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Adenine aminohydrolase: Occurrence and possible significance in trypanosomid flagellates

(*Crithidia*/*Leishmania*/coformycin/deoxycoformycin)

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ABSTRACT **Adenine aminohydrolase (EC 3.5.4.2) from four species of *Leishmania* and from *Crithidia fasciculata* was examined for specific activities, affinity for substrate (adenine), and stability to heat. All were found to be strongly and noncompetitively inhibited by both coformycin and deoxycoformycin, two tight-binding inhibitors of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4). Deoxycoformycin is the more potent inhibitor of the two. Neither inhibitor was active against the purine phosphoribosyltransferases. When deoxycoformycin was added to the defined growth medium containing hypoxanthine as the purine source, the growth of *C. fasciculata* was unaffected, but when adenine was the purine source for the organism, severe inhibition resulted. This implies that hypoxanthine is the obligatory base for nucleotide synthesis and that the adenine phosphoribosyltransferase (AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) is, in some manner, denied access to exogenous substrate.**

Since our earlier report (1) on the occurrence and properties of adenine aminohydrolase (EC 3.5.4.2) (adenine deaminase) in the trypanosomid flagellate *Crithidia fasciculata*, the enzyme has been reported from *Leishmania tropica* (2), *L. donovani*, and *L. braziliensis* (3). We have extended our investigations of this somewhat unusual enzyme and have found it to be present also in *L. tarentolae* and *L. mexicana*, and have shown that it is subject to drastic inhibition by two tight-binding adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) inhibitors, coformycin and deoxycoformycin. By the use of these potent inhibitors, it has been possible to assess to some degree the role of the enzyme as a control mechanism in these purine-requiring organisms.

MATERIALS AND METHODS

[8-^{14}C]Adenine and [8-^{14}C]hypoxanthine (both at 50 Ci/mmol, 1 Ci = 3.70×10^{10} becquerels) were obtained from Amersham/Searle. All chemicals of the defined medium used for *C. fasciculata* were obtained from Sigma; the ingredients of the semi-defined medium of Berens *et al.* (4) were obtained from GIBCO. Generous samples of coformycin (3-β-D-ribofuranosyl-6,7,8-trihydroimidazo[3,4-*d*][1,3]diazapin-8-(*R*)-ol) and 2′-deoxycoformycin were a gift from H. W. Dion (Warner-Lambert/Parke-Davis).

C. fasciculata, a mosquito parasite, is the strain that has been used in this laboratory for the past 20 years (5–6). *L. tarentolae*, a lizard parasite, was obtained through the courtesy of Larry Simpson (University of California, Los Angeles). This is the strain originally used by Trager (7). *L. donovani*, *L. braziliensis*, and *L. mexicana*, all human pathogens, were kindly supplied by J. J. Marr (St. Louis University). These strains have been used by Marr and his associates for the past several years (3).

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C. fasciculata was grown in the defined medium of Kidder and Dutta (5), in low profile flasks when large amounts of tissue were needed or in side-arm Nephelo flasks (Bellco, Vineland, NJ) or 25 × 125 mm optically clear tubes for the growth studies. Growth was followed by reading optical densities at 650 nm, with uninoculated medium as the standard. In all cases hemin, glucose, and inhibitors (if any) were filter sterilized and added to the bulk of the heat-sterilized medium.

L. tarentolae was grown in low profile flasks in heat-sterilized Beef-Heart Infusion (Difco) to which filter-sterilized hemin (6 μg/ml) had been added or in the filter-sterilized HOSMEM medium of Berens *et al.* (4) containing 8% fetal calf serum.

The other three species of *Leishmania* were grown in disposable 1-liter plastic flasks in the HOSMEM medium, in an atmosphere of 5% CO_2/95% air. In our hands the somewhat more defined medium of Berens and Marr (8), which contains no fetal calf serum, produced such low yields as to make it entirely impractical.

Cell extracts were prepared from harvested, washed cells (Sorvall sealed assemblies were used on 250-ml bottles for the first sedimentation), as has been described (9). After sonification, the cell debris was removed by centrifugation at 40,000 × *g* for 1 hr and the extracts were used without further treatment except for dilution, to equalize protein concentration, with 10 mM phosphate buffer containing 20 mM 2-mercaptoethanol at pH 7.5. When the human pathogens were being used, all necessary manipulations were carried out under a BIOGARD (Baker) hood.

Coformycin and deoxycoformycin, being labile at low pH, were dissolved in sodium phosphate buffer at pH 8.0 and used within 3–4 days, being stored in the meantime at −20°C.

Enzyme activities were determined by paper chromatography or by paper electrophoresis as has been described (1) after incubations of reactants with 8-^{14}C-labeled substrate. When inhibitors were used, they were added to the enzyme/buffer solution at pH 8 and preincubated for 10 min at 22°C. After equilibrium was reached at 35°C, the radioactive substrate was added. The reaction was stopped at the appropriate time by addition of a drop of glacial acetic acid and the reaction mixture was streaked on Whatman no. 1 paper for descending chromatography or for electrophoretic separation. Radioactive peaks were located with the aid of a Tracerlab 4π scanner and identified by cochromatographs of authentic samples. Quantitation was accomplished by determining the areas under the peaks by planimetry.

RESULTS

The amounts and activities of the adenine deaminases vary with the organism, with the specific activity of the *Crithidia* enzyme being the highest and *L. braziliensis* the lowest (Table 1). These activities were determined with preparations all prepared in the same manner and adjusted to approximately equal protein content. Although *L. braziliensis* produces far less of the en-

Table 1. Kinetic data on the adenine deaminases of a number of trypanosomid flagellates

Enzyme source	Specific activity*	K_m†	Inhibitors, mM	
			Coformycin‡	Deoxycoformycin‡
C. fasciculata	0.71	1.0–0.8	6.2×10^{-4}	7.4×10^{-6}
L. tarentolae	0.58	1.1–1.4	6.7×10^{-3}	5.8×10^{-5}
L. donovani	0.64	2.0–2.2	7.6×10^{-4}	1.5×10^{-5}
L. mexicana	0.22	1.7–2.0	1.7×10^{-3}	2.6×10^{-5}
L. braziliensis	0.012	0.7–0.8	4.0×10^{-3}	4.2×10^{-5}

* Micromoles of product per min per mg of protein of the supernatants of cell sonicates after centrifugation at 40,000 × *g* for 1 hr. Amounts of protein in the reaction tubes were adjusted to approximate equivalence.

† Determined from Lineweaver–Burk plots with substrate (adenine) concentrations from 5 to 70 nmol/tube (0.11 ml).

‡ Amount (mM) of inhibitor required for half-maximal inhibition.

zyme than the other organisms, its affinity for the substrate is highest (K_m 0.7–0.8, Table 1).

As was reported earlier (1), the *Crithidia* adenine deaminase is quite stable to heat. The enzymes from the *Leishmania* are heat labile; 95% of the activity is lost after holding the preparations at 50°C for 1 min. Storage at 4°C of the *Crithidia* and *L. tarentolae* enzymes results in minimal losses even after 1–2 weeks whereas the enzymes from human pathogens rapidly lose activity upon storage.

All of the adenine deaminases reported here act optimally over a rather broad and high pH range. For that reason and because the inhibitors used are most stable at high pH, the enzymatic reactions were all run at pH 8.0.

The most interesting finding, and the most unexpected, is that the tight-binding inhibitors of adenosine deaminase, coformycin and deoxycoformycin (Fig. 1), also inhibit the five trypanosomid adenine deaminases. These inhibitors have been extensively studied, and there are vast numbers of reports on them in both the nonclinical and clinical literature. An excellent summary of the subject has been published recently (10). It has been pointed out that calculation errors of inhibitory activities may occur with tight-binding, noncompetitive compounds of this type and that results may best be appreciated, as a first approximation, by reporting the amounts required to bring about half-maximal inhibition (I_{50}) rather than K_i (10). We have used this approach; the results are presented in Table 1. The enzyme from *C. fasciculata* is from approximately 2-fold to 10-fold more sensitive to coformycin than those from the *Leishmania*, and from 2-fold to 8-fold more sensitive to deoxycoformycin. In all cases the amounts were calculated from reaction mixtures in which the inhibitor had been preincubated for 10 min with the enzyme and buffer before addition of the substrate (adenine). The amount of substrate was at a sufficient level not to be rate limiting. When the amount of substrate was lowered (20 nmol/tube) so that it was deaminated to hypoxanthine completely within the 5-min period of incubation by the more active enzymes (those from *C. fasciculata* and *L. donovani*) and when only one concentration each of coformycin and deoxycoformycin was used in the preincubation, comparisons of inhibitory actions can be seen at a glance (Fig. 2). The data for *C. fasciculata* were omitted because they closely resemble those for *L. donovani* (Fig. 2*B*). Deoxycoformycin approaches or exceeds 100 times the activity of coformycin under these conditions. These differences between the activities of the two compounds against adenine deaminase are greater than those found with the adenosine deaminases (10).

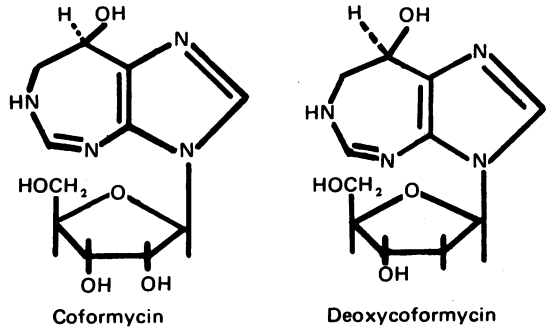

FIG. 1. Structures of inhibitors.

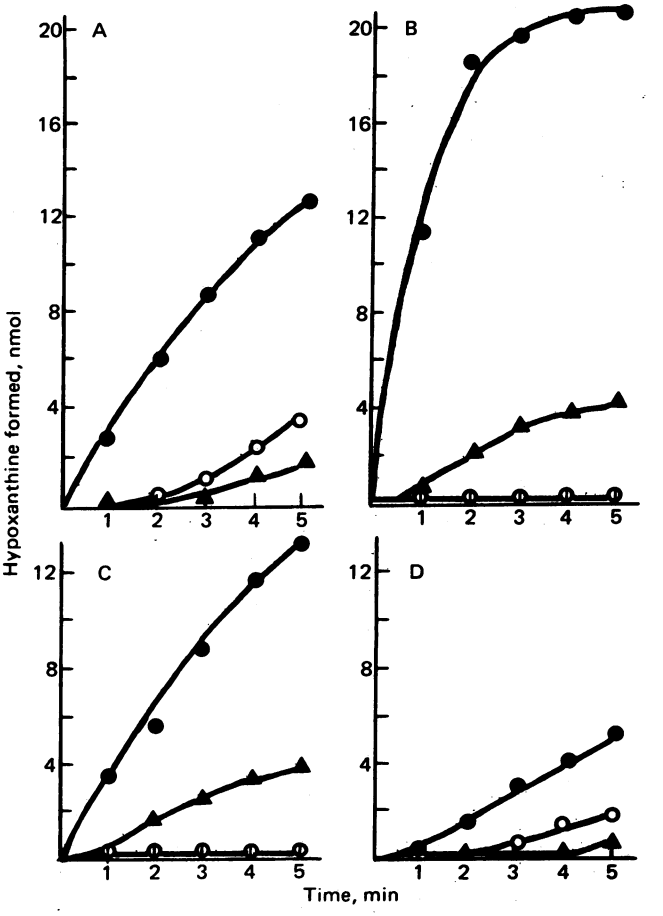

FIG. 2. Time course of inhibition of adenine deaminases from four species of *Leishmania*: (*A*) *L. tarentolae*; (*B*) *L. donovani*; (*C*) *L. mexicana*; (*D*) *L. braziliensis*. ●, Control (no inhibitor); ○, coformycin (1.5×10^{-3} mM for *A*, 1.3×10^{-2} mM for all others); ▲, deoxycoformycin (2.9×10^{-5} mM for *A*, 1.3×10^{-4} mM for all others). Protein in all enzyme preparations was approximately 10 μg/tube, preincubated with inhibitor for 10 min at 22°C.

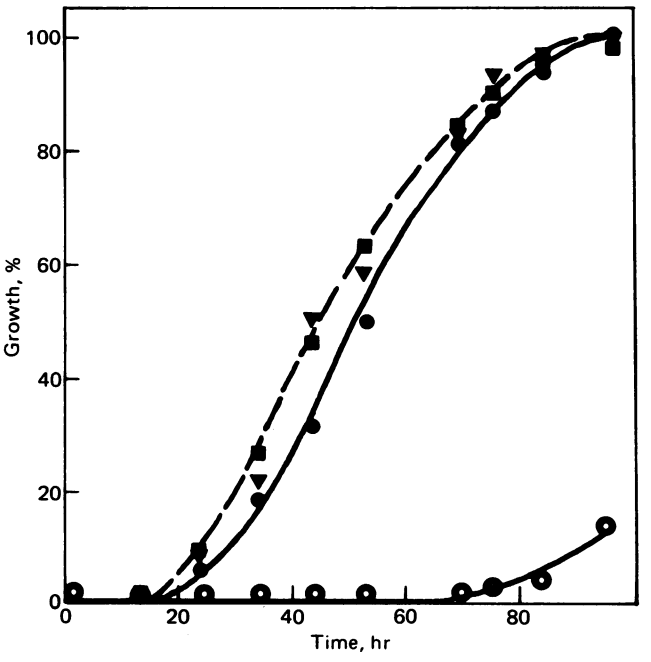

FIG. 3. Growth of *C. fasciculata* in defined medium plus 8 μg of hypoxanthine per ml (▲, – –); 8 μg of hypoxanthine per ml plus 0.05 mM deoxycoformycin (■); 8 μg of adenine per ml (●, —); 8 μg of adenine per ml plus 0.05 mM deoxycoformycin (○).

In order to evaluate the consequences of adenine deaminase inhibition as a function of the growth of whole cells, we studied *C. fasciculata* in the defined medium of Kidder and Dutta (5) with either adenine or hypoxanthine as the sole purine source and with and without deoxycoformycin. Growth is almost completely inhibited during the earlier phase, but only in the adenine-containing medium (Fig. 3). These inhibited cells "recover" to some extent, but never reach the level of the controls over the 4-day period.

DISCUSSION

There is a striking similarity between adenosine deaminase and adenine deaminase as to their affinities for coformycin and deoxycoformycin. There is a growing conviction that the cells containing adenosine deaminase build up a protection against excess adenine nucleotides (10). Some such regulator mechanism in those cells possessing only adenine deaminase therefore seems likely. We are unaware of any cell that has been shown to have both enzymes. Only adenine deaminase occurs in the trypanosomids we have studied, but we have also found it to be present in dark-grown *Euglena gracilis* (unpublished data) so that it may be a characteristic of flagellates in general and not confined to trypanosomids, as we recently suggested (12).

The use of deoxycoformycin in the growth studies has made it possible to formulate one explanation for the importance of adenine deaminase in the economy of these purine-dependent cells. Adenine and hypoxanthine are equal in growth-promoting activity when added as such to a purine-free medium. However, if the cells' ability to deaminate adenine to hypoxanthine is blocked, then adenine becomes virtually useless. This can only be interpreted as meaning that of the two bases, hypoxanthine is the obligatory one for nucleotide synthesis.

If, of course, these organisms were deficient in adenine phosphoribosyltransferase (EC 2.4.2.7), a ready answer would be at hand. We have examined the problem of the purine phosphoribosyltransferases in some detail and found that, in *C. fasciculata*, adenine phosphoribosyltransferase is present and active (12). In addition, neither coformycin (1 mM) nor deoxycoformycin (1 mM) is the least inhibitory to any of the purine phosphoribosyltransferases when tested *in vitro*. Preliminary tests indicate that the inhibitors do not affect the transport (5 sec) system (11) of either *Crithidia* or *L. tarentolae*, but more work will have to be done on this subject before one can be certain of this point. At present, it seems that we have the possibility of either the *in vivo* (in contrast to the *in vitro*) blockage of the adenine phosphoribosyltransferase of *Crithidia* by deoxycoformycin or "compartmentation" of the enzyme, which, in some manner, never becomes exposed to adenine transported into the cell. At the same time we must recognize that no such compartmentation exists for hypoxanthine phosphoribosyltransferase.

One possible explanation for the "recovery" of inhibited cells after a period of hours in contact with the inhibitor is the lability of the inhibitor at 25°C. Although the initial pH of the growth medium is 8.0, this drops significantly if there is appreciable cell multiplication. With the breakdown of inhibitor to below effective levels and the synthesis of fresh adenine deaminase toward the end of the 96-hr growth period, there may be little inhibitor left.

The surprising finding that nucleosides (coformycin and deoxycoformycin) will bind tightly to the active site of a purine base-deaminating enzyme, as well as to adenosine deaminase, raises questions regarding the role of the sugar moiety of the inhibitors. That the sugar is, in some manner, taking part in the binding to the adenine active site is evident from the differences in inhibitory activity of the two compounds. A direct test will have to await the availability of the aglycone.

The similarities of the adenine deaminases of the trypanosomid flagellates and the contrast between these cells and those of their hosts (which contain adenosine deaminase) may at some time be used to advantage in the chemotherapy of these important pathogens.

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1. Kidder, G. W., Dewey, V. C. & Nolan, L. L. (1977) *Arch. Biochem. Biophys.* **183**, 7–12.
2. Königk, E. & Rascul, S. A. (1978) *Tropenmed. Parasitol.* **28**, 319–322.
3. Marr, J. J., Berens, R. L. & Nelson, D. J. (1978) *Biochim. Biophys. Acta* **544**, 360–371.
4. Berens, R. L., Brun, R. & Krassner, S. M. (1976) *J. Parasitol.* **62**, 360–365.
5. Kidder, G. W. & Dutta, B. N. (1958) *J. Gen. Microbiol.* **18**, 621–638.
6. Kidder, G. W., vol. ed. (1967) in *Chemical Zoology*, eds. Florkin, M. & Scheer, B. (Academic, New York), Vol. 1, pp. 93–159.
7. Trager, W. (1957) *J. Protozool.* **4**, 269–276.
8. Berens, R. L. & Marr, J. J. (1978) *J. Parasitol.* **64**, 160.
9. Dewey, V. C., Kidder, G. W. & Nolan, L. L. (1973) *Arch. Biochem. Biophys.* **157**, 380–387.
10. Agarwal, R. R., Cha, S., Crabtree, G. W. & Parks, R. E., Jr. (1978) in *Chemical and Biological Nucleosides and Nucleotides*, eds. Harmon, R. E., Robins, R. K. & Townsend, L. B. (Academic, New York), pp. 159–198.
11. Kidder, G. W., Dewey, V. C. & Nolan, L. L. (1978) *J. Cell Physiol.* **96**, 165–170.
12. Kidder, G. W., Nolan, L. L. & Dewey, V. C. (1979) *J. Parasitol.*, in press.