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ARTICLE in ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS · MAY 1994

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Interaction of the High-Affinity Inhibitor Tetrahydro-dUMP with the Allosteric Enzyme Deoxycytidylate Aminohydrolase¹

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Received July 6, 1993, and in revised form October 28, 1993

Tetrahydro-dUMP, an analog of the putative transition state in aminohydrolysis of deoxycytidine monophosphate (dCMP) inhibits the allosteric enzyme deoxycytidylate aminohydrolase with high affinity. The inhibition is reversible, and its kinetics is consistent with the analog binding at the substrate site only to one and the same conformation that binds the substrate dCMP. Such kinetics is what would be expected for a transition state analog interacting in an allosteric "K system." © 1994 Academic Press, Inc.

yuridine (THdurd) (3). This is useful for metabolic studies and might have applications in cancer chemotherapy. One hope, based on the fact that dCMPase levels are high in cancer cells, is to use THdurd together with other drugs affecting pyrimidine nucleotide metabolism to achieve protocols less toxic to noncancerous cells (4).

On the other hand, dCMPase is an allosteric enzyme, controlled by deoxypyrimidine nucleoside triphosphate concentrations, and with cooperative substrate kinetics (literature reviewed in Refs. 5–7). A high-affinity inhibitor could be of value in investigations of the allosteric mechanism, particularly if the dissociation of the inhibitor was slow. A dissociation slow compared to the time of kinetic measurements is equivalent to irreversible binding. Some questions about allosteric mechanisms difficult or impossible to answer by studies with reversible modifiers could be solved using irreversibly bound substrate or modifier analogs.

Many transition-state analog high-affinity inhibitors do combine with and dissociate from their target enzymes quite slowly. Examples are THurd with human liver cytidine aminohydrolase (8, 9) and coformycin or deoxycoformycin and their 5'-phosphates with adenosine aminohydrolase and AMP aminohydrolase, respectively (10–13). These enzymes are very probably related to dCMPase. Up to now only reversible modifiers of dCMPase have been studied (reviewed in Ref. 7).

From the enzymological, metabolic, and pharmacological points of view, therefore, it is of some interest to characterize the nature of the inhibition of dCMPase produced by THdUMP.

MATERIALS AND METHODS

Enzyme purification and assay. dCMPase was purified from donkey spleens and assayed spectrophotometrically by the procedure reported by Nucci *et al.* (14).

¹ This work was supported by CNR "Progetto Finalizzato: Chimica Fine" and by Commission of the European Communities.

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³ Abbreviations used: THdUMP, tetrahydro-dUMP; dCMP, deoxycytidine monophosphate; dCMPase, deoxycytidylate aminohydrolase; THurd, tetrahydouridine; THdurd, tetrahydrodeoxyuridine; dCTP, deoxycytidine triphosphate; dAMP, deoxyadenosine monophosphate.

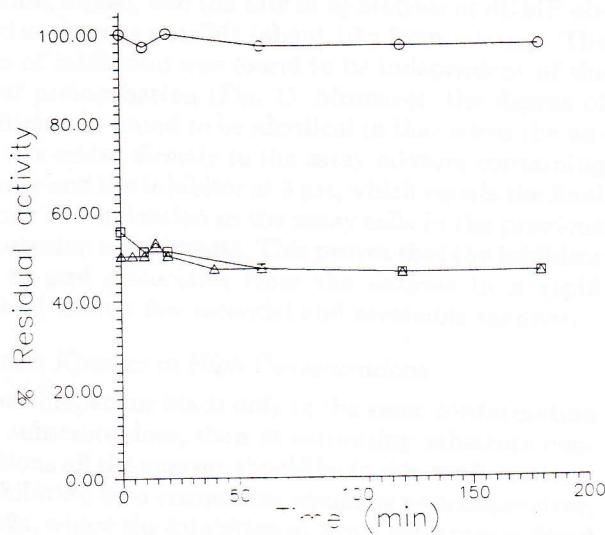


FIG. 1. Demonstration that inhibition of dCMPase by THdUMP is rapid and rapidly reversible. The enzyme was preincubated with and without the inhibitor in 0.1 M sodium phosphate, pH 7.0, 50% glycerol, at 0°C. O, Incubation without inhibitor, assay with 1.5 mM dCMP; □, incubation without inhibitor, assay with 1.5 mM dCMP, 3 μM THdUMP; △, incubation with 90 μM THdUMP, assay with 1.5 mM dCMP (concentration of THdUMP in assay, 3 μM).

Synthesis of THdUMP. The synthesis of THdUMP was performed using Hanze's (15) procedure slightly modified; in our case the 2'-deoxyuridine-5'-monophosphate (dUMP) was first hydrogenated to 5,6-dihydroderivative (DHdUMP) by reaction with H₂ in the presence of rhodium as catalyst and then reduced in the C-4 position by reaction with sodium borohydride thus affording, after purification, a mixture of C-4 hydroxyisomers of the tetrahydroderivative THdUMP.

DHdUMP: 352 mg (1 mmol) of dUMP (disodium salt), dissolved in water (8 ml) was hydrogenated with H₂ over rhodium on alumina (5% Rh, 170 mg) under stirring at room temperature and atmospheric pressure. After 8 h the catalyst was removed by filtration and the eluate, taken to dryness, afforded 283 mg of almost pure DHdUMP (80% yield).

¹H NMR (270 MHz, D₂O) δ: 6.13 (1H, dd, H-1'), 4.33 (1H, m, H-3'); 3.87 (1H, m, H-4'); 3.71 (2H, t, J = 8.3 Hz, H-6); 3.45 (2H, m, H₂-5'); 2.61 (2H, t, J = 8.3 Hz, H₂-5); 2.22 (1H, m, Ha-2'); 1.96 (1H, m, Hb-2').

THdUMP: to a ice-cold solution of DHdUMP (178 mg, 0.5 nmol) in 5 ml of water (pH 7.5–8.0) 19 mg (0.5 nmol) of sodium borohydride were added. After 2 h thin-layer chromatography (TLC, silica gel 0.25 mm, eluent isopropanol/NH₄OH concentrate/water, 55/35/10 v/v/v) showed the almost complete disappearance of DHdUMP (*R*_f 0.65) and the formation of THdUMP (*R*_f 0.5). The excess of sodium borohydride was destroyed with acetic acid and the mixture dried *in vacuo*. The mixture, dissolved in water, was chromatographed on preparative TLC (silica gel 0.5 mm, eluent isopropanol/NH₄OH concentrate/water, 55/35/15). The band *R*_f 0.5, scratched from the plates, was suspended in water/methanol (95/5) and then centrifuged. The collection of the supernatants of three centrifugations afforded, after drying, 124 mg of almost pure THdUMP (70% yield). Finally, 10 mg of the product, dissolved in water, was further purified by filtration through a RP-18 Sep-Pak filter (Merck).

¹H NMR (270 MHz, D₂O) significant proton at δ: 6.33 and 6.24 (1H, dd, H-1'), 5.02 and 5.10 (1H, t, H-4); 4.37 (1H, m, H-3'); 3.76 (2H, t, H₂-6); 2.50–1.80 (2H, complex signals, H₂-2').

All other chemicals used were commercial products.

The treatment of data applying the equation

$$\frac{v}{V} = \frac{x}{(x + ay)} f(x + ay) \quad [1]$$

was as explained in Mastrantonio *et al.* (16).

RESULTS

Interaction of the Inhibitor with the Enzyme Is Rapid and Reversible

In direct assays, inhibition of dCMPase by THdUMP was achieved when this inhibitor is about 1000-fold less concentrated than the substrate, e.g., at 2 mM dCMP and 2 μM THdUMP (Fig. 2a). In order to determine whether inhibition was time dependent in the seconds-to-hours range, the inhibitor was incubated with the enzyme without substrate in 100 mM sodium phosphate, pH 7.0, 50% glycerol at 90 μM, i.e., about 30-fold the concentration

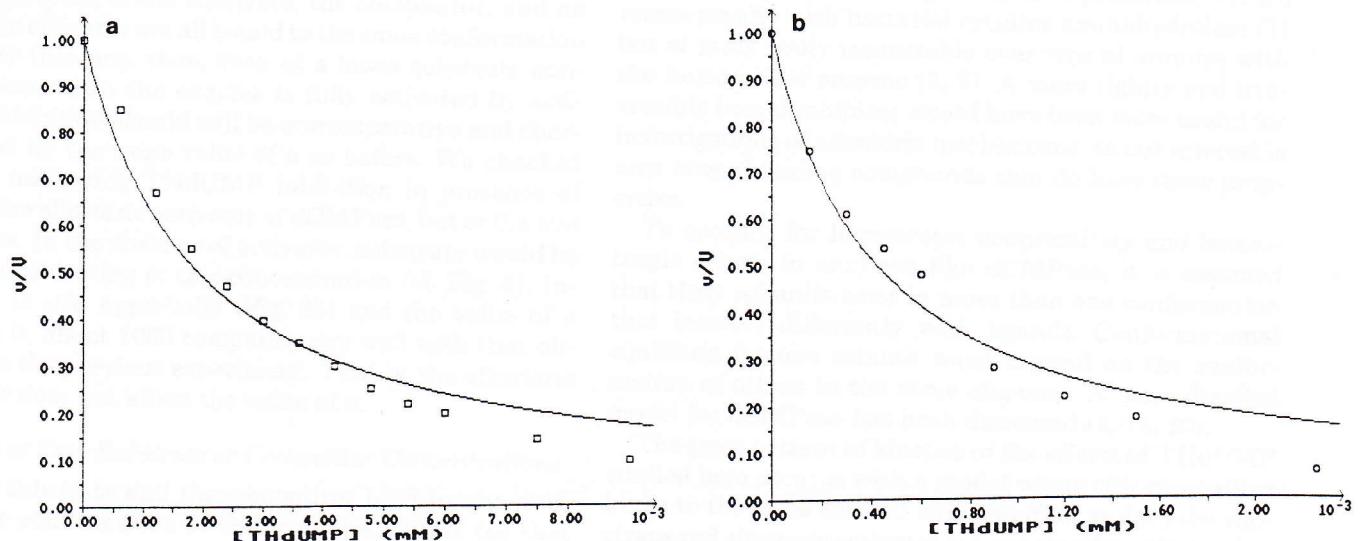


FIG. 2. Inhibition of dCMPase by THdUMP at high substrate concentration or in the presence of activator dCTP. (a) Inhibition at 2 mM dCMP. The curve represents Eq. [2] with *a* = 1047. (b) Inhibition at 0.4 mM dCMP, 2 μM dCTP. The curve represents Eq. [2] with *a* about 973.

required for half-inhibition, and 10 μl of the preincubation mixture diluted into the (300 μl) assay cell containing substrate, mixed, and the rate of hydrolysis of dCMP observed as soon as possible (about 10 s from mixing). The degree of inhibition was found to be independent of the time of preincubation (Fig. 1). Moreover, the degree of inhibition was found to be identical to that when the enzyme was added directly to the assay mixture containing substrate and the inhibitor at 3 μM , which equals the final inhibitor concentration in the assay cells in the previous preincubation experiments. This proves that the inhibitor binds to and dissociates from the enzyme in a rapid (within at most a few seconds) and reversible manner.

Inhibition Kinetics at High Concentrations

If the competitor binds only to the same conformation as the substrate does, then at saturating substrate concentrations all the enzyme should be in this conformation, and inhibition by a competitor would be noncooperative. Figure 2a, where the inhibition at 2 mM substrate is fitted to a hyperbolic inhibition curve, shows that this is what happens experimentally, at least at a first approximation. To obtain a constant characterizing the inhibition, the data are fitted to the equation,

$$\frac{v}{V} = \frac{x}{x + ay}, \quad [2]$$

where x , y are substrate and competitor concentrations, respectively. Equation [2] can be regarded on the one hand as the classical competition formula at high concentrations and on the other hand as what Eq. [1] reduces to when $f = 1$. a corresponds to the classical K_m/K_i ; its experimental value found for the THdUMP, dCMP pair is close to 10^3 . (If, as is probable, there is stereospecificity for the transition state analog, the value for the active species is close to 2×10^3 of course).

Effect of Allosteric Activator

Furthermore, if the substrate, the competitor, and an allosteric activator are all bound to the same conformation and only that one, then, even at a lower substrate concentration, when the enzyme is fully activated by activator, inhibition should still be noncooperative and characterized by the same value of a as before. We checked this by measuring THdUMP inhibition in presence of dCTP, the allosteric activator of dCMPase, but at 0.4 mM substrate. In the absence of activator, substrate would be far from saturating at this concentration (cf. Fig. 3). Inhibition is still hyperbolic (Fig. 2b) and the value of a that fits it, about 1000 compares very well with that obtained in the previous experiment. That is, the allosteric activator does not affect the value of a .

Kinetics at Low Substrate or Competitor Concentrations

If the substrate and the competitor bind to one-and-the-same conformation of the enzyme subunits (so that

the binding of either to a substrate site has an identical effect at another substrate site), then the overall kinetics is predicted to follow Eq. [1] (17, 18). In this equation, $f(x)$ is any function that fits the curve of v/V against substrate concentration in the absence of the competitor, i.e., any function giving a fit like the curve of Fig. 3a.

Equation [1] can be obtained from the above assumptions by Linkage (17, 18) or equivalent (19) arguments. The advantages of this approach have been discussed previously (16, 7). It involves determining a single physical constant, a , which can be estimated reliably; no attempt is made to estimate the physical constants that determine cooperativity (i.e., the shape of f) which cannot be done reliably from steady-state measurements, indeed can be quite misleading. a and f being experimentally known from separate experiments, there are no free parameters when the predictions of the equation at low concentrations are compared with experiment.

A qualitative prediction of Eq. [1] is that the competitor should actually *activate* the enzyme at low substrate concentrations. Experimentally this activation is observed (Figs. 3b, 3c).

Quantitatively the experimental data agree with the predictions of Eq. [1] (Figs. 3b, 3c) at least to a first approximation. It is hard to say without further investigation whether such deviations as are observed are really significant. There may be a limited amount of binding of the competitor to some conformation of subunits other than the substrate-binding conformation. A certain amount of deviation from ideal noncooperativity seen in Fig. 2a might arise from the same cause.

DISCUSSION

THdUMP inhibits dCMPase with fairly high affinity (1000-fold that of the substrate—up to 2000-fold for the active enantiomer of THdUMP if the enzyme distinguishes them), interacting with the enzyme in a rapid and reversible fashion. Whether it would have interacted rapidly or slowly could hardly have been predicted; THdUMP reacts rapidly with bacterial cytidine aminohydrolase (1) but at rates easily measurable over tens of minutes with the human liver enzyme (8, 9). A more tightly and irreversibly bound inhibitor would have been more useful for investigations of allosteric mechanisms, so our interest is now toward finding compounds that do have these properties.

To account for homotropic cooperativity and heterotropic effects in enzymes like dCMPase, it is assumed that their subunits exist in more than one conformation that interact differently with ligands. Conformational equilibria for one subunit must depend on the conformation of others in the same oligomer. A more detailed model for dCMPase has been discussed (7, 14, 20).

The gross pattern of kinetics of the effects of THdUMP studied here accords with a model where this competitor binds to the same subunit conformation as does the substrate and allosteric activator but not significantly to other

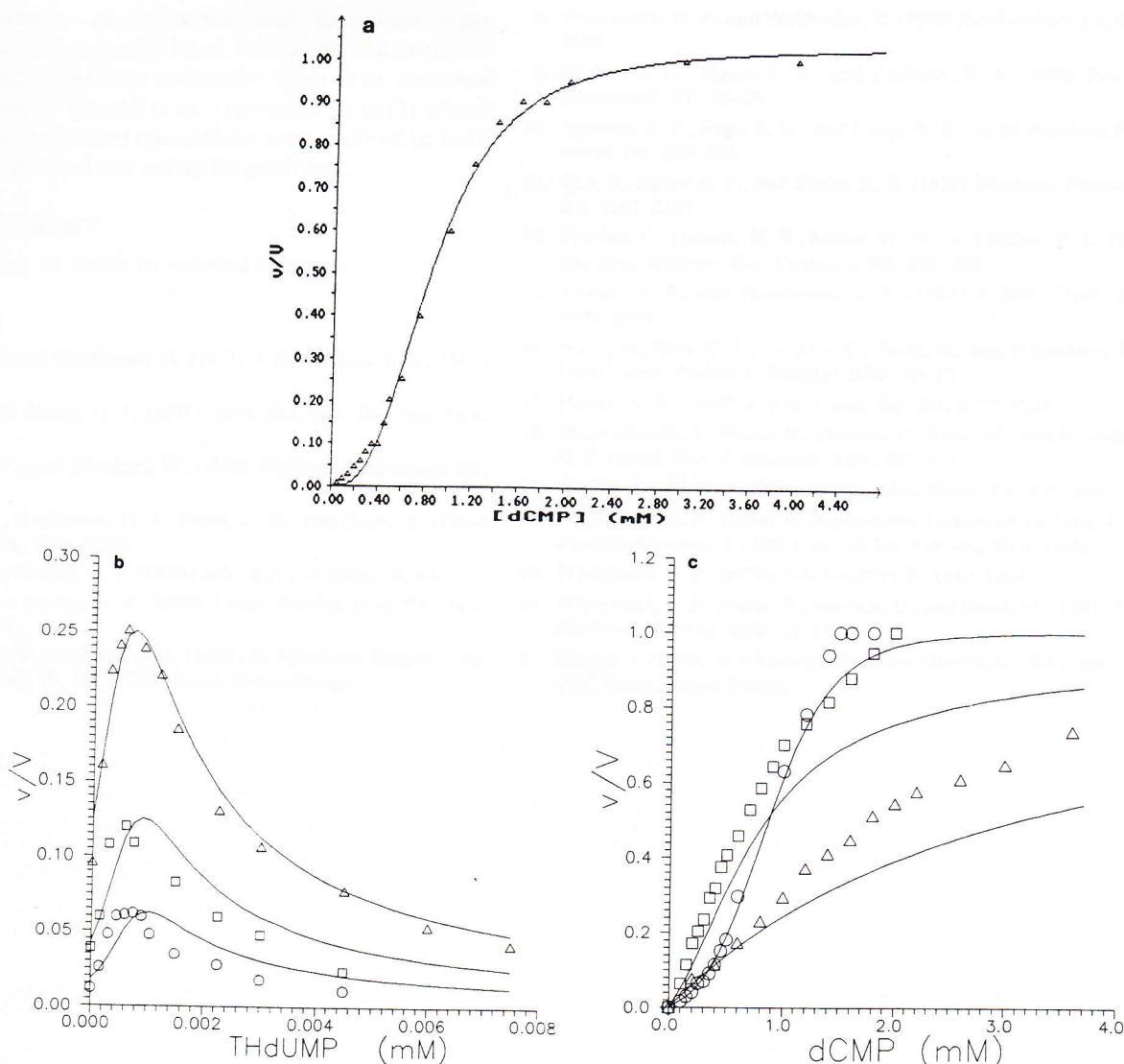


FIG. 3. Experimental results and theoretical predictions for kinetics at low substrate and inhibitor concentrations. (a) Dependence of v/V on substrate concentration in the absence of the inhibitor. The curve that fits the experimental points represents $f(x)$, the function used to predict the curves of (b). (b) Experimental points and curves predicted using Eq. [1] and the values of a and f obtained from the curves of Figs. 2a and (a) for effects of THdUMP concentration on v/V at the following fixed dCMP concentrations: \circ , 0.1 mM; \square , 0.2 mM; \triangle , 0.5 mM. (c) Experimental points and curves predicted by Eq. [1] for v/V dependence on substrate (dCMP) concentration at the following fixed THdUMP concentrations: \circ , 0.0 μ M; \square , 0.6 μ M; \triangle , 3.0 μ M.

subunit conformation(s). Such other conformations must be present in significant amounts at low substrate concentrations. This is how one would expect a transition-state analog to behave in an allosteric "K system," of which dCMPase is a classical example.

So far as the resolution of the present and other (7) allosteric kinetic investigations are concerned, a subunit conformation exists that binds dCMP, dCTP, THdUMP, and dAMP. The kinetics can be accounted for considering this conformation a single entity, and that other conformations do not bind these ligands.

Strictly speaking, of course, a transition state analog does not bind to the same conformation as the substrate,

but to a conformation similar to that present as the reaction proceeds through the transition state. (The analog binds with high affinity because the unperturbed enzyme conformation is more complementary to the transition state than to the substrate ground state).

What the observed behavior means, to the extent that the above simple model accounts for it, is that the difference between the transition state conformation and the ground state conformation at a substrate site is not felt at a different substrate site nor does it extend as far as the allosteric activator site. In other words, unlike the transitions between substrate-binding and nonbinding conformations discussed in the previous paragraphs, the

transition between ground-state and transition-state conformations is essentially local, limited to the environment of a single substrate molecule. This is at variance with the models of Ricard *et al.* [reviewed in (21)] where the same conformational transitions are involved in both allosteric control and the catalytic pathway.

ACKNOWLEDGMENT

We thank Mrs. A. M. Cirillo for technical assistance.

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