T4 PHAGE-CODED DEOXYCYTIDYLATE HYDROXYMETHYLASE: PURIFICATION AND STUDIES ON INTERMOLECULAR INTERACTIONS[1]

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SUMMARY

DNA precursor biosynthesis in T4 coliphage-infected bacteria is catalyzed by a complex of virus-coded enzymes, with some host cell components. As one way to explore the nature of this complex, we are purifying its constituent enzymes, so as to attempt partial or complete reconstitution of the complex. This communication describes purification to homogeneity of one of these enzymes, deoxycytidylate hydroxymethylase. The enzyme has a molecular weight of about 60,000 and consists of two subunits of identical molecular weight. Two approaches are described for studying intermolecular interactions involving this enzyme protein.

Infection of *Escherichia coli* by bacteriophage T4 leads to a severalfold increase in the rate of DNA synthesis per cell. Several lines of evidence (1,2) indicate that DNA precursors in this system are synthesized by a specific enzyme complex which exists in juxtaposition to the replication forks. Enzyme aggregation provides a means for maintaining high local concentrations of precursors at their sites of utilization, even though their average intracellular concentrations are relatively low (3). Recently our laboratory has described an aggregate of several phage-coded enzymes and at least one bacterial enzyme, nucleoside diphosphokinase (2). Kinetic properties of this aggregate suggest that it does represent a specific complex which serves as a substrate shuttle.

As one of several approaches to understanding the nature of this complex, we would like to attempt its partial or complete reconstitution from purified components. One enzyme in the aggregate is dCMP hydroxymethylase, which catalyzes formation of the virus-specific DNA precursor, 5-hydroxymethyl-dCMP (4). Complete purification of this enzyme has not previously been described,

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although Mathews *et al.* (5) did obtain a highly purified preparation of the T6 enzyme, which was used in an experiment showing that dCMP hydroxymethylase is synthesized *de novo* after infection. This communication describes purification of the T4 enzyme to homogeneity, plus some approaches to the study of intermolecular interactions involving the enzyme.

METHODS. Experiments were carried out with *E. coli* B and phage strains T4D (wild type), plus amber mutants T4 *am*B24 (gene 1), T4 *am*BL292 (gene 55), T4 *am*N122, and T4 *su2am*NG485 (genes *reg*A and 62). Phage and bacteria were grown and stocks maintained as described previously (6).

For purification of dCMP hydroxymethylase *E. coli* B was infected with T4 *su2am*NG485, an overproducer of early enzymes (7). Procedures for large-scale cell growth and infection were as described previously (8). One hundred grams of cell paste was subjected to a procedure essentially identical to that used for the T6 enzyme by Mathews *et al.* (5). After the final step of that procedure, namely, CM-Sephadex chromatography, the pooled active fractions were concentrated by ultrafiltration and applied to a 2.5 x 50-cm Sephadex G-100 column in 0.1 M potassium phosphate buffer, pH 6.5. The column was developed at 0.45 ml/minute. The pooled active fractions were concentrated by ultrafiltration to 10 ml.

Affinity chromatography involving columns of immobilized dCMP hydroxymethylase was carried out by an adaptation of Ratner's procedure (9). The purified protein (1.0 mg/ml) was affixed to CNBr-activated Sepharose 4B exactly as described by Ratner for constructing RNA polymerase affinity columns. Control columns for analyzing nonspecific binding were prepared identically, with either bovine serum albumin or carbonic anhydrase (2.0 mg/ml each) being linked to the Sepharose. Analysis of labeled proteins binding to each affinity substance was essentially as described by Ratner (9).

RESULTS. In our earlier experiments on the T6 dCMP hydroxymethylase (5) it was necessary to purify the enzyme to the point where it was free of contamination with host cell proteins. This was established, but we did not determine the absolute homogeneity of the preparation. After carrying the T4 enzyme through a purification scheme similar to that used previously, we found the T4 enzyme to have a specific activity of 4850 nmole/min/mg protein, as compared with 4450 for the earlier T6 preparation. However, the preparation contained two minor contaminants, as judged by SDS-polyacrylamide disc gel electrophoresis[3] (Fig. 1). Accordingly, the preparation was subjected to an additional step, namely, gel filtration on Sephadex G-100. Although this step

[3]Abbreviation : SDS, sodium dodecyl sulfate

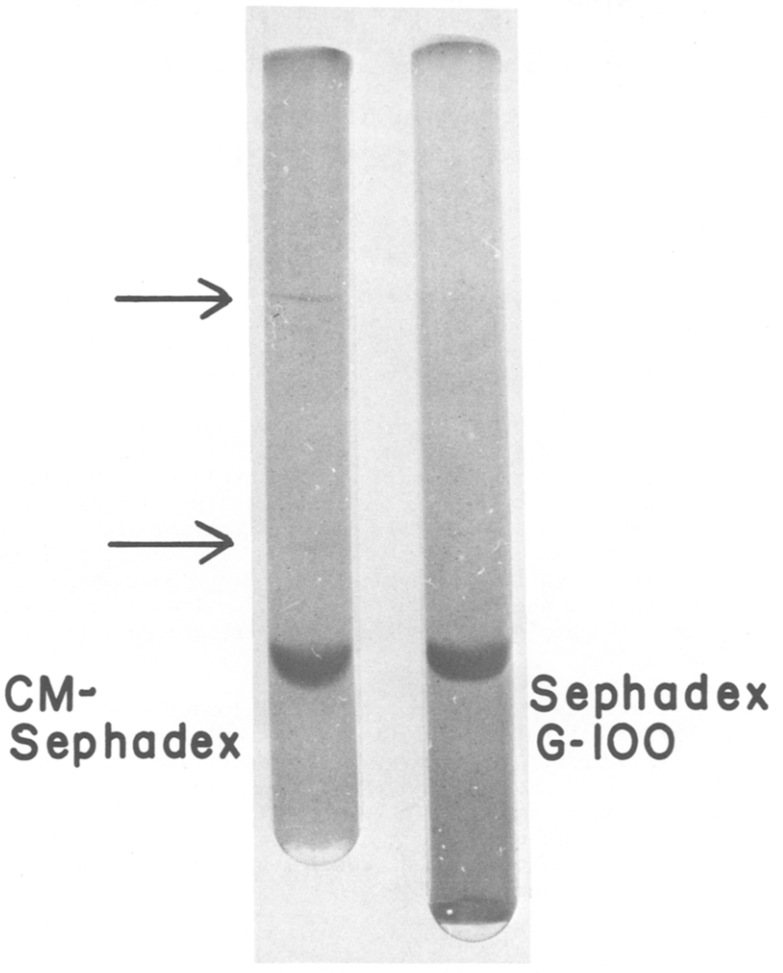

Figure 1. Electrophoretic analysis of enzyme purity. The last two fractions from the purification scheme described in Table I were analyzed by SDS-polyacrylamide gel electrophoresis in tubes (13). The arrows denote contaminants which were detected after the penultimate step in the fractionation.

gave little increase in specific activity (Table I), it did result in a preparation showing but one band on disc gel electrophoresis (Fig. 1).

The Sephadex G-100 column used for the final purification step was calibrated by running through it proteins of known molecular weight. From this plus the elution volume of the hydroxymethylase, we could estimate that its molecular weight is about 57,000 (data not shown). The molecular weight of the native enzyme was determined also by sedimentation equilibrium. In each of

Table I

Purification of T4 Phage-coded dCMP Hydroxymethylase

Purification step	Total protein	Specific activity	Recovery of activity
	mg	nmoles/min./mg	percent
Ammonium sulfate	2700	78	100
DEAE-cellulose	95	1070	48
Calcium phosphate	39	1800	33
CM-Sephadex	12	4850	27
Sephadex G-100	10	4900	13

several runs a plot of absorbance at 280 nm *vs.* squared distance from the center of rotation gave a straight line (data not shown), confirming the homogeneity of the preparation. The average value of the slopes determined in four separate runs yielded a molecular weight of 63,000 (10).

The subunit molecular weight, estimated from electrophoretic mobility on calibrated SDS-polyacrylamide slab gels, is about 27,000 (10), slightly higher than the value of 25,000 reported by O'Farrell *et al.* (11). The molecular weight of the native enzyme is either 2.1 or 2.3 times the subunit molecular weight, depending upon the value chosen for native molecular weight. Thus, the enzyme probably consists of two subunits of equal molecular weight. Since only one T4 cistron, namely, gene 42, is known to code for dCMP hydroxymethylase, and since genetic mapping suggests a cistron length consonant with the subunit molecular weight reported here (12), it appears that dCMP hydroxymethylase is a dimer of identical subunits.

Affinity chromatography. To explore protein-protein interactions involving dCMP hydroxymethylase, we carried out affinity chromatographic analysis of

labeled proteins binding to a column containing immobilized hydroxymethylase as the affinity adsorbent. Our approach was based upon that of Ratner (9), who analyzed proteins binding to RNA polymerase isolated from T4-infected cells, and detected several specific associations. After attachment of dCMP hydroxymethylase to the CNBr-activated Sepharose, the bound enzyme had a specific activity of 625 nmoles/min/mg protein, as compared with 4900 for the initial preparation. This compares with 20-25% of the RNA polymerase activity retained in Ratner's procedure. One might expect the much larger RNA polymerase to be bound with fewer structural constraints and, hence, less loss of activity. However, from the protein concentrations of the affinity resins and the respective molecular weights, we can estimate that the molar concentration of active dCMP hydroxymethylase in our affinity adsorbent is quite close to that of RNA polymerase in Ratner's column (10).

Extracts containing labeled proteins were passed through the affinity column, and bound proteins were analyzed, after elution, by polyacrylamide slab gel electrophoresis and radioautography. Extracts analyzed included proteins labeled early in infection, late in infection, labeled in uninfected cells, and labeled in uninfected cells followed by infection in nonradioactive medium. In each case several proteins were bound to the column. However, identical patterns were seen with proteins bound to control columns containing serum albumin or carbonic anhydrase (data not shown). Thus, we were not able to demonstrate specific binding of proteins to dCMP hydroxymethylase by this technique. This does not preclude the existence of such interactions, but it does state that any binding affinities are either relatively low or else that additional components are required for binding to occur, such as substrates or other metabolites.

Analysis of membrane proteins. The above approach would probably not detect interactions stabilized primarily by hydrophobic bonds. However, if DNA replication occurs at the membrane, as shown by others, and if the precursor-synthesizing complex is juxtaposed with the replication complex, then hydrophobic interactions might be important. To approach this question we

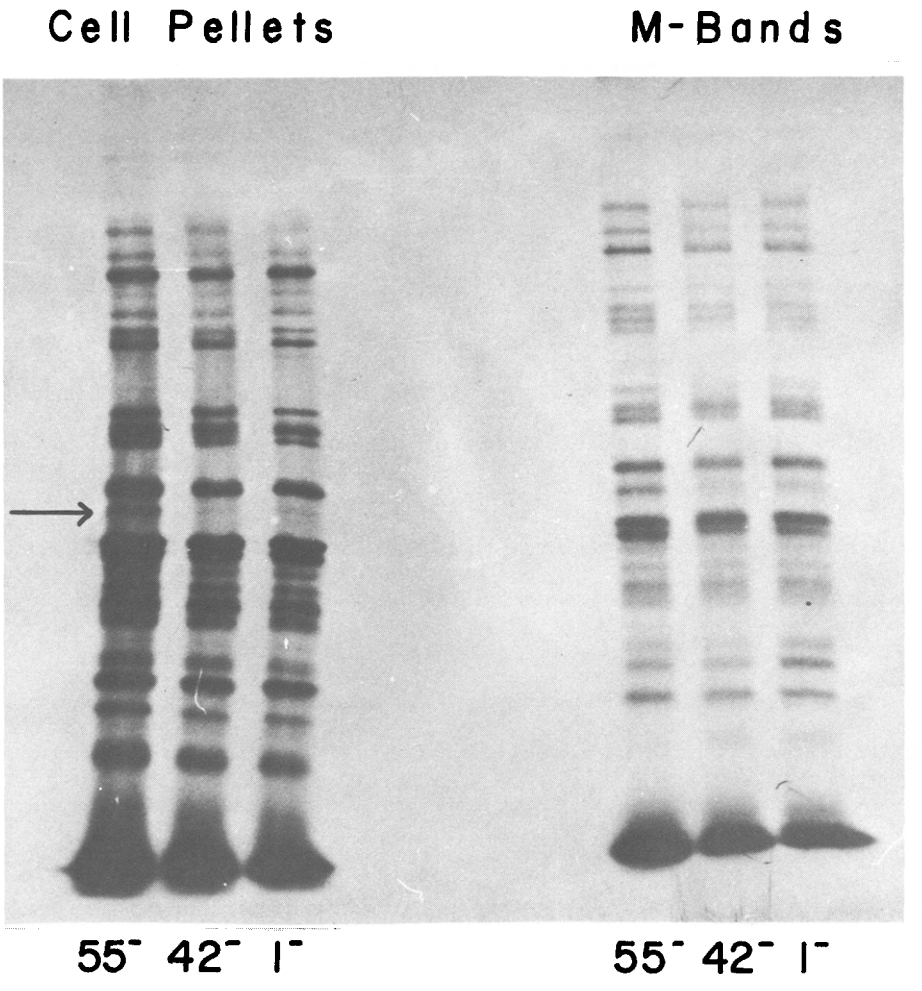

Figure 2. Electrophoretic analysis of membrane proteins. Proteins were labeled from 0 to 10 minutes after infection of *E. coli* B with T4 mutants *am*BL292 (55⁻), *am*N122 (42⁻), or *am*B24 (1⁻). Each culture was divided in half, and membranes were prepared from one half as described by Huang (14; "Cell Pellets") and from the other half as described by Earhart *et al.* (15; "M-Bands"). The proteins in each preparation were analyzed by SDS-polyacrylamide slab gel electrophoresis (6). The arrow denotes a protein band, of molecular weight about 40,000, which is much more abundant in the 55⁻ membrane preparations than in the others.

asked whether dCMP hydroxymethylase and/or deoxyribonucleoside 5'-monophosphate kinase, the products of genes 42 and 1, respectively, are associated with cell membranes after infection. Proteins labeled after infection with 42⁻ and 1⁻ mutants were compared with those of a 55⁻ mutant. The gene 55 mutant, which

synthesizes early proteins and DNA but no late proteins, serves essentially as a wild-type control. Membranes were prepared by two different methods, and the labeled membrane proteins were analyzed by SDS polyacrylamide gel electrophoresis. If the gene 42 product were present in the membrane, one would expect to see an altered or absent band in the 42^- preparations, corresponding to a molecular weight of 27,000. To identify the kinase as a membrane protein, one would have to see an alteration in the 22,000-dalton region (11). However, the only difference noted among the preparations was a band in the 55^- extract which was much more heavily labeled than in the corresponding 42^- and 1^- patterns (Fig. 2). This band, which has not been identified, corresponds to a molecular weight of about 40,000, so that it cannot represent the products of genes 42 or 1. Therefore, we must conclude either that these gene products are not membrane components or else that they are attached very loosely.

In summary, T4 dCMP hydroxymethylase has been purified to homogeneity and partially characterized. Two experimental approaches failed to demonstrate intramolecular interactions involving this protein. However, other lines of evidence (1,2) make it clear that such interactions exist, and the availability of homogeneous enzyme makes several other approaches possible.

REFERENCES

1. Tomich, P. K., Chiu, C., Wovcha, M. G., and Greenberg, G. R. (1974) J. Biol. Chem. *249*, 7613-7618.
2. Reddy, G. P. V., Singh, A., Stafford, M. E., and Mathews, C. K. (1977) Proc. Natl. Acad. Sci. U.S.A., in press.
3. Mathews, C. K. (1972) J. Biol. Chem. *247*, 7430-7438.
4. Flaks, J. G., and Cohen, S. S. (1957) Biochim. Biophys. Acta *25*, 667-672.
5. Mathews, C. K., Brown, F., and Cohen, S. S. (1964) J. Biol. Chem. *239*, 2957-2963.
6. North, T. W., Stafford, M. E., and Mathews, C. K. (1976) J. Virol. *17*, 973-978.
7. Wiberg, J. S., Mendelsohn, S., Warner, V., Hercules, K., Aldrich, C., and Munro, J. L. (1973) J. Virol. *12*, 775-782.
8. Erickson, J. S., and Mathews, C. K. (1971) Biochem. Biophys. Res. Commun. *43*, 1164-1171.
9. Ratner, D. (1974) J. Mol. Biol. *88*, 373-387.
10. North, T. W. (1976) Ph.D. Dissertation, University of Arizona.
11. O'Farrell, P. Z., Gold, L. M., and Huang, W. M. (1973) J. Biol. Chem. *248*, 5499-5505.
12. Holmes, G. E. (1975) J. Virol. *16*, 1085-1089.
13. Laemmli, U. K. (1970) Nature *227*, 680-682.
14. Huang, W. M. (1975) Virology *66*, 508-515.
15. Earhart, C. F., Tremblay, G. Y., Daniels, M. J., and Schaechter, M. (1968) Cold Spr. Hbr. Symp. Quant. Biol. *33*, 707-712.