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ADENYLATE DEAMINASE: POTENT INHIBITION BY 2'-DEOXYCOFORMYCIN 5'-PHOSPHATE

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SUMMARY: The nucleotide 2'-deoxycoformycin 5'-phosphate has been enzymatically synthesized from 2'-deoxycoformycin and found to be a potent stoichiometric inhibitor of adenylate deaminase from rabbit muscle. It is shown that the inhibitor binds to the active site and may be considered as a possible transition state analog. The inhibition is time dependent which may reflect an inhibitor induced conformational change.

There are a number of enzymes which bind analogs of the substrate(s) with extremely high affinity (1-3). Some of these compounds are considered to be possible transition state analogs because they may mimic the structure of the transition state of the true substrate and as a consequence bind to the enzyme tightly. Since they are unable to be utilized as substrates, they serve as potent inhibitors for the enzymatic reaction. Coformycin and 2'-deoxycoformycin [(R)-3-(2-deoxy-β-D-erythro-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-d][1,3]diazepin-8-ol] have both been found to be potent inhibitors of adenosine deaminase (4,5). Since these compounds have a tetrahedral carbon at the position which normally is deaminated, it has been suggested that they are possible transition state inhibitors for adenosine deaminase.

Both coformycin and 2'-deoxycoformycin have also been tested as inhibitors of rabbit muscle adenylate deaminase and found to be strong non-competitive inhibitors with inhibition constants (approximately equal K_{is} and K_{ii} values) of about 5×10^{-8} and 3×10^{-6} M, respectively (6). Since coformycin and 2'-deoxycoformycin bind to adenosine deaminase with much greater affinity than to adenylate deaminase, it seemed likely that the 5'-phosphate derivative

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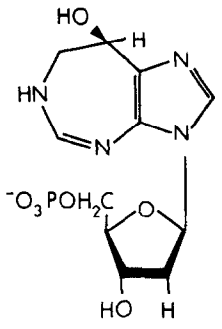


Fig. 1. 2'-deoxycoformycin 5'-phosphate.

would serve as a better inhibitor. For this reason, 2'-deoxycoformycin 5'-phosphate (Fig. 1) was synthesized. In this communication, we show that it is indeed a much more potent inhibitor of the adenylate deaminase than the corresponding nucleoside.

EXPERIMENTAL METHODS

Adenylate Deaminase - Adenylate deaminase was prepared from rabbit muscle as previously described (7). The enzyme concentration was determined from the absorbance at 280 nm using $A^{1\%}_{280} = 9.13$ (8). The molecular weight was taken to be 280,000 (8) and the enzyme is considered to contain four identical subunits (9). Enzyme was stored in 0.5 M KCl at pH 6.5 in 0.01 M imidazole/HCl buffer containing 1 mM β-mercaptoethanol. For use in the experiments described here, this enzyme was dialyzed against 0.15 M KCl at pH 6.5 in 0.01 M imidazole/HCl buffer. Activity measurements were performed in 0.15 M KCl, 0.01 M imidazole/HCl buffer, pH 6.5, using 100 μM AMP. The absorbance change was followed spectrophotometrically at 265 nm where the molar extinction coefficient difference between AMP and IMP is 8.86×10^3.

Chemicals - 2'-Deoxycoformycin was obtained from the Developmental Therapeutics Program, Chemotherapy, National Cancer Institute. 3-Iso-AMP (3-β-D-ribofuranosyladenine) 5'-phosphate was synthesized from 3-isoadenosine using method B as described by Leonard and Laursen (10). We are indebted to Dr. Linda Kurz for the synthesis of the 3-isoadenosine and 3-iso-AMP.

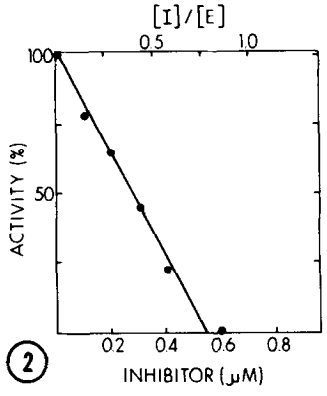
2'-Deoxycoformycin 5'-phosphate was enzymatically synthesized from 2'-deoxycoformycin using a preparation of specific 5'-nucleoside phosphotransferase from Serratia marcescens (11). The reaction mixture (6 ml; 37°) contained 20 mM 2'-deoxycoformycin, 100 mM p-nitrophenylphosphate, 100 mM sodium acetate, pH 5.4, and 9.5 mg of the phosphotransferase preparation. After 16 hr, the reaction was extracted with 12 ml of diethylether. The resulting aqueous phase, after being adjusted to pH 8 with NaOH, was centrifuged at 20,000 x g for 15 min and applied to a column containing 20 ml of Bio-Rad AG1X2 resin which had been equilibrated with 50 mM ammonium bicarbonate. The compound was eluted with a 400 ml linear gradient (50 mM to 500 mM) of ammonium bicarbonate. Fractions containing the 2'-deoxycoformycin 5'-phosphate (6.5 mg, 14% yield) were pooled and stored at -70°. This material was used without further purification.

The 2'-deoxycoformycin 5'-phosphate prepared in this way showed a single symmetrical peak which was eluted in the nucleoside monophosphate region on ion exchange high pressure liquid chromatography (12); a single spot on cellulose thin layer chromatography developed in n-propanol/15 M NH_4OH/H_2O (6:3:1) (R_f of 2'-deoxycoformycin 5'-phosphate = 0.26; R_f of 2-deoxycoformycin = 0.70); and the same uv spectrum as 2'-deoxycoformycin. In addition, the compound was hydrolyzed rapidly to 2'-deoxycoformycin (as determined by thin layer chromatography) by E. coli alkaline phosphatase and slowly by 5'-nucleotidase from Crotalus adamanteus.

RESULTS AND DISCUSSION

Experiments to measure the stoichiometry and the time dependence of inhibition by 2'-deoxycoformycin 5'-phosphate were performed by incubating enzyme with inhibitor at 25° in 0.15 M KCl, 0.01 M imidazole buffer, pH 6.5, for a given period of time and then measuring the activity of a dilution from the incubation mixture. Fig. 2 shows the results of such an experiment when the incubation mixture contained 50 μg/ml (0.74 μM) adenylate deaminase and varying amounts of 2'-deoxycoformycin 5'-phosphate. The mixture was allowed to incubate 15 min before assaying enzymatic activity. Inhibition was complete at a ratio of 0.77:1 for inhibitor to subunit concentration. While this result is slightly lower than expected, it appears reasonable that the inhibition is stoichiometric and that each of the four subunits in the active enzyme contains a single binding site for the inhibitor.

Fig. 3 (lower curve) shows the time dependence of inhibition by 2'-deoxycoformycin 5'-phosphate at levels which are slightly greater than stoichiometric (1.2:1). In these experiments, 0.14 μM of inhibitor and 0.12 μM enzyme were incubated at 23° and, at timed intervals, the activity was measured with an aliquot of the mixture diluted 100-fold into the assay mixture. The loss of activity did not follow first order kinetics since the concentration of inhibitor was not present in large excess of that of the enzyme. Enzyme in the absence of inhibitor loses little activity over the time course of the experiment (upper curve). Fig. 3 also shows (middle curve) the protection against the 2'-deoxycoformycin 5'-phosphate inhibition by iso-AMP, a competitive inhibitor for the substrate AMP (8,13).



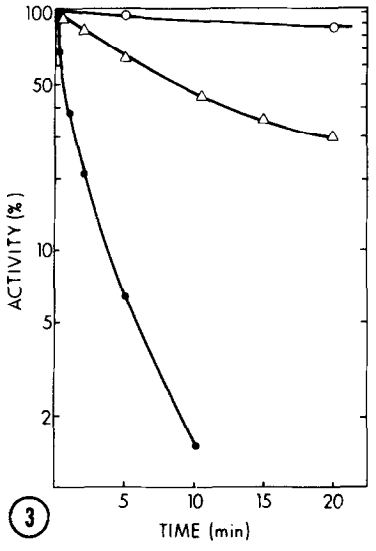


Fig. 2. Stoichiometry of inhibition by 2'-deoxycoformycin 5'-phosphate. Adenylate deaminase (0.7 μM based on a subunit molecular weight of 70,000) and 2'-deoxycoformycin 5'-phosphate were incubated for 15 minutes in 0.01 M imidazole buffer, 0.15 M KCl, pH 6.5, at 25°. Activity was measured by dilution of an aliquot into the assay mixture.

Fig. 3. Time course of inhibition by 2'-deoxycoformycin 5'-phosphate. Enzyme (0.12 μM) and inhibitor (0.14 μM) were incubated in 0.01 M imidazole buffer, 0.15 M KCl, pH 6.5, at 23°. The lower curve (-●-) is the time course in the presence of the inhibitor; the middle curve (-Δ-) in the presence of inhibitor and iso-AMP, and the upper curve (-○-) is in the absence of inhibitor or iso-AMP.

The inhibition by 2'-deoxycoformycin 5'-phosphate may be simulated by a mechanism of the type

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \qquad (1)$$

where the best fit of the data is given by a value of 9×10^4 sec^{-1} $moles^{-1}$ for k_1 assuming that k_{-1} is zero. This value is much lower than that expected for a diffusion controlled second order rate constant for a small molecule binding to a protein (14). Therefore, it is likely that the mechanism of inhibition is

$$E + I \rightleftharpoons EI \rightleftharpoons E'I \qquad (2)$$

with the time dependence representing an inhibitor-induced conformational change.

The possibility existed that the binding of the inhibitor is irreversible due to covalent modification. This was examined in the following way. Approximately equal amounts of enzyme and inhibitor were preincubated until complete inhibition was established. When more enzyme was added to this mixture, it was not inhibited, indicating that all the inhibitor was bound. This solution and an identical one containing inhibitor with no enzyme were boiled for 2 min and rapidly cooled to 25°. Addition of a stoichiometric amount of fresh enzyme to the boiled solutions resulted in 70-80% inhibition of the enzyme in both cases. Thus denaturation of the enzyme by boiling released the inhibitor. Similar results were obtained when the enzyme solution was raised to pH 12 for a few minutes. These experiments seem inconsistent with any covalent modification by the inhibitor. Presumably, therefore, the inhibitor is bound tightly, albeit reversibly to adenylate deaminase. Preliminary experiments measuring the rate of recovery of enzymatic activity after dilution of a 1:1 enzyme-inhibitor complex indicate a value between 10^{-9} and 10^{-10} M for the overall inhibition constant. It should be noted that coformycin is a stronger inhibitor of the adenylate deaminase than 2'-deoxycoformycin (6). It therefore might be expected that coformycin 5'-phosphate would be an even more potent inhibitor of this enzyme than is 2'-deoxycoformycin 5'-phosphate.

Adenylate deaminase is a highly regulated enzyme whose activity is influenced by a number of metabolites which bind to sites distinct from the active site (15). In spite of that, the physiological role of the enzyme is not understood. The finding that 2'-deoxycoformycin 5'-phosphate is a potent inhibitor of the enzyme may help to clarify this role as well as questions relating to the mechanism of deamination.

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