cyano compounds to the corresponding amides (**13** & **14**) (Scheme 4). Although the direct substitution of the cyano group with a water molecule seems straightforward, the desired product, 6-OH-1,3-DMU (**11**), was not observed.



reagents and conditions:
(a) *n*-$BuNH_2$, reflux, 3 hr, 84 %; (b) 1 N HCl / EtOH, reflux, 12 hr, 68 %.

Scheme 3

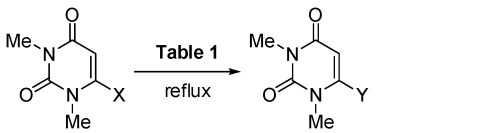


Scheme 4

Alternatively, when 6-CN-1,3-DMU (**7**) was treated with alkoxides in alcohols, nucleophilic substitution reactions took place to give 6-alkoxy-1,3-dimethyluracils (**8** & **16**). This direct substitution reaction of 6-CN-1,3-DMU (**7**) could be considered as an ideal model for the enzymatic transformation of 6-CN-UMP (**3**). To clarify the function of the Asp residue in the transformation, acetate was used to mimic the Asp residue in the model reactions. When 6-CN-1,3-DMU (**7**) was heated in the presence of NaOAc in ethanol, 6-EtO-1,3-DMU (**16**) was obtained in a good yield. The same result was found with Na_2CO_3 in ethanol. A catalytic amount of NaOAc or Na_2CO_3 in ethanol afforded the same product in comparable yields. These results revealed that the ethanolysis of 6-CN-1,3-DMU (**7**) is a general base catalyzed nucleophilic substitution reaction and acetate plays the role of the general base in the chemical reaction (Table 1, X = CN). Meanwhile, the reaction of 6-Cl-1,3-DMU (**15**) was also exemplified as a chemical model for the enzymatic transformation of 6-I-UMP (**5**). Nucleophilic displacement with *n*-$BuNH_2$ proceeded readily to give 6-*n*-Bu-NH-1,3-DMU (**9**), which represented the formation of a Lys-UMP adduct from 6-I-UMP (**5**) in the active site of wild-type *Mt*ODCase. On the other hand, subjecting 6-Cl-1,3-DMU (**15**) to NaOAc or Na_2CO_3 in ethanol afforded 6-EtO-1,3-DMU (**16**) as the only product, which suggests that the nucleophilic substitution is substantially catalyzed by a general base as well (Table 1, X = Cl).

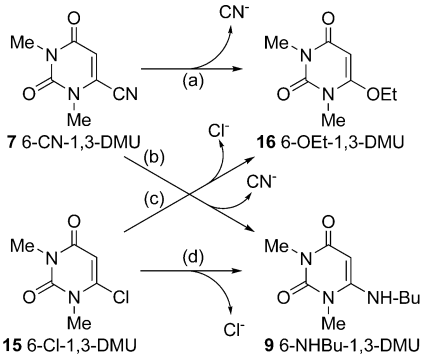
Kotra *et al.* recently reported the crystal structures for the complexes of several *Mt*ODCase mutants with 6-CN-UMP (**3**) and 6-I-UMP (**5**).[17] The D70A and D75N mutants formed a covalent bond between the C-6 position of UMP and the Lys residue when these mutants were incubated with 6-CN-UMP (**3**) (Scheme 1(B)(b)). This result indicated that, in the absence of the Asp residue in the active site, the transformation of 6-CN-UMP (**3**) to BMP (**4**) could not proceed and that the Lys side chain underwent the substitution reaction to form the Lys-UMP complex instead (Scheme 1(B)(b)). In contrast,

Table 1 Reactions of 6-CN-1,3-DMU under nucleophilic conditions



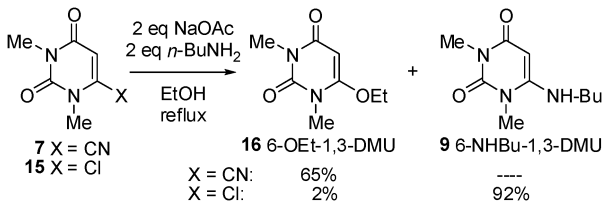
Entry	X	Reagent (eq)	Solvent	Time/h	Products (yield)
1	CN		*n*-$BuNH_2$	3	**9** (84%)
2	CN	NaOMe (1)	MeOH	1	**8** (64%)
3	CN	NaOEt (1)	EtOH	1	**16** (73%)
4	CN	NaOAc (2)	EtOH	1	**16** (54%), **12**[a] (5%), **17** (27%)
5	CN	NaOAc (0.2)	EtOH	1	**16** (35%), **12**[a] (2%), **17** (52%)
6	CN	Na_2CO_3 (2)	EtOH	1	**16** (75%)
7	CN	Na_2CO_3 (0.2)	EtOH	1	**16** (60%), **17** (26%)
8	Cl	*n*-$BuNH_2$ (2.2)	DMF	18	**9** (96%)
9	Cl	NaOAc (2)	EtOH	24	**16** (8%), recovered **15** (69%)
10	Cl	Na_2CO_3 (2)	EtOH	28	**16** (96%)

[a] 5-CN-1,3-DMU (**12**).



reagents and conditions:
(a) NaOAc in EtOH, reflux; (b) *n*-$BuNH_2$, reflux; (c) NaOAc in EtOH, reflux; (d) *n*-$BuNH_2$ in DMF, reflux.

Scheme 5



Scheme 6

without the Lys residue in the active site, the K72A mutant transformed 6-I-UMP (**5**) to BMP (**4**) which is similar to how 6-CN-UMP (**3**) was hydrolyzed by the wild-type *Mt*ODCase (Scheme 1(B)(c)).

Our studies are in agreement with Kotra's observations that both 6-CN-1,3-DMU (**7**) and 6-Cl-1,3-DMU (**15**) underwent ethanolysis in the presence of NaOAc, and that their reactions with *n*-$BuNH_2$ resulted in only 6-*n*-Bu-NH-1,3-DMU (**9**) (summarized in Scheme 1(B) and Scheme 5). Furthermore, in a competing experiment with a 1 : 1 mixture of both NaOAc and *n*-$BuNH_2$ in ethanol under reflux, to mimic the existence of both Asp and Lys residues in the ODCase active site, 6-CN-1,3-DMU (**7**) only gave 6-EtO-1,3-DMU (**16**) while 6-Cl-1,3-DMU (**15**) predominantly afforded 6-*n*-Bu-NH-1,3-DMU (**9**) (Scheme 6). The competing experiment has revealed the distinct preferential reactivities of 6-CN-1,3-DMU (**7**) and 6-Cl-1,3-DMU (**15**), which could account for the different transformations of 6-CN-UMP (**3**) and 6-I-UMP (**5**) catalyzed by the same enzyme (Scheme 1(B), (a) & (d)).

Our results from the chemical models may indicate that the Asp residue in the active site could assume a role similar to that of acetate in the chemical models. Thus, the model studies suggest that the transformation of 6-CN-UMP (**3**) to BMP (**4**) is catalyzed by *Mt*ODCase with the Asp residue as a general base. While most of the model studies for ODCase have focused on the explanation of transition state stablization of the OMP decarboxylation,[18–23] our chemical models have established the role of the Asp residue in the ODCase active site.[24] It remains to be further investigated how the general acid–base chemistry effects the decarboxylation of *Mt*ODCase.

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24 During the preparation of this manuscript, Rudolph *et al.* reported that the cocrystallization of the *human* ODCase (*h*ODCase) with 6-CN-UMP (**3**) resulted in the presence of UMP (**2**) in the active site, instead of BMP (**4**) (ref. 13). The proposed mechanism involved the hydrolysis of 6-CN-UMP (**3**) to OMP (**1**), followed by a normal decarboxylation. In our model studies, we have observed the formation of ethyl 1,3-dimethyluracil-6-carboxamidate (**17**) as a by-product from the base-catalyzed ethanolysis (Table 1, entries 4, 5 and 7), and 1,3-dimethyluracil-6-carboxamide (**13**) as a result of base-catalyzed hydrolysis. (Scheme 4) Accordingly, we anticipated that the Asp residue also plays the role of a general base for the *h*ODCase-catalyzed hydrolysis of 6-CN-UMP (**3**).