Regulatory Properties and Subunit Structure of Chick Embryo Deoxycytidylylate Deaminase*

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SUMMARY

Evidence has been presented that chick embryo deoxycytidylylate deaminase (EC 3.5.4.12) is capable of undergoing structural changes under a variety of conditions. Rate studies revealed that inhibition of the enzyme by ethylenediaminetetraacetate markedly diminishes at 10°C and below. Although EDTA does not inhibit the deaminase at 10°C, the inhibition by urea, N-ethylmaleimide, and 5,5'-dithiobis(2-nitrobenzoic acid) is dramatically enhanced in its presence. End product inhibition by deoxythymidine 5'-triphosphate, shown to be both pH and concentration dependent, is completely eliminated at 10°C by EDTA. The reversal of deoxythymidine 5'-triphosphate inhibition by deoxycytidine 5'-triphosphate is markedly sigmoidal, emphasizing the regulatory interaction of these ligands. Hill constants for the reaction of the enzyme with substrate in the presence of deoxycytidine 5'-triphosphate and deoxythymidine 5'-triphosphate are 1.0 and 4.0, respectively. The Hill constant for the homotropic reaction of deoxycytidine 5'-monophosphate with the deaminase at 30°C is 4.0, whereas that at 10°C is 2.8. The apparent $K_m$ value for the activation of the deaminase by deoxycytidine 5'-triphosphate is $2.12 \times 10^{-4}$ mM.

The subunit structure of the deaminase is emphasized by its conversion from an $d_{18}$ value of 6.78 in the presence of deoxycytidine 5'-triphosphate to a value of 3.63 S in the presence of deoxycytidine 5'-triphosphate. The latter form of the enzyme is inactive when assayed in the absence of mercaptoethanol.

Deoxycytidine 5'-triphosphate and, to a lesser extent, deoxycytidine 5'-monophosphate and deoxyguanosine 5'-monophosphate protect the deaminase rather strikingly from inactivation by trypsin. The low molecular weight, inactive form of the enzyme is not protected under similar circumstances.

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Our initial observation (1) describing the regulation of deoxycytidylylate deaminase through the interplay of dCTP and dTTP was shown subsequently to be an extremely effective method of directing the contribution of this enzyme to the pyrimidine deoxyribonucleotide pathway in animal cells (2, 3). Extension of these studies to the deaminase induced in T2 bacteriophage-infected Escherichia coli (4, 5) revealed a similar but less effective inhibition by dTTP and a complete dependence on dCTP for the deamination of dCMP. The regulation of deoxycytidylylate deaminase would appear in both cases to be related to the efficient utilization of dCMP.

The extreme sensitivity of the chick embryo deaminase (6) to feedback regulation by dCTP and dTTP provided an excellent opportunity to study the mechanisms by which these regulatory agents exert their influence. In earlier studies (2, 6) we described the cooperative nature of the deaminase reaction in the absence of dCTP, an effect that could be eliminated by adding dCTP and enhanced by adding dTTP. Similar results have been obtained with a deoxycytidylylate deaminase from donkey spleen (7). Whether the regulatory effects are associated with alterations in enzyme subunit interaction resulting from conformational changes induced by the regulatory ligands cannot be stated with absolute certainty at present. Experiments with the phage deoxycytidylylate deaminase have definitely shown this enzyme to be composed of subunits (5), but analogous results with the chick embryo deaminase have been more difficult to obtain because of the instability of the latter enzyme. However, even in this case, evidence for an inactive, low molecular weight form of the deaminase could be provided by the use of mercaptoethanol (8). The requirements for restoration of the inactive, low molecular weight form of deoxycytidylylate deaminase to the higher molecular weight, active enzyme will be described in the following paper (9). Evidence for different conformational forms of the deaminase was suggested from the marked stabilization of the deaminase by dCTP against heat, denaturing agents, and mercurials (6).

As shown previously (8), the stabilizing and activating properties of dCTP could be dissociated by the use of mercaptoethanol in the reaction mixtures. Thus, in the absence of dCTP, little or no activity could be shown in the presence of

1 The most probable effectors are Mg-dCTP and Mg-dTTP, but for simplicity only the nucleotides will be presented.
Materials and Methods

Reagents—Nucleotides were purchased from Calbiochem, Schwarz BioResearch, and Sigma. Trizma base, used to prepare Tris-HCl, was obtained from Sigma. Urea was recrystallized before use. Sephadex G-200 was purchased from Pharmacia Fine Chemicals, Inc. All other chemicals were reagent grade.

Chick Embryo Deoxycytidylate Deaminase—The deoxycytidylate deaminase was prepared from chick embryo extracts essentially as described by Maley and Maley [6], but with a second cellulose phosphate column (10). The specific activity of the enzyme used in these studies was about 300 μmoles of dCMP deaminated per mg of protein in 10 min at 37°C.

Assay 1 for Deoxycytidylate Deaminase—Initial velocity determinations were obtained with a Beckman DU spectrophotometer equipped with a Gilford model 2000 multiple absorbance recorder. The routine reaction mixtures contained dCMP, 0.5 μmole; dCTP, 0.02 μmole; MgCl₂, 1 μmole; Tris-HCl, pH 8.0, 10 μmole; enzyme; and water to a final volume of 1.0 ml. The specific activity of the enzyme used in these studies was about 300 μmoles of dCMP deaminated per mg of protein in 10 min at 37°C.

Assay 2 for Deoxycytidylate Deaminase—Some of the measurements of enzyme activity at 10°C were performed as described earlier (11). Perchloric acid was used to terminate the reactions at various time intervals and the decrease in absorbance at 290 nm was followed at 10°C or 30°C and recorded automatically. To convert to micromoles of dUMP formed (x), the relationship

\[ y = 2.33(a - x) + 0.358x \]

was used (10), where y is absorbance at 290 nm, and a is initial concentration of dCXP, in micromoles. At substrate concentrations above 1 μM, cuvettes with a light path of 5 and 2 mm were used.

Effect of EDTA on Deoxycytidylate Deaminase Activity—The requirement for a divalent metal ion to show the activating and stabilizing effects of dCTP was observed initially with the chick embryo enzyme (6) and was later found to hold for the spleen (7) and T2-induced (5) deaminases. Since EDTA is an irreversible inhibitor of the deaminase under the usual assay conditions (30°C or 37°C), it was considered possible that the metal requirement could be shown more definitively at temperatures where the enzyme is more stable. As seen in Fig. 1, an effect contrary to that anticipated was obtained, the inhibition by EDTA was virtually eliminated at 10°C and below. The results indicate that the stabilizing effect of the Mg-dCTP complex is unnecessary at the lower temperatures. Similar results were obtained in the presence of EDTA at higher temperatures (30–37°C), when either mercaptoethanol or dithiothreitol was used as the stabilizing agent.

Urea Inactivation—EDTA, as shown in Fig. 2, has a marked potentiating effect on the inactivation of the deaminase at 10°C by urea. At concentrations where neither alone affects the enzyme, the two together appear to act in concert to impair deaminase activity.

N-Ethylmaleimide and 5,5'-Dithiobis(2-nitrobenzoic Acid) Inhibition—Our initial studies (11) revealed that embryo deoxycytidylate deaminase was markedly sensitive to p-chloromercuribenzoate, an inhibition later shown to be limited by the presence of dCTP (6). That the sulphydryl groups involved in the impairment of enzyme activity were of the “masked” or

Fig. 1. The effect of EDTA on deoxycytidylate deaminase activity at various temperatures. The reaction mixtures contained 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2 mM dCMP, and 10 units of enzyme, in a total volume of 2.50 ml. At the indicated temperatures (0, 10, 17.5, 22, 30, and 37°C), a 0.3-ml aliquot was removed at zero time and at least three later times after the addition of enzyme, and added to 2.7 ml of 1 N perchloric acid. The initial velocity (V₀) of enzyme activity in the presence of EDTA was divided by the initial velocity (V₅₀) in the absence of EDTA to derive the ordinate.

Fig. 2. Enhancement of urea inhibition of deoxycytidylate deaminase by EDTA. Assay 2 (see “Materials and Methods”) was used without added dCTP and MgCl₂. The concentration of EDTA was 4 mM. The incubation time, at 10°C, was 30 min. V₀ was 0.250 μmole at 30 min.
Fig. 3. EDTA requirement for N-ethylmaleimide (NEM) inhibition of deoxycytidylate deaminase. Assay 2 (see "Materials and Methods") was used, with conditions similar to those in Fig. 2. \( V_0 \) was 0.244 \( \mu \)mole at 30 min.

Table I

Effect of EDTA on 5,5'-dithiobis(2-nitrobenzoic acid) inhibition at 10°

The reaction was measured by Assay 2 (see "Materials and Methods"). The concentration of EDTA was 10 mM and the concentration of 5,5'-dithiobis(2-nitrobenzoic acid) as indicated. \( V_0 \) was 0.244 \( \mu \)mole at 15 min.

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<thead>
<tr>
<th>5,5'-Dithiobis(2-nitrobenzoic acid)</th>
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"buried" variety was indicated by the resistance of the enzyme to inhibition by N-ethylmaleimide and iodoacetamide (6). The reactivity of these masked sulfhydryl groups can, however, be made more accessible to N-ethylmaleimide by the presence of EDTA, as shown in Fig. 3. Little or no inhibition is evident in the absence of EDTA. Similar results were obtained with 5,5'-dithiobis(2-nitrobenzoic acid) (Table I).

Effect of EDTA on dTTP Inhibition—Aside from its effect on "loosening" or "relaxing" the enzyme structure, EDTA also eliminated the end product inhibition by dTTP, a result that emphasizes the requirement for a divalent cation in addition to dTTP to obtain an inhibitory response. This effect was seen most dramatically at high dCMP levels (>2 mM), where dCTP was not required for the demonstration of deaminase activity (Fig. 4). Unlike the activation by dCTP, the inhibition by dTTP was pH dependent (Fig. 5), an effect probably related to binding of the latter to a specific site on the enzyme. Similar findings have been reported for the spleen deaminase and murine neoplasms (12, 13). As indicated in Fig. 5, the extent of inhibition by dTTP, as well as its pH dependence, was related to the concentration of dTTP.

Apparent \( K_m \) for dCTP—The instability of the deaminase at low dCTP concentrations (<0.001 mM) initially made it impractical to determine the apparent \( K_m \) value for dCTP, but when mercaptoethanol was found to stabilize the deaminase in the absence of dCTP (8), the information presented in Fig. 6 could be obtained. The data indicated an apparent \( K_m \) value for dCTP of 2.1 \( \times 10^{-4} \) mM, a value well within the physiologically significant range.

Fig. 4. Effect of EDTA on dTTP inhibition of dCMP deaminase in the absence of dCTP. \( \bullet \), reaction mixtures contained the following components, in a final volume of 0.5 ml: 5 mM dCMP, 10 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, and 1.25 units of enzyme. \( \circ \) was the same as \( \bullet \), but contained 5 mM EDTA. The reaction rates were measured at 30° by Assay 1 (see "Materials and Methods").

Fig. 5. Effect of pH on dTTP inhibition of deoxycytidylate deaminase. The reaction mixtures contained the following components, in a final volume of 1.0 ml: 0.5 mM dCMP, 0.02 mM dCTP, 1 mM MgCl₂, 20 mM mercaptoethanol, 25 mM acetate phosphateborate, wide range buffer at the pH indicated, and 2.5 units of enzyme; dTTP was present at the concentrations indicated. The reaction rates were measured at 30° by Assay 1 (see "Materials and Methods").
was not felt immediately, but gradually approached a maximum. The reversal of inhibition, however, was associated with a much more rapid response as indicated in Lines 3 and 4, with the reaction rates approaching that of the uninhibited sample (Line 1) almost immediately. The addition of an excess of dTTP to Cuvette 1 a minute or two after the deamination was initiated elicited a response similar to that obtained in Cuvette 4. An awareness of the gradual nature of this response in deriving the kinetics is important, as difficulty may be encountered in obtaining initial velocity measurements at high inhibitor concentrations unless maximum scale expansion with the Gilford multiple absorbance meter is used (Assay 1).

The sigmoidal kinetic response to dTTP inhibition is dramatically emphasized in Fig. 8, where a striking reversal of inhibition was effected by a 2- to 3-fold increase in the level of dCTP. The extreme degree of cooperativity introduced into this enzyme reaction through the interplay of dCTP and dTTP marks chick embryo deoxycytidylate deaminase as one of the enzymes most sensitive to end product regulation. Even the T2-induced (5) and spleen (7) deaminases, which exhibit similar sigmoidal kinetics, do not appear to be as sensitive to dCTP and dTTP regulation.

Hill Plots for dCTP Activation—Fig. 9 typifies the effect of an activator on a cooperative reaction. The sigmoidal response of the deaminase to dCMP was completely eliminated by dCTP with the restoration of normal Michaelis-Menten kinetics to the reaction. This effect is associated with a conversion of the Hill constant from 4.0 in the absence of dCTP to 1.0 in its presence. A comparison of the deaminase reaction at 10°, in the absence of dCTP, with that at 30° reveals less cooperativity at the lower temperature. An explanation for this effect might reside in a difference in configuration of the enzyme at the two temperatures, a difference that could contribute to the marked change in sensitivity of the enzyme to EDTA at 30° and 10°. However, other explanations certainly cannot be ruled out at the present time.

Hill Plot for dTTP Inhibition—As shown in Fig. 10 and also suggested by the results presented in Fig. 8, the net effect promoted by the presence of dTTP is an apparent enhancement of cooperative subunit interaction. It is not known whether the restoration of the Hill constant to 4.0 signifies that the configuration of the enzyme in the presence of dCMP alone is similar to that in the presence of dTTP. However, the much greater stability of the enzyme in the presence of dTTP is not in agreement with such a proposal.
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TUBE NUMBER

FIG. 11. Sucrose density gradient centrifugation of deoxycytidylate deaminase in the presence of dCTP or dTTP. Gradient tube I contained 4.6 ml of a 5 to 20% linear sucrose gradient, 50 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, and 0.04 mM dCTP. Gradient tube II contained the same components as I, plus 0.12 mM dTTP. Four units of deoxycytidylate deaminase in 0.05 ml were added to the top of each gradient tube and centrifuged at 40,000 rpm for 12.6 hours in the SW 39 head of the Spinco model L ultracentrifuge. The tubes were then punctured and 10-drop fractions were collected. The fractions were assayed by a modification of Assay 2 (8) after the addition of 5 μmoles of mercaptoethanol to each fraction from Gradient II. The incubation was for 40 min at 37°C.

Subunit Structure of Deoxycytidylate Deaminase—Although previous studies (8) indicated that the chick embryo deaminase undergoes a change in subunit aggregation during the course of a sucrose gradient centrifugation in the presence of dTTP, the interpretation of these findings is questionable in the light of more recent experiments. As indicated in Fig. 11, two distinct enzyme peaks can be obtained, one dependent on the presence of dCTP (1) and the other on the presence of an inhibitory excess of dTTP (II). Although the recovery of enzyme activity is usually low in the latter case (not more than 20%), these results have been consistently recorded in over a dozen centrifuge runs. In each case, the low molecular weight form of the deaminase observed in the presence of dTTP was completely inactive unless mercaptoethanol was included in the incubations. The originally reported 1 to 2 S form (6) found in centrifuge runs containing 5 mM dCMP appears to have been an artifact resulting from the deamination of dCMP during the centrifugation.

FIG. 9. Hill plots showing the dependence of deoxycytidylate deaminase on substrate concentration and the effect of dCTP. All reaction mixtures contained 10 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, and the following additions, in a final volume of 1.0 ml: O, dCMP alone (the concentration of substrate was varied from 0.4 to 5 mM, with 2.5 units of enzyme per ml; reaction temperature, 30°C); X, same as O, but at 10°C and with 5 units of enzyme per ml; •, dCMP in the presence of 0.02 mM dCTP and 1.0 mM MgCl₂ (the concentration of substrate was varied from 0.025 to 5 mM, with 2.5 units of enzyme per ml; reaction temperature, 30°C). Assay 1 was used (see "Materials and Methods").

FIG. 10. Effect of dTTP on the Hill plot of deoxycytidylate deaminase. All reaction mixtures contained 10 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, and 2.5 units of enzyme per ml. O, dCMP alone; the concentration of substrate was varied from 0.4 to 5 mM. △, plus 1 mM dTTP; ●, plus 2 mM dTTP; X, plus 3 mM dTTP. The reactions were measured at 30°C by Assay 1 (see "Materials and Methods").

FIG. 12. Protection of deoxycytidylate deaminase against trypsin inactivation by dCTP, dTTP, and dGMP. Digestion mixtures contained the following components, in a volume of 0.20 ml: dCMP deaminase, 25 units; 50 mM mercaptoethanol; 7.5 mM potassium phosphate, pH 7.5; 0.02 mM nucleotide; and 25 μg of trypsin. At the indicated time intervals, 10-μl aliquots were removed and assayed for enzyme activity by Assay 1 (see "Materials and Methods").

The value calculated for 15 determinations was 6.78 ± 0.10; that for the dTTP form was 3.63 ± 0.41, based on 10 deter-
restored by mercaptoethanol. It has been found, since, that irreversible inactivation of the unstable, dissociated form of the deaminase can be effected by the inclusion of mercaptoethanol in the assay mixture. That dTTP is effective in protecting the deaminase against inactivation was shown previously (6). Evidence favoring this proposal is presented in the following paper (9).

Protection against Trypsin Inactivation—That ligands can alter the conformation of a protein, making it less susceptible to the action of a proteolytic enzyme, was shown for hemoglobin (14). Similarly, it has now been found that dCTP dramatically stabilizes the deaminase against the action of trypsin (Fig. 12). Some protection was afforded by dTTP and dGMP but, as in the heat stabilization studies (6), neither was as effective as dCTP. Even after incubation for 48 hours at room temperature little inactivation of the deaminase was found. However, neither dCTP nor dTTP protected against the proteolytic denaturation of the inactive form (Peak II of Fig. 11) of the deaminase.

DISCUSSION

Conditions known to effect conformational transitions in proteins were shown previously (6) to alter the properties of chick embryo deoxycytidylate deaminase. To supplement these findings, it has now been shown in low temperature experiments (10° and below) that EDTA contributes to a relaxation in enzyme structure, as evidenced by the contribution of this chelating agent to the inactivation of the deaminase by urea and N-ethylmaleimide. Normally, no effect was observed in the absence of EDTA (Figs. 3 and 4). The weakening of hydrophobic forces at the lower temperatures, an effect that may be accentuated by urea (15), could have contributed to these results, as well as to the apparent shift of the Hill constant from 4.0 at 30° to 2.8 at 10°.

Also emphasizing the flexibility in enzyme structure are the results showing the striking protection by dCTP against trypsin inactivation and the restoration of enzyme activity to a disaggregated, inactive form of the deaminase by mercaptoethanol supplemented with dCTP or dTTP (8). It was shown recently that dCTP or dTTP, the activator and inhibitor, respectively, for E. coli deoxycytidylate kinase, can effect a dimerization of this enzyme (16). It would be of interest to determine if this protein is also more resistant to proteolysis in the presence of its allosteric effectors.

The presence of dTTP was shown previously to favor a disaggregated form of the deaminase (8). Although this effect has been obtained numerous times, current results indicate that thiol reagents prevent the dissociation of chick embryo deoxycytidylate deaminase in the presence of dTTP. Thus, it appears that dTTP exerts its effect mainly by preventing the irreversible inactivation of the unstable, dissociated form of the enzyme during the course of the gradient centrifugation. A thiol reagent, such as mercaptoethanol or dithiothreitol, not only prevents the disaggregation but, in these cases where it has already occurred, promotes time-dependent restoration to the active, high molecular weight form of the deaminase provided dTTP or dCTP is present (9). The latter results would therefore furnish an explanation for the presence of the high molecular weight form of the deaminase in those gradient tubes containing both dTTP and mercaptoethanol, in contrast to those containing dTTP alone. It is thus problematical whether dTTP inhibits the enzyme by promoting an inactive, low molecular weight form of the enzyme, since this nucleotide inhibits even under conditions that favor the aggregated state. These results are, however, somewhat at variance with those recently described for the more stable T2 deaminase where, even after the inclusion of mercaptoethanol in the sucrose gradient, the disaggregated form of the deaminase was obtained. Even in this case, however, inhibition could be observed under assay conditions that favored rather strongly the aggregated structure. The most likely explanation at present for the existence of two forms of the enzyme is that the deaminase is subject to a conformational change effected by a sulfhydryl-disulfide interchange reaction. The extent to which the deaminase is affected depends on the type of ligands and chelating reagents to which the enzyme is exposed (9). It is of interest to note that the pH dependence of the dTTP inhibition (Fig. 5) is also related to the concentration of dTTP. Such an effect could be mediated by a structural change in the deaminase effected by dTTP.

The deaminase appears to belong to a growing list of end product-regulated enzymes, most of which seem to be composed of subunits (17). In most instances, however, the aggregate subunit structure is not altered in the presence of regulatory ligands and in some cases (18) even the Hill constant, which is often taken as a measure of subunit interaction, does not vary. For this reason, the sequential model of enzyme regulation (19), which proposes a gradual or sequential change in protein subunits by the regulatory ligands, is somewhat more attractive than the more restrictive symmetry model (17). However, as indicated recently by Sweany and Fisher (20), cooperative subunit interactions are not necessarily responsible for sigmoidal kinetics if conformational changes affecting rate constants in a reaction pathway are introduced. Since, as indicated above, a number of possible alternatives may be responsible for the regulation of enzymes by end products, all that can be claimed for the chick embryo deoxycytidylate deaminase at this time is that dCTP and dTTP appear to alter the conformation of the enzyme protein. Whether this effect influences attendant changes in the quaternary structure of the protein or involves primarily an alteration in the molecular volume of the enzyme, as shown recently for aspartate transcarbamylase (21), remains to be determined.

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REFERENCES
Regulatory Properties and Subunit Structure of Chick Embryo Deoxycytidylate Deaminase
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