Cytidine Deaminase from Escherichia coli

PURIFICATION, PROPERTIES, AND INHIBITION BY THE POTENTIAL TRANSITION STATE ANALOG 3,4,5,6-TETRAHYDOURIDINE*

(Received for publication, July 19, 1971)

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SUMMARY

Cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) has been purified approximately 160-fold from extracts of Escherichia coli B. The enzyme shows constant activity between pH 6 and 11. No significant change in rate is observed when D2O replaces water as the solvent. In addition to the deamination of cytidine, the purified deaminase catalyzes slow hydrolysis of N4-methylycytidine. The enzyme is competitively inhibited by 3,4,5,6-tetrahydrouridine, previously shown to inhibit the deamination of 1-β-D-arabinofuranosylcytosine by preparations of human liver. The bacterial enzyme exhibits an affinity for this inhibitor exceeding by more than four orders of magnitude its combined affinity for the products uridine and ammonia.

The hydrolytic deamination of cytosine nucleosides is catalyzed by enzymes widely distributed in microorganisms (1, 2), plants (3), and animals (4-8). Enzymes of this kind catalyze the interconversion of derivatives of C and U, two letters of the genetic code. The net reaction is measurably reversible, as shown in the accompanying paper. The position of cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) in catabolic and "salvage" pathways suggests that it may determine the availability of nucleic acid precursors and their antagonists.

This paper describes the purification and kinetic properties of the cytidine deaminase originally detected by Wang et al. (1) in extracts of Escherichia coli. 3,4,5,6-Tetrahydrouridine (9), previously shown to inhibit the deamination of 1-β-D-arabinofuranosylycytosine by preparations of human liver (10), is found to be an extraordinarily potent competitive inhibitor of the purified bacterial enzyme. The effectiveness of this inhibitor, to which brief reference was made in a preliminary communication (11), suggests that it may be considered a potential "transition state analog." Other mechanistic information is consistent with the likelihood that cytidine deaminase may catalyze displacement of ammonia by water through an addition-elimination mechanism analogous to that proposed for adenosine deaminase (12, 13).

MATERIALS AND METHODS

3,4,5,6-Tetrahydrouridine (1-β-D-ribofuranosyl)-4-hydroxytetrahydro-2-pyrimidone (9) was the generous gift of Dr. Arthur H. Hanze. 4-O-Methyluridine was kindly provided by Dr. Peter Cerutti. The following compounds were synthesized according to published procedures: 4-N-methylycytidine (14), 4-N,N-dimethylycytidine (14), 3-N-methylcytidine (15), and 1-β-D-arabinofuranosyl)-4-sulfonyl-2-pyrimidone (16). 5,6-Dihydrouridine and streptomycin B sulfate were obtained from Calbiochem. 4-Thiouridine was prepared by sodium bisulfite reduction (17) of 4,4'-di (thiouridine) obtained from Cyclo Chemical Co. Other nucleosides and nucleotides were purchased from Sigma and Calbiochem. Sephadex and Blue Dextran 2000 were obtained from Pharmacia; cellulose ion exchangers from Gallard Schlesinger Corp.; hydroxylapatite, as a dry powder, from Bio-Rad Corp.; bacterial alkaline phosphatase and bovine serum albumin from Sigma; calf duodenal adenosine deaminase from Boehringer-Mannheim; and deuterium oxide (99.75%) from Merck, Sharpe and Dohme of Canada, Ltd.

Cytidine deaminase activity was routinely determined by a direct spectrophotometric assay based on the loss of absorbance when cytidine is converted to uridine, at 282 nm where Δε = -3600 for a 1-cm light path at pH 7.5 (Table I). The standard assay solution contained enzyme, cytidine (1.67 × 10^-4 M), and Tris-HCl buffer (0.05 M, pH 7.5). One unit of activity was defined as the amount of enzyme required to deaminate 1 μmol of cytidine per min in the standard assay solution at 25°C, based on initial rates obtained during the initial 5 to 10% of reaction. Protein concentration was determined by the method of Lowry et al. (19), with bovine serum albumin as a standard. For the determination of K_m and V_max, initial rates were measured as a function of substrate concentrations covering the range at least from 0.5 to 5 × K_m, with quartz cuvettes of light path ranging from 0.1 to 1 cm as required. The effects of pH were determined in potassium bicarbonate, Tris-HCl, imidazole-HCl, potassium acetate, and glycine-KOH buffers, all at a concentration of 0.05 M. Determinations of pH were made before and after reaction with the use of the glass electrode of a Radiometer model 4 pH meter. No specific buffer effects were observed when rates were
buffer (0.01 M) removed by centrifugation and discarded, and the supernatant stirred for an additional 30 min. The resulting precipitate was added slowly over a period of 50 min, and the solution was covered by centrifugation, redissolved in a minimal volume of distilled water, dialyzed exhaustively against Tris-HCl buffer (0.01 M, pH 7.5), and applied to a column of carboxymethylcellulose (75 ml bed volume) equilibrated with the same buffer.

Deaminase activity was not retained appreciably on this column, which removed some inactive protein. The eluted enzyme solution was applied to a column of hydroxypatite (100 ml bed volume) and eluted with a linear gradient, the first reservoir containing Tris-HCl buffer (0.03 M, pH 7.5), the second reservoir containing Tris-HCl buffer (0.5 M, pH 7.5). Active fractions were pooled, dialyzed against potassium phosphate buffer (0.01 M, pH 8.8), and concentrated on a column of DEAE-cellulose (5 ml bed volume) equilibrated with the same buffer. The enzyme, in its final state of purification, was eluted with potassium phosphate buffer (1 M, pH 6.5). Over-all purification, summarized in Table II, was approximately 175-fold.

**RESULTS**

**Properties of Enzyme**—Cytidine deaminase, purified as described, was found to be reasonably stable, retaining 55% of its activity after 3 weeks at 4°. Disc gel electrophoresis in polyacrylamide (7.5%) containing Tris-HCl (0.1 M, pH 8.1) (20, 21) showed that the enzyme, here purified 175-fold (Table II), was still contaminated with much inactive protein.

The elution behavior of the enzyme on a calibrated column of Sephadex G-100 (3.2 x 60 cm), determined according to the procedure of Siegel and Monty (22), indicated a single peak of activity, with a Kd value of 0.147 in potassium phosphate buffer (0.1 M, pH 7.5). Corresponding values for bovine serum albumin, calf duodenal adenosine deaminase, and bacterial alkaline phosphatase were 0.237, 0.206, and 0.106, respectively. Blue Dextran 2000 was used to determine the void volume. From these Kd values, the effective pore radius for the column was calculated as 11.3 A according to the procedure of Ackers (23). From these data, the apparent Stokes' radius of the enzyme was found to be approximately 40 A. Sucrose gradient centrifugation of the enzyme, according to the procedure of Martin and Ames (24) with bacterial alkaline phosphatase as a standard, yielded a sedimentation coefficient of 4.4 S. Together, these results suggest an approximate molecular weight of 73,000, assuming a partial specific volume of 0.725 cm³ per g.

No loss of activity was encountered when the enzyme was exhaustively dialyzed against EDTA (10⁻⁴ M) nor did EDTA at this concentration affect activity when included in the assay. The enzyme was not inactivated by incubation with sodium borohydride (10⁻⁴ M) for short periods in the presence or absence of the substrate. No significant change in activity was encountered when uracil, adenine, hypoxanthine, guanine, or their respective nucleosides and N¹ nucleotides were included in the standard assay at concentrations equivalent to that of the substrate (2 x 10⁻⁴ M).

**Effects of pH and D⁰**—Double reciprocal plots of the initial
rate of cytidine deamination against substrate concentration were consistently linear, yielding $K_m = 2.1 \times 10^{-4}$ M at pH 7.5. When both $V_{max}$ and $K_m$ were measured as a function of pH, little variation was observed in the range from 6.5 to 10.7 (Fig. 1). Below pH 6.5 there was a gradual reduction in $V_{max}$. Irreversible loss of activity occurred at pH values below 4. When deuterium oxide (90 atom % excess) was substituted for water as solvent at several pH values, there was a slight increase in $V_{max}$ and no significant change in $K_m$ (Table III).

Shapiro and Klein (25) have shown that the citrate-catalyzed deamination of cytidine is accompanied by incorporation of solvent deuterium into position 5 of the nucleoside. It was therefore of mechanistic interest to determine whether a similar reaction occurs with cytidine deaminase. When cytidine was completely hydrolyzed in D$_2$O in the presence of cytidine deaminase, the nuclear magnetic resonance spectrum of the product uridine showed that no exchange had occurred.

Inhibitors—The product uridine and its reduced derivatives, 5,6 dihydrouridine and 5,4,5,6 tetrahydrouridine, were found to serve as competitive inhibitors of cytidine deamination under the usual assay conditions. The strong absorbance of uridine in the ultraviolet spectrum, together with its relatively weak binding, made it necessary to carry out rate studies with this inhibitor at 290 nm, where the extinction coefficients for cytidine and uridine are 2500 and 300, respectively (26). The other inhibitors were studied at 282 nm, the wavelength used for the standard assay. Fig. 2 shows results obtained with tetrahydrouridine. This compound ($K_i = 2.4 \times 10^{-3}$ M) was more than four orders of magnitude more effective as an inhibitor than uridine ($K_i = 2.5 \times 10^{-4}$ M) or dihydrouridine ($K_i = 3.4 \times 10^{-4}$ M). Inhibition by tetrahydrouridine was found to be instantaneous, purely competitive and fully reversed by dilution within the time (approximately 5 s) required to initiate the standard assay.

The following compounds, tested at a concentration ($3 \times 10^{-4}$ M) in excess of the $K_m$ value of the substrate, failed to show significant inhibition in the standard assay with 1.67 $\times 10^{-4}$ M cytidine: 3-N-methyleytidine, 4-O-methyluridine, 4-thiouridine, 4-N,N-dimethyleytidine, and 1-(β-D-ribofuranosyl)-4-sulfonyl-2-pyrimidone. 4-N-Methyleytidine, previously reported to be a substrate of cytidine deaminase (27), was indeed found to be a substrate, but a poor one, with $K_m : V_{max}$ at least three orders of magnitude higher than that of cytidine.

The product ammonia showed no significant inhibition at quite high concentrations, an indication that its $K_i$ value is probably well in excess of 1 M. Inhibition was not observed in ammonia-ammonium chloride buffers at concentrations as high as 1 M at pH 9.2, at which ammonia and its conjugate acid are

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Fig. 2. Reciprocal plot of the rate of deamination of cytidine at 25°, in 0.05 M Tris-HCl buffer, pH 7.5, with 0.05 unit per ml of deaminase in the presence of varying concentrations of 3,4,5,6-tetrahydrouridine.

Fig. 1. pH dependence of $K_m (\triangle)$ and $V_{max} (\bullet)$ for cytidine at 25° with 0.06 unit per ml of deaminase in glycine, acetate, phosphate, imidazole, bicarbonate, and Tris-HCl buffers.
Dedicated carbon atom at the same position, this inhibitor
adenosine deaminase, and may be contrasted with fungal adeno-
desine deaminase which shows measurable inhibition by ammonia
at a concentration of $10^{-3}$ M (12).

**DISCUSSION**

Cytidine deaminase from *E. coli*, prepared as described here, exhibits a $K_m$ value for cytidine similar to that obtained by Wang et al. (1) for the activity in crude extracts of *E. coli* and similar to that of the enzyme from sheep liver (8). The specific activity of the enzyme, still grossly impure, is more than an order of magnitude higher than purified enzymes from bakers' yeast (2) and sheep liver (8).

The bacterial enzyme resembles fungal and mammalian ade-
nosine deaminases in the nature of the reaction catalyzed and in
the absence of any apparent requirement for cofactors (28). It is
similarly subject to competitive inhibition by the nucleoside product (12), and is similarly inhibited by organic mercurials
(28). Like adenosine deaminase from *Aspergillus oryzae*, it shows a relatively insignificant solvent deuterium isotope effect (12), and shares the ability of adenosine deaminases to catalyze hydrolytic removal of both ammonia and methylamine (29-31). Like adenosine deaminases (13), it is subject to unusually potent competitive inhibition by a nucleoside possessing a tetrahedral carbon atom at the position where substitution occurs. These common characteristics suggest that deaminases for cytidine and adenosine may act through rather similar mechanisms.

The reaction catalyzed by the nucleoside deaminases involves displacement of amine-leaving groups by water. Like other enzyme catalyzed nucleophilic displacements at electron-deficient carbon, this reaction may proceed through an addition-elimina-
tion mechanism involving a tetrahedral intermediate of high
energy, and there is considerable evidence for adenosine deam-
inases which is consistent with this mechanism (12).

The unusual affinity of cytidine deaminase for the inhibitor 3,4,5,6-tetrahydrouridine (exceeding that for the product uridi-
ne by four orders of magnitude) is readily understandable on
the basis of its resemblance to an intermediate in direct water
attack on cytidine (Fig. 3). In addition to possessing an sp3-
hybridized carbon atom at the same position, this inhibitor
shares with the proposed intermediate a hydroxyl substituent in
the required position, C4. The C4 hydrogen atom is then in
the position occupied by the variable leaving group. The inhibitor,
unlike the intermediate, is reduced across the 5-6 doublebond,
and this might be regarded as an alternative source of its
high affinity. However 5,6-dihydrouridine, which shares this
structural feature, is even less tightly bound than uridine. It is
therefore reasonable to suppose that the effectiveness of tetra-
hydrouridine is mainly based on its resemblance to the proposed intermediate. This provides evidence in favor of the proposed
mechanism, and supports the view that catalysis is achieved by
stabilization, through tight binding, of the altered substrate.

For reasons considered in detail elsewhere (32), it is appropriate
in reactions with more than one substrate that compounds con-
sidered as potential transition state analogs compare favorably
in binding properties with the product of the binding affinities of
individual substrates. Otherwise, tight binding might result
merely from combining the binding properties of the individual
substrates in a single molecule. The $K_i$ value for the product uridine is approximately 104 higher than that of tetrahydrouridine
as a competitive inhibitor whereas the $K_i$ value for the product ammonia was too high to determine, well in excess of 1 M.
As compared with the combined apparent dissociation constants of
the products (substrates for the reverse reaction), the low dis-
sociation constant of tetrahydrouridine is therefore particularly
impressive. It is more difficult to make comparisons for the reaction in the forward direction, since the true dissociation
constant of cytidine may be higher or lower than the observed
$K_m$, while the $K_o$ of water has not been established. However
it may be noted that studies on other hydrolases in mixed solvents
suggest that water saturation never occurs. Instead, rates of
enzymatic hydrolysis tend to increase in proportion to water.
activity, suggesting that $K_m$ for water may exceed 55 M (33-36).
If this is also the case for cytidine deaminase, it would be appro-
priate to compare the $K_i$ of tetrahydrouridine with a number
very much larger than the observed $K_m$ of cytidine.

Camiener has shown that tetrahydrouridine is an effective
inhibitor of *ara*-cytidine deaminase from human liver and several
other sources (10). This inhibition was used by Hanze as an
assay procedure in the original purification of tetrahydrouridine
from crude products of catalytic reduction of cytidine in water
(9). Inhibition of the enzyme from human liver was found to be
complex, giving nonlinear double reciprocal plots of velocity
against substrate concentration in the presence of inhibitor (10).
The onset of inhibition of the enzyme from human liver was
relatively slow, suggesting that the conditions required for the
usual steady state analysis of the nature and magnitude of
inhibition may not have been fulfilled (37, 38), and constants
resulting from an analysis based on double reciprocal plots are
not simple to interpret. However, at low substrate concentration
tetrahydrouridine was observed to produce approximately
50% inhibition at a concentration of $1.7 \times 10^{-6}$ M (10), suggest-
ing that its affinity for the enzyme from human liver may equal
or exceed its affinity for the bacterial enzyme.

The present results obtained with the bacterial enzyme showed
inhibition to be fully reversible by dilution, purely competitive,
and instantaneous relative to the time scale of initial velocity
measurements. Inhibition of cytidine deaminase might conceivably result from binding at a site other than that directly
involved in catalysis, however experiments with added nucleo-
tides give no evidence for an allosteric site on the enzyme, and

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**Fig. 3.** The cytidine deaminase reaction and the structure of inhibitors.
the substrate saturation curve of the enzyme is hyperbolic. Tetrahydrouridine is a potent inhibitor of enzymes from human liver, mouse kidney, and streptomycetes (10) as well as E. coli; it appears unlikely that such diverse organisms would have maintained a site for binding of tetrahydrouridine if it were not related to catalytic interactions so efficient that they were never abandoned during evolution. The potency of this inhibitor equals or exceeds that of any of several inhibitors presently considered as potential transition state analogs (32); binding of tetrahydrouridine exceeds the combined affinity for substrates for the reverse reaction by a factor in excess of $10^4$.

An ideal transition state analog might be expected to be bound even more tightly, since comparison of the minimal turnover number of the enzyme at its present degree of purification with an estimate of the rate of the nonenzymatic reaction (39) suggests that the enzyme produces a rate enhancement exceeding $10^{10}$. It may be possible to obtain even better inhibitors, but the inevitable differences in structure between any real analog and the altered substrate in the transition state will probably place limits on the improvements which can be realized.

Acknowledgments—We wish to thank Mr. Steve Hohf and Mr. Larry Byers for their assistance in performing the nuclear magnetic resonance studies.

REFERENCES
23. Ackers, G. K., Biochemistry, 6, 723 (1964).
25. Shapiro, R., and Klein, R. S., Biochemistry, 6, 3575 (1967).