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Partial Purification and Properties of Cytidine Deaminase from Baker's Yeast*

Pier Luigi Ipata, Giovanni Cercignani, and Ettore Balestreri†

ABSTRACT: Enzymological studies on partially purified preparations of baker's yeast cytidine deaminase are described. The enzyme is inhibited both by cytidine 5′-monophosphate (CMP) and substrate excess. Plots of kinetic data for CMP take the form of sigmoidal inhibition curves. Substrate saturation curves show first-order kinetics at low cytidine concentrations, suggesting the absence of interaction between catalytic sites; however, substrate inhibition seems to involve cooperative binding of cytidine at inhibitory sites. Cooperativity is also observed between CMP and cytidine inhibitory sites. Heating the enzyme preparation above 50° abolishes both CMP and substrate excess inhibition, while catalytic activity is left unchanged even upon heating at 60°. The data obtained are interpreted as indicating the allosteric nature of inhibition by both CMP and cytidine; as a mere hypothesis, the same kind of inhibitory sites could be thought to be implied in the binding of both compounds, thus providing a satisfactory explanation for the kinetic data reported.

Cytidine deaminase (cytosine nucleoside aminohydrolase, EC 3.5.4.5) was originally found by Schmidt (1932) in dog liver extracts and has since been identified in a variety of biological sources (Conway and Cooke, 1939; Greenstein *et al.*, 1947; Creasey, 1963; Tomchick *et al.*, 1968; Wang *et al.*, 1950; Wang, 1955; Wisdom and Orsi, 1967; Achar *et al.*, 1966). The enzyme has been partially purified from mouse kidney (Creasey, 1963; Tomchick *et al.*, 1968), sheep liver (Wisdom and Orsi, 1967), and green-gram seedlings (Achar *et al.*, 1966). An enzyme activity in yeast, catalyzing the deamination of cytidine, was originally found in crude extracts by Wang *et al.* (1950) and appears to be distinct from cytosine deaminase. The properties of the enzyme, however, have never been studied in purified systems. The present paper reports some kinetic properties of yeast cytidine deaminase. The data show that the enzyme is allosterically inhibited both by substrate excess and by CMP.[1] Cytosine deaminase activity, in accordance with the results obtained by Lochmann (1965), was also found in yeast extracts; in the course of purification, however, the ratio of the two enzymatic activities changes from step to step; after gel filtration on G-100 Sephadex two distinct peaks are obtained for cytidine and cytosine deaminase activities.

Experimental Section

Materials. Nucleosides and nucleotides were obtained either from Sigma Chemical Co. or from Boehringer und Soehne. Whale skeletal myoglobin was obtained from Seravac Laboratories. Pancreatic ribonuclease was obtained from Sigma Chemical Co. Adenosine deaminase from calf intestinal mucosa was obtained from Boehringer und Soehne. Tris (Sigma) was used as a buffer. Other chemicals were of reagent grade or of the highest quality available.

Assay Procedure. A spectrophotometric assay, based on the differential absorption of cytosine and uracil and their nucleosides, was used. The assay was conducted in microcuvets with 1-cm light path and was monitored at 286 mμ with a Zeiss PQM II absorbance recording spectrophotometer, at room temperature. The assay mixture contained, in a final volume of 1 ml, 0.45 M Tris-HCl buffer (pH 7.2), different amounts of enzyme preparation, and substrate solution; the reaction was started by addition of the enzyme preparation and the decrease in optical density at 286 mμ was recorded against a reference cuvet in which substrate was substituted by water. The molarities of all substrate and inhibitors solutions were measured spectrophotometrically from the extinction coefficients at 260 mμ at pH 7 (Cohn, 1955). Modifications of the standard conditions are described in the presentation of ex-

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[1] Abbreviation used is: *p*-MB, *p*-mercuribenzoate.

TABLE I: Purification of Cytidine Deaminase from Baker's Yeast.

Fraction	Vol (ml)	Protein (mg)	Cytidine Deaminase (Units)[a]	Sp Act. (Units/mg of Protein)	Act. Ratio Cytidine Deamination: Cytosine Deamination
1	1582	17,700	276,000	15.6	0.5
2	74	8,000	293,000	36.7	1.88
3	55	765	79,000	103	12.5
4	37	186	42,300	227	18
5	27	47.5	8,700	395	26.1
6	63	6.3	4,100	650	∞

[a] One unit equals an activity equivalent to a decrease of 0.001 optical density unit/min per mg of protein.

perimental data. This assay method, a slight modification of that of Wang (1955), is a sensitive one, and final substrate concentrations as high as 1.5 mM can be used; the difference absorptivities at 286 mμ for cytidine and uridine, and for cytosine and uracil, are 3.14 μm, and 0.937 cm² per μmole, respectively; thus, a change in optical density of 1 optical density unit corresponds to the formation of 0.318 μmole/cm² of uridine from cytidine, and to the formation of 1.067 μmoles/cm² of uracil from cytosine.

Preparation of Partially Purified Cytidine Deaminase. In the fractionation procedure reported below, the temperature was maintained between 0 and 4° unless otherwise stated. The purification scheme is summarized in Table I.

PREPARATION OF YEAST AUTOLYSATE. Baker's yeast Vulcania was plasmolyzed according to the method of Kunitz (1947) by swelling with toluene (2 kg/l.) at 45°, left at room temperature for 2–3 hr, added with cold distilled water (1 l./kg of yeast), and transferred to separatory funnels in the cold. After 18 hr the aqueous phase, containing the cellular homogenate, was collected and centrifuged for 20 min at 10,000 rpm; the precipitated fraction was discarded and any turbidity from the supernatant eliminated by filtration through Whatman No. 3MM paper. The filtered supernatant was considered as crude extract (fraction 1 of Table I).

AMMONIUM SULFATE FRACTIONATION. Fraction 1 was precipitated by adding solid ammonium sulfate up to 67% saturation. The pellet was solubilized in the minimal volume of 0.05 M Tris-HCl buffer (pH 7.2, fraction 2). The residual supernatant still retained some cytosine deaminase activity, while cytidine deaminase activity was found only in the precipitated fraction.

G-100 SEPHADEX FRACTIONATIONS. Fraction 2 (20 ml) was applied to the top of a G-100 Sephadex column (120 × 6 cm), equilibrated with 0.05 M Tris-HCl buffer (pH 7.2), and eluted with the same buffer in 9-ml fractions at a flow rate of approximately 15 ml/hr; this procedure was repeated several times in order to process the entire fraction 2. The elution profile is shown in Figure 1A; the most active fractions (77–85) were pooled and are referred to as fraction 3.

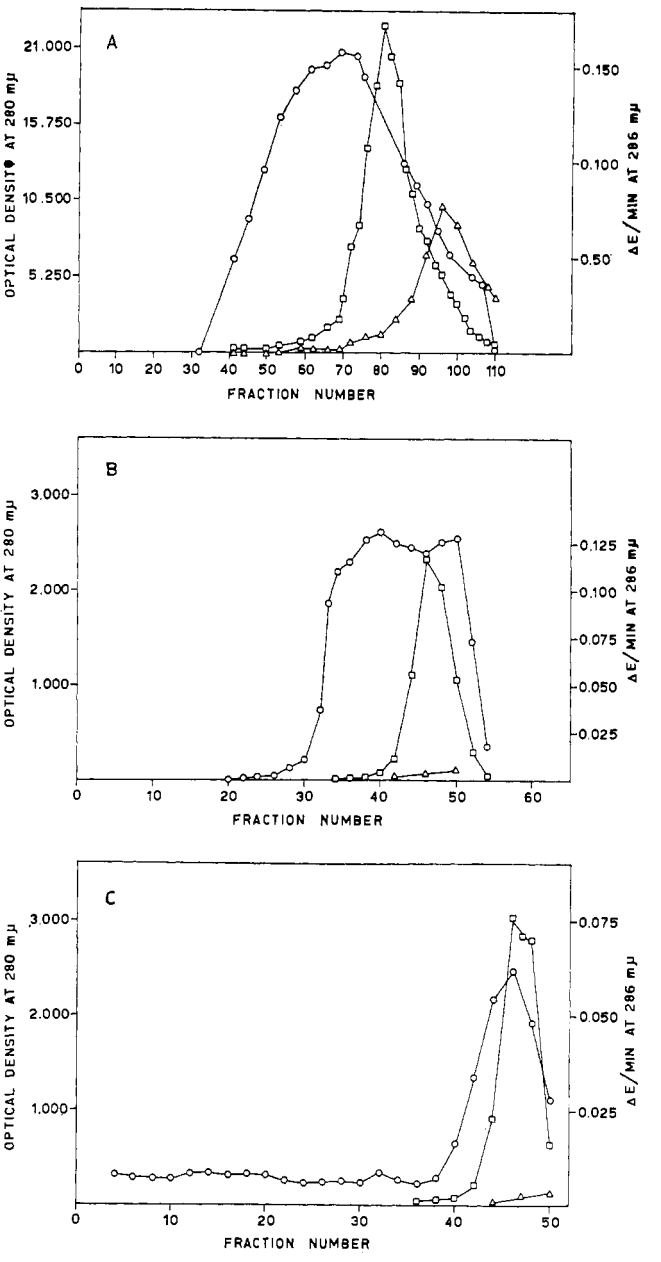

FIGURE 1: Elution profiles on G-100 Sephadex columns for fraction 3 (A), fraction 4 (B), and fraction 5 (C). (○—○) Optical density at 280 mμ, (□—□) cytidine deaminase activity, and (△—△) cytosine deaminase activity. Enzyme activities were assayed as described under Experimental Section and are expressed as Δ absorbance per minute at 286 mμ. 100 μl of each fraction was used in the assay.

Subsequent steps in purification procedure were carried out trying to make the most of the possibilities of gel filtration technique. Thus, fraction 3 was precipitated with ammonium sulfate up to 67% saturation, solubilized in the minimal amount of 0.05 M Tris-HCl buffer (pH 7.2) and then passed through a second G-100 Sephadex column (95 × 3 cm), eluted as before in 6-ml fractions, at a flow rate of about 9 ml/hr (Figure 1B); the pool of the active fractions (45–49) is referred to as fraction 4. The procedure was repeated a third time, with the same column of step 4 (flow rate 7.5 ml/hr;

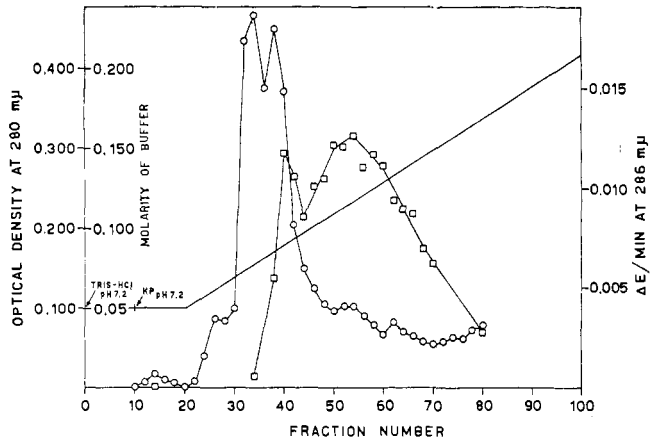

FIGURE 2: Elution profile of cytidine deaminase on DEAE-cellulose column (fraction 6). (○—○) Optical density at 280 mμ and (□—□) cytidine deaminase activity. Enzyme activity was assayed and expressed as in Figure 1.

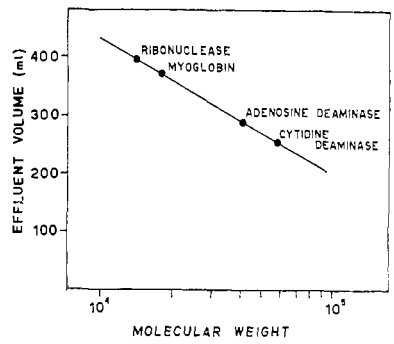

FIGURE 4: Plot of elution volume against log (molecular weight) for cytidine deaminase and other marker proteins on G-100 Sephadex. Elution was carried out according to Andrews (1964). Void volume for myoglobin was estimated by the absorption at 407 mμ; void volumes for adenosine deaminase and ribonuclease were estimated by assaying the effluent by the methods of Kalckar (1947) and of Ipata and Felicioli (1968), respectively. Cytidine deaminase was located by assaying the effluent as in Figure 1.

fraction volume 6 ml); tubes containing the most active fractions (45–47) were collected and pooled (fraction 5) (Figure 1C).

DEAE-CELLULOSE CHROMATOGRAPHY. Fraction 5 was absorbed on a DEAE-cellulose column (27 × 2 cm), which was then eluted in 6-ml fractions with the following buffers: 50 ml of 0.05 M Tris-HCl (pH 7.2), 50 ml of 0.05 M potassium phosphate (pH 7.2), and a linear gradient between 0.05 and 0.2 M potassium phosphate (pH 7.2). A flow rate of approximately 23 ml/hr was maintained. The enzyme protein was eluted around a concentration of 0.1 M phosphate buffer (fraction 6). As can be seen in Figure 2, although most of the inactive proteins were removed, the purification factor of this step (Table I) is not so high as it would be expected, and some inactivation seems to have occurred. Indeed, higher purification factors in this step were obtained during some preparations, when the eluting buffers of the DEAE-cellulose column contained 1 mM glutathione.

During gel filtration steps, inactivation never occurred, showing that the yeast enzyme is not sensitive to this treatment, in contrast with the mouse kidney enzyme (Tomchick *et al.*, 1968).

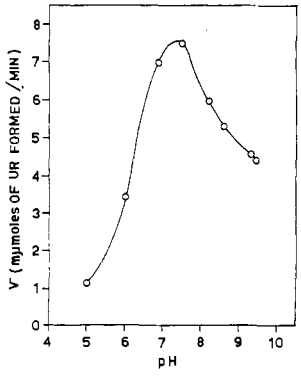

FIGURE 3: Effect of hydrogen ion concentration on initial velocity of baker's yeast cytidine deaminase. The velocity is expressed as millimicromoles of uridine formed per minute.

The enzyme preparation was usually stored at −25°. No appreciable loss of activity was detected in the stored preparation after 1 month. The final preparation did not catalyze the deamination of adenosine, guanine, AMP, and CMP.

Proteins were determined spectrophotometrically (Warburg and Christian, 1942).

Results

Effect of pH. Figure 3 shows the variation of reaction rate as a function of hydrogen ion concentration. Tris-acetate buffer (0.5 M) was used. It must be noted that the difference molar absorptivity for cytidine and uridine is practically independent of hydrogen ion concentration in the pH range studied. As can be seen, a single activity optimum is found near pH 7. At pH values lower than 5, rapid inactivation and precipitation of the enzyme protein occurs.

Molecular Weight. This determination was carried out according to Andrews (1964) by gel filtration on a G-100 Sephadex column (90 × 3 cm), equilibrated with 0.025 M Tris-HCl buffer (pH 7.2). A mixture of enzyme preparation and marker proteins of known molecular weight was applied to the top of the column, which was then eluted with the same buffer, at a flow rate of about 15 ml/hr. In Figure 4, the effluent volume is plotted against the logarithm of molecular weight. The elution volume corresponding to cytidine deaminase led to a calculation of 57,000 as its molecular weight.

Sensitivity to p-MB. Cytidine deaminase from baker's yeast is highly sensitive to mercurial agents. *p*-MB significantly reduces reaction rate at concentrations as low as 0.1 μM (Figures 5 and 8), that is, nearly in stoechiometric amounts with respect to enzyme protein.

Reaction Kinetics. Yeast cytidine deaminase displays a peculiar substrate saturation curve (Figure 6): accord with Michaelis–Menten kinetics is found up to 0.500 mM cytidine; increasing concentration of substrate causes a reduction of catalytic activity, which is reduced almost to zero beyond 1.5 mM. Evaluation of the K_m (app) from Lineweaver–Burk double-reciprocal plot gives a value of 0.25 mM (inset in Figure 6). Several determinations ranged between 0.2 and 0.3 mM.

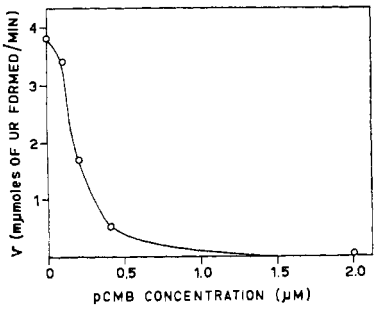

FIGURE 5: Effect of *p*-MB concentration on initial velocity. *p*-MB was added in the cuvet simultaneously with substrate, before the addition of the enzyme preparation. The velocity is expressed as in Figure 3.

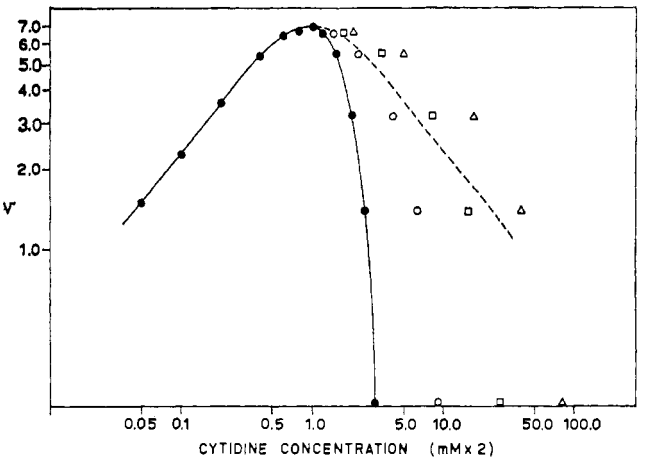

FIGURE 7: The same data of Figure 6 are reported on a double-logarithmic plot. (●—●) Abscissa = (S), (○- -○) abscissa = (S^2), (□- - -□) abscissa = (S^3), and (△- -△) abscissa = (S^4). The dotted line indicates symmetrical extrapolation of the rising part of the graph.

When substrate saturation data are reported on a logarithmic plot, according to Augustinsson (1949; see also Laidler, 1958), they fail to fall on a symmetrical bell-shaped curve (Figure 7), as it would be expected from a first-order dependence of inhibition on substrate concentration; rather, the right part of the graph is much steeper than the left one. However, if points beyond 0.500 mM are plotted as two, three, and four times the logarithm of cytidine concentration, a mixed fit is obtained on a symmetrical curve, indicating an increasing order of inhibition rate with respect to substrate concentration.

Inhibitors. Cytidine deaminase from baker's yeast is strongly inhibited by CMP. The following compounds, tested at 0.350 mM final concentration, had no inhibitory effect on enzyme activity: ATP, ADP, ITP, IDP, IMP, XMP, CTP, UDP, adenosine, and cytosine.

The inhibition exerted by CMP was further investigated. The shape of substrate saturation curves is maintained in the presence of constant CMP concentrations, but the maximum velocity attainable and the corresponding substrate concentration are lowered by increasing concentration of the inhibitor. An appreciable decrease in the K_m (app) evaluated from double-reciprocal plots is also observed (Figure 8). The theoretical V_{max} is also altered in the same sense.

When cytidine deaminase is assayed in the presence of increasing concentrations of CMP, a sigmoidal inhibition curve is obtained. A slight but constant activation at low inhibitor concentration was always observed. The interaction coefficient (n') for CMP, calculated from the Hill plot, was 4.6 (Figure 9).

Heat Sensitivity. Cytidine deaminase preparations in 0.05 M Tris-HCl buffer (pH 7.2) were held at various temperatures for 5 min, rapidly chilled and tested under three conditions for each preincubation temperature: (1) at a noninhibitory cytidine concentration; (2) at an inhibitory cytidine concentration; (3) at a noninhibitory cytidine concentration, but in the

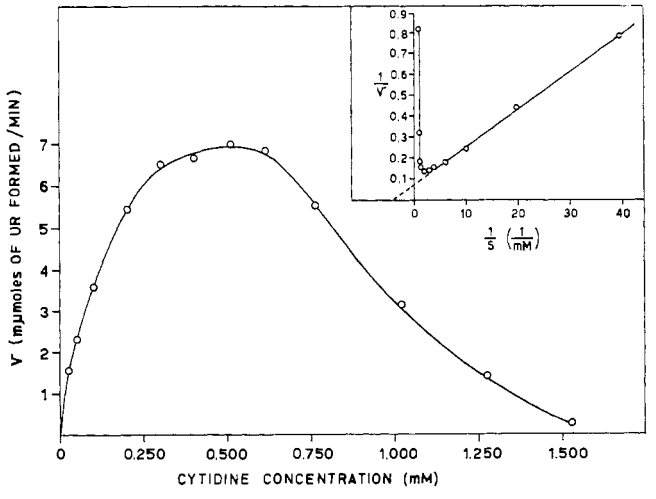

FIGURE 6: Effect of cytidine concentration on initial velocity. The inset shows the plot of $1/v$ *vs.* 1/(S) for the evaluation of the K_m (app) by extrapolation of the linear part of the graph. The velocity is expressed as in Figure 3.

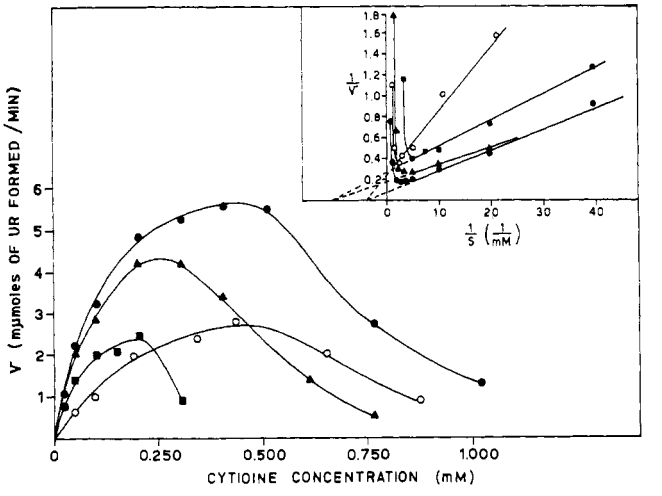

FIGURE 8: Effect of cytidine concentration on initial velocity in the absence and in the presence of inhibitors. (●—●) No addition, (▲—▲) 0.225 mM CMP, (■—■) 0.375 mM CMP, and (○—○) 0.25 μM *p*-MB. The inset shows double-reciprocal plots. The velocity is expressed as in Figure 3.

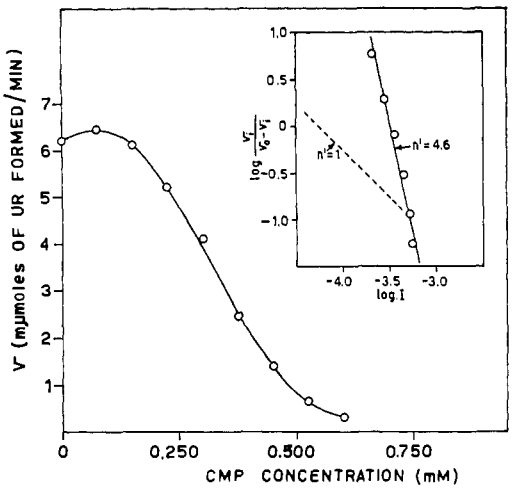

FIGURE 9: Effect of CMP concentration on initial velocity. The final cytidine concentration was 0.300 mM. The inset shows the Hill plot for the evaluation of the interaction coefficient, n'. The velocity is expressed as in Figure 3.

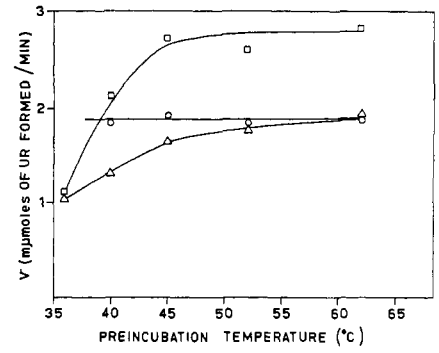

FIGURE 10: Effect of heating on catalytic activity, substrate inhibition, and CMP inhibition of cytidine deaminase. The enzyme preparations were held at different temperatures for 5 min, rapidly chilled, and then assayed with 0.290 mM cytidine (○—○); with 0.870 mM cytidine (□—□); with 0.290 mM cytidine in the presence of 0.630 mM CMP (△—△). The velocity is expressed as in Figure 3.

presence of CMP. The results are reported in Figure 10, where initial velocity rates are plotted against preincubation temperature: as can be seen, cytidine deaminase from baker's yeast is desensitized to both substrate and CMP inhibition upon heating at temperatures above 50°, while catalytic activity is practically left unchanged by the treatment in this temperature range; the two desensitization curves show an almost parallel temperature dependence.

Discussion

The data presented above indicate the presence in yeast plasmolysates of a cytidine deaminase with a molecular weight of about 57,000, characterized by sensitivity to mercurial agents, a common property of all nucleoside aminohydrolases (Creasey, 1963; Tomchick *et al.*, 1968; Achar *et al.*, 1966; Wisdom and Orsi, 1967; Ronca *et al.*, 1967; Wolfenden *et al.*, 1967), and by inhibition by CMP and substrate excess. The kinetic data are noteworthy enough to deserve brief discussion.

It is generally assumed that substrate inhibition might be due to one of the following mechanisms: (1) direct interaction between two substrate molecules at the active site; (2) binding of substrate molecules at a second site, resulting in the formation of an inactive complex (Laidler, 1958; Mahler and Cordes, 1969); (3) an allosteric transition between two states of the enzyme protein "with different affinities for the substrate, the one with higher affinity being catalytically inactive," as proposed by Monod *et al.* (1965). Our data show that, at inhibitory substrate concentration (beyond 0.500 mM), several substrate molecules interact with cytidine deaminase at noncatalytic sites. Although the exact number of sites could not be determined on the basis of kinetic experiments, this statement is supported by the data reported in Figure 7.

Interaction of more than one CMP molecule with the enzyme at noncatalytic sites is suggested by the strongly sigmoidal shape of the inhibition curves. Furthermore, any direct interaction at the catalytic site between cytidine and CMP could be excluded *a priori*, on the basis of kinetic data, showing that the nucleotide does not behave as a competitive inhibitor (Figure 8).

Finally, the assumption that the inhibitory sites, at which cytidine and CMP bind, are topographically distinct from the catalytic site(s) is supported by the fact that heat treatment causes a desensitization of cytidine deaminase to both substrate and CMP inhibition. Even though further data are required for supporting this hypothesis, the almost parallel temperature dependence of the two inhibitory effects seems to indicate that cytidine and CMP probably bind at the same sites.

It must be finally emphasized that the shapes of substrate saturation curves obtained in the presence of a purely noncompetitive inhibitor, such as *p*-MB, are somewhat different from those observed in the presence of CMP, as shown by the fact that the substrate concentration corresponding to the maximum attainable rate is lowered by increasing the nucleotide concentration; thus, in the absence of inhibitors or in the presence of *p*-MB, the maximum rate occurs around 0.450 mM cytidine, while, at 0.225 and at 0.375 mM CMP, around 0.250 and 0.200 mM, respectively (Figure 8).

To account for this result, it might be assumed that the binding of CMP at the inhibitory sites causes a conformational change of the enzyme protein, leading to an increased affinity of cytidine for the inhibitory, noncatalytic sites.

A tentative metabolic role of CMP inhibition on cytidine deaminase could be suggested, but additional data on the specificity of the inhibitor are still required, along with further investigation on the kinetic effects of other metabolites.

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Purification and Properties of Cytochrome c_3 of *Desulfovibrio salexigens**

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ABSTRACT: Cytochrome c_3 of *Desulfovibrio salexigens* was purified to homogeneity as judged by disc gel electrophoresis and by ultracentrifugation. The molecular weight calculated from sedimentation-diffusion data is 13,387 and from amino acid composition it is 13,901. It has a partial specific volume of 0.72 ml/g, an isoionic point of 10.8, a redox potential of -205 ± 5 mV at pH 7.0, and contains three heme groups per mole of protein. *D. salexigens* cytochrome c_3 differs from the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris* in its amino acid composition and electrophoretic mobility on polyacrylamide gel at pH 6.6. Immunodiffusion data demonstrate that it does not share a common precipitating antigenic determinant with the cytochrome c_3 of *D. desulfuricans* or *D. vulgaris*.

As part of a program on the comparative biochemistry of the nonsporulating, sulfate-reducing bacteria, we have conducted studies to determine if the differences in the deoxyribonucleic acid base composition (Saunders *et al.*, 1964) of the physiologically related species of *Desulfovibrio* (Postgate and Campbell, 1966) would be reflected in differences in the cytochrome c_3 of these organisms. This protein was chosen because of its small size and its role in the metabolism of these organisms (Postgate, 1956, 1965).

In previous papers we have examined the properties of the cytochrome c_3 of *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris*. The cytochromes c_3 of these two species are similar in their redox potentials, sedimentation constants, molecular weights, partial specific volumes, and polypeptide chain lengths, and contain three heme groups per mole of protein (Drucker and Campbell, 1969; Drucker *et al.*, 1970a). They differ, however, in their isoionic points, amino acid compositions (Drucker *et al.*, 1970a), optical rotation properties (Drucker *et al.*, 1970b), and electrophoretic properties, and do not share a common precipitating antigenic determinant (Drucker and Campbell, 1969).

LeGall *et al.* (1965) and Bruschi-Heriaud and LeGall (1967) have reported that the cytochrome c_3 of *Desulfovibrio gigas* has some properties in common with the cytochrome c_3 of *D. vulgaris* but differs from the latter in its electrophoretic behavior and amino acid composition. By use of the data of LeGall *et al.* (1965) and Bruschi-Heriaud and LeGall (1967) and a molecular weight of 13,425 we have calculated that *D. gigas* cytochrome c_3 also contains three hemes per mole of protein (Drucker *et al.*, 1970a). *D. gigas* cytochrome c_3 has been shown not to cross-react with antisera against the cytochrome c_3 of *D. desulfuricans*, *D. vulgaris*, or *D. salexigens* (E. Trousil and L. L. Campbell, unpublished data).

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