Enzymatic synthesis of deoxy-5-methylcytidylic acid replacing deoxycytidylic acid in *Xanthomonas oryzae* phage Xp12 DNA

PHAGE Xp12 on *Xanthomonas oryzae* was isolated from the irrigation water of a rice field. In the DNA of this phage, cytosine is completely replaced by 5-methylcytosine[1]. Isotope tracing studies on the biosynthesis of this unusual pyrimidine demonstrated that methylation of this cytosine is different from that of the 5-methylcytosine which is found in trace amounts in most plant and animal DNAs. The latter is methylated by methionine. On the other hand, the methyl group of 5-methylcytosine residues of Xp12 is derived from the 3-carbon of serine and not from the thiomethyl carbon of methionine[2]. In this investigation, the synthesis of deoxy-5-methylcytidylic acid (d5MCMP) was found to take place at the level of nucleotide, with deoxycytidylic acid (dCMP) and formaldehyde as substrates in the presence of 5,6,7,8-tetrahydrofolic acid (THFA) and an enzyme preparation from *X. oryzae* infected with Xp12 phage. This reaction seems to account for the synthesis of the unique pyrimidine, 5-methylcytosine, which occurs in the DNA of phage Xp12.

Exponential phase *X. oryzae* strain 604 grown in PS medium[3] (potato, 200 g; peptone, 5 g; sucrose, 15 g, $Ca(NO_3)_2.4H_2O$, 0.5 g; $Na_2HPO_4.12H_2O$, 2 g in 1 l of distilled water) was infected with a fivefold excess of Xp12, which has a latent period of 120 min in these conditions; aeration was continued for 40 min after infection. The cultures were rapidly chilled to 2 °C and the organisms were collected by centrifugation. After washing and resuspension in 0.05 M Tris-HCl buffer, *p*H 7.5, the cells were disrupted by using a Blacktone Model BP-2 Ultrasonic Probe. Unbroken cells and other particles were removed by centrifugation at 8,000*g* for 5 min. The supernatants were dialysed against 0.05 M Tris-HCl, *p*H 7.5 at 4 °C for 4 h. To show the induction by the phage of synthesis of the enzyme with methylating activity, protein synthesis was inhibited by the addition of chloramphenicol (30 μg ml^{-1}) just before phage infection. Extracts from uninfected and chloramphenicol-treated bacteria were prepared by the same method from parallel cultures. The protein concentration was approximately 13 mg ml^{-1} as measured by the method of Lowry *et al.*[4].

The reaction mixture (150 μl) contained 50 μl enzyme extract, 10 μmol dCMP, 0.8 μmol THFA, 2 μmol ^{14}C-formaldehyde (2 m Ci $mmol^{-1}$), 50 μmol $MgCl_2.6H_2O$, 32 μmol 2-mercaptoethanol and 40 μmol Tris-HCl, *p*H 7.5. The mixtures were incubated at 30 °C for 30 min. The reaction was stopped by adding 150 μl of 1.0 M sodium acetate, which dissociated 'active formaldehyde' to formaldehyde and tetrahydrofolate[5], followed by 100 μl of 0.4 M dimedone (in 50% ethanol) to precipitate formaldehyde[6]. The mixture was heated in boiling water for 5 min and the precipitated protein and formaldehyde were removed by centrifugation at 8,000*g* for 5 min. The supernatant and authentic d5MCMP, prepared from Xp12, were applied respectively to Whatman No. 1 chromatography paper and developed with isopropanol–HCl–H_2O (65: 16.6: 18.4).

Table 1 Incorporation of $H^{14}CHO$ into deoxy-5-methylcytidylic acid by extracts of Xp12 infected *X. oryzae*

Reaction mixture	Specific activity (nmol d5MCMP formed per mg protein in 30 min)
Complete system*	20.0
Complete system $-Mg^{2+}$	15.7
Complete system − THFA	1.5
Complete system − dCMP	0.6
Reagent mixture + boiled enzyme†	0.8
Reagent mixture + extract of chloramphenicol-treated cells‡	0.8
Reagent mixture + extract of uninfected cells	1.6

*Complete system as described in text.
†Extract from infected bacteria was heated for 5 min in boiling water.
‡Extract from infected bacteria treated with chloramphenicol. Chloramphenicol was added just before phage infection.

Table 1 shows that the extract of infected cells promotes the synthesis of d5MCMP. A sharp peak of radioactivity was detected at the position corresponding to authentic d5MCMP. For general enzyme assays, the area round the peak was cut out from the paper, placed in a vial, and the radioactivity counted with a Packard Model 3375 scintillation spectrometer. To identify the reaction product, the spot containing the radioactivity was cut out and eluted with water. The eluate was dried and hydrolysed with 50 μl of 90% formic acid at 175 °C for 30 min. Acid hydrolysis of the nucleotide yielded a base with R_f identical to that of authentic 5-methylcytosine in three solvent systems: isopropanol–HCl–H_2O (170 : 40 : 39), *n*-butanol–H_2O (86 : 14) and *n*-butanol–H_2O (86 : 14) with 5% NH_4OH in air. For further identification, a small amount of the substance obtained by evaporation of the eluate from a chromatogram spot was deaminated with HNO_2. Deamination was carried out by adding 50 μl of 2 M $NaNO_2$ and 10 μl of glacial acetic acid to the eluate. After standing overnight at 25 °C, the solution was applied directly to Whatman No. 1 paper. A sample of authentic 5-methylcytosine was treated in the same way. Both the unknown and authentic 5-methylcytosine were found to convert into a substance identical to thymine in its chromatographic properties.

On the basis of these observations, we conclude that an enzyme found in the infected bacteria catalyses the direct methylation of dCMP, but that this enzyme, deoxycytidylate methyltransferase, is absent from uninfected cells. The reaction required the presence of THFA and Mg^{2+}, in addition to dCMP and formaldehyde as substrates. Deoxy-5-methylcytidylic acid can thus be synthesised in phage Xp12-infected *X oryzae* by the following reaction

$$dCMP + HCHO \xrightarrow{THFA,\ Mg^{2+}} d5MCMP$$

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Definitions of free energy levels in biochemical reactions

IN considering the energetics of an enzyme-catalysed reaction it is of interest to enquire into what constitutes the "drive", in a thermodynamic sense, for the reaction, particularly when the enzyme is involved in an energy-coupled process. This often

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