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## Transition-state discrimination by adenosine deaminase from *Aspergillus oryzae*

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Adenosine deaminase from *Aspergillus oryzae* resembles mammalian adenosine deaminases in its ability to catalyze the hydrolytic removal of many substituents from C-6, and in the chirality at C-6 of the active isomer of the transition-state-analogue inhibitor 6-hydroxymethyl-1,6-dihydropurine ribonucleoside. The 5'-OH group of adenosine has been found to contribute a factor of  $5 \cdot 10^4$  to transition-state stabilization by calf intestinal adenosine deaminase, and crystallographic observations suggest that a zinc-histidine 'bridge' is formed between the 6-OH and the 5'-OH groups of the substrate in the transition state for its deamination. The present paper describes experiments indicating that this bridge is not present during the action of adenosine deaminase from *Aspergillus oryzae*. We find (1), that the fungal enzyme catalyzes deamination of adenosine and 5'-deoxyadenosine with  $k_{\text{cat}}/K_{\text{m}}$  values that are almost identical; (2), that the  $K_{\text{i}}$  value of the transition-state-analogue inhibitor 2'-deoxycoformycin is much higher for the fungal enzyme ( $2.7 \cdot 10^{-9}$  M) than for the mammalian enzyme ( $2 \cdot 10^{-12}$  M) and (3), that this difference in binding affinities arises mainly from a difference in rates of enzyme-inhibitor association. Thus, the onset of inhibition was markedly slower for the fungal enzyme ( $k_{\text{on}} = 1.3 \cdot 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) than for the calf intestinal enzyme ( $k_{\text{on}} = 2.6 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ). Effects of chelating agents and divalent cations suggest that the fungal enzyme, like other deaminases for adenosine and cytidine, contains essential zinc.

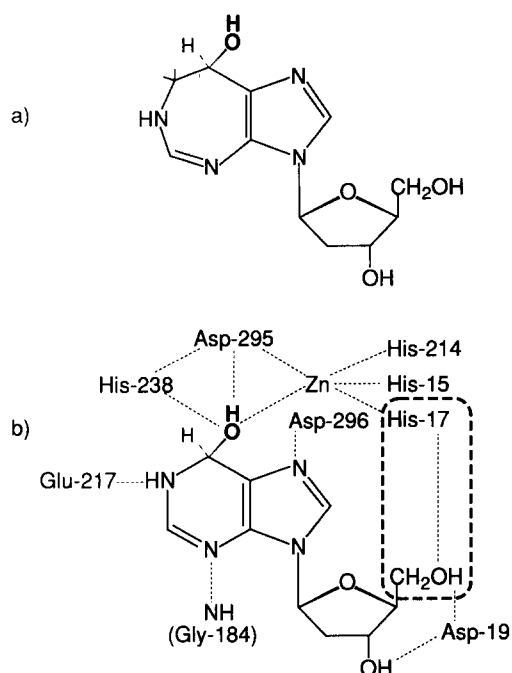
### Introduction

Mammalian adenosine deaminases show extremely high levels of discrimination between ligands, depending on the presence or absence of a single functional group. Thus, adenosine deaminase from calf duodenum binds the hydroxyl-containing transition-state-analogue inhibitor nebularine hydrate  $2 \cdot 10^7$ -fold more tightly than it binds the hydrogen-containing analogue, 1,6-dihydropurine ribonucleoside [4,5]. Binding discrimination is also very strong in some parts of the substrate that are not directly involved in the chemistry of substrate transformation. Thus, 5'-deoxyadenosine is deaminated with a  $k_{\text{cat}}/K_{\text{m}}$  value lower than that of adenosine by a factor of  $2 \cdot 10^{-5}$  [6]. X-ray diffraction from single crystals shows that the oxygen atom of the 6-OH group of the transition-state-analogue nebularine hydrate interacts with a zinc atom at the active site of the mammalian enzyme. This zinc atom, shown in Scheme I, is coordinated by several histidine residues,

including His-17 which also serves as an H-bond donor to the oxygen atom of the 5'-OH group of nebularine hydrate [9]. Evidently, His-17 serves, with zinc, as a 'bridge' between oxygen atoms at C-6 and C-5' of the substrate in the transition state for its deamination by mammalian adenosine deaminase, furnishing a plausible explanation for the striking contribution of the C-5' hydroxyl group to substrate reactivity.

Unlike these mammalian enzymes, adenosine deaminase from *Aspergillus oryzae*, first described by Mitchell and McElroy [7], acts not only on adenosine, but also on AMP and oligonucleotides, and is a dimer of 103 kDa subunits [13]. Both fungal and mammalian enzymes are active on substrates in which halogens or methoxyl groups replace the leaving 6-amino group of the substrate adenosine [12], and both are strongly inhibited by the same diastereomer of the transition-state-analogue 6-hydroxymethyl-1,6-dihydropurine ribonucleoside, which was designed to resemble a 1,6-hydrated intermediate in direct attack by water on adenosine [2]. This paper describes experiments aimed at determining whether a zinc-histidine bridge is likely to play a role in the action of the adenosine deaminase of *Aspergillus oryzae*, similar to that which it plays in the mammalian enzymes. Specifically, we wished to

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Scheme I. Schematic views of (a), (8R)-2'-deoxycoformycin and (b), the complex formed between nebularine hydrate and a mammalian adenosine deaminase [9], showing the interaction between His-17 and the 5'-hydroxyl group.

learn whether enzyme interactions with the 5'-OH group of adenosine make a significant contribution to catalysis by the fungal enzyme, and whether the fungal enzyme interacts with 2'-deoxycoformycin in a way that differs from that of the mammalian enzymes.

## Materials and Methods

2'-Deoxycoformycin (*R*-isomer, Scheme I) was a gift from Dr. David C. Baker, of the Department of Chemistry, University of Tennessee, and was quantitated by its UV absorption ( $\lambda_{\max} = 282 \text{ nm}$ ,  $\epsilon_{282} = 8250 \text{ cm}^{-1} \text{ M}^{-1}$ ). Radiolabeled [ $^{14}\text{C}$ ]deoxycoformycin was kindly donated by Dr. Peter W.K. Woo, Parke-Davis Pharmaceutical Division (Ann Arbor, MI, USA). [ $^3\text{H}$ ]2'-deoxycoformycin ( $2 \cdot 10^4 \text{ mCi/mmol}$ ) was obtained from Moravsek (Brea, CA, USA) as a solution in 50% ethanol. Nitrocellulose filters (type HA,  $0.45 \mu\text{m}$  pore size) were purchased from Millipore. 1,10-Phenanthroline and 2,6-dipicolinic acid were purchased from Aldrich and adenosine and 5'-deoxyadenosine were purchased from Sigma. Adenosine deaminase was purified to a specific activity of 40 U/mg (740-fold) from takadiastase (Fluka), as described by Wolfenden et al. [13]. For determination of dissociation rates of the enzyme complex with 2'-deoxycoformycin, the enzyme was further purified by gel

filtration with Sephadex G-150 (Pharmacia) to 89 U/mg (1650-fold). The buffer used for gel filtration was 0.01 M potassium phosphate (pH 7.4) containing 0.1 M NaCl.

Enzyme activity was measured spectrophotometrically in a volume of 0.5 ml of potassium phosphate buffer (0.1 M, (pH 6.5)) at 25°C. The reaction was initiated by addition of enzyme, or in the case of preincubation of enzyme with inhibitor, by addition of the substrate adenosine. For substrate concentrations lower than  $300 \mu\text{M}$ , the wavelength used was 260 nm, where  $\Delta\epsilon_{\text{M}}$  for conversion of adenosine to inosine is  $-7930 \text{ cm}^{-1} \text{ M}^{-1}$ . For concentrations of  $300 \mu\text{M}$  or more, the wavelength used was 280 nm, where  $\Delta\epsilon_{\text{M}}$  for conversion of adenosine to inosine is  $-330 \text{ cm}^{-1} \text{ M}^{-1}$ .

The time-course for enzyme association with 2'-deoxycoformycin was determined by incubating the enzyme with a substantial molar excess of inhibitor, and then determining the enzyme activity remaining after various periods of time at 23°C. In all experiments, protein ( $1 \mu\text{g}$ ) was incubated with inhibitor in 3.0 ml buffer for a measured interval, and adenosine was added to produce a final concentration of  $2 \cdot 10^{-4} \text{ M}$  in the assay mixture. The time required for assay of activity (1–2 min) was small compared with the time of preincubation, except for the earliest values, measured after 3–5 min. In control experiments, the enzyme was incubated with no inhibitor, in order to estimate the rate of nonspecific inactivation.

To determine the rate of enzyme-inhibitor dissociation, enzyme was first incubated with radiolabeled inhibitor at 4°C to allow formation of the inhibitory complex. This mixture was then incubated at room temperature with or without addition of a large excess of unlabeled 2'-deoxycoformycin, in the presence of bovine serum albumin added to prevent enzyme degradation. The enzyme-ligand complex remaining labeled after various intervals was determined by filtration using a nitrocellulose filter. [ $^3\text{H}$ ]2'-Deoxycoformycin ( $5 \mu\text{Ci}$ , final concn.  $2.5 \cdot 10^{-7} \text{ M}$ ), enzyme ( $20 \mu\text{g}$ ) and bovine serum albumin ( $1 \text{ mg}$ ) were incubated in buffer ( $1 \text{ ml}$ ) for 4 days at 4°C. The solution was incubated at room temperature, and sufficient unlabeled deoxycoformycin was added to produce a final concentration of  $9.4 \cdot 10^{-6} \text{ M}$ . Aliquots ( $15 \mu\text{l}$ ) were removed after various time intervals and tested as follows: A Millipore filter (type HA,  $2.5 \text{ cm}$  diameter,  $0.45 \mu\text{m}$  pore size) was moistened with buffer (about  $0.5 \text{ ml}$ ), and vacuum was applied and removed. The enzyme sample ( $15 \mu\text{l}$ ) was then pipetted onto the filter and vacuum was applied and removed. The filter was then washed 5 times with buffer ( $300 \mu\text{l}$ ) and finally transferred to a scintillation counter for analysis. The rate constant for release,  $k_{\text{off}}$ , was determined from the slope of a plot of the logarithm of the radioactivity remaining bound as a function of time.

## Results

### 5'-deoxyadenosine as a substrate

When double-reciprocal and Eadie-Hofstee plots were made of the rate of deamination as a function of the concentration of 5'-deoxyadenosine, varying from 50 to 2000  $\mu\text{M}$ , simple linear relationships were observed.  $K_m$  values were  $2.4 \cdot 10^{-4}$  M for adenosine and  $4.7 \cdot 10^{-4}$  M for 5'-deoxyadenosine, and  $V_{\max}$  values were 510 mmol/min per mg for adenosine and 840 mmol/min per mg for 5'-deoxyadenosine. The resulting quotient,  $V_{\max}/K_m$ , was 1.2-fold larger for 5'-deoxyadenosine than for adenosine.

### Rate of enzyme association with 2'-deoxycoformycin

Fig. 1 shows the results obtained when a solution of adenosine deaminase was incubated with 2'-deoxycoformycin, and the catalytic activity of aliquots was determined after various time intervals. Linear regression analysis of the pseudo-first-order rate constants obtained from these results yielded the first-order rate constant for association:  $k_{\text{on}} = (1.3 \pm 0.15) \cdot 10^4 \text{ s}^{-1} \text{ M}^{-1}$ .

### Rate of enzyme dissociation from 2'-deoxycoformycin and the value of $K_i$

When enzyme containing radioactive inhibitor was exposed to a large excess of unlabeled inhibitor for various intervals, and the radioactivity remaining bound was measured as a function of the time of exposure to unlabeled inhibitor, results were obtained as shown in Fig. 2. Of the radioactivity that was bound at the earliest times in a typical experiment (38000 cpm), approx. 2900 cpm remained bound after long periods of time; this value included approx. 1050 cpm that remained bound by the filter in the absence of protein.

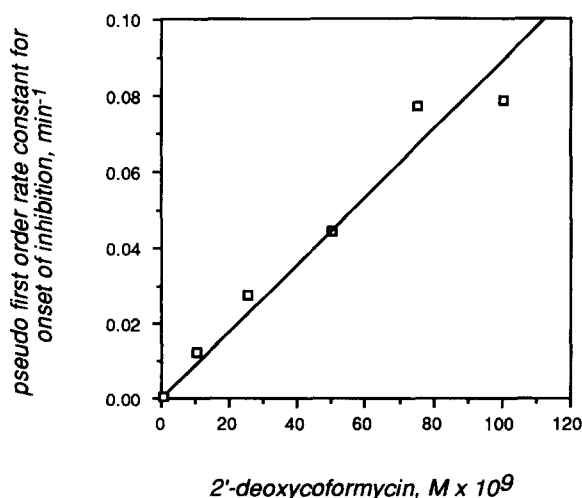


Fig. 1 Apparent first-order rate constants for the onset of inhibition, plotted as a function of the concentration of 2'-deoxycoformycin at 25°C, in potassium phosphate buffer (0.01 M (pH 7.4)).

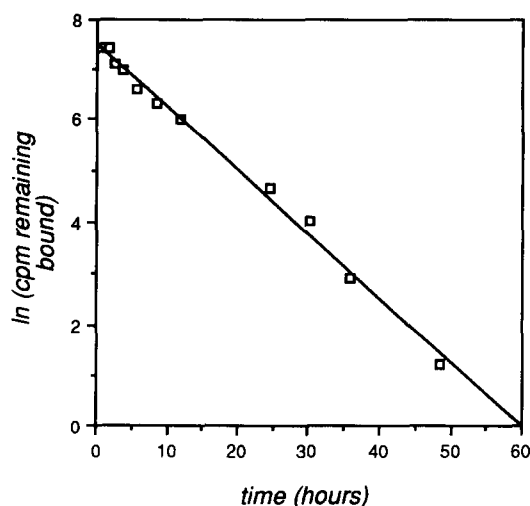


Fig. 2 Release of radiolabeled 2'-deoxycoformycin from fungal adenosine deaminase, plotted as a function of time at 25°C, in potassium phosphate buffer (0.01 M (pH 7.4)).

A plot of the logarithm of the counts remaining bound after various time periods, after correction for the radioactivity that remained bound after very long time periods (Fig. 2), followed simple first-order kinetics. The corresponding rate constant,  $k_{\text{off}}$ , was  $3.5 \cdot 10^{-5} \text{ s}^{-1}$ . Combining this value with the rate constant for enzyme-inhibitor association described above, it is evident that 2'-deoxycoformycin serves as a strong inhibitor of the fungal enzyme, with  $K_i = 2.7 \cdot 10^{-9}$  M.

Bovine serum albumin (1 mg/ml) was included in the above experiments to enhance protein stability during long incubations. In control experiments, bovine serum albumin (1 mg/ml) was incubated in the absence of adenosine deaminase with the same concentration of radiolabeled 2'-deoxycoformycin, for three days at 4°C, and unlabeled 2'-deoxycoformycin was then added to produce a total concentration of  $9.4 \cdot 10^{-6}$  M. After incubation at room temperature, the amount of radioactivity remaining bound by bovine serum albumin was found to be extremely small, approx. 30 cpm above background, and did not change detectably with the passage of time.

### Tests for essential zinc

To obtain some indication of whether a Zn atom might be involved in catalysis by the adenosine deaminase of *Aspergillus oryzae*, solutions of the fungal enzyme were incubated with  $\text{Zn}^{2+}$  chelating agents. Dipicolinic acid or 1,2-phenanthroline at various concentrations were incubated with enzyme (16  $\mu\text{g}$ ) in potassium phosphate buffer (0.5 ml, 10 mM (pH 6.5)) at 4°C, and the activity remaining was tested at intervals. In the presence of dipicolinic acid ( $8 \cdot 10^{-3}$  M), 50% of activity was lost after 24 h. In the presence of saturated 1,2-phenanthroline, 58% of activity was lost in the same period of time. Similar experiments yielded simi-

lar results with calf intestinal adenosine deaminase, known to contain an essential zinc atom [9].

In another test for the possible involvement of zinc in enzyme activity, the fungal enzyme was incubated with cobalt and cadmium. In these experiments, the enzyme (0.5 ml) was dialysed for 20 h against buffer (50 ml 0.4 M imidazole-HCl (pH 7), containing metal chloride, 10 mM), with one change of buffer, at 4°C. When  $\text{Co}^{2+}$  was present, 68% of activity was retained. When  $\text{Cd}^{2+}$  was present, 20% of activity was retained. When  $\text{Zn}^{2+}$  was present, 96% of activity was retained. In control experiments with no added metal ion, 100% of activity was retained.

## Discussion

The mechanisms of action of fungal and mammalian enzymes appear to be similar in several important respects. Thus, both types of deaminase catalyze hydrolysis of substrates with several leaving groups other than ammonia [12], and exchange of  $^{18}\text{O}$  from solvent water into inosine [11]. The fungal enzyme, like the mammalian enzyme, is strongly inhibited by 6-hydroxymethyl-1,6-dihydropurine ribonucleoside, which was designed to resemble a hydrated adduct that might be formed during direct attack by water on the substrate [2]. A more searching test of this resemblance is that both fungal and mammalian enzymes bind the same stereoisomer of 6-hydroxymethyl-1,6-dihydropurine ribonucleoside preferentially, suggesting that water attack is mounted from the same side of the purine ring by both enzymes [2]. The critical hydroxyl-group of the hydrated intermediate is believed to be situated on the side of the ring that faces the viewer in Scheme I, as indicated by the stereochemistry of the more tightly bound forms of inhibitors [8] and by the location of the 6-hydroxyl group in bound nebularine hydrate [9]. The present findings suggest a further resemblance between these enzymes, in that the fungal enzyme probably contains a zinc atom at the active site, like the mammalian enzyme [8] and *Escherichia coli* cytidine deaminase [14].

In the complex that is formed between a specific mammalian adenosine deaminase and nebularine hydrate, a zinc-bound histidine residue at the active site bridges the oxygen atoms at C-6 and C-5' of the inhibitor [9], which is believed to resemble the transition state for deamination by direct water attack on the substrate [4,5]. In accord with the implied involvement of the 5'-hydroxyl group in transition state stabilization, 5'-deoxyadenosine is deaminated with second order rate constant ( $k_{\text{cat}}/K_m$ ) 50 000-fold smaller than the value for adenosine, offering a striking example of the role in transition-state stabilization of substrate substituents that are not involved electronically in bond making or breaking [6].

The present results show that for the nonspecific adenosine deaminase of *A. oryzae*, the reactivities of the substrates adenosine and 5'-deoxyadenosine do not differ from each other appreciably. These results indicate that a histidine residue at the active site of fungal adenosine deaminase does not play an important bridging role analogous to that of His-17 at the active site of human adenosine deaminase.

We find that 2'-deoxycoformycin serves as a strong inhibitor of the fungal enzyme ( $K_i = 2.7 \cdot 10^{-9}$  M), but that its affinity falls far short of its affinity for the enzymes from calf intestinal mucosa ( $K_i = 2 \cdot 10^{-12}$  M [3]) or human erythrocytes ( $K_i = 2.5 \cdot 10^{-12}$  M [1]). It seems reasonable to infer that this reduced affinity may be based, at least in part, on the absence of attractive interactions between the fungal enzyme and the ligand's 5'-hydroxyl group.

From a more general point of view, it is of interest that this difference in thermodynamic binding affinities arises mainly from differences in the rate of enzyme-inhibitor association, not dissociation. Thus, the rate of inhibitor binding is approx. 200-fold smaller for the fungal ( $k_{\text{on}} = 1.3 \cdot 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) than for the erythrocyte enzyme ( $k_{\text{on}} = 2.6 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ). This extremely slow rate of enzyme-inhibitor association is unusual, but not unprecedented, a few cases being known in which enzyme inhibitors exhibit  $k_{\text{on}}$  values of  $10^4 \text{ M}^{-1} \text{ s}^{-1}$  or less. Such behavior is consistent with a slow change in enzyme conformation following initial binding of the inhibitor in a loosely-formed complex. Alternatively, slow binding may arise from the frequency of nonproductive encounters, possibly involving release of site-bound water molecules (for a review, see Ref. 10).

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