

ON THE DEFECT OF A dCMP HYDROXYMETHYLASE MUTANT OF BACTERIOPHAGE T4
SHOWING ENZYME ACTIVITY IN EXTRACTS*

Paul K. Tomich and G. Robert Greenberg

Department of Biological Chemistry
The University of Michigan
Ann Arbor, Michigan 48104

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SUMMARY

Infection by Ll3, a temperature-sensitive mutant of gene 42 of phage T4, the structural gene for dCMP hydroxymethylase, previously was shown not to form T4 DNA at nonpermissive temperatures. Yet the enzyme activity was found in extracts. Since inactivation of the enzyme was not reversible, we have examined acid-soluble extracts of cells infected at nonpermissive temperature by tsLl3 for 5-hydroxymethyl dCMP in order to determine whether the enzyme functioned in vivo. A double mutant of tsLl3 and amB24 (5-hydroxymethyl dCMP kinase) did not form the nucleotide at nonpermissive temperature, but the control, amB24, formed large quantities. From these results and previous temperature-shift studies it is suggested that the enzyme is normally activated to function in vivo between 5 and 8 minutes after infection.

Our earlier studies (1) gave compelling evidence that the T4 phage-induced enzyme, dCMP hydroxymethylase, has a complex nature. It was shown that tsLl3, a T4 phage carrying a temperature-sensitive mutation in the structural gene for dCMP hydroxymethylase, had enzymatic activity in vitro at both permissive and nonpermissive (42°) temperatures, but DNA synthesis occurred only at permissive temperatures. Evidence was presented that thermal inactivation of the enzyme was irreversible in extracts or in vivo. It was also demonstrated that some other component(s) (called the 5-8 minute component based on the time of appearance after infection) could activate the enzyme at permissive temperature so that DNA synthesis and normal phage yield occurred at nonpermissive temperatures. In our first report we postu-

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lated an additional function for dCMP hydroxymethylase: either direct involvement in the DNA replication machinery, or the necessity to interact with another component to form a complex allowing catalytic activity.

In order to determine whether cultures infected by tsL13 at 42° showed dCMP hydroxymethylase activity in vivo, we have measured the formation of the enzyme product, 5-hydroxymethyl-dCMP (HMdCMP). Since amb24 (gene 1, HMdCMP kinase)-infected cultures were shown by Warner and Hobbs (2) to accumulate far greater amounts of HMdCMP than occurred - as HMdCTP - in gene 43 mutants (DNA polymerase) we constructed a double mutant, tsL13/amb24, to determine whether HMdCMP accumulated after infection by this phage.

MATERIALS AND METHODS

Escherichia coli B was the nonpermissive host for amber mutants and E. coli CR63 the permissive host. The T4 phage used in these studies were provided by Dr. Robert S. Edgar. A double mutant was constructed by crossing tsL13 and amb24 in E. coli CR63 and selecting for a recombinant which would grow only on E. coli CR63 at 30°, but not on E. coli B or at 42°C.

The 5-hydroxymethyl-dCMP used as a standard was prepared enzymatically by Dr. Ronald L. Somerville and isolated by Dowex-50-H⁺ and then Dowex-1-formate column chromatography. It was further characterized by ultraviolet spectra, C, H, N and P analysis, and by conversion to 5-hydroxymethyldeoxycytidine with snake venom 5'-nucleotidase followed by deamination with nitrous acid and comparison of the product with authentic 5-hydroxymethyldeoxyuridine (Calbiochem).

Radioactivity was measured in a Beckman scintillation spectrometer as described previously (6). Paper chromatograms were scanned on a paper-strip counter. 3-¹⁴C-L-serine was purchased from Calbiochem and 3-³H-L-serine from Amersham-Searle Co.

In order to label the -CH₂OH group of HMdCMP (3), L-serine labeled in the 3 position was added one minute after infection. Serine synthesis is feedback-inhibited (4), and the most pertinent measurements were made using

0.45 mM L-serine, far in excess of the level required to fully inhibit the pathway (5).

All infections were performed with E. coli B grown at 37° to a titer of 5×10^8 cells/ml in Bonner-Vogel minimal salts medium containing 0.2% glucose. Before infection the cultures were transferred to a 42° water bath and equilibrated. L-tryptophan was then added to 50 µg/ml, and the cultures were infected one minute later by phage at a multiplicity of infection of 8:1.

The infection was stopped after twelve minutes by addition of perchloric acid (at 42°) to a final concentration of 2%. Cell debris was removed by centrifugation. Nucleotides were adsorbed to acid-washed Norite A charcoal (0.2 g per 100 ml culture), stirring for about 20 minutes. The charcoal was collected on Schleicher and Schuell 0.45 micron membrane filters, washed with 100 ml of deionized water, and the nucleotides were desorbed with 0.4 M NH₄OH in 80% ethanol v/v. The recovery of nucleotides from the charcoal averaged 65%. The ammonia-ethanol eluant was evaporated to dryness at 30° under vacuum and dissolved in 0.01 M formic acid, applied to a Dowex-50-H⁺ column previously equilibrated with 0.01 M formic acid, and the compounds were eluted with the same solution. The elution pattern for the amb24 infection is shown in Figure 1. The 2',3' and 5' CMP derivatives elute between about 11 to 13 bed volumes. The labeled HmdCMP peak is at about 12 column volumes. 5'-dCMP is eluted at about 22 bed volumes (not shown). Fractions II and III are guanosine monophosphates. Other nucleoside phosphate derivatives do not adsorb (I).

RESULTS

In order to verify that labeled HmdCMP was formed in an amb24 infection, tube numbers 76 to 88 in the CMP-HmdCMP fraction (Figure 1) eluted from the Dowex-50 column were combined and evaporated to dryness at 30° under vacuum. The nucleotides were then treated with snake venom 5'-nucleotidase (7), and the resulting mixture was chromatographed with two

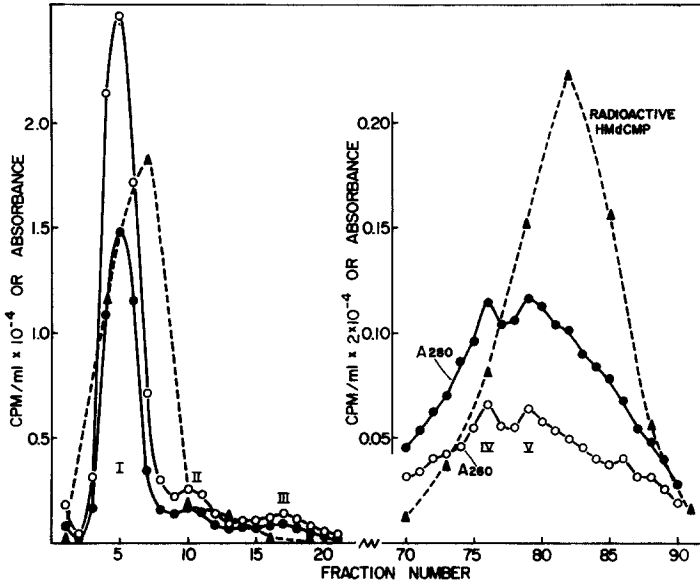


Fig. 1: Elution pattern from a Dowex-50 column of a trichloroacetic acid extract of a culture infected by amB24 (hydroxymethyl dCMP kinase). The column had the dimensions $0.7 \text{ cm}^2 \times 30.5 \text{ cm}$, and the fractions were 3.2 ml. The Roman numerals refer to absorbance peaks.

solvents. The R_f values of the radioactive compound, along with those of authentic nucleosides, are reported in Table I. In both instances the radioactive product of the nucleotidase activity corresponds to the position of 5-hydroxymethyldeoxycytidine. In each solvent a spot occurred corresponding to cytidine and deriving from contaminating 5'-CMP.

The amounts of HMDcMP formed after infection by amB24, amB24/tsL13 and by tsL13 are shown in Table II. Infection by amB24 did indeed lead to the accumulation of HMDcMP, a total of 4.0 μmoles per 100 ml culture, uncorrected for about a 35% loss at the charcoal step. This compares favorably to a value of 6.0 μmoles reported by Warner and Hobbs who used $2\text{-}^{14}\text{C}$ -uracil as a label with the same phage mutant and the same host (2). On the other hand HMDcMP could not be detected with the double mutant amB24/tsL13.

Thus the dCMP hydroxymethylase formed by tsL13 infection at nonpermissive temperature showed no activity *in vivo* even in the presence of the amB24 mutation which normally accumulates elevated levels of HMDcMP. The same

The quantity of dCMP present at 12 minutes after infection by tsL13 at 42° and isolated from the Dowex 50 column was estimated by A_{280} measurement and was found to be 28 μ moles per 100 ml of culture, uncorrected for loss. Thus more than sufficient dCMP was available to allow dCMP hydroxymethylase to function (see also ref. 8).

DISCUSSION

Since cultures infected by tsL13/amB24 at 42° do not form HMcMP, we may conclude that tsL13 dCMP hydroxymethylase is not functional in vivo at nonpermissive temperature. This conclusion is in keeping with the original suggestion of Mathews and Kessin (9). However these findings must be considered together with our earlier experiments with tsL13 which show that between 5 and 8 minutes after infection an interaction occurs at permissive temperature which allows prolonged and normal DNA synthesis on shifting to a nonpermissive temperature (1). In addition a short 30° pulse (1 to 3 min) 15 minutes after infection at 42° by tsL13 allows DNA synthesis at nonpermissive temperature. Since the low temperature pulse required protein synthesis, we concluded that new native dCMP hydroxymethylase had to be formed in order to interact with the 5-8 component (see Introduction). That is, the dCMP hydroxymethylase of tsL13 cannot interact with the 5-8 component at nonpermissive temperatures. Recent studies from this laboratory show that the tsL13-induced enzymes formed after infection at 42° and at 30° have different stability and kinetic properties.²

Accordingly these findings argue that the dCMP hydroxymethylase product of gene 42 must first interact with another component between 5 and 8 minutes after infection to be activated to function in vivo. Our present studies are directed at a kinetic analysis of the activity of this enzyme in vivo and a search for the nature of the 5-8 event.

The present findings do not preclude a dual function of the activated dCMP hydroxymethylase. The enzyme could function more directly in nucleic acid synthesis or regulate de novo synthesis of ribo- or deoxyribonucleotides.

² Che-Shen Chiu and G. R. Greenberg, in manuscript.

Thus amN122 (gene 42)-infected cultures show very low levels of incorporation of labeled uracil into the total uridine and cytidine phosphate pools compared to amB24 or T4 infection (2).

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