Communications to the Editor

A Tight Binding Inhibitor of 5-Aminoimidazole Ribonucleotide Carboxylase

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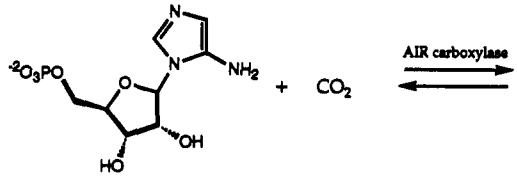
The *de novo* biosynthesis of purines, a central process to all life forms except protozoans, involves a diverse array of enzymatic transformations. Because of the essential role that the pathway plays in rapidly proliferating tissues, enzymes in this pathway have become important targets for new chemotherapeutic agents.[1] Unique among the transformations in purine biosynthesis is a carbon-carbon bond forming reaction at C4 of 5-aminoimidazole ribonucleotide (AIR, 1) to form 4-carboxy-5-aminoimidazole ribonucleotide (CAIR, 2). Although this reaction can occur spontaneously, the AIR carboxylase from *Escherichia coli* serves to accelerate the conversion of 1 to 2 by a factor of 10^6.[2] Carboxylases commonly utilize cofactors or divalent metals to activate or stabilize reactive intermediates.[3] AIR carboxylase has no known cofactor requirement, a fact that poses fundamental mechanistic questions.[2,4] Furthermore, additional catalytic functions are associated with AIR carboxylases depending upon the source of protein. For example, the avian protein is a bifunctional enzyme that also catalyzes the subsequent condensation of 2 with aspartic acid to give 4-(*N*-succinocarboxamido)-5-aminoimidazole ribonucleotide (SAICAR).[5-7] As part of our effort to identify the key catalytic features of AIR carboxylase, a synthetic analog has been prepared and used as a mechanistic probe. This report is the first example of a tight-binding AIR carboxylase inhibitor which has important implications for a catalytic mechanism.[8]



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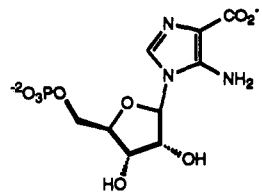

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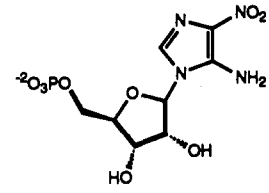
Previous mechanistic studies of AIR carboxylase have been partially limited by the instability of aminoimidazole ribonucleosides in aqueous solution. In fact, the natural substrate and product of this enzyme polymerize *in vivo*.[9] For this reason, our approach has focused on product analogs with predictable stability and inhibitory properties that could be enhanced by further synthetic elaboration. To this end, 5-amino-1-(5'-phospho-β-D-ribofuranosyl)-4-nitroimidazole (3) was selected as an initial target molecule because of its proposed isoelectronic and isosteric relationship with product 2.[10] The nucleoside precursor to 3 was synthesized using a reported route.[11] Direct phosphorylation of the nucleoside followed by ion-exchange chromatography provided 3 which was pure by HPLC analysis and proved stable under conditions relevant to this study.



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In initial steady-state inhibition studies of the decarboxylation of 2 catalyzed by avian AIR carboxylase-SAICAR synthetase,[12] 3 exhibited apparent competitive inhibition kinetics. However, the estimate of K_i = 1.3 nM in the presence of 0.5 nM enzyme suggested a tight binding phenomenon. A second estimate of inhibitor binding was determined by fitting steady-state velocity data as a function of total enzyme concentration to a general rate equation accounting for changes in free inhibitor concentrations, and this analysis gave a K_i = 1.6 nM.[13] In order to characterize the enzyme-inhibitor binding equilibrium, which was found to occur on the steady-state time scale, data from progress curves of the enzyme reaction in the presence of 3 were fitted to the integrated rate equation.[14] Secondary plots of k_{obs} versus inhibitor concentration indicated a hyperbolic relationship, consistent with a binding model that describes an initial rapid equilibrium of enzyme-inhibitor followed by conversion to a second, tighter complex, as shown in Table I. Examples of the progress curves shown in Figure 1, display the increase and diminution of inhibition, both of which approach a similar steady-state velocity confirming the

Table I. Results of Kinetic Studies for the Inhibition of Avian AIR Carboxylase by 3

	K_i (nM)	K_i^* (nM)	k_6 (s^{-1})	k_5 (s^{-1})
Lineweaver-Burk	1.3			
E_{total} vs I	1.6			
k_{obs} vs I[a]	1.4	0.34	8.8×10^{-3}	2.8×10^{-2}
k_p(complex)[b]	1.2	0.34	8.2×10^{-3}	2.0×10^{-2}

[a] Values derived from data where protein was added to initiate reactions in the presence of 2 and 3 under the conditions defined in the legend of Figure 1. Data were fitted to the integrated equation $k_p = v_s t + (v_o - v_s)(1 - e^{-kt})/k$ and the results were analyzed using the equations $k_6 = kv_s/v_o$ and $k_5/k_6 = K_i/K_i^* - 1$ according to the model.[14]

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

[b] Reactions were initiated by the addition of enzyme-3 complex (Figure 2) to a final concentration of 1 nM. Values were derived by fitting to the integrated equation above and the results were analyzed using $k_6 = kv_o/v_s$.

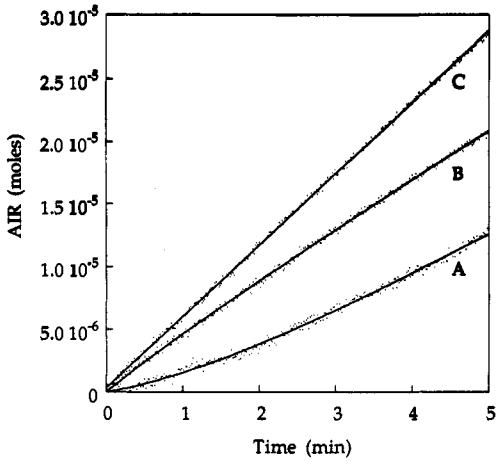


Figure 1. Progress curves for the decarboxylation of **2**. All assays were conducted at 30 °C, with 50 mM Tris-HCl, 0.5 mM EDTA, pH 8.0, in the presence of 200 μM **2** and 0.5 nM protein. Conversion of **2** to **1** was observed as a decrease in absorbance at 260 nm. Curve A, preincubation of enzyme with 4 nM **3** at 20 °C for 10 min before addition of **2**; curve B, addition of enzyme mixture to an assay mixture containing 4 nM **3**; curve C, addition of enzyme to a reaction in the absence of inhibitor.

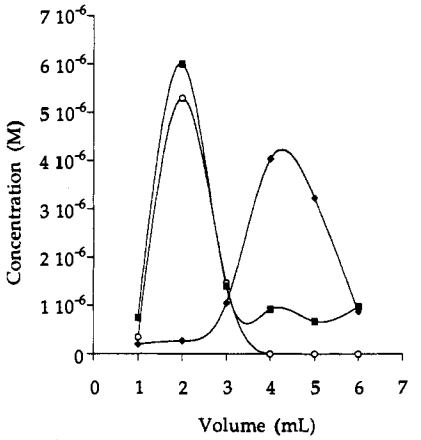


Figure 2. Isolation of AIR carboxylase-**3** complex by gel-filtration chromatography. Inhibitor **3** (10 nmol) and enzyme (10 nmol) were mixed in 1 mL of 50 mM Tris-HCl, 1 mM EDTA, pH 8.0, and incubated at 4 °C for 25 min. The sample was loaded onto a Sephadex G-25 column (1.3 cm × 2.7 cm) and eluted with the same buffer. Fractions (1 mL) were analyzed for protein[19] and inhibitor concentration using absorbance at 366 nm (pH 1.0, ϵ = 14, 700). (○) Concentration of avian AIR carboxylase (MW 47 245)[5] after incubation with **3**. (■) concentration of **3** with the identical incubation. Fraction 2 was used in the subsequent determination of k_p in Table I. (◆) Elution of **3** in the absence of enzyme.

reversible binding of **3**.[15] An independent estimate of the kinetic constants for inhibition was determined using a 1:1 enzyme–**3** complex which was isolated at 4 °C by gel-filtration chromatography (Figure 2). The stoichiometry of the complex was verified by the correlation of protein concentration with the unique absorbance at 366 nm due to **3**. Progress curve data for the complex in the presence of **2** are consistent with values obtained using varied amounts of inhibitor, as indicated in Table I.

The nature of the tight-binding complex between **3** and AIR carboxylase was determined by a combination of methods. Perturbations of the aromatic nucleus of **3** (λ_{max} 366 nm) were investigated in the enzyme-**3** complex by UV–vis spectrophotometry which indicated a +10-nm shift but no significant alteration of the extinction coefficient (Figure 3).[11,16] Compound **3** released from the enzyme–**3** complex and separated from the protein by centrifugal ultrafiltration was shown to have the same HPLC retention time as an authentic sample. Also, the protein recovered from incubation with a 100-fold excess of **3**, following extensive dialysis, was found to retain the same catalytic activity as a control sample that had not been incubated with **3**. These data are consistent with no alterations of the inhibitor or protein as a result of the formation of the enzyme–**3** complex.

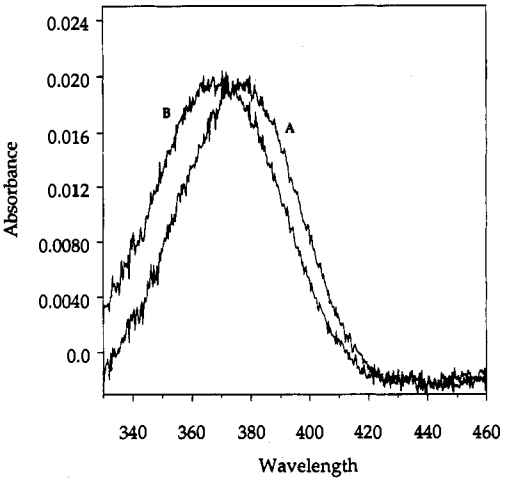
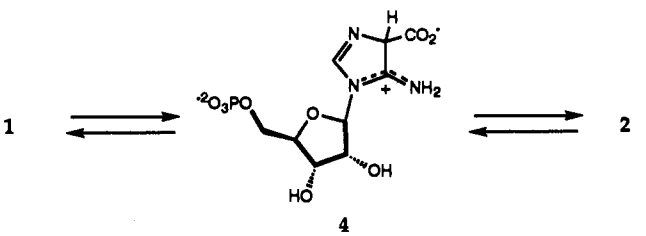


Figure 3. UV–vis spectra of **3** in the presence and absence of avian AIR carboxylase: (A) Inhibitor **3** was added to a cuvette containing 1.53 μM AIR carboxylase referenced against an equal molar concentration of enzyme, until no change in absorbance at 366 nm was observed (final spectrum); (B) Inhibitor **3** in the absence of enzyme at the concentration of the final spectrum in A (1.47 μM).

Enzyme substrate analogs that use an isosteric nitro substitution to mimic carboxylic acids have been described in a number of cases.[10] Several of these are tight-binding inhibitors that undergo enzyme-mediated cleavage of a critical carbon–hydrogen bond to result in nitronate mimics of reactive intermediates on the normal catalytic pathway.[17] In the case of **3**, the K_i is 10^4 less than the K_m for **2**, indicating that the inhibitor is not a simple substrate analog. Preliminary modeling suggests that the overall size and shape of **3** and **2** are comparable. The noncovalent nature of the enzyme–**3** complex and the slow, tight binding phenomenon suggest that **3** might be a mimic of a reactive intermediate or transition state.[18] Comparison of UV data for **3** and **2** indicate a difference of 100 nm for the absorbance maxima, consistent with extended conjugation in **3**, and as a consequence altered charge density at C-4. One plausible interpretation is that **3** is a mimic of a transition state related to a proton elimination from intermediate **4**. The intermediate **4** is tetrahedral at C4, and would result from direct carboxylation. Current studies on the details of the interaction of **3** with AIR carboxylase are focused on the mechanistic relationship of this inhibition to catalysis, and should provide a basis for further rational inhibitor design.



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Supplementary Material Available: The procedures for the synthesis and spectral characterization of **3** are available (4 pages). Ordering information is given on any current masthead page.

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