Bacteriophage PBS2-Induced Deoxycytidine Triphosphate Deaminase in *Bacillus subtilis*

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The dCTP deaminase induced by *Bacillus subtilis* bacteriophage PBS2, whose DNA contains uracil instead of thymine, requires metal ion and thiol activators and has a molecular weight of 125,000. The enzyme displays sigmoidal substrate saturation kinetics and inhibition by dUTP, consistent with the deaminase's proposed role of providing balanced levels of dUTP and dCTP for PBS2 uracil-DNA synthesis.

Bacteriophage PBS1 and its clear-plaque mutant PBS2 are unique because their DNA contains uracil instead of thymine (5, 11). These phages induce several enzymes which appear to modify the deoxyribonucleotide metabolism of the bacterial host, *Bacillus subtilis*. Tomita and Takahashi (12) have described the appearance and some of the kinetic properties of a dCTP deaminase (dCTP aminohydrolase, EC 3.5.4.13) activity induced after PBS1 phage infection. As part of our investigations on the enzymatic mechanism of uracil-DNA synthesis (5, 6), we have continued the study of the induction and the properties of PBS2 dCTP deaminase in relation to its proposed role in vivo of providing dUTP for PBS2 uracil-DNA synthesis.

Reaction mixtures for dCTP deaminase assays contained 50 mM potassium phosphate (pH 7.1), 0.3 mM MgCl₂, 10 mM 2-mercaptoethanol (MSH), 0.42 mM [5-³H]dCTP (7 μ Ci/ μ mol), and enzyme in a total volume of 100 μ liters. After incubation for 15 to 60 min at 37 C, the assay tubes were held in a boiling water bath for 1 min. Any precipitate was removed by brief centrifugation, and 50 μ liters of the supernatant fluid (containing about 20,000 counts/min) was spotted on Whatman no. 1 paper for descending chromatography for 20 h in isobutyric acid-1 M NH₄OH-0.1 M EDTA (100:60:1.6, by volume). The area corresponding to dUTP (R_f = 0.15) was cut from the paper and counted in 15 ml of toluene-based scintillation fluid (6). Blank values of about 200 counts/min (from reactions lacking enzyme or containing enzyme inactivated by boiling) were subtracted.

Methods for the growth of *B. subtilis* SB19 and PBS2 phage, including the preparation and dialysis of extracts of infected cells, have been described previously (5). The PBS2 dCTP de-

aminase from crude extracts was passed through a DEAE-cellulose column as described by Tomita and Takahashi (12). The active pooled fractions were concentrated by ultrafiltration in buffer G (50 mM Tris-chloride, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol) and applied to a Sephadex G100 column. The deaminase activity was eluted in fractions corresponding to a molecular weight of 125,000. The specific activity of the pooled fractions was 2.5 μ mol of dUTP produced per hour per milligram of protein, about 10-fold greater than that of dialyzed crude extracts. This preparation was free of contamination by the bacterial dUTP nucleotidohydrolase, which binds to DEAE-cellulose and has molecular weight of about 45,000 (A. Price, unpublished data); it was also free of any dCTP-hydrolyzing activities. All deaminase preparations were unstable, losing 50% of their activity per month at 4 or -20 C (or all activity in 2 weeks at 4 C in the absence of thiol compounds).

The dCTP deaminase appears to be a new PBS2-induced enzyme. Uninfected *B. subtilis* extracts (and DEAE-cellulose column fractions) only degrade dCTP to dCDP and some dCMP, producing essentially no deoxyuridine-containing compounds (less than 5% of infected extracts activity). Addition of chloramphenicol (100 μ g/ml) or actinomycin D (10 μ g/ml) at the time of PBS2 infection prevents the subsequent appearance of dCTP deaminase, indicating that RNA and protein synthesis are essential for enzyme induction. In addition, rifampin (100 μ g/ml) does not affect deaminase induction; this antibiotic has been previously shown to block host (but not phage) RNA synthesis (7, 8), suggesting that dCTP deaminase is indeed a PBS2 phage-coded enzyme.

In confirmation of the results of Tomita and

Takahashi (12), we find that partially purified PBS2 deaminase is specific for dCTP, has a pH optimum of 6 to 8, is inhibited by high Pi concentrations, and is stimulated about twofold by magnesium ions. In addition, we find that 3 mM EDTA blocks all activity, whereas elimination of MSH reduces the activity by 30%. All activity in the absence of MSH is lost on addition of 1 mM HgCl_2 , CuCl_2 , or *p*-hydroxymercuribenzoate; this inhibition is prevented by MSH.

Incubation of increasing amounts of partially purified dCTP deaminase results in the progressive deamination of dCTP to dUTP to an apparent limit at about 55% conversion. Tomita and Takahashi (12) showed an apparently linear, stoichiometric conversion of 60% of dCTP to dUTP in 1 h; but they did not indicate whether the reaction would proceed further. This limit does not appear to result from reversal of the reaction, since incubation of [^3H]dUTP with ammonium chloride did not lead to the production of labeled dCTP nor to dUDP and dUMP. Likewise, addition of [^3H]dUMP to the standard assay mixture with unlabeled dCTP did not produce any labeled dUTP. Instead, we believe the reaction is limited by product inhibition of the dCTP deaminase.

Under conditions in which dUTP production was proportional to enzyme concentration and to assay time at each dCTP concentration, partially purified dCTP deaminase displayed sigmoidal substrate saturation kinetics over the range of 5 μM to 1.5 mM dCTP. The nonlinear double reciprocal plot (Fig. 1) is suggestive of positive cooperativity (4) in dCTP binding sites. Extrapolation of such plots from eight independent experiments to the *x*-intercept gave approximate K_m 's for dCTP of 50 to 100 μM . A Hill plot (4) of these data was linear and gave a Hill number of 1.20. Further work to obtain a stable, pure enzyme preparation for physical studies will be required to relate these data to the number of nucleotide-binding sites and possible subunits in the enzyme.

Our results differ from those of Tomita and Takahashi (12), whose data indicate linear saturation kinetics with a K_m for dCTP of 360 μM . (F. Tomita and I. Takahashi [personal communication] indicate that the legend to their Fig. 5 [ref. 12] is correct, but that the $1/S$ values on the figure should all be divided by 450 to obtain the correct units [in μM^{-1}].) However, they used a direct spectrophotometric assay for dCTP deamination over only the short range of 90 to 360 μM . Their assay is relatively insensitive, since the difference in molar extinction

FIG. 1. Double reciprocal plot of PBS2 dCTP deaminase activity dependence on substrate concentration. Partially purified enzyme (10 μliters) was incubated in assays containing bovine serum albumin (100 $\mu\text{g/ml}$) and the indicated concentrations of [^3H]dCTP (0.3 μCi per assay) for 15 min, allowing only 5 to 10% conversion of substrate (*v* expressed as micromoles of dUTP per hour per milliliter of enzyme).

coefficients at 280 nm between dCTP (7,000) and dUTP (3,900) results in absorbance changes of only 2.3 or 11.7% when 5 or 25% conversion of dCTP to dUTP has occurred (ignoring the absorbance of the enzyme preparation itself). Furthermore, the data in Fig. 4 of reference 12, which show linear conversion of 60% of the dCTP over a 60-min period from an initial concentration of 380 μM , are inconsistent with the calculated K_m of 360 μM (ref. 12).

In assays containing dCTP at a concentration below the K_m for dCTP, various nucleotides were found to inhibit the PBS2 dCTP deaminase (Table 1). The most potent inhibitor was dTTP, with dTDP, dTMP, and deoxythymidine decreasingly effective, in agreement with Tomita and Takahashi (12). These authors found no inhibition by 55 μM dUDP nor dUMP (dUTP was not tested) at 250 μM dCTP concentration. In contrast, we find that deoxyuridine nucleotides are effective inhibitors, although they are about fivefold less potent than the corresponding deoxythymidine nucleotides. Furthermore, we show in Table 1 that dCDP, dCMP, CTP, UTP, and other compounds do not inhibit dCTP deamination (and are not substrates). None of the nucleotides were inhibitory at these levels in the standard assay with 420 μM dCTP, and the ^3H -assay is too tedious to make K_i determinations worthwhile.

Figure 2 indicates that the inhibitory effects of dTTP and dUTP can be overcome by high dCTP concentrations. The data of Tomita and Takahashi (12) indicate that 10 to 20 μM dTTP inhibits noncompetitively with a K_i of 26 μM in

TABLE 1. Inhibitors of PBS2 dCTP deaminase

Added compound*	Concn (μ M)	Inhibition (%)
dUTP	20	80
dUDP	20	20
dUMP	20	15
Deoxyuridine	100	5
dTTP	4	75
dTDP	4	20
dTMP	10	25
Deoxythymidine	10	15
dCDP	100	0
dCMP	100	5
Deoxycytidine	100	0
CTP	100	5
dGTP	100	5
ATP	100	10
UTP	100	15
PPi	100	10

* The indicated compounds (at pH 7) were added to standard assay mixtures containing 15μ M [3 H]dCTP and partially purified enzyme as in Fig. 1. Control reactions produced 2,300 counts/min (0.15 nmol) of dUTP.

their direct spectrophotometric assay (but see discussion above). In addition to differences in assay techniques, our results could differ from those of Tomita and Takahashi (12) because of differences between the enzyme preparations employed or possibly between PBS1 and PBS2 phage-induced deaminases. We have not detected any large size changes in the presence of inhibitors; sedimentation of partially purified dCTP deaminase through sucrose gradients (in the absence or presence of 10μ M dUTP or dTTP) indicated a value of about 6S, consistent with a molecular weight of 125,000 (see above).

PBS1 and PBS2 DNA contains only deoxyuridine and no deoxythymidine (5, 11). Since PBS2 induces a potent dTMP phosphohydrolase (6), and since PBS2 DNA polymerase can utilize dUTP and dTTP at similar rates in vitro (5), we suggest that PBS2-infected cells must contain dUTP but no dTTP. Thus, the strong inhibition of PBS2 dCTP deaminase by dTTP observed in vitro (ref. 12 and this paper) is unlikely to occur in vivo. Inhibition by dTTP in vitro may instead reflect the evolution of this PBS2 enzyme from a cellular dCTP (2, 3) or dCMP (1, 9, 10) deaminase which was feedback inhibited by cellular dTTP. However, we do find that dUTP does inhibit PBS2 dCTP deaminase, to prevent conversion of more than 60% of the dCTP to dUTP. This product inhibition is consistent with the proposed role of the PBS2 dCTP deaminase in vivo of providing

FIG. 2. Double reciprocal plot of PBS2 dCTP deaminase inhibition by dTTP and dUTP. Incubations were performed as in Fig. 1, in the presence of 1.8μ M dTTP or 12μ M dUTP as indicated. The amount of dUTP formed (1 to 5% of substrate converted) is expressed (v) as micromoles per hour per milliliter of enzyme.

dUTP (but leaving sufficient dCTP) for the synthesis of uracil-containing phage DNA.

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