Properties of 5′-AMP Deaminase and its Inhibitors with the Aid of a Continuous Fluorimetric Assay with Formycin-5′-phosphate as Substrate

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A new continuous fluorimetric assay for AMP deaminase activity is described. The method makes use of a fluorescent analog of 5′-AMP, formycin-5′-phosphate (5′-FMP), which undergoes deamination to formycin B-5′-phosphate, not fluorescent at neutral pH. The pH-dependence for deamination of 5′-FMP is similar to that for 5′-AMP, but shifted about 0.2 units to more acidic pH. Deamination of 5′-FMP may also be followed spectrophotometrically at 306 nm, permitting better assays of crude extracts. Some kinetic results obtained by means of the new method for AMP deaminase from chick and rabbit skeletal muscle are presented. In particular it was found that the natural product of deamination, 5′-IMP exhibited allosteric inhibition of the chick enzyme with K_i values 1.6 mM, 1.2 mM and 1.0 mM at pH 5.8, 6.5 and 7.3, respectively. Activation by diadenosine tetraphosphate, Ap_4A, reported for mouse muscle AMP deaminase, has not been noted for the chick enzyme. Inhibition by the transition state analogs, coformycin and 2′-deoxycoformycin, was observed for both rabbit and chick deaminases with K_i values ~ 1 μM and ~ 1.6 μM respectively. Kinetic data for coformycin-5′-phosphate show it to be a tight-binding inhibitor with $K_i < 0.6 \times 10^{-9}$ M as compared to 1×10^{-9} M for 2′-deoxycoformycin-5′-phosphate.

Introduction

AMP deaminase (EC 3.5.4.6) is a cytoplasmic enzyme which catalyzes irreversible hydrolytic deamination of AMP to generate IMP and ammonia. The enzyme is the point of entry of AMP into the purine nucleotide cycle and is widely distributed among mammalian cells; in skeletal muscles it is present at a high concentration [1]. Although the physiological function of the enzyme remains unclear, it has been found to play a regulatory role in various cellular processes such as: regulation of intracellular nucleotide pools [2], stabilization of adenylate energy charge [3], and control of glycolysis [4, 5]. Moreover it has been noted that a marked reduction of activity is associated with human Duchenne dystrophy [6] and unknown forms of myotonia [7], and is also observed in the muscles of dystrophic animals [8, 9].

We now present a new continuous fluorimetric assay for AMP deaminase based on deamination of the fluorescent formycin A-5′-phosphate (a close structural analogue of 5′-AMP) to formycin B-5′-phosphate (a close structural analogue of 5′-IMP), which is not fluorescent at neutral pH. Inhibition constants for transition state analogs, coformycin and 2′-deoxycoformycin, against chick and rabbit skeletal muscle AMP deaminases, obtained by means of the new assay, are compared with literature results based on traditional methods. Other kinetic properties of both enzymes, including product inhibition by 5′-IMP, hitherto not reported, are described. Tight-binding inhibition by coformycin-5′-phosphate, and associated kinetic data, are also reported.

Materials

Chick skeletal muscle adenylate deaminase, partially purified as described elsewhere [10–12], with a specific activity of 15 U/mg, under standard conditions (described below) at 25 °C and at a substrate concentration 70 μM, was a kind gift of Dr. W. Makarewicz (Institute of Biochemistry, Medical

Abbreviations: F, formycin A or simply formycin; 5′-FMP, formycin-5′-phosphate; FB, formycin B; 5′-FBMP, formycin B-5′-phosphate; CF, coformycin; 2′-DCF, 2′-deoxycoformycin; CF-5′-MP, coformycin-5′-phosphate; 2′-DCF-5′-MP, 2′-deoxycoformycin-5′-phosphate; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; Ap_2A, adenosine(5′)diphospho(5′)adenosine, with similar connotations for Ap_3A and Ap_4A; DDT, dithiothreitol.

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School, Gdansk, Poland). The corresponding enzyme from rabbit muscle, obtained from Sigma (St. Louis, Mo., U.S.A.) had a specific activity of 41 U/mg under the same conditions.

5′-AMP and 5′-IMP were products of Waldhof (Stuttgart, F.R.G.). Formycin A was obtained from Meiji Saika Kaishi (Kawasaki, Japan), formycin B from Calbiochem (Zurich, Switzerland), sodium cacodylate from BDH (Poole, U.K.), dithiothreitol from Serva (Heidelberg, F.R.G.). Coformycin and 2′-deoxycoformycin were generous gifts of Dr. V. Narayanan of the National Cancer Institute (Bethesda, Md., U.S.A.).

The 5′-phosphates of formycin, formycin B, coformycin and 2′-deoxycoformycin were prepared by phosphorylation of the parent nucleosides essentially by the method of Yoshikawa *et al.* [13], as described elsewhere [14, 15].

Methods

Ultra-violet absorption spectra were run on Zeiss (Jena, G.D.R) UV-VIS and Specord UV-VIS spectrophotometers.

Emission spectra and fluorescence intensities were recorded on an Aminco-Bowman SPF spectrofluorimeter, fitted with a Hanovia 901 xenon lamp as the source, and a Hammamatsu 1P28 photomultiplier as detector. Emission spectra are non-corrected.

Measurements of pH were performed with a Radelkis (Budapest, Hungary) type OD-201 digital pH-meter. For measurements on small samples (~ 3 ml), a Mera-Elwro (Wrocław, Poland) type N-517 instrument was employed.

Adenylate deaminase activity was monitored in an incubation medium of 2–3 ml containing 50 mM cacodylate buffer pH 6.5 (unless otherwise indicated), 100 mM KCl, 2.5 mM dithiothreitol, and substrate, inhibitor and enzyme concentrations as indicated. Because of the known lability of the enzyme (see below), incubation parameters were selected so that observations of activity were limited to 30 min at 25 °C, during which activity of a control decreased by 10% at most. Enzyme activity was followed by two procedures:

Spectrophotometrically, by classical methods [16], based on the difference in extinction between substrate and product. For substrate concentrations below 100 μM in 10-mm pathlength cuvettes, the reaction was followed at 265 nm with 5′-AMP as substrate ($\Delta\epsilon = 8.9 \times 10^3$), and 306 nm with 5′-FMP as a substrate ($\Delta\epsilon = 6.6 \times 10^3$). With higher initial substrate concentrations, 2-mm pathlength cuvettes were employed, and the wavelength of observation selected so that the initial optical density did not exceed 1.

The deamination reaction was usually followed to completion. With substrate concentrations of the order of K_m or more, this made possible evaluation of K_m^{app} without the use of a series of measurements on samples with various initial substrate concentrations. With lower substrate concentrations, it is possible to measure the pseudo first-order rate constant, V_{max}/K_m, since K_m appreciably exceeds the initial substrate concentration.

Spectrofluorimetrically, with 5′-FMP as substrate, permitting of fairly accurate (± 5%) determinations of reaction rates with substrate concentrations in the range 1–100 μM. The excitation wavelength was selected so that the initial optical density at $\lambda = \lambda_{exc}$ did not exceed 0.3 (substrate concentration ≤ 100 μM). For substrate concentrations ≤ 35 μM, $\lambda_{exc} = 306$ nm; for concentrations of 35–100 μM, $\lambda_{exc} = 318$ nm. Fluorescence emission was followed at 355 nm, close to the maximum of the emission band (see below), but sufficiently displaced from λ_{exc} to avoid Raman scattering.

Analysis of kinetic data

Time-dependent decreases in substrate concentration during the entire course of deamination were calculated from changes in optical density, with 5′-AMP and 5′-FMP as substrates, or from the decrease in fluorescence intensity with 5′-FMP as substrate. For the latter method, when the optical density at λ_{exc} exceeded 0.05 (under which conditions the fluorescence intensity is not linearly proportional to concentration of the fluorescent substrate), an appropriate correction was made for the inner-filter effect [17], with the aid of an iterative procedure elsewhere described [14, 18]. This made possible measurements of concentrations of 5′-FMP to an accuracy of 1% over the concentration range 1–100 μM.

The kinetic parameters, K_m^{app} and V_{max}^{app}, were calculated with the use of the method of least squares, from the equation:

$$t(S) = (K_m^{app}/V_{max}^{app})\ln(S_o/S) + \\ + (S_o/V_{max}^{app})(1 - S/S_o) \quad (1)$$

where S_o is initial substrate concentration, and S is the concentration at time t.

In those instances where substrate concentrations were much lower than the value of K_m (pseudo first-order reactions), the equation employed was:

$$S(t) = S_o \exp(-t\, V_{max}^{app}/K_m^{app}) \quad (2)$$

Both of these are simply the integrated forms of the Michaelis equation [19]. These calculations, as well as those for inhibition constants, K_i^{app} (see below), were carried out on a Minc 11 minicomputer with an RT 11 system, using a program described elsewhere [20].

Inhibition constants, K_i^{app}, were measured at low substrate concentrations ($S_o \ll K_m$), so that the pseudo first-order rate constant of Eqn. (2) becomes $V_{max}/K_m(1 + I/K_i^{app})$, where I is the inhibitor concentration. Under these conditions, the calculated value of $K_{iapp} = K_i$ for both competitive and true non-competitive inhibition, but does not distinguish between the two.

The foregoing procedure may, on the other hand, distinguish more complex mechanisms of inhibition, *e.g.* allosteric regulation, where K_i^{app} is dependent on the inhibitor concentration I, as follows:

$$K_i^{app} = K_i \frac{1 + (I\, k_2\, K_s)/(K_i\, k_i\, K_{si})}{1 - (k_2\, K_s)/(k_1\, K_{si})} \quad (3)$$

where K_s and K_{si} are dissociation constants of enzyme-substrate and enzyme-substrate-inhibitor complexes, and k_1 and k_2 are the turnover constants for these complexes.

Results and Discussion

Spectral properties of 5′-FMP

The absorption spectra of 5′-FMP, and its deamination product 5′-FBMP, are exhibited in Fig. 1, upper panel. It will be noted that the largest difference in extinction between the two is at 306 nm, with $\Delta\epsilon = 6.6\ 10^3\ M^{-1}\ cm^{-1}$, somewhat lower than the maximal difference between the absorption spectra of 5′-AMP and its deamination product 5′-IMP, $\Delta\epsilon = 8.9\ 10^3$ at 265 nm. It is, however, more than adequate for following deamination of 5′-FMP spectrophotometrically, as for 5′-AMP [16] and, furthermore, possesses the additional marked advantage that the reaction may be followed at 306 nm, where absorption of proteins and nucleic acids is low, permitting better assays of crude extracts.

The differences in emission spectra are much more pronounced, as displayed in Fig. 1, lower panel. The

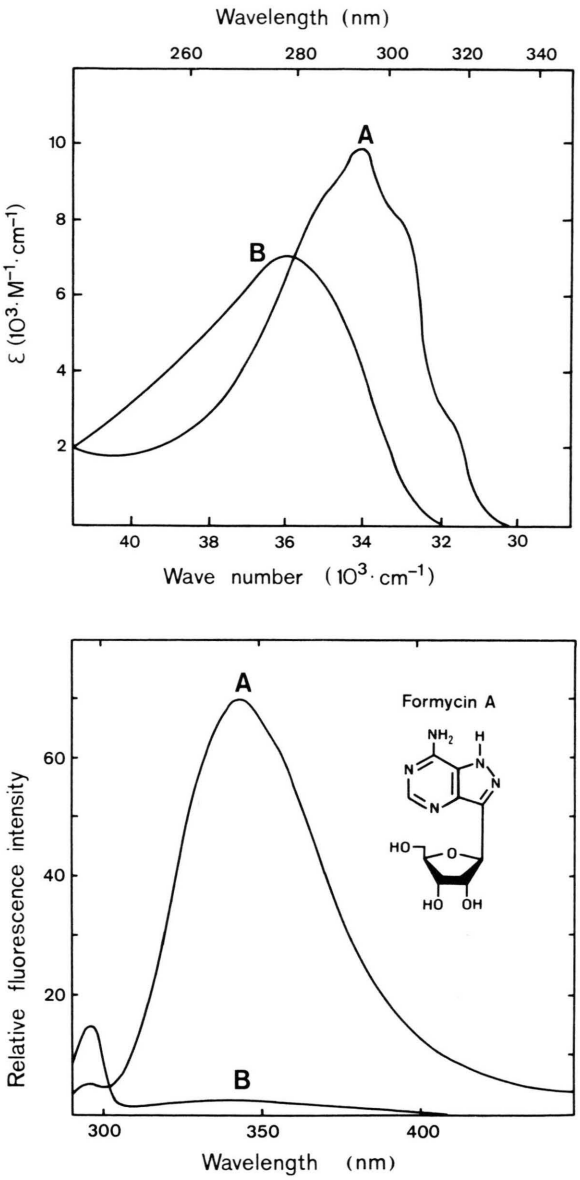

Fig. 1. Absorption (upper panel) and emission (lower panel) spectra of 5′-FMP at pH 6.5 prior to (A) and following (B) complete deamination to 5′-FBMP by chick skeletal muscle AMP deaminase as described in Materials and Methods. For the emission spectra $\lambda_{exc} = 295$ nm.

fluorescence quantum yield of 5′-FMP is approximately $\phi = 0.048$ [21] and 5′-FBMP is not fluorescent at neutral pH; hence following deamination, fluorescence intensity decreases to background, and is adequate to follow the concentration of 5′-FMP during the course of deamination to an accuracy of 1%. The

sensitivity of the fluorimetric procedure permits the use of substrate concentrations as low as 1 μM in standard 10-mm cuvettes.

Substrate properties of 5′-AMP and 5′-FMP

Using the spectrophotometric method for following deamination of 5′-AMP by the chick enzyme, with various initial substrate concentrations, the time-dependent decreases in substrate concentration correspond to Michaelis kinetics, but the fitted values of K_m^{app} increased non-linearly with an increase in initial substrate concentration (Fig. 2). This pointed to the existence of non-competitive product inhibition, a finding further supported by use of the fluorimetric method (see next section).

The value of K_m was determined from measurements of initial velocities, to give K_m = (0.34 ± 0.10) mM, and V_{max} = (0.22 ± 0.03) mmol min^{-1} ml^{-1} = (63 ± 6) μmol min^{-1} mg^{-1}. This value of K_m corresponds to that obtained by continuous monitoring of the reaction with an initial substrate concentration of 0.6 mM, *i.e.* under conditions where the concentration of product formed is 2-fold less than the K_m for product inhibition (see next section). The foregoing value is twice that reported by Frieden *et al.* [22], K_m = 0.18 mM (for the rabbit enzyme), using a stopped-flow method with an initial substrate concentration of 0.2 mM, but is close to that found by Kaletha [23], 0.2–0.3 mM for the chick enzyme. It differs appreciably from the value of 2.2 mM reported by Stankiewicz [24].

With 5′-FMP, also following the entire course of deamination spectrophotometrically, the fitted K_m^{app} values were, as for 5′-AMP, non-linearly dependent on initial substrate concentration, again pointing to inhibition by the product, 5′-FBMP, and an allosteric character of inhibition. Measurements of initial velocities led to K_m = (1.0 ± 0.3) mM, and V_{max} = (0.65 ± 0.08) mmol min^{-1} ml^{-1} = (173 ± 20) μmol min^{-1} mg^{-1}.

It will be noted that, although both K_m and V_{max} for 5′-FMP are higher than those for the natural substrate, the rate constants V_{max}/K_m are almost identical, 0.17 and 0.19 min^{-1} mg^{-1}, respectively.

For purposes of convenience, the kinetic parameters of both substrates, as well as the inhibition constants for the product and transition-state inhibitors (see below) are listed in Table I.

pH optima

Measurements of rate constants, V_{max}/K_m, as a function of pH, with substrate concentrations well below K_m, demonstrated that the pH optimum for deamination of 5′-AMP was in the range 5.8–7.3. The pH-dependence for deamination of 5′-FMP was strikingly similar, but shifted about 0.2 pH units to more acidic pH.

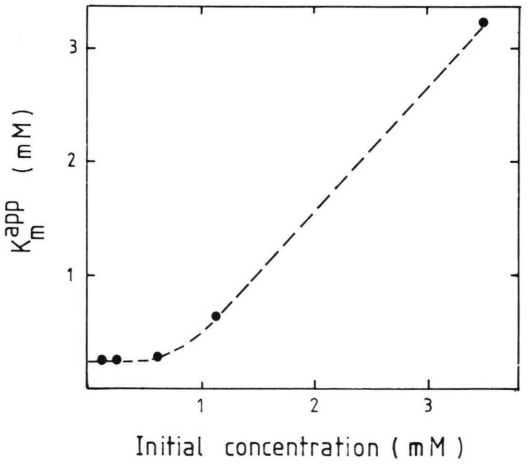

Fig. 2. Effect of initial substrate concentration on the apparent Michaelis constant K_m^{app} for deamination of 5′-AMP by chick skeletal muscle AMP deaminase. All measurements were carried out in 50 mM cacodylate buffer, pH 6.5 at 25 °C in the presence of 100 mM KCl and 2.5 mM DTT.

Product inhibition

To our knowledge, product inhibition in the adenylate deaminase system (with 5′-AMP as substrate, see above) has not hitherto been noted. With 5′-FMP as substrate, and using the fluorimetric procedure, potential inhibition by 5′-IMP was examined with the chick enzyme at three pH values, 5.8, 6.5 and 7.3, with inhibitor concentrations in the ranges 0.5–5.1 mM, 1.1–9.0 mM and 0.6–9.0 mM, respectively. The pH of the incubation medium was checked after completion of each reaction. K_i^{app} was found to increase approximately linearly with an increase in inhibitor concentration (see Fig. 3), suggestive of an allosteric nature of inhibition (see Methods). With this in mind, the linear relationships were used to obtain K_i values at each pH: 1.6 mM, 1.2 mM and 1.0 mM, respectively. Inhibition is enhanced at pH 7.3, where its allosteric nature is also most evident (difference between $\delta_1 = k_1/K_s$ and $\delta_2 = k_2/K_{si}$, see

Table I. Kinetic parameters for some substrates and inhibitors of chick skeletal muscle AMP deaminase in 50 mM cacodylate buffer, at 25 °C, determined spectrofluorimetrically unless otherwise indicated[a].

Compound	pH	K_m [mM]	V_{max} [μmol min^{-1} mg^{-1}]	V_{max}/K_m [min^{-1} mg^{-1}]	K_i [M]
5′-AMP	6.5	~0.34[b]	63[b]	0.19[b]	–
5′-FMP	6.5	~1.0[b]	173[b]	0.17[b]	–
	6.5	–	–	0.15	–
5′-IMP	5.8	–	–	–	1.6×10^{-3}
	6.5	–	–	–	1.2×10^{-3}
	7.3	–	–	–	$\sim1.0\times10^{-3}$
2′-DCF	6.5	–	–	–	$\sim1.6\times10^{-6}$
CF	6.5	–	–	–	$\sim11.6\times10^{-8}$
CF-5′-MP	6.5	–	–	–	$<0.6\times10^{-9}$

[a] Errors are ±15% unless otherwise indicated (~).
[b] Parameters determined spectrophotometrically by initial velocity method.
[c] With 2′-DCF-5′-MP, Frieden *et al.* [32] reported $K_i \sim 10^{-9}$ M.

Fig. 3). Although product inhibition appears weak (relative to potent exogenous inhibitors, see below), it cannot be ignored since it is of the same order of magnitude as K_m (~ 0.3 mM). The small, but clear-cut, pH-dependence of K_i may be related to dissociation of the secondary phosphate hydroxyls of substrate and/or product (pK ~ 6.5).

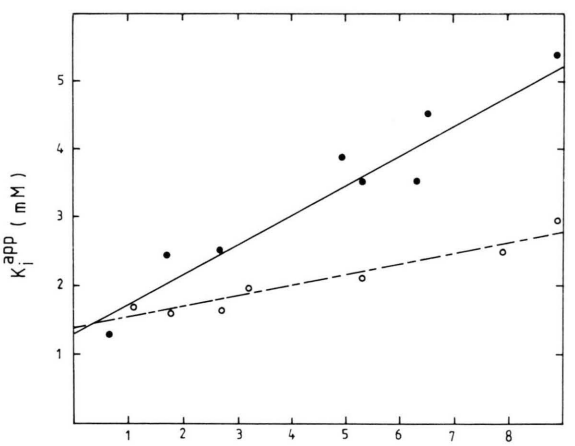

Fig. 3. Effect of inhibitor concentration, 5′-IMP, on the apparent inhibition constant K_i^{app} for deamination of 5′-FMP by chick skeletal muscle AMP deaminase. All measurements were in 50 mM cacodylate buffer at 25 °C, in the presence of 100 mM KCl, 2.5 mM DTT and various inhibitor concentrations, and the reaction was followed spectrofluorimetrically (see Materials and Methods). Points are experimental data and lines are theoretically derived from Eqn. (3) using the following values of K_i and $K_s k_2/K_{si}k_1$: (●) pH 7.3, K_i = (0.96 ± 0.19) mM, $K_s k_2/K_{si}k_1$ = (0.30 ± 0.04); (○) pH 6.5, K_i = (1.22 ± 0.12) mM, $K_s k_2/K_{si}k_1$ = (0.14 to 0.03); pH 5.8 (data not shown for purposes of clarity) K_i = (1.64 ± 0.15) mM, $K_s k_2/K_{si}k_1$ = (0.12 ± 0.04).

Because of the lack of an adequate quantity of 5′-FBMP, inhibition by this product was examined at only one concentration, 0.7 mM, to give K_i^{app} = (1.7 ± 0.2) mM at pH 6.5. While this single measurement does not permit evaluation of K_i, it is clearly comparable to that observed with 5′-IMP.

The above results are consistent with a model for AMP deaminase from rabbit muscle [25], in which three types of binding sites were identified: an active site specific for AMP and two other inhibitory (activatory) sites, one specific for various triphosphates (NTP), the other for various nucleotides (NXP), which binds IMP as well.

Lability of AMP deaminase

Adenylate deaminase, whatever its source, is known to be fairly labile at the dilutions and temperatures normally employed for following enzyme kinetics. With tight-binding inhibitors this leads to considerable technical difficulties in evaluation of kinetic constants [26, 27].

The time-dependent loss in activity of the chick skeletal muscle enzyme (following the 1000-fold dilution) may be described as a two-stage process, as follows:

$$V_{max}/K_m = c_1 \exp(-k_1 t) + c_2 \exp(-k_2 t) + c_0 \qquad (4)$$

where $c_1 = 0.74 \cdot min^{-1}$, $c_2 = 0.48 \cdot min^{-1}$, $c_0 = 0.14 \cdot min^{-1}$, $k_1 = 3.0 \cdot h^{-1}$, $k_2 = 0.18 \cdot h^{-1}$.

This may be due to two independent inactivation processes or, alternatively, to the existence of more than one active form of the enzyme with different stabilities. The latter appears the more likely, since inactivation proceeds to a plateau level of about 10%, which is then stable for a long period. This residual activity was found to behave like the freshly diluted enzyme toward various inhibitors, including 5′-IMP and 2′-deoxycoformycin.

The enzyme may be partially stabilized by addition to the medium of 2.5 mM dithiothreitol (k_1 = $0.55 \cdot h^{-1}$, $k_2 = 0.037 \cdot min^{-1}$).

The rabbit muscle enzyme, under the foregoing conditions, was slightly more labile.

Influence of diadenosine oligophosphates

Fernandez *et al.* [28] reported that mouse muscle AMP deaminase is stimulated by Ap_4A which, at a concentration of 25 μM, increased the pseudo first-order rate constant about 2.3-fold. Ap_3A at the same concentration did not affect the reaction rate.

With the aid of the fluorimetric procedure, and 5′-FMP as substrate, we have examined the effect, on chick skeletal muscle AMP deaminase, of Ap_2A, Ap_3A and Ap_4A at concentrations of 80 μM for the first and ~ 30 μM for latter two in the presence of low (5 μM) substrate concentrations. No detectable activation of the enzyme was observed. On the other hand Ap_2A under these conditions, gave about 35% inhibition, corresponding to an apparent $K_i \geqslant 0.2$ mM. The dinucleoside oligophosphates themselves were not substrates for the enzyme.

AMP deaminase inhibitors
2′-deoxycoformycin

Inhibition of the chick skeletal muscle enzyme was studied by means of emission spectroscopy with 5′-FMP as substrate. The calculated value of K_i^{app} for a range of inhibitor concentrations of 1–17 μM was found to be independent of the time of prior incubation of enzyme with inhibitor (up to 35 min), and of the pH of the medium (in the range 5.8–7.3, about the pH optimum), but was significantly dependent on inhibitor concentration. With 1–8 μM DCF, K_{iapp} increased linearly with inhibitor concentrations, whereas at a DCF concentration ⩾ 19 μM it attained a plateau value of 2.5 μM. From this behaviour it is difficult to characterize the type of inhibition involved, but it is clearly not simple competitive or true non-competitive since, under the present experimental conditions, K_i^{app} should equal K_I, independent of inhibitor concentration. We evaluate $K_i = (1.6 \pm 0.7)$ μM. This is close to the value 2 μM reported by Agarwal & Parks [29] for the rabbit muscle enzyme, but significantly higher than the value of 0.36 μM calculated by Frieden *et al.* [22] also for the rabbit enzyme.

Coformycin

With the aid of the fluorimetric procedure, and following the reaction to completion, inhibition constants were measured for the chick and rabbit skeletal muscle enzymes. Enzyme was initially incubated with inhibitor for 15 min; longer incubation periods did not affect observed inhibition constants.

In this instance there is no observable dependence of K_i^{app} on inhibitor concentration, so that $K_i^{app} = K_i$, and inhibition is simple competitive or true noncompetitive (our procedure is unable to distinguish between them, see Methods). The measured values of K_i were $(11.6 \pm 1.9) \times 10^{-8}$ M for the chick muscle enzyme and $(9.4 \pm 1.1) \times 10^{-8}$ M for the rabbit enzyme. The average of these two close values, 10^{-7} M differs significantly from the value of 0.2×10^{-7} M obtained by Frieden *et al.* [22] and is about twice that reported by Agarwal & Parks [29], in both cases for the rabbit enzyme.

Effect of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA)

Henderson *et al.* [30] reported significant (70%) inhibition of rabbit muscle AMP deaminase by 7–15 μM EHNA. By contrast, Agarwal & Parks [28], found no detectable inhibition with up to 110 μM EHNA.

We have examined the effect of EHNA at concentrations up to 50 μM on the chick skeletal muscle enzyme, simultaneously taking account of the known lability of the enzyme during the course of the reaction. Assuming that the presence of EHNA does not affect enzyme stability, no significant inhibition could be detected.

Coformycin-5′-phosphate

In initial experiments on inhibition by coformycin-5′-phosphate, the rabbit or chick enzyme, at a concentration of 10^{-9} M in cacodylate buffer pH 6.5 at 25 °C, was incubated with increasing concentrations

of the inhibitor for 10 min, and the samples then assayed for adenylate deaminase activity with the aid of the emission method. Assuming that the system enzyme-inhibitor-substrate is in equilibrium, and utilizing the Henderson equation [26] for inhibition under conditions where the substrate concentration (5 μM 5′-FMP) is much lower than K_m (~ 1 mM):

$$\frac{I}{1 - v_o/v_i} = E_t + K_i\,(v_1/v_0$$

the value of the inhibition constant is about 0.5×10^{-9} M and 0.9×10^{-9} for rabbit and chick enzymes respectively (both values are the average of 5 experiments with 5′-CFMP concentration in the range 3.5×10^{-9} M-17×10^{-9} M). This is at best an upper limit for K_i, since equilibrium between enzyme and inhibitor is most likely not fully attained.

The concentration of active enzyme in our preparations was evaluated by comparing the specific activity with that of a reported homogeneous rabbit enzyme, 155 μM min^{-1} mg^{-1} with 5′-AMP as substrate [31]. The validity of this procedure is testified to by our observation that coformycin and coformycin-5′-phosphate do not bind to inactive enzyme molecules.

We then performed the same experiments as Frieden *et al.* [32] for inhibition by 2′-DCF-5′-phosphate. Aliquots of the rabbit enzyme (0.072 μM, expressed in terms of enzyme subunits) were incubated in cacodylate buffer pH 6.5 at 18 °C for 15 min with increasing concentrations of CF-5′-MP (0−0.1 μM). Each sample was then diluted about 20-fold, and the rate of deamination of 5′-FMP determined, to give values of V_{max}/K_m. From these results, shown in Fig. 4, it follows that: (a) CF-5′-MP is a tight-binding inhibitor, since inhibition of the rate of deamination v_i/v_o, with a substrate concentration $S \ll K_m$, corresponds to the relationship [26]:

$$v_i/v_o = (E_o - I)/E_o$$

and not to the form:

$$v_i/v_o = 1/(1 + I/K_i);$$

(b) binding of the inhibitor to the enzyme is stoichiometric, *i.e.* one molecule of inhibitor binds one subunit of the enzyme; (c) the inhibition constant, K_i, is 2−3 orders of magnitude below the concentration of enzyme employed (0.072 μM), since only under such conditions can the enzyme in equilibrium bind virtually 100% of the inhibitor [26].

To determine the rate of association of inhibitor with enzyme, equimolar (0.072 μM) concentrations of the two were incubated at 18 °C. At various time intervals, aliquots were withdrawn, diluted about 20-fold, and remaining enzyme activity determined as in the previous section. The results are shown in Fig. 5. Assuming that the association reaction is second-order, *i.e.*

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

and that $k_{-1} = 0$, as in the case of binding of 2′-DCF-5′-MP [32], we have:

$$1/E = 1/I = k_1\,t + 1/E_o,$$

from which the value of k_1 is calculated as $(1.5 \pm 0.2) \times 10^5$ M^{-1} s^{-1}. For the binding of 2′-DCF-5′-MP to the enzyme from the same source, the value of k_1 was found to be 0.9×10^5 M^{-1} s^{-1} [32]. The latter authors concluded that, since such a value is much lower than that expected for a diffusion-controlled second-order rate constant for binding of a small

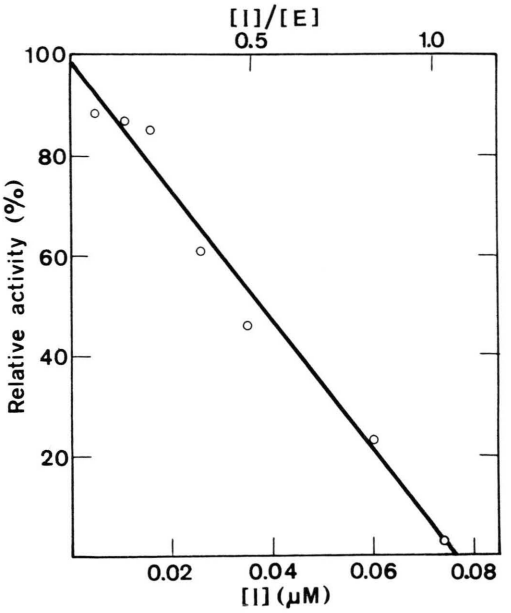

Fig. 4. Stoichiometry of inhibition of rabbit skeletal muscle AMP deaminase by coformycin-5′-phosphate. Rabbit enzyme (0.072 μM, expressed in terms of enzyme subunits) and increasing concentrations of inhibitor were incubated for 15 min in 50 mM cacodylate buffer pH 6.5, 100 mM KCl and 2.5 mM DTT at 18 °C. Aliquots were then diluted 20-fold for assay of enzyme activity.

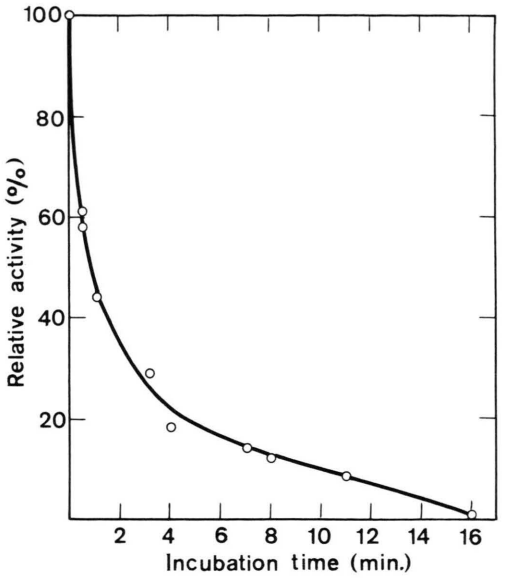

Fig. 5. Time-course of inhibition of rabbit muscle AMP deaminase by coformycin-5′-phosphate. Enzyme and inhibitor at equimolar concentrations (0.072 μM) were incubated in 50 mM cacodylate buffer, 100 mM KCl, 2.5 mM DTT, at 18 °C. At various time intervals, aliquots were withdrawn, diluted 20-fold, and enzyme activity assayed spectrofluorimetrically.

ligand to a protein, the time dependence must be due to an inhibitor-induced change in conformation.

Our results show that phosphorylation of coformycin leads to a decrease in the inhibition constant *vs.* AMP deaminase of at least 2 orders of magnitude, so that $K_i < 0.6 \times 10^{-9}$ M. Under our experimental conditions, loss of enzyme activity during measurements was 10%. As pointed out elsewhere [26], accurate measurement of K_i for tight-binding inhibitors is possible only with enzyme systems which are fully stable. In the present instance, the inhibitor, CF-5′-MP also exhibits some lability at pH 6.5 [15], optimal for adenylate deaminase activity. It may, nonetheless, be concluded that both CF-5′-MP and 2′-DCF-5′-MP are tight binding inhibitors of adenylate deaminase, with K_i values of approximately 10^{-9} M or lower.

Relevant to the foregoing results are the findings that both coformycin [33] and 2′-deoxycoformycin [34] undergo intracellular phosphorylation. The cellular enzymes responsible for this have not been identified, but it has been shown that 2′-DCF is not a substrate for either deoxyadenosine kinase or deoxycytidine kinase [34], although the latter enzyme is known to also phosphorylate deoxyadenosine. Another possibility is phosphorylation by nucleoside phosphotransferases, which have recently been reported to exist in a variety of human tissues [35].

The demonstrated intracellular phosphorylation of CF and 2′-DCF, in conjunction with the fact that the 5′-phosphates of both are potent tight-binding inhibitors of adenylate deaminase, leads to the conclusion that these compounds probably operate intracellulary not only as inhibitors of adenosine deaminase, but also of adenylate deaminase.

Concluding Remarks

Formycin and its nucleotides have been widely used as analogues of adenosine and adenosine nucleotides in various enzymatic systems such as adenosine deaminase [18], 5′-nucleotidase [14], adenosine kinase [36, 37], adenylate cyclase [38], and fluorescence of these compounds has been exploited for purposes of detection. The procedure described here for AMP deaminase can profit from the changes in emission, as well as ultra-violet absorption, during the course of deamination. Since the rates of deamination of 5′-AMP and 5′-FMP are comparable, the fluorimetric and absorption procedures based on 5′-FMP are particularly advantageous in experiments with cell extracts which exhibit high optical density; measurements in the range of pseudo first-order kinetics ($S_0 \ll K_m$), since the fluorimetric assay is sensitive even with S_0 of the order of a few μM; and when studying properties of inhibitors (or activators) with high molar extinctions near 260 nm, *i.e.* close to the maximum of 5′-AMP.

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