# The Rate Constant Describing Slow-Onset Inhibition of Yeast AMP Deaminase by Coformycin Analogues Is Independent of Inhibitor Structure†

David J. Merkler,‡ Michael Brenowitz, and Vern L. Schramm*,§
*Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461*
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ABSTRACT: (*R*)- and (*S*)-2′-deoxycoformycin, (*R*)-coformycin, and the corresponding 5′-monophosphates were compared as inhibitors of yeast AMP deaminase. The overall inhibition constants ranged from 4.2 mM for (*S*)-2′-deoxycoformycin to 10 pM for (*R*)-coformycin 5′-monophosphate, a difference of $3.8 \times 10^8$ in affinities. (*R*)-Coformycin, (*R*)-2′-deoxycoformycin 5′-monophosphate, and (*R*)-coformycin 5′-monophosphate exhibited both rapid and slow-onset inhibition. The *S* inhibitors and (*R*)-2′-deoxycoformycin exhibited classical competitive inhibition but no time-dependent onset of inhibition. The results indicate that the presence of the 2′-hydroxyl and 5′-phosphate and the *R* stereochemistry at the C-8 position of the diazepine ring are necessary for the optimum interaction of inhibitors with yeast AMP deaminase. This differs from the results for rabbit muscle AMP deaminase [Frieden C., Kurz, L. C., & Gilbert, H. R. (1980) *Biochemistry 19*, 5303-5309] and calf intestinal adenosine deaminase [Schramm, V. L., & Baker, D. C. (1985) *Biochemistry 24*, 641-646], in which a tetrahedral hydroxyl at C-8 in the *R* stereochemistry is sufficient for slow-onset inhibition with the coformycins. The results suggest that the transition state contains a tetrahedral carbon with the *R* configuration as a result of the direct attack of an oxygen nucleophile at C-6 of AMP. Slow-onset inhibition of yeast AMP deaminase is consistent with the mechanism

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EI^*$$

in which the combination of E and I is rapidly reversible. For these inhibitors, $K_i$ varied by a factor of $3 \times 10^3$, and the overall inhibition constant ($K_i^*$) varied by a factor of $2 \times 10^5$. The rate $k_5$, which induces slow-onset inhibition, was similar for the three slow-onset inhibitors while $k_6$ varied by a factor of 650, suggesting that $k_5$ is independent of inhibitor structure. The ability to act as a slow-onset inhibitor is a result of the ability of the inhibitor to stabilize the EI* complex. These findings argue against inhibitor-induced transition-state conformations for the slow-onset inhibitors and suggest that the enzyme attains the transition-state configuration at a low rate independent of the inhibitor structure.

AMP deaminase (adenosine 5′-monophosphate deaminase, EC 3.5.4.6) catalyzes the hydrolytic cleavage of AMP to yield IMP and ammonia. ATP is an allosteric activator and GTP

$$AMP + H_2O \xrightarrow[-GTP,\ -PO_4]{+ATP} IMP + NH_3$$

and inorganic phosphate are allosteric inhibitors. The enzyme has been proposed to play a role in the maintenance of the adenylate energy charge (Champan & Atkinson, 1973; Manfredi & Holmes, 1984; Yoshino & Murakami, 1985) and in purine–pyrimidine interconversion (Bennett et al., 1986). Lowenstein (1972) has identified AMP deaminase as the regulatory step in the purine nucleotide cycle.

Previous work on the yeast enzyme has characterized its kinetic and regulatory properties (Murakami, 1979; Yoshino et al., 1979; Yoshino & Murakami, 1980, 1986; Merkler et al., 1989) and has demonstrated the complex interplay of the allosteric regulators (Merkler & Schramm, 1990). However, little mechanistic work has been carried out for yeast AMP deaminase. Mammalian adenosine deaminases (Agarwal et al., 1977; Kati & Wolfenden, 1989a,b) and muscle AMP deaminase (Frieden et al., 1980; Bzowska & Shugar, 1989) are potently inhibited ($K_m/K_i \geq 10^5$) by purine analogues that are tetrahedral at positions equivalent to the C-6 position of the adenine ring. These inhibitors are proposed to mimic the transition state that results from the direct attack of water at C-6 of the substrate.

In this paper, we describe the inhibition of yeast AMP deaminase by the series of coformycin analogues. Expansion of the pyrimidine ring of adenine to a seven-membered diazepine ring and the tetrahedral secondary alcohol in the position near C-6 of the adenine ring make these compounds transition-state analogues of purine deaminases (Sawa et al., 1967). The availability of six analogues, three of which exhibit slow-onset inhibition, makes yeast AMP deaminase a useful enzyme for understanding the mechanism of slow-onset inhibition. Unlike adenosine deaminase and muscle AMP deaminase (Frieden et al., 1980; Schramm & Baker, 1985), the dissociation and rate constants can be experimentally determined for every step in the process of slow-onset inhibition. The results indicate that the slow onset of inhibition is a result of an inhibitor-independent enzyme conformational change into the transition state. Slow-onset inhibition is only observed when the inhibitor, which happens to be bound at the catalytic site, is capable of stabilizing the transition-state configuration. This mechanism can also explain several previously reported patterns of slow-onset inhibition (see Discussion).

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* Corresponding author.
‡ Present address: Division of Protein Chemistry, Unigene Laboratories, Inc., 110 Little Falls Rd., Fairfield, NJ 07004.
§ Preliminary reports of this work have been presented at the 197th National Meeting of the American Chemical Society (Merkler & Schramm, 1989).

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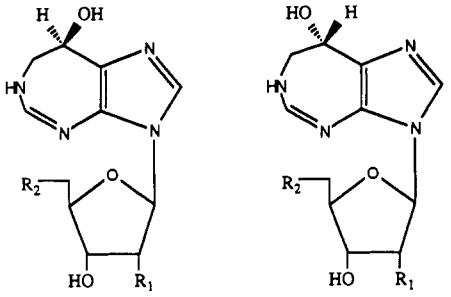



| Compound | $R_1$ | $R_2$ |
|---|---|---|
| dCoformycin | H | OH |
| dCof 5'-$PO_4$ | H | $OPO_3$ |
| Coformycin | OH | OH |
| Cof 5'-$PO_4$ | OH | $OPO_3$ |

FIGURE 1: Inhibitor structures. The asymmetric carbon that determines the *R* or *S* configuration is C-8 of the molecule.

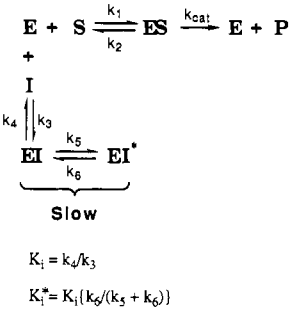
## Materials and Methods

*Materials.* Coformycin was obtained from Calbiochem. (*R*)-Deoxycoformycin was the natural product isolated from *Streptomyces antibioticus* and (*S*)-deoxycoformycin was synthesized by the procedure of Chan et al. (1982). Both compounds were gifts of Dr. D. C. Baker, University of Alabama. (*R*)-Deoxycoformycin 5'-monophosphate was a gift of Drs. J. C. Hanvey and R. J. Suhadolnik, Temple University School of Medicine. (*R*)-Coformycin 5'-monophosphate and (*S*)-deoxycoformycin 5'-monophosphate were synthesized by the phosphorylation of (*R*)-coformycin and (*S*)-deoxycoformycin following the procedures developed by Bennett et al. (1986) for the preparation of neplanocin 5'-monophosphate (Figure 1). (*R*)-Coformycin 5'-monophosphate was purified by preparative TLC (Bennett et al., 1986), and (*S*)-deoxycoformycin 5'-monophosphate was purified by HPLC using a reverse-phase $C_{18}$ μBondapak column (5 mm × 30 cm, flow rate 1.0 mL/min of 30 ammonium acetate, pH 5.0). Purified (*R*)-coformycin 5'-monophosphate and (*S*)-deoxycoformycin 5'-monophosphate were single, symmetric peaks by reverse-phase HPLC, which coeluted with their corresponding nucleosides after alkaline phosphatase treatment. The $^{31}P$ NMR spectrum of ribavirin 5'-monophosphate synthesized by this procedure on a larger scale is a triplet, indicating a single phosphate is esterified to the 5'-hydroxyl of (deoxy)ribose. ATP and AMP were of the highest grade available from Sigma. Calf intestinal alkaline phosphatase was from P-L Biochemicals. Bakers' yeast AMP deaminase was purified by the method of Merkler et al. (1989). The specific activity of homogeneous, purified AMP deaminase exceeds 500 μmol $min^{-1}$ $mg^{-1}$ when purified, with a slow loss of specific activity on storage. All experiments used enzyme with specific activity >200 μmol $min^{-1}$ $mg^{-1}$. All other materials were of the highest quality commercially available. Water was deionized and distilled.

*Evaluation of Kinetic Parameters.* Initial rate studies that resulted in reversible, competitive inhibition were analyzed according to the equation

$$v = \frac{V_{MAX}A}{K_m(1 + I/K_i) + A} \quad (1)$$

Scheme I: Kinetic Model for Slow-Binding Inhibition




where $K_m$ is the Michaelis constant, $V_{MAX}$ is the catalytic rate at saturating substrate concentration ($A$), $K_i$ is the dissociation constant for the enzyme–inhibitor complex, and $I$ is the inhibitor concentration (Cleland, 1979).

A model consistent with the time-dependent inhibition of yeast AMP deaminase is shown in Scheme I. The progress curves for this model are described by eq 2, as discussed by

$$P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \quad (2)$$

Morrison and Stone (1985). In this equation, $P$ represents the product, IMP or $NH_3$, $v_s$ and $v_0$ are the final and initial steady-state rates, and $k$ is the apparent first-order rate constant for the establishment of the final steady-state equilibrium. For Scheme I, the relationship between $k$ and the rate and kinetic constants is given by eq 3. The progress curves were

$$k = k_6 + k_5\left(\frac{I/K_i}{1 + A/K_m + I/K_i}\right) \quad (3)$$

fit to eqs 2 and 3 by using methods of nonlinear least-squares parameter estimation to determine the best-fit values, their 65% confidence limits, and the variance of the fit (Johnson & Frasier, 1985). The overall inhibition constant for Scheme I is defined as

$$K_i^* = \frac{(E)(I)}{(EI) + (EI^*)} = K_i\left(\frac{k_6}{k_5 + k_6}\right) \quad (4)$$

where $K_i = k_4/k_3$.

For the time-dependent inhibitors used in these studies, there exists a time range in the process curves in which the formation of EI* is small (e.g., see Figure 2). Within this time range, it is possible to directly measure the effect of the inhibitor on $v_0$, i.e., to measure $K_i$ directly. Values for $K_i$ were obtained from Dixon analysis at a constant substrate concentration (eq 5).

$$\frac{1}{v} = \frac{1}{V_{MAX}} + \frac{K_m}{V_{MAX}A}(1 + I/K_i) \quad (5)$$

The rate of enzyme–inhibitor dissociation, $k_6$, was measured directly for the time-dependent inhibitors. Small volumes of concentrated enzyme and inhibitor were incubated to reach equilibrium, followed by large dilutions (typically 1–20 000) in assay mixtures containing near-saturating substrate (30 mM AMP). The rate of enzymatic activity regain was measured by the rate of product formation.

*AMP Deaminase Assays.* Deamination of AMP to IMP and $NH_3$ was measured by the spectrophotometric assay described previously (Merkler et al., 1989) or the colorimetric assay for ammonia (Chaney & Marbach, 1962). The buffer

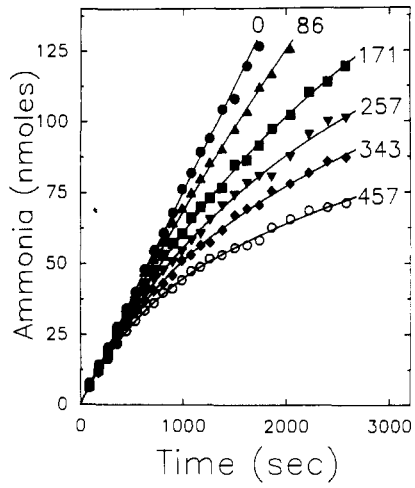



FIGURE 2: Inhibition by (*R*)-coformycin 5′-monophosphate. Reactions were initiated at 30 °C by the addition of 0.45 μg (5.6 pmol of active sites) of yeast AMP deaminase (specific activity = 272 μmol $min^{-1}$ $mg^{-1}$) into 2.8 mL of 30 mM HEPES-$K^+$, pH 7.0, 100 mM KCl, 100 μM ATP, 100 μM DTT, 30 mM AMP, and the indicated concentrations of (*R*)-coformycin 5′-monophosphate. At the appropriate time, 100-μL aliquots were removed and assayed for ammonia. The lines are the curves predicted by eqs 2 and 3 using the best-fit values specified in Table I. The concentrations of inhibitor indicated on the graph are nanomolar.

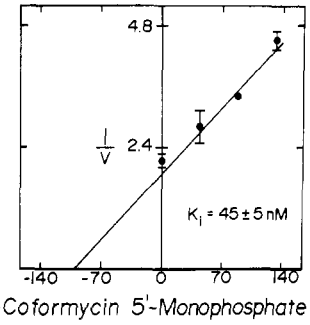
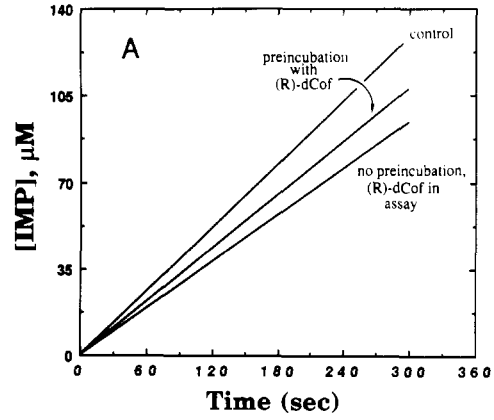



FIGURE 3: Initial rate inhibition constant for (*R*)-coformycin 5′-monophosphate. Reactions were initiated at 30 °C by the addition of 91 ng of enzyme (specific activity = 784 μmol $min^{-1}$ $mg^{-1}$) into 30 mM HEPES-$K^+$, pH 7.0, 100 mM KCl, 100 μM DTT, 100 μM ATP, and 0.5 mM AMP. The $K_m$ for AMP under these conditions was determined to be 0.30 ± 0.02 mM. The error bars represent the standard deviation of two to five initial rate determinations. Velocities are in micromoles per minute per milligram.

for enzyme assays was 50 mM triethanolamine hydrochloride, pH 7.0 (or 30 mM HEPES,[1] pH 7.0), 100 mM KCl, 100 μM ATP, and 100 μM DTT.

## Results

*Reaction Rates with Slow-Onset Inhibitors.* In the absence of inhibitor, the steady-state rate of yeast AMP deaminase is reached rapidly and is maintained during significant conversion of AMP to IMP. In the presence of (*R*)-coformycin 5′-monophosphate, there is a time-dependent decrease in the steady-state rate, which is a function of (*R*)-coformycin 5′-monophosphate concentration (Figure 2). Thus, (*R*)-coformycin is a slow-binding inhibitor (Morrison & Stone, 1985; Morrison & Walsh, 1988). Time-dependent inhibition similar to that shown in Figure 2 was also observed with (*R*)-coformycin and (*R*)-2′-deoxycoformycin 5′-monophosphate.



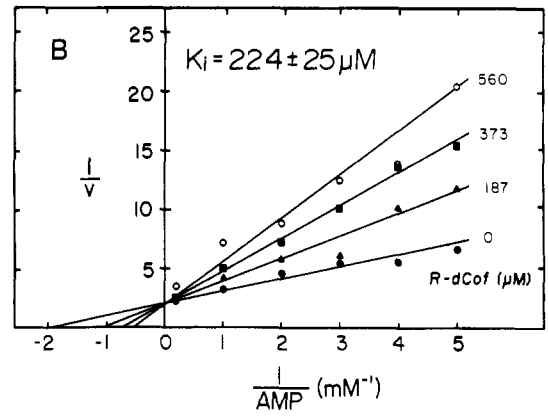




FIGURE 4: Initial rates in the presence of (*R*)-2′-deoxycoformycin. (A) The control represents the initial rate obtained upon the addition of 1.1 mM AMP to 0.38 μg/mL yeast AMP deaminase (specific activity = 489 μmol $min^{-1}$ $mg^{-1}$) preincubated for 20 min at room temperature in the assay buffer without AMP. The "no preincubation" experiment is the initial rate obtained upon the addition of 0.38 μg/mL enzyme to a solution of 1.1 mM AMP and 156 μM (*R*)-2′-deoxycoformycin preincubated for 20 min at room temperature in assay buffer without AMP. The "preincubation" experiment represents the initial rate obtained upon the addition of 1.1 mM AMP to 0.38 μg/mL enzyme and 156 μM inhibitor, which were preincubated for 20 min at room temperature in assay buffer without AMP. The differences in rate are within normal error limits for preincubation experiments. Rates were measured by the continuous recording method at 235 nm. (B) Determination of the $K_i$ for (*R*)-2′-deoxycoformycin. Initial rates were measured at 30 °C, and the assay mix for experiments in panel A was 50 mM triethanolamine hydrochloride, pH 7.0, 100 mM KCl, 50 μM DTT, and 50 μM ATP. Velocities are in micromoles per minute per milligram.

Examination of the progress curves (Figure 2) for each of the slow-binding inhibitors revealed a time range where the conversion of EI to EI* was minimal. For a low concentration of (*R*)-coformycin 5′-monophosphate, this time range is 0–250 s. Within this time range, classical competitive inhibition experiments can be used to determine $k_4/k_3$, the $K_i$ for a competitive inhibitor (eq 5). From such experiments, the inhibition constant for (*R*)-coformycin 5′-monophosphate was determined to be 45 ± 5 nM (Figure 4). Similar experiments with (*S*)-deoxycoformycin and (*S*)-deoxycoformycin 5′-monophosphate gave inhibition constants of 4.2 mM and 40 μM, respectively (Table I).

*Reaction Rates with Classical Competitive Inhibitors.* In contrast to the slow-onset inhibitors, the inhibition by (*R*)- and (*S*)-2′-deoxycoformycin and (*S*)-deoxycoformycin 5′-monophosphate did not show time dependence of inhibition. These compounds were "slope-linear" competitive inhibitors with respect to AMP. The kinetic and rate constants determined for these inhibitors are summarized in Table I.

[1] Abbreviations: Cof, coformycin; dCof, 2′-deoxycoformycin; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

Table I: Comparative Inhibition Constants for AMP and Adenosine Deaminases[a]

| | yeast AMP deaminase | | | | | muscle AMP deaminase | | | adenosine deaminase | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| inhibitor | $K_i$[b] | slow onset inhibition | $K_i^*$ | $k_5$ ($s^{-1}$) | $k_6$[b,f] ($s^{-1}$) | $K_i$ (mM) | slow onset inhibition[d] | $K_i^*$[c] | $K_i$ | slow onset inhibition | $K_i^*$[c] (pM) |
| (*S*)-dCof | 4.2 ± 0.8 mM | no | | | | | | | 33 μM | no | |
| (*R*)-dCof | 224 ± 25 μM | no | | | | >0.1 | yes | 360 nM | >0.1 mM | yes | 2.5 |
| (*S*)-dCof-5′-$PO_4$ | 40 ± 8 μM | no | | | | | | | | | |
| (*R*)-dCof-5′-$PO_4$ | 730 ± 120 nM | yes | 41 nM | 0.06 | $3.4 \pm 0.3 \times 10^{-3}$ | >0.1 | yes | 1 nM | | | |
| (*R*)-Cof | 70 ± 8 μM | yes | 2 μM | 0.05 | $1.8 \pm 1.6 \times 10^{-3}$ | >0.1 | yes | 20 nM | >0.1 mM | yes | 220 |
| (*R*)-Cof-5′-$PO_4$ | 45 ± 5 nM | yes | 10 pM | 0.02 | $5.2 \pm 1.1 \times 10^{-6}$ | >0.1 | yes | 55 pM | | | |

[a] Values for the rate constants for yeast AMP deaminase were calculated from Scheme I at 30 °C in 30 mM HEPES, pH .0, 100 mM KCl, and 100 μM DTT. Except where noted, the errors are the calculated standard errors of the fit. [b] Values for $K_i$ and $k_6$ were determined in separate experiments. These values were then fixed in eqs 2 and 3 in order to obtain $K_i^*$ and $k_5$. [c] Values for muscle AMP deaminase were obtained from Frieden et al. (1980). [d] Although there was no evidence for a fast initial step followed by a slow isomerization from stopped-flow studies, the $k_{on}$ rates for these inhibitors were $10^3$–$10^5$ slower than expected for diffusion on-rates. [e] Values for calf intestinal adenosine deaminase were obtained from Frieden et al. (1980) and Schramm and Baker (1985). [f] Errors are standard deviations from multiple experiments except for (*R*)-Cof-5′-$PO_4$ (Figure 5).

The relatively poor inhibition by (*R*)-2′-deoxycoformycin ($K_m/K_i \sim 1$) was unexpected, since it has been shown that (*R*)-2′-deoxycoformycin is a slow, tight-binding inhibitor for rabbit muscle AMP deaminase, $K_M/K_i \sim 500$ (Frieden et al., 1980). Evidence that (*R*)-2′-deoxycoformycin is not a time-dependent inhibitor of yeast AMP deaminase is presented in Figure 4A. The control experiment shows the initial rate obtained by the addition of AMP to yeast AMP deaminase. The "no preincubation" experiment is the initial rate obtained on the addition of enzyme to a solution of AMP and (*R*)-2′-deoxycoformycin. The "preincubation" experiment demonstrates the initial rate obtained on the addition of AMP to enzyme and (*R*)-2′-deoxycoformycin that had been preincubated prior to substrate addition.

If (*R*)-2′-deoxycoformycin were a slow-binding inhibitor, preincubation would cause the initial rate to be substantially less than that for the experiment without preincubation. The observation that this is not the case (Figure 4A) demonstrates that (*R*)-2′-deoxycoformycin is not a slow-binding inhibitor for yeast AMP deaminase. Similar experiments have also shown that (*R*)-2′-deoxycoformycin is a slow-binding inhibitor of human erythrocyte adenosine deaminase (Agarwal et al., 1977) and calf intestinal adenosine deaminase (Schramm & Baker, 1985). (*R*)-2′-Deoxycoformycin inhibited yeast AMP deaminase as a slope-linear competitive inhibitor with an inhibition constant of 224 ± 25 μM (Figure 4B). Even with concentrations of (*R*)-2′-deoxycoformycin of 560 μM, no slow-onset inhibition was observed in the inhibition experiments of Figure 4B.

*Analysis of Reaction Rate Progress Curve Data.* An independent method to determine $k_6$, the rate constant for the conversion of EI* to EI, involves preincubating relatively high concentrations of enzyme and inhibitor for sufficient time to allow the system to reach equilibrium. Dilution into a relatively large volume of assay mix containing saturating substrate causes dissociation and regeneration of activity. Under these conditions, $v_0$ and the effective inhibitor concentration are approximately equal to zero and the rate of activity regeneration will provide $k_6$ (Morrison & Stone, 1985). After preincubating yeast AMP deaminase with (*R*)-coformycin 5′-monophosphate, the enzyme–inhibitor mixture was diluted 20 000-fold into assay mix containing AMP at 150 $K_m$. A value of $k_6 = 5.2 \pm 1.1 \times 10^{-6}$ $s^{-1}$ for (*R*)-coformycin 5′-monophosphate was obtained by least-squares minimization of eq 2 to the data for recovery of enzyme activity (Figure 5). Equation 2 was used with the assumption that the inhibitor concentration = 0, $v_0$ = 36 μmol $min^{-1}$ $mg^{-1}$, and $v_s$ = 793 μmol $min^{-1}$ $mg^{-1}$. The final steady-state rate, $v_s$, is from the control that was preincubated without inhibitor.

The values of $k_6$ for (*R*)-deoxycoformycin 5′-monophosphate and (*R*)-coformycin were determined in enzyme recovery

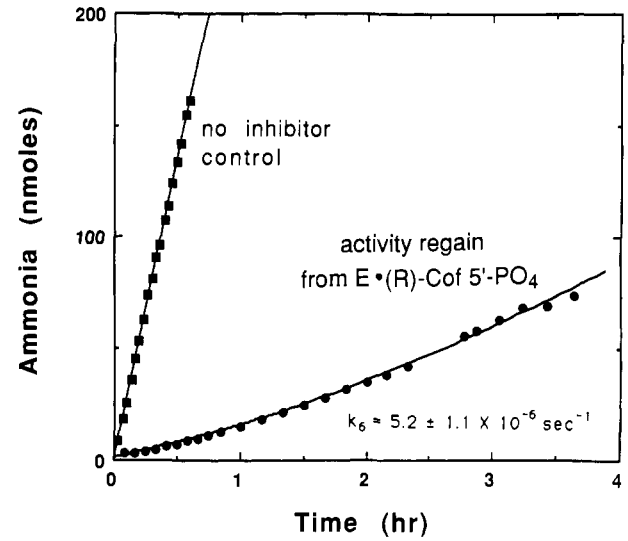



FIGURE 5: Dissociation rate ($k_6$) for the AMP deaminase–(*R*)-coformycin 5′-monophosphate complex. Enzyme (1.1 mg/mL, 14 μM subunits) with (●) or without (■) 200 μM (*R*)-coformycin 5′-monophosphate was preincubated for 130 min on ice in 30 mM HEPES-$K^+$, pH 7.0, 70 mM KCl, and 100 μM DTT. After the preincubation, 1 μL was removed and added to 20 mL of 30 mM HEPES-$K^+$, pH 7.0, 100 mM KCl, 100 μM DTT, 100 μM ATP, and 30 mM AMP. At the indicated times, 150-μL aliquots were removed and assayed for ammonia. The off-rate ($k_6$) was determined as described in the text.

experiments of the type shown in Figure 5. The values of $k_5$ for all inhibitors exhibiting slow-onset inhibition were obtained from fits of eq 3 to the onset of inhibition data of the type shown in Figure 2. The experimentally determined values of $K_i$ and $k_6$ were used to obtain $k_5$ using eq 3. These results are summarized in Table I.

Morrison and Stone (1985) and Erion and Walsh (1987) discuss two alternative models to Scheme I that will also result in time-dependent inhibition. An inhibition model in which the binding of the inhibitor to the enzyme is slow and tight, but occurs in a single step, is eliminated by the data of Table I because the inhibitors have a measurable effect on the initial reaction rate before the onset of slow-binding inhibition. An inhibition model in which the inhibitor binds only to a form of the free enzyme that has slowly adopted the transition-state configuration can be eliminated by the observed rates of onset of inhibition shown in Figure 2. This mechanism also predicts that slow-binding inhibitors will not inhibit the initial rate of product formation in a rapidly reversible manner.

## Discussion

*Mechanistic Implications of Tight-Binding Inhibitors.* Comparison of the substrate properties and inhibition of yeast

AMP deaminase by (*R*)-coformycin 5′-monophosphate ($K_i^*$ = 10 pM, Table I) gives a $K_m/K_i^*$ ratio of $2 \times 10^7$, one of the more tightly bound inhibitors that are considered to be transition-state mimics (Morrison & Walsh, 1988). The result suggests that the transition state is tetrahedral, resulting from the direct attack of water on C-6 of AMP. This mechanism is similar to that proposed for mammalian adenosine deaminase (Evans & Wolfenden, 1973; Frieden et al., 1980; Frick et al., 1986; Weiss et al., 1987; Jones et al., 1989) rabbit muscle AMP deaminase (Frieden et al., 1980), cytosine deaminase (Kornblatt & Tee, 1986), and cytidine deaminase (Wentworth & Wolfenden, 1975). The possibility that AMP deamination occurs via a double-displacement mechanism with the initial formation of a tetrahedral enzyme–nucleotide intermediate, followed by water attack, is also compatible with the results presented here. However, the finding that purine ribonucleoside is hydrated by adenosine deaminase (Kruz & Frieden, 1987) supports the direct attack of water at C-6 for this enzyme. Slow-onset inhibition by the *R* isomers but not the *S* isomers of these inhibitors supports the proposal that the water attack comes from the *re* face of the ring.

*Steps in Tight-Binding Inhibition.* A general mechanism for slow-onset tight-binding inhibition is shown in Scheme I. The inhibitor binds rapidly to form a ground-state complex, followed by a second slower step that involves reorganization of the protein toward the transition-state structure. The nature of these changes has been discussed (Jencks, 1975; Wolfenden, 1976), and the changes have been observed in the adenosine deaminase–inhibitor complex (Kurz et al., 1985; Philips et al., 1987).

The ground-state (EI) complexes of AMP deaminase with all of the coformycin derivatives of Table I were observed experimentally in initial rate studies. The affinity of the initial complexes varied from 4 mM to 45 nM, a factor of $9 \times 10^5$. The complexes formed by (*S*)-deoxycoformycin, (*R*)-deoxycoformycin, and (*S*)-deoxycoformycin 5′-monophosphate did not exhibit the slow onset of inhibition, while the complexes of (*R*)-deoxycoformycin 5′-monophosphate, (*R*)-coformycin, and (*R*)-coformycin 5′-monophosphate caused the time-dependent onset of a more strongly inhibited complex indicated by EI* in Scheme I. Although the usual pattern of slow-onset inhibition is for tight binding, this is not a necessary consequence of the change. The extent of EI* formation depends on the ratio of the first-order rate constants $k_5$ and $k_6$ in Scheme I. The overall observed inhibition depends on the affinity of the EI complex and the relative rates of formation of EI* and the relaxation of the EI* complex to EI. The properties of yeast AMP deaminase provide a unique opportunity to quantitate these rates and affinities.

Formation of EI is too rapid to be measured by steady-state kinetics and is likely to be near diffusion control, as is found for most ground-state inhibitors. The rate of EI* formation is slow and is relatively independent of the stability of the EI complex or of the ability of the inhibitor to stabilize the EI* complex. For the slow-onset inhibitors in Table I, $k_5$ ranges from 0.02 to 0.06 $s^{-1}$, while the catalytic turnover number of the enzyme is $10^3$ $s^{-1}$. For the slow-onset inhibitors of yeast AMP deaminase shown in Table I, the value of $k_6$ varies by a factor of 650. For (*R*)-coformycin 5′-monophosphate, $k_6$ is $5.2 \times 10^6$ $s^{-1}$, which gives a half-time for reactivation of 37 h for the EI* complex. Half-times for reactivation of the EI* complexes with (*R*)-coformycin and (*R*)-deoxycoformycin 5′-monophosphate are 6 and 3 min, respectively. The $K_i^*$ of 2 μM for (*R*)-coformycin demonstrates that slow-onset inhibition need not be tight-binding inhibition. The conclusion from this analysis is that the major variable for the phenomenon of slow-onset inhibition for AMP deaminase is $k_6$, the first-order rate at which the EI* complex relaxes to EI, the form which releases the inhibitor. An equivalent statement is that $K_i^*$ depends on the ability of the inhibitor to stabilize the EI* complex.

*Inhibition and Slow-Onset Specificity.* (*R*)-Coformycin 5′-monophosphate is the strongest inhibitor of yeast AMP deaminase, and any structural change resulted in decreased inhibition (Table I). The 2′-hydroxyl, the 5′-phosphate, and the stereochemistry about C8 of the diazepine ring (Figure 1) are critical determinants for inhibition. (*S*)-2′-Deoxycoformycin, which lacks both the 2′-hydroxyl and the 5′-phosphate and is the opposite diastereomer relative to (*R*)-coformycin 5′-phosphate, has an inhibition constant of only 4.2 mM. Thus, (*R*)-coformycin 5′-monophosphate binds $3.8 \times 10^8$ times as tightly to yeast AMP deaminase as does (*S*)-2′-deoxycoformycin. This corresponds to a difference in binding energy of 11.9 kcal/mol.

The relative affinities of (*R*)-coformycin and (*R*)-coformycin 5′-phosphate indicate that phosphate contributes 4.4 kcal/mol binding energy to $K_i$ and 7.3 kcal/mol binding energy for stabilization of $K_i^*$. Comparison of the inhibition constants for (*R*)-2′-deoxycoformycin 5′-phosphate and (*R*)-coformycin 5′-phosphate establishes that the 2′-hydroxyl contributes only 1.7 kcal/mol to $K_i$, but 5.0 kcal/mol to $K_i^*$. For significant stabilization of the EI* complex, the stereochemistry must be *R*, as none of the *S* isomers caused slow-onset inhibition. The formation of EI* also requires the presence of the 5′-phosphate *or* the 2′-hydroxyl, since (*R*)-2′-deoxycoformycin did not cause slow-onset inhibition, but (*R*)-coformycin did. Even though (*R*)-coformycin induces slow-onset inhibition, the complex is poorly stabilized, with a $K_i^*$ of 2 μM. This emphasizes the importance of the 5′-phosphate in stabilizing the transition-state configuration EI*.

*Comparative Inhibition of AMP Deaminases and Adenosine Deaminase.* Frieden et al. (1980) have measured the inhibition of calf intestinal adenosine deaminase and rabbit muscle AMP deaminase by several of the compounds used in this study. For these enzymes, the initial binding of all inhibitors was weak, with $K_i$ ($k_4/k_3$) estimated to be $10^{-4}$–$10^{-3}$ M (see Table I). The measured inhibition constants for (*S*)-2′-deoxycoformycin ($K_i$ = 33 μM) and 8-ketodeoxycoformycin ($K_i$ = 43 μM) with calf intestinal adenosine deaminase were near this range and did not produce slow-tight binding (Schramm & Baker, 1985). Since the $K_i$ values and initial binding energies are approximately the same for both ground-state and tight-binding inhibitors of rabbit muscle AMP deaminase and calf intestinal adenosine deaminase, the binding energy of the ground state does not predict which of the inhibitors is capable of stabilizing the EI* complex of Scheme I. However, for both enzymes, only those inhibitors with a tetrahedral (*R*)-hydroxyl at the C-8 position of the diazepine ring are capable of causing $k_6 < k_5$. This proposal is consistent with the observation that all inhibitors with a tetrahedral (*R*)-hydroxyl at C-8 tested against muscle AMP deaminase (Frieden et al., 1980; Bzowska & Shugar, 1989) and mammalian adenosine deaminase (Agarwal et al., 1977; Frieden et al., 1980) are slow-onset, tight-binding inhibitors.

Yeast AMP deaminase differs considerably from both adenosine deaminase and muscle AMP deaminase because of large differences in $K_i$ for the initial binding of the inhibitors. The $K_i$ ($k_4/k_3$) values ranged from 4.2 mM for (*S*)-deoxycoformycin to 45 nM for (*R*)-coformycin 5′-monophosphate. Thus, the initial binding energy for the inhibitors listed in Table

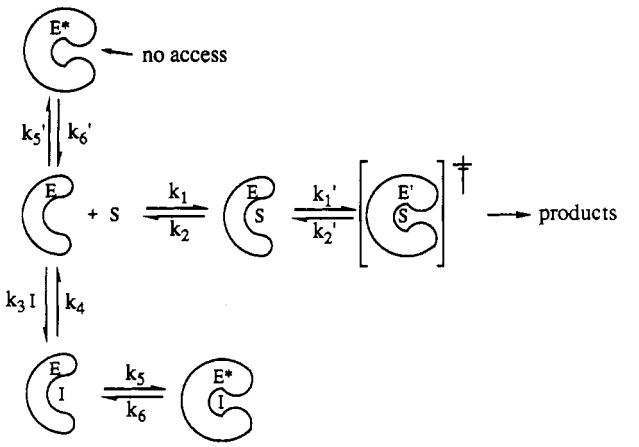
I varies from 3.3 to 10.2 kcal/mol.

Another important distinction between yeast AMP deaminase and calf intestinal adenosine deaminase is the effect of the pentose ring. For adenosine deaminase, the deoxyribose is favored since (*R*)-deoxycoformycin binds 100-fold tighter than (*R*)-coformycin in the overall formation of EI*. With yeast AMP deaminase, ribose is favored since (*R*)-coformycin 5′-phosphate binding is 4000-fold tighter than (*R*)-2′-deoxycoformycin 5′-phosphate binding (see $K_i$* in Table I). This preference is also reflected in the substrate specificity of yeast AMP deaminase, which has a $V_{MAX}/K_m$ ratio of 170 for AMP relative to 2′-deoxy-AMP (Merkler and Schramm, unpublished observations). In contrast, adenosine deaminase catalyzes deamination of adenosine and 2′-deoxyadenosine with nearly equal efficiency (Wolfenden et al., 1967). Therefore, the ribose 5′-phosphate of (*R*)-coformycin 5′-phosphate plays an important role in stabilizing EI* in yeast AMP deaminase.

*Mechanistic Implications for Slow-Onset Inhibition.* The slow-onset inhibition of yeast AMP deaminase by the coformycin family can be accommodated by the steps of Scheme I. However, the observed pattern of ground-state and slow-onset inhibitors in Table I indicates that the inhibitors stabilize EI*, rather than induce EI* formation. In this mechanism, all inhibitors bind initially to the enzymatic ground-state configuration that normally recognizes AMP. The affinity for the ground state is reflected in the steady-state inhibition constant, $K_i$. The enzyme–inhibitor complex remains primarily in the ground-state configuration, but a small fraction attains the transition-state configuration EI* with a rate constant of 0.02–0.06 $s^{-1}$, independent of the nature of the EI complex. Thus, a small fraction of the enzyme is constantly sampling the transition-state configuration but rapidly returns to the ground state without proper ligands to maintain that structure. The experimental evidence consistent with this mechanism is the nearly constant value of $k_5$ while both $K_i$ and $k_6$ vary by over 3 orders of magnitude with the family of coformycin inhibitors. The conversion of enzyme to the EI* complex is unlikely to be differentially induced by the slow-onset inhibitors, since the $K_i$ values vary by over 3 orders of magnitude while the $k_5$ changes only 3-fold. Inhibitors that do not exhibit slow-onset inhibition are also proposed to reside at the substrate site while the enzyme samples the transition state (EI* complex). Without the ability to bind tightly to this complex, the ground-state inhibitors permit the rapid return of EI* to the EI complex, and no time-dependent inhibition is observed. The large rate differences between catalysis (1000 $s^{-1}$) and $k_5$ (0.02–0.06 $s^{-1}$) explain the slow onset of inhibition. The fraction of enzyme in EI* can only be significant when the inhibitor diminishes $k_6$ to values lower than $k_5$ and the slow-onset constant for $k_5$ requires significant time to accumulate EI*. This mechanism also explains the slow-onset weak inhibition of 2 $\mu$M with (*R*)-coformycin. The constant simply reflects the poor ability of the compound to diminish the rate constant $k_6$.

The normal catalytic cycle involves the substrate-dependent entry of the enzyme–substrate complex, ES, into the transition state with a rate constant equal to or greater than 1000 $s^{-1}$ (Merkler et al., 1989). This process differs intrinsically from the process described by $k_5$ and must occur by substrate-facilitated entry to the transition-state complex. These differences support the proposal that most transition-state inhibitors are rather poor mimics of the catalytically functional transition state. Those that are good mimics are expected to trigger the rapid entry into the transition-state complex.

Scheme II: Proposed Enzyme Conformations for Slow-Onset Inhibition of Yeast AMP Deaminase[a]




[a] Enzyme (E) has an open catalytic site at which substrate (S) or inhibitor (I) can bind to form the readily reversible complexes ES and EI. The enzyme adopts a conformation (E*) related to the transition state with closely related rate constants $k_5$ and $k_5'$ in the presence or absence of inhibitor. These constants are slow, 0.02–0.06 $s^{-1}$ for yeast AMP deaminase. The E* complex has an inaccessible catalytic site and does not permit entry or release of substrate or inhibitor. Inhibitors that stabilize the E*I complex cause its accumulation and slow-onset inhibition. "Ground-state" inhibitors also can enter the E*I complex but are not kinetically detectable since they do not decrease the rate of $k_6$. Both $k_6$ and $k_6'$ are >0.02–0.06 $s^{-1}$ for the ground-state inhibitors and for free enzyme but are much slower for slow-onset inhibitors. The presence of substrate induces the rapid and specific formation of the transition-state complex in step $k_1'$. This rate is >1000 $s^{-1}$, the turnover number for the enzyme. Transition-state analogues, which are closely related to the [E′S]* complex, are expected to induce the rapid onset of inhibition.

*Implications from Other Slow-Onset Inhibition Systems.* The experimental observation for several slow-binding enzyme inhibitors is that $k_{on}$ ($k_5$ in Scheme I) is nearly constant for a given enzyme even when the slow-binding inhibitors vary widely in $K_i$ and $K_i$*. Thus, $k_{on}$ for four inhibitors of muscle AMP deaminase varies by a factor of 11 while $k_{off}$ and $K_i$* both vary by more than $10^3$ [Frieden et al., 1980; analyzed in Morrison and Walsh (1988)]. Five slow-binding inhibitors of dihydrofolate reductase show a similar pattern. $K_i$* varies by $10^4$ and $K_i$ varies by $>10^3$, but $k_5$ varies by only a factor of 14 [summarized in Morrison and Walsh (1988)]. A remarkable feature of this constancy is that one of the inhibitors, methotrexate, is bound upside down in the catalytic site (Bolin et al., 1982). These findings do not support the assumption that slow-binding inhibitors induce the conformational change, since an induced change would be expected to occur with substantial differences in rate, depending on the $K_i$ or $K_i$* for the inhibitor. The observation that three well-characterized enzyme systems exhibit onset of slow-binding inhibition that is independent of the affinity of the inhibitor indicates that, for these enzymes, the onset of slow-binding inhibition is caused by a normal conformational mode of the enzyme–inhibitor complex that attains the transition-state configuration. The effectiveness of the slow-binding inhibitor reflects the ability of the inhibitor to stabilize the transition-state complex and, thus, slow its return to the ground state. Slow-binding inhibitors have little effect on the probability that the protein will attain the transition-state configuration. Free enzyme is also likely to attain the transition-state configuration without inhibitor bound; however, in this configuration, the binding site in inaccessible. The mechanism for slow-onset inhibition for yeast AMP deaminase is illustrated in Scheme II.

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