

## AN ABSTRACT OF THE THESIS OF

Christian Ungermann for the degree of Master of Science in Biochemistry and Biophysics presented on May 4, 1993.

Title: Protein-Protein Interactions of the Bacteriophage T4-coded dCTPase-dUTPase

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Abstract approved: Christopher K. Mathews, Ph.D.

The bacteriophage T4 forms a deoxynucleoside triphosphate (dNTP) synthesizing complex in its host *Escherichia coli* (*E.coli*) during the infection cycle. This complex provides dNTPs to the replication machinery and consists of at least ten proteins, including enzymes encoded by the host cell genome. The enzyme deoxycytidine triphosphatase-deoxyuridine triphosphatase (dCTPase-dUTPase), which is encoded by gene 56 of the bacteriophage, was purified from an overexpressing strain of *E.coli*. The enzyme was immobilized on an affinity column and used for affinity chromatography. The specifically retained proteins belong to the dNTP synthetase complex and, furthermore, to the replication machinery. Purified protein was injected in rabbits to generate polyclonal antisera. The purified antibodies were tested, and then again injected in rabbits to raise antiidiotypic antibodies. The antiidiotypic antibodies had specificity for deoxynucleoside monophosphokinase. Native gel electrophoresis with dCTPase-dUTPase was applied to study protein-protein interactions in a native environment. In this *in vitro* approach, the enzyme dCMP deaminase was shown to interact with dCTPase-dUTPase and dCMP hydroxymethylase.

In a second project, the purification of the dNTP synthetase complex was

attempted in a novel approach. An affinity tag consisting of six consecutive histidines was added to the N- and the C-terminus of the enzyme dCMP hydroxymethylase, one of the constituent enzymes of the complex. This tag interacts specifically with a  $\text{Ni}^{2+}$ -column. The modified enzyme displays approximately the same activity as the wild-type form. I attempted the purification of complex by infecting the overexpressing bacteria with a T4-strain deficient in dCMP hydroxymethylase and applying the lysate to the  $\text{Ni}^{2+}$ -column. However, the copurification of the dNTP synthetase complex failed.

Protein-Protein Interactions in the Bacteriophage T4-coded dCTPase-dUTPase

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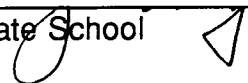
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# PROTEIN-PROTEIN INTERACTIONS INVOLVING THE BACTERIOPHAGE T4-CODED DCTPASE-DUTPASE

## CHAPTER 1

### Introduction

Since the early 1940s, major breakthroughs in the fields of biochemistry, virology, and molecular biology have originated from research on the bacteriophage T4. The bacteriophage T4 is a large DNA virus with a 171-kbp genome, which encodes a large amount of phage specific proteins and is not like other smaller viruses dependent on many host proteins. Some significant advantages made it a favorite virus to work on. These include the high efficiency for infection of its host *E.coli*, a large burst size of up to 400 phages produced in each infected cell, and unusual characteristics, such as the specific glucosylation of its DNA. In addition, the phage has several mechanisms to arrest the synthesis of nucleic acids and proteins in the host cell. Thus, isotopic precursors applied to a T4-infected cell are almost exclusively detected in virus-specific macromolecules. The isolation of conditional lethal mutants by Edgar and Epstein (1963), affecting almost all functions essential for the virus multiplication, has contributed to its popularity as well.

The bacteriophage T4 has an additional feature that could possibly serve as a model to understand the DNA precursor synthesis and its organization in eucaryotic cells: a multienzyme complex that is formed shortly after infection by the bacteriophage of its host. For over a decade, our laboratory has focused on this phenomenon. In this thesis, I will describe some of the individual protein-protein interactions within the complex that may contribute to its efficient synthesis of dNTPs.

In the following, I would like to introduce the infection cycle of the phage on its host and will outline some of the specific adaptations, by which the phage modifies the bacterial metabolism.

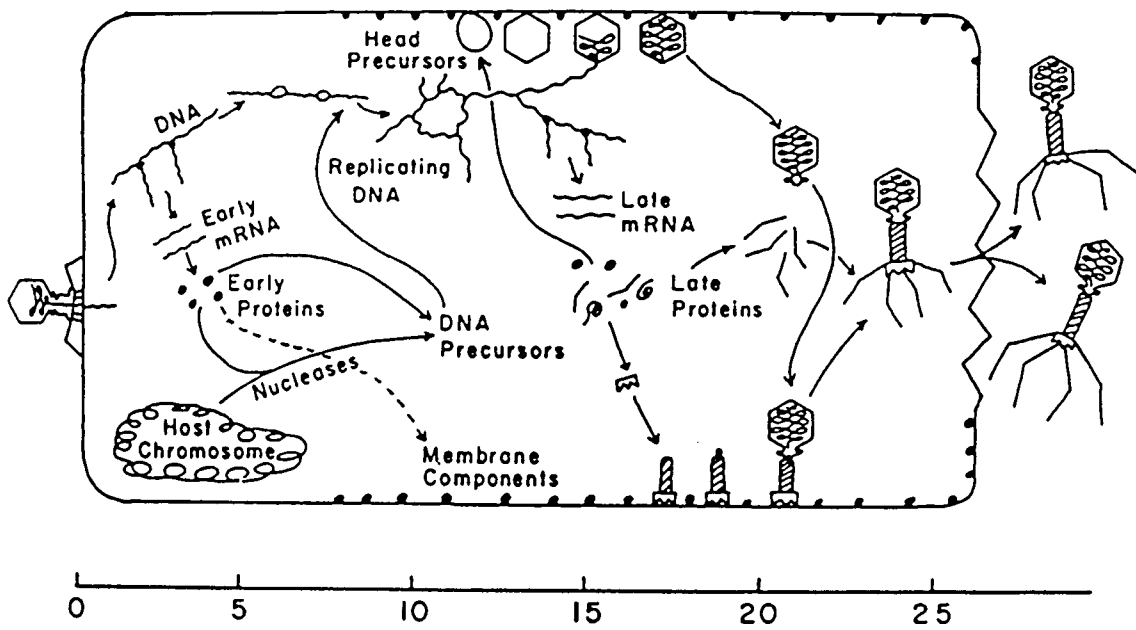


Figure I-1. The infection cycle of Bacteriophage T4 in *E. coli* (from Mathews, 1977)



### The bacteriophage T4 — from infection to lysis

The phage T4 interacts specifically with a receptor protein or polysaccharides on the surface of the bacterium *E. coli*. It injects its DNA into the cell with the help of a membrane potential (Bayer, 1968). In the cell, a phage protein (gp2=product of gene 2) protects the DNA from degradation, and the host DNA-directed RNA polymerase begins with the transcription of the early genes. The phage DNA is glucosylated at every cytosine, which helps to protect the DNA from degradation by host endonucleases. Most of the early expressed proteins are not found in the final phage particle, but code for replication and transcription proteins. These are, for example, RNA polymerase-binding proteins, nucleases, and the enzymes of dNTP *de novo* synthesis, which form a multienzyme complex. DNA polymerase, DNA polymerase-accessory proteins, single-stranded DNA-binding proteins, topoisomerases, and some other replication proteins are produced in the early phase as well and allow DNA replication to begin six minutes after infection. The bacteriophage DNA is unique in that it contains hydroxymethylated cytosine which, in addition, is glucosylated. The change in the transcription pattern from early, to delayed-early, and then to late genes is associated with the modification of the host RNA polymerase by ADP-ribosylation and the replacement of the host  $\sigma^{70}$ -factor by several phage-encoded transcription factors (Mathews, 1993).

The proteins produced in the last part of the infection cycle are mostly structural proteins. The syntheses of the tail, head and the tail fibers occur independently (Epstein et al, 1963, Edgar and Wood, 1966). These subassembly processes are membrane-linked. Finally, DNA packaging appears to be energy driven and is limited by the size of the DNA molecule. The phage cannot pack more than 171 kbps into a normal size head. The final assembly of the whole phage particle is a spontaneous process that is not dependent on additional energy supply. After at least 25 min the cell is lysed and about 200-400 phages are released into the medium (Figure I-1).

In the next two sections I will focus on multienzymatic aggregation in order to introduce the main topic of the thesis.

### **Multienzyme complexes**

Postulating some level of organization within the cell is necessary in order to account for a regulated cell metabolism. One way to obtain organization is to bring into close proximity those proteins which catalyze sequential reactions in a metabolic pathway. Organization of these enzymes into complexes may further enhance the rate of catalysis in enzymatic pathways. This makes it attractive to think of multienzymatic complexes. Enzymatic organization is maintained (i) by multiple activities in one protein, such as in DNA polymerase, which is able to catalyze different reactions with different catalytic sites, or (ii) by tightly associated enzymes like the fatty acid synthetase complex or the pyruvate dehydrogenase complex. Those two are completely noncontroversial because the tight binding makes it possible to even visualize the complete complex by electron microscopy (Mathews and van Holde, 1990).

However, a third and much more controversial group of multienzymatic aggregations consists of enzymes that associate through weak and noncovalent interactions. The isolation of this group of complexes often is more difficult, because their stability may strongly depend on the protein concentration and ionic composition inside the cell. A drop in the protein concentration during the isolation may destabilize the system and prevent an isolation of a complete aggregate. Therefore, even though the stability of some of the complexes *in vitro* is weak, it is unlikely to represent the strength of binding *in vivo*. Complementarity of surfaces, as proposed for many of the involved enzymes, might not be enough to hold the purified system together. In addition, to prove their existence, the weak protein-protein interactions have to be clearly differentiated from artificial aggregations of proteins, which remains a difficult task. Initially, multienzyme complexes have been proposed to explain

the discrepancy between the small amount of free substrate and the large amount of product produced at the observed catalytic rate. An example for this discrepancy was the calculated apparent concentration of oxaloacetate in mitochondria that was not sufficient to account for the rate of oxidation observed in the tricarboxylic acid cycle (Srere, 1972; Lopes-Cardoso et al., 1978). In this system, for example, the flux rate of observed catalysis could not be explained by a simple diffusion theory of metabolites (for a review see Srere, 1987). Although the possibility of artificial aggregation cannot be ruled out for some of the systems, there are multiple examples supporting this postulate: (i) the multitude of complexes found in several metabolic systems (Srere, 1987) and (ii) the copurification of enzymes that catalyze sequential reactions (e.g., the copurification of DNA precursor synthesis enzymes, reviewed by Mathews, 1993). In addition, the reduction of transient times to shuttle a substrate from one enzyme to the next in these systems are convincing evidence to discount the "non-existence" argument.

An even more controversial topic involves transient complexes. In these cases the complex formation should occur only when the substrate is provided (Srivastava and Bernhard, 1985). Some dehydrogenases transfer the NADH cofactors from one enzyme to the next in a stereospecific manner. This process can be followed with an enzyme buffering assay, where the free NADH concentration is lowered by buffering with excessive concentration of the donor dehydrogenase (Srivastava and Bernhard, 1985). The results of Srivastava and Bernhard indicate that the NADH is transferred from one dehydrogenase binding site to another via an  $E_1$ -NADH- $E_2$  complex, but only when the active sites of the two dehydrogenases accept the NADH molecule in opposite configurations.

In sum, the multienzyme complexes seen in nature differ widely from each other in stability. Their presence in procaryotic systems, however, may allow inferences about the organization within eucaryotic cells.

### The dNTP synthetase complex

Initially, the T4 dNTP synthetase multienzyme complex has in part been proposed to explain the discrepancy between the small amounts of free DNA precursors and the large amount of DNA produced at the observed catalytic rate. An uninfected rapidly growing bacterial cell has six DNA-replication forks, compared to about 60 forks and ten times the rate of DNA synthesis in a T4-infected cell (Werner, 1968). Qualitatively, one can argue that the dNTPs for DNA replication cannot be provided to these sites by simple diffusion from remote sites. For the phage system, one can further propose that the DNA precursor synthesis ought to be very well organized, in order to allow for the phage's rapid DNA synthesis (Mathews, 1993). The copurification of dNTP synthesis enzymes which carried out sequential reactions from T4-infected *E.coli* cells (Reddy et al., 1977) contributed to the postulation of a multienzyme complex near the replication sites in the T4-infected bacterial cell (Mathews et al., 1979). Further observations helped to confirm this postulate. Kinetic experiments were carried out with permeabilized T4-infected cells. In those studies it was found that deoxyribonucleoside monophosphates (dNMPs) and ribonucleoside diphosphates (rNDPs) were incorporated into the DNA two to three times more efficiently than were dNTPs (Allen et al., 1980), suggesting a preferential utilization of distal precursors by a complex for dNTP synthesis. Furthermore, when radioactively labeled dNMPs were supplied to these cells, the dNTP pool did not accumulate radioactivity, consistent with the idea that the replication apparatus utilizes the dNTPs from small pools (Reddy and Mathews, 1978). Additional evidence for the existence of the complex came from the *in vivo* flux rate determination for thymidylate synthase and dCMP hydroxymethylase by Flanagan and Greenberg (1977). These authors showed that the ratio of the dTMP to hmdCMP flux rate was 2:1, close to the relative presence of these nucleotides in the T4 DNA.

Two groups were successful in partially purifying a multienzyme aggregate consisting of at least ten enzymes of the dNTP biosynthesis (Chiu et





al, 1982, Moen et al., 1988). The complex has a molecular weight of about 1300 kDa (gel filtration) and catalyses sequential reactions very efficiently. It consists of at least eight phage enzymes and two host enzymes (see Table I-1). In the following, three of these enzymes will be introduced (see also Figure I-3).

*dCMP hydroxymethylase (gp42)* — The enzyme hydroxymethylates dCMP. After this modified base is incorporated into T4-DNA, it is glucosylated at this hydroxymethyl-group. As a result, the T4 DNA differs significantly from the host DNA. The hydroxymethylase is a central part of the dNTP synthetase complex as shown by various lines of evidence. (i) The infection of *E.coli* cells with T4-mutants coding for a truncated gp42 prevented the isolation of a functional complex (Thylén and Mathews, 1989). (ii) Immobilized hydroxymethylase specifically retained several enzymes of DNA precursor synthesis (Wheeler et al., 1992). Furthermore, a mutation in gp42 can be suppressed by a mutation in gp43 (DNA polymerase), suggesting an intracellular interaction of these enzymes (Chao et al., 1977).

*dCMP deaminase (gpca)* — This hexameric enzyme plays a crucial role in the dNTP synthetase complex. It is feedback-inhibited by dTTP and allosterically activated by dCTP. The enzyme contains 2 mol of Zn per mol of dCMP deaminase subunit, which is essential for the activity of the protein. However, the role of the metal ion in catalysis remains to be determined (Moore et al., 1993).

*Deoxynucleoside monophosphokinase (gp1)* — This protein is unusual because it phosphorylates hmdCMP, dTMP and dGMP, but is insensitive to dAMP, which is substrate to (d)AMP kinase, one of the host enzymes of the dNTP synthetase complex. Brush and Bessman (1993) have carried out biochemical and genetic studies with this protein, indicating that a single catalytic site is capable of catalyzing all phosphorylation steps. Deoxynucleoside monophosphokinase was found to dissociate easily from the complex during the purification carried out in our laboratory. Therefore, the protein is probably

only loosely bound to the complex.

It is often asked whether the bacteriophage T4 system can be taken as an example for eucaryotic cells. As C.K. Mathews (1993) points out, the nuclear pools of dNTPs for repair and replication differ. A comparable organization of the enzymes for dNTP synthesis has not been detected in these cells. The compartmentation in eucaryotic cells poses a different challenge to the organization of dNTP synthesis than is created by a phage during the infection cycle. The phage's successful replication depends on a highly adapted system to ensure rapid dNTP synthesis. Therefore, one should see the dNTP synthetase complex of T4 as an example of a phage's adaptation to a host bacterium. The challenge in studying this system is to understand the three-dimensional structure of a complex, and to comprehend how the interactions are capable of mediating a tight regulation of synthesis of the four dNTPs.

### **dUTPases and their relations to pseudoproteases**

*Sequence comparison* — The dUTPase is a highly conserved protein. Sequence comparisons with available sequences from the data base led to the discovery that the pseudoproteases of several viruses and retroviruses are dUTPases (McGeoch, 1990). This was a surprise, because it was thought for a long time that the pseudoproteases have evolved by duplication of an oncovirus protease gene and subsequent diversification (McClure et al., 1987). However, the strong homology of the pseudoproteases of retroviruses and the dUTPase from *E.coli* indicated their relatedness. The argument was further strengthened by the analysis of the cDNA sequence of the human dUTPase (McIntosh et al., 1992). The amino acid sequence of the human protein exhibits a 53% identity with the dUTPase from *Saccharomyces cerevisiae* and a 35% identity with the *E.coli* enzyme. In addition, several motifs displayed by the human enzyme were homologous to the dUTPase from *E. coli*, herpesvirus, and also to putative pseudoproteases from poxviruses, indicating that those are dUTPases as well.

*Conservation and role in the cell* — The conservation of dUTPase in

evolution is obviously related to its function in the cell. Although Warner et al. (1977) demonstrated that uracil-containing DNA can be efficiently transcribed, the exclusion of uracil from DNA is essential to maintain the conservation of the genetic information. Uracil needs to be excluded from the DNA because: (i) Spontaneous deamination of cytosine in the DNA forms uracil, which can be mistaken for thymine. If no correction takes place, adenine may be incorporated in the complementary strand of the DNA during replication, thereby causing a point mutation at this position. The cell has developed a special repair system to avoid this. The enzyme uracil-DNA-glycosylase cleaves uracil out of the DNA, leaving an apyrimidinic site. This is recognized by the apyrimidinic endonuclease that removes the remaining sugar residue through a cleavage on the 5' site of the phosphodiester bond. The DNA polymerase I and DNA ligase finally fill the gap again (Mathews & van Holde, 1990). (ii) dUTP, when present in sufficiently high levels, is readily accepted by the DNA polymerase and incorporated into the replicating DNA. When thymine is replaced by uracil, guanine can be misincorporated into the complementary strand in the place of adenine when replication takes place. This leads to a transition mutation. To avoid these problems, the dUTPase maintains an extremely low dUTP level and thereby excludes this component as a substrate for DNA polymerase. As has been demonstrated for *E.coli* and *Saccharomyces cerevisiae* dUTPase-deficient mutants, the enzyme is necessary for the viability of the cell (El-Hajj et al., 1988; Haynes et al., unpublished). These findings are supported by recent work about the dUTPases encoded by retroviruses. It has been suggested that the enzyme may serve to decrease the incorporation of dUTP into the intermediate DNA, which is generated by reverse transcriptase, thereby increasing the genetic stability (Elder et al., 1992). If, for some reason, the retrovirus is exposed to high levels of dUTP in its natural host cell environment, the attempted genome replication could lead to uracil substitution in both DNA strands during reverse transcription. Consequently, the convergent excision repair of both DNA strands would result in an irreversible fragmentation of this

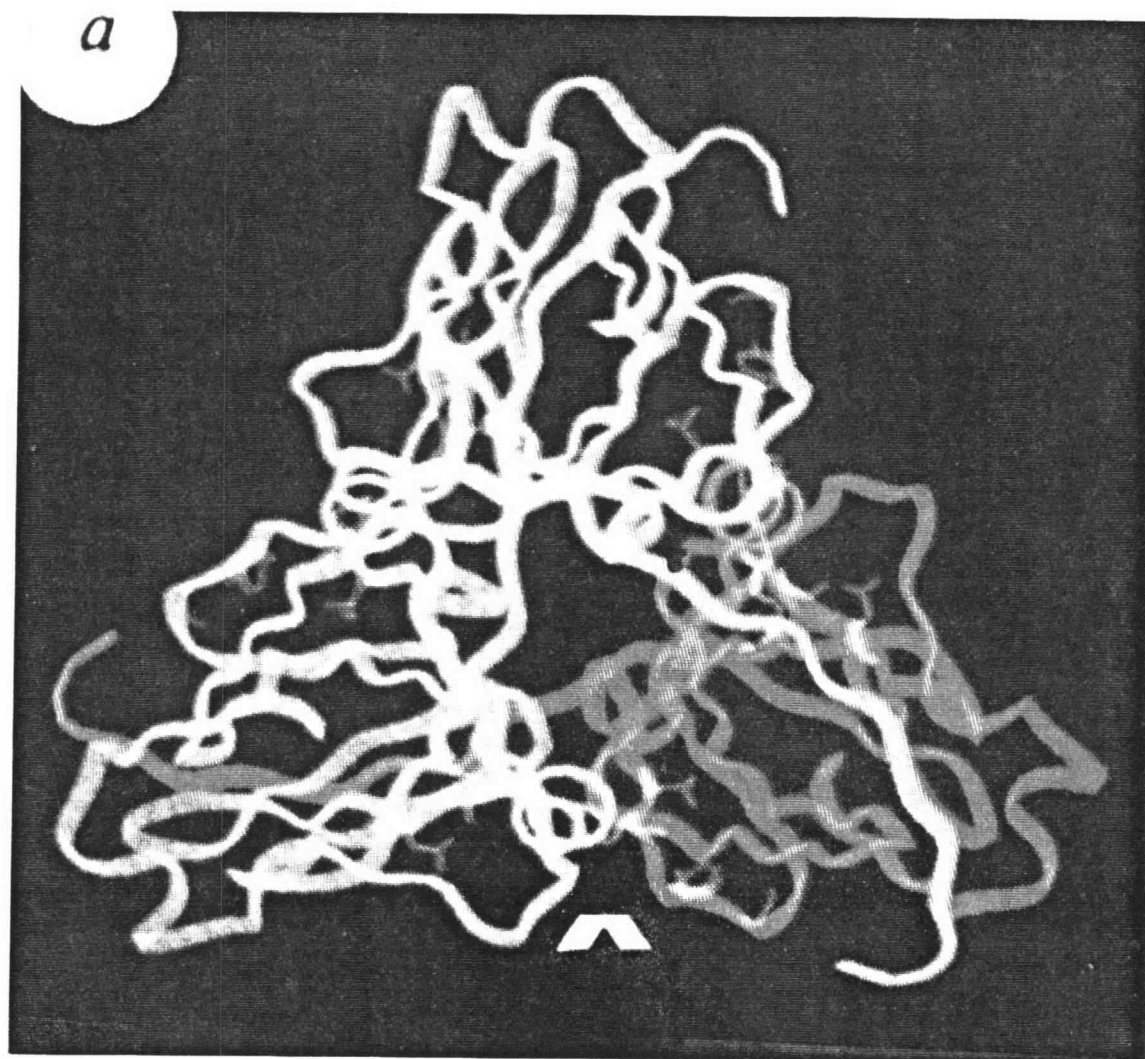


Figure I-4. Crystal structure of the *E.coli* dUTPase from Cedergren-Zeppezauer et al., 1992. For details see text.

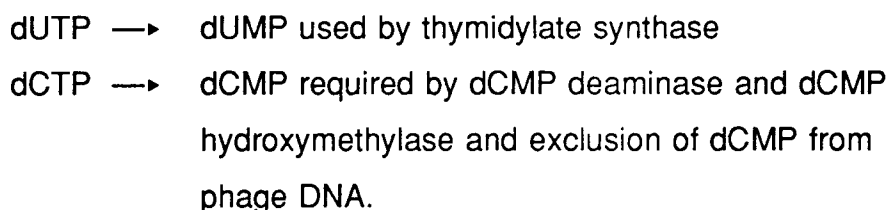
vital replication intermediate. In contrast, hypermutable retroviruses, such as the human and simian immunodeficiency viruses, do not encode a dUTPase (Elder et al., 1992). These viruses might, however, use the host dUTPase for their purposes.

Finally, it has been shown in that a developmentally regulated inhibitor for dUTPase exists in *Drosophila melanogaster*, which may constitute a mechanism for programmed cell death (Nation et al., 1989). Therefore, it appears that the conservation of the dUTPase presents an evolutionary advantage for small viruses as well.

*Structural considerations* — In 1992, the crystal structure of the dUTPase from *E.coli* was published (Cedergren-Zeppezauer et al., 1992). This was the first picture of the three-dimensional structure of a dUTPase (Figure I-4). The protein is a trimer around the threefold axis of the crystal. It comprises the same stoichiometry as the mammalian dUTPase and the T4-encoded dUTPase. The strong homology in the dUTPase family makes it intriguing to propose that all dUTPases contain this trimeric composition. Additionally, the enzyme does not show the 'classical' nucleotide binding domain (Rossman fold). Unfortunately, the position of the active site was not detected in this study (Cedergren-Zeppezauer et al., 1992).

### **The dCTPase-dUTPase of the bacteriophage T4**

As one of the proteins of the dNTP synthetase complex, the dCTPase-dUTPase (gp56) is linked to several enzymes in the dNTP synthesis complex. The enzyme mainly catalyzes the following reactions:



In addition, this enzyme accepts dCDP and dUDP as substrates.

The enzyme was partially purified by Warner and Barnes (1966). They

detected dCTPase and dUTPase activity in the same fractions, along with dUDPase and dCDPase activities. Similar results were obtained by Greenberg (1966) with bacteriophage T2. Finally, Munroe and Wiberg (1968) established that gene 56 is the structural gene for the dCTPase and the dUTPase. The complete description of the dCTPase-dUTPase of T4 and its role in the infection cycle was provided by Price and Warner (1969). They found that the protein is very acidic and, in the native form, has a molecular weight of approximately 60 kDa. The dUTPase activity prevents the incorporation of uracil into the DNA, and provides dUMP for the dTMP synthesis through thymidylate synthase. However, if the host is deficient in uracil-DNA-glycosylase, the uracil-containing phage DNA is still competent for RNA transcription and does not impair the functioning of the T4 DNA (Warner and Duncan, 1978). Furthermore, the enzymatic activity is extended to hydrolyze dCTP as well. The dCTPase is essential not only to exclude cytosine from incorporation into the phage DNA, but also to give a precursor to the synthesis of hydroxymethyl-dCMP by the dCMP hydroxymethylase. The widening of the dUTPase activity by the dCTPase activity also reflects the remarkable adaptation of the enzyme through evolution. In fact, the high affinity for dCTP ( $K_m=4.2 \mu\text{M}$ ) and dCDP ( $K_m=2.2 \mu\text{M}$ ) is in the same range as for dUTP (Zimmermann et al., 1960). Therefore, both the dCTP and dCDP pools, as well as the dUTP and dUDP pools, remain very small after infection by the phage.

### **Present work**

The ability of the complex to channel intermediates needs to be related to its organization. Protein-protein interactions must play a crucial role in the proper arrangement of the constituents.

Most of the work described in this thesis concentrates on the role of the T4-encoded enzyme dCTPase-dUTPase within the complex. Several approaches to identify protein-protein interaction involving this enzyme were attempted. One of the more promising methods to test for direct interaction of purified enzymes of the dNTP synthetase complex was native gel electrophoresis.

In a second project, I have focused on the isolation of the dNTP synthetase complex. An affinity tag was added to an enzyme of the complex, dCMP hydroxymethylase. By infecting the overexpressing bacteria with a gp42-deficient mutant, I attempted the isolation of the complex.

## CHAPTER 2

**The dCTPase-dUTPase Of The Bacteriophage T4 Interacts With Two Phage Enzymes In The Deoxynucleotide Synthesizing Complex**

## INTRODUCTION

To recall some of the information given in the first chapter, I will give a brief introduction. The bacteriophage T4 is a large DNA virus which degrades the DNA of its host *E.coli* and captures the deoxyribonucleotides precursors and the dNTPs of *E.coli* for the synthesis of its own DNA (Warner et al., 1983). The *de novo* synthesis of the dNTPs is mediated by a dNTP synthesizing multienzyme complex which is formed shortly after the infection of the phage on its host (Mathews, 1993). This complex has been partially purified by this laboratory and others (Moen et al., 1988, and Chiu et al., 1982). It consists of 10 virus encoded enzymes and two host enzymes with a total mass of 1300kDa (Moen et al., 1988). The purification alone, however, did not provide sufficient evidence for the direct interaction of proteins within the multienzyme aggregate, partially because the complex was found to be very unstable. In fact, individual interactions of proteins in the complex seem to play a crucial role for stability and the final balance of the four dNTPs that constitute the DNA (Thylén and Mathews, 1989). Therefore, our laboratory has focussed on alternative approaches to study individual protein-protein interactions.

The phage-encoded enzyme dCTPase-dUTPase is one of the key enzymes preventing misincorporation of uracil into the DNA (Greenberg, 1966). It is a 20.1-kDa protein and is active as a trimer ( Ungermann & Mathews, unpublished observations). This enzyme acts on dUTP, dCTP, dUDP, and dCDP (Warner and Barnes, 1966). Its ability to do so plays important roles in the dNTP synthesis of the phage. The dCMP formed through the dCTPase activity is substrate both for dCMP deaminase and for dCMP hydroxymethylase. The dUTPase activity of the enzyme is essential in excluding dUTP from



incorporation into the replicating DNA, and in providing dUMP as a substrate to the thymidylate synthase (Price and Warner, 1969). Finally, the dUDPase and dCDPase activities are needed in the salvage pathways of dNTPs, but do not play a major role in the infection cycle.

In multienzyme complexes protein-protein interactions are supposed to be essential for metabolic channeling. Support for this hypothesis comes from previous work in this laboratory, where it has been demonstrated that gp42 and td interact *in vivo* (Young et al, 1992). In the present study I demonstrate additional protein-protein interactions in the dNTP synthetase complex by affinity chromatography, the antiidiotypic antibodies and native gel electrophoresis. This includes the interaction of dCTPase-dUTPase with cd and gp1. Additionally, I have found a strong interaction of cd with the gp42.

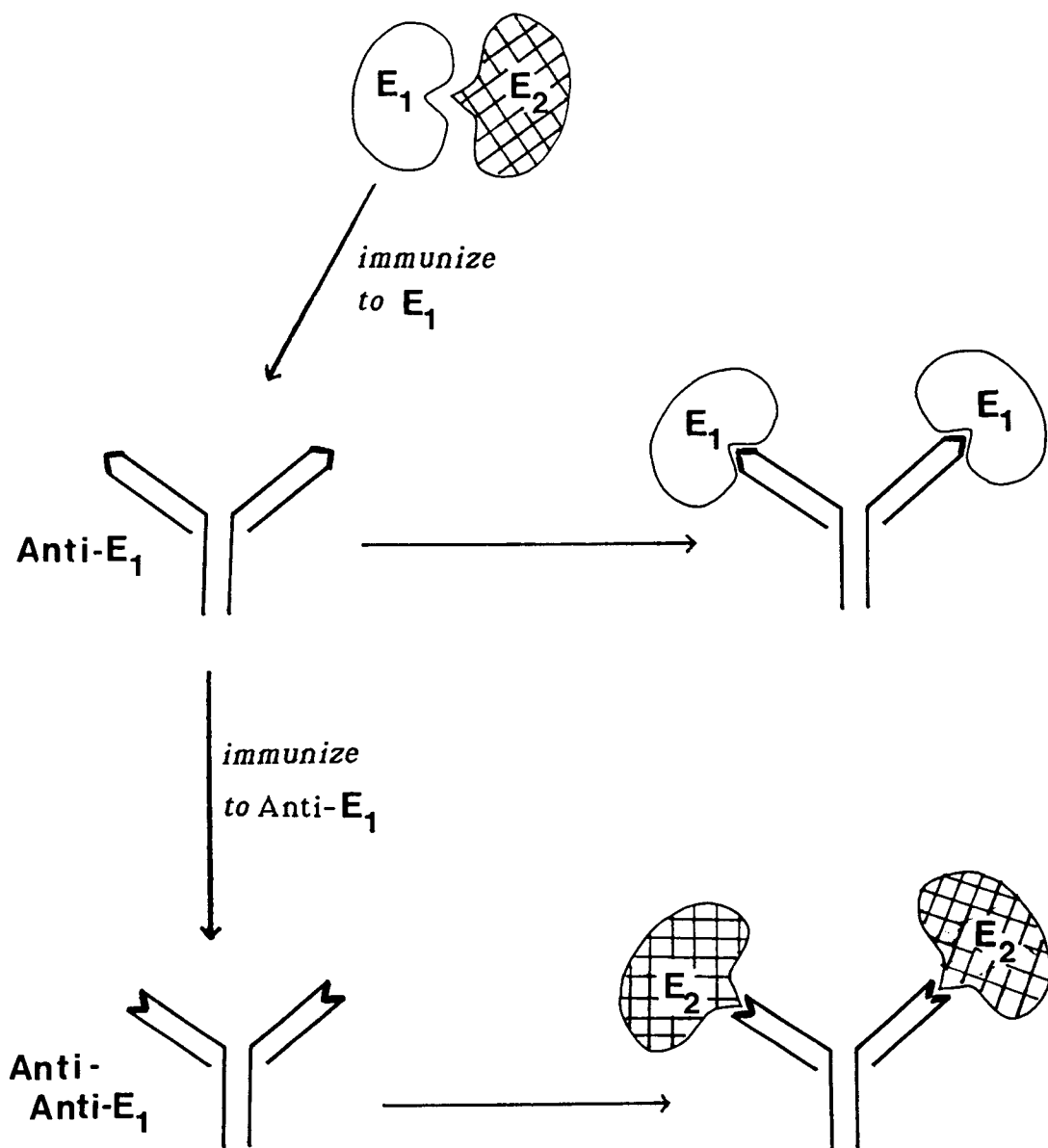


Figure II-1: The identification of protein-protein interactions by anti-idiotypic antibodies. Polyclonal antibodies to a protein  $E_1$  might contain idiotypic determinants that can induce an anti-idiotypic response. These antibodies may be specific to the protein  $E_2$  that interacts specifically with  $E_1$ .

## EXPERIMENTAL PROCEDURES

**Materials** — The plasmid carrying gene 56 in *E. coli* strain M5219 was a gift from Gisela Mosig, Vanderbilt University. The MPL+TDM (Monophosphoryl lipid A+Trehalose dicorynomycolate) adjuvant used for the generation of antibodies was purchased from RIBI ImmunoChem Research Inc., MT; Protein A sepharose was purchased from Sigma, and Affi-gel 10 from Bio-Rad. The purified enzymes were kindly provided by: Frank Maley (dCMP deaminase), Linda Wheeler (dCMP hydroxymethylase and dihydrofolate reductase), and Melissa Clason (deoxynucleoside monophosphokinase).

**Purification of the dUTPase-dCTPase** — The T4 phage-coded dUTPase-dCTPase was purified from 12 g (wet weight) of a heat-induced *E. coli* strain carrying a plasmid with gene 56 downstream of a controllable T7 phage promoter. After a streptomycin sulfate cut, the proteins were passed over a DEAE cellulose column, a Superose 12 gel exclusion column, and a Phenyl Superose hydrophobic interaction column. The procedure was modified following Warner et al. (1966) and yielded 8 mg of purified protein.

**Affinity Chromatography** — Affinity chromatography with immobilized dUTPase-dCTPase followed a protocol described by Wheeler et al. (1992). Samples were analyzed by two-dimensional gel electrophoresis (Formosa et al., 1983).

**Preparation of antibody columns** — Four antibodies were crosslinked to protein A beads with dimethylpimelimidate following a protocol by Harlow et al. (1988). Five hundred  $\mu$ l of beads were loaded on each column and pre-equilibrated with 0.1 M potassium phosphate buffer, pH 7.4, 5% glycerol, 2 mM EDTA. T4-infected *E. coli* bacteria were gently lysed (Moen et al., 1988), and the supernatant was applied to the antibody column. The proteins were circulated for 1 h. The columns were washed with 20 column volumes of the same buffer as mentioned above and then eluted with 3 volumes of 0.1 M glycine, pH 2.5. The samples were concentrated for several hours in a

Centricon 10 device (Amicon). Equal amounts of proteins were applied to a SDS-polyacrylamide gel and then transferred to nitrocellulose. The gels were immunoblotted with four different antibodies.

*Immunoprecipitation* — [ $^{35}\text{S}$ ] methionine-labeled proteins from T4-infected and uninfected *E.coli* were immunoprecipitated to test the specificity of the antibodies raised against the dUTPase-dCTPase, and to identify the proteins precipitated by the raised antibodies.

The proteins were labeled by adding [ $^{35}\text{S}$ ] methionine to an infected culture. Bacteriophage T4-infected *E.coli* was labeled from 3 to 8 min post-infection, and *E.coli* cells were labeled for 20 min. The cells were pelleted, sonicated, and the supernatant was spun for 10 min at 15,000 x g. Immunoprecipitation was carried out in RIPA buffer (150 mM NaCl, 10% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 8.0 ). The sera and the antigen were incubated at room temperature for 1 h, then protein A beads were added and incubation was continued for an additional hour. The samples were centrifuged for 20 sec at 10,000 x g, and the pellet was washed 3 times in RIPA buffer. Twenty ml of SDS sample buffer were applied to the dried protein A beads. The samples were boiled for 10 min in a water bath and loaded on a 12.5% SDS polyacrylamide gel, prepared as described by Laemmli (1972). The gel was fixed, soaked in 1M salicylic acid, dried, and placed on film.

*Native gel electrophoresis* — Two variations of non-denaturing gels were applied. The method described by Edison et al. (1992), with agarose as the gel material, was used in the one approach, in a second one polyacrylamide was substituted for agarose. Proteins in a nondenaturing environment will migrate according to their acidity (pI) and their molecular weight. A 10% polyacrylamide gel with a buffer, containing 25 mM Tris-base, 190 mM glycine, and 2 mM EDTA (pH=8), termed TGE-buffer, was poured, proteins of interest were mixed for 30 min at room temperature or overnight at 4°C, and a total of 4 µg of protein was loaded on each lane. The gel was run for 4 hrs at 100 V at 20° C. In the agarose system, a 0.7% gel with the same buffer was used; it was run

for 1.5 hrs at 100 V. The proteins were transferred onto a nitrocellulose membrane and immunoblotted for the carried protein.

*Antibodies against dUTPase-dCTPase* — Three hundred µg of purified enzyme were injected into New Zealand White rabbits, along with a synthetic adjuvant, MPL+TDM emulsion. After 4 week, each rabbit was boosted with an additional 300 µg of purified enzyme. One week after the boost, a bleed (40 ml) was taken and analyzed for antibody production by immunoprecipitation (Harlow et al., 1988). The rabbits were bled (8 ml) before the injection with dUTPase-dCTPase to control for a possible preimmune response.

*Antiidiotypic antibodies to dCTPase-dUTPase antibodies* — The antibodies against dCTPase-dUTPase were tested by immunoprecipitation with labeled T4 proteins. The antibodies were purified on a protein A column following a protocol by Harlow et al. (1988). The rabbits were immunized with about 1 mg of purified IgG antibodies per boost, in the same way as described for the primary antibodies.

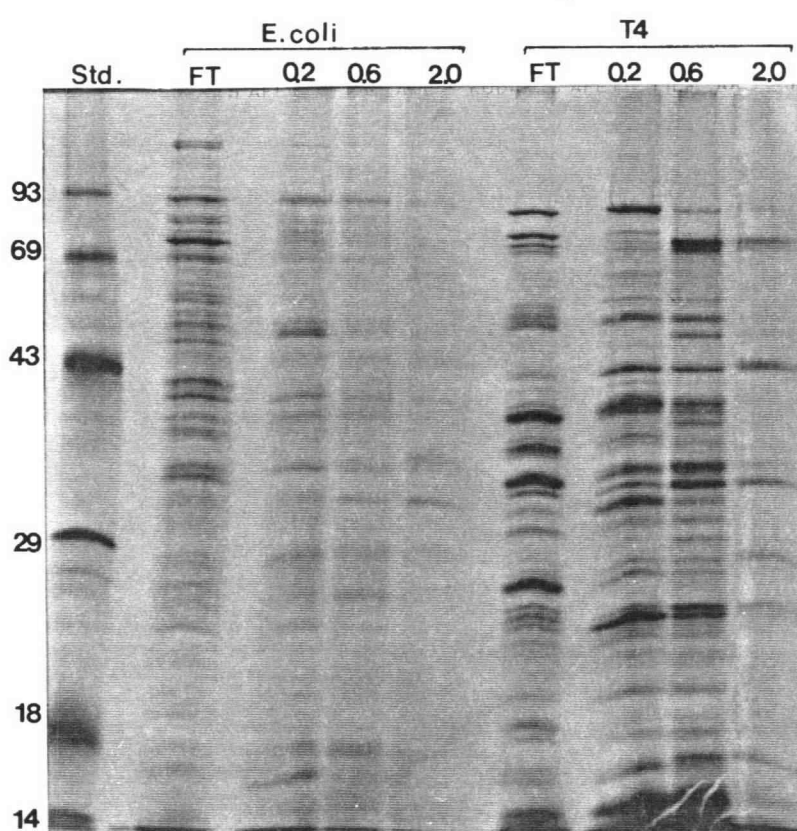


Figure II-2. One dimensional gel electrophoretic analysis of radiolabeled *E. coli*-proteins (left) and T4-proteins (right) eluted from the dCTPase-dUTPase affinity column. The numbers on top of the lanes indicate the molarity of the applied NaCl in the elution buffer and lane FT is the flow-through of the column.

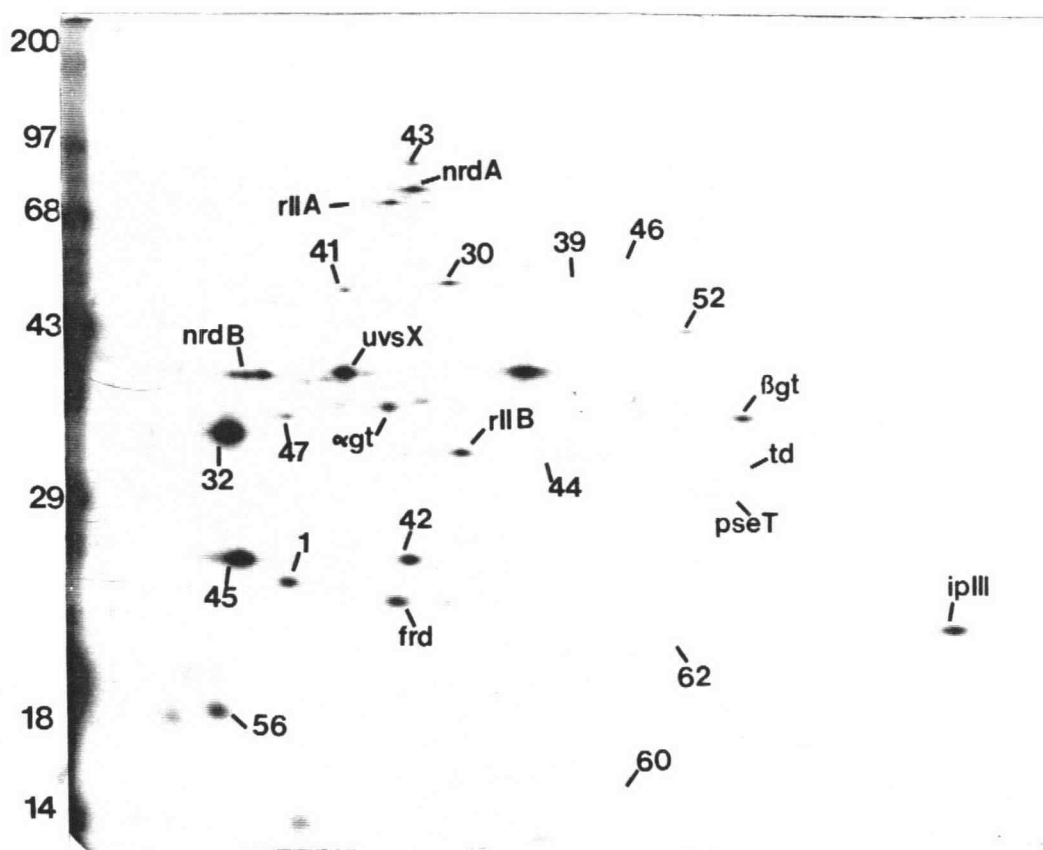


Figure II-3. Two-dimensional gel electrophoretic analysis of radiolabeled total T4-proteins. The proteins were separated by charge in the horizontal and by mass in the vertical direction. The numbers on the left side of the autoradiogram give the molecular weight in kilodaltons.

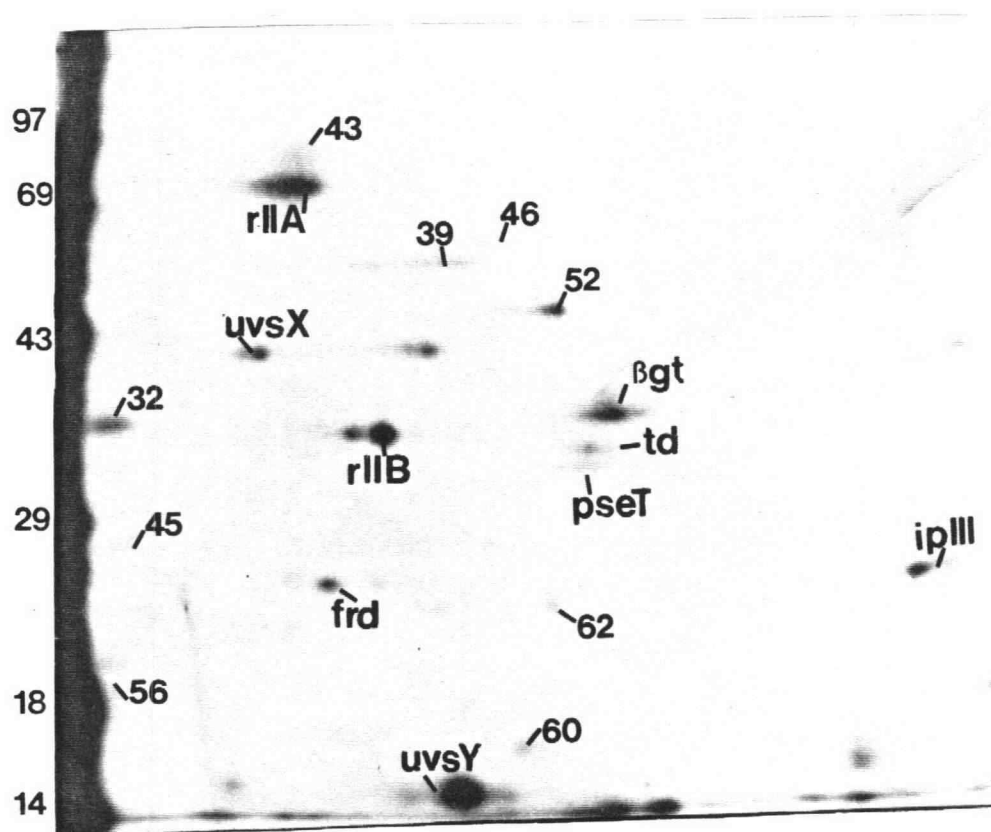


Figure II-4. Two-dimensional gel electrophoretic analysis of radiolabeled T4-proteins retained by immobilized dCTPase-dUTPase. Proteins were bound at 0.2 M and eluted at 0.6 M NaCl. In the first dimension, the most acidic proteins are seen on the left side of the gel. The numbers on the left identify the molecular weight markers in kilodalton along the second dimension.



## RESULTS

*Two-dimensional gel electrophoretic analysis of dCTPase-dUTPase-bound T4 proteins* — T4 proteins, labeled with [<sup>35</sup>S]-methionine between 3 and 8 min post infection, were identified on a two-dimensional gel by analysis of purified proteins on stained gels, by immunoblotting with specific antibodies, or through the analysis of radioautograms of extracts of cells infected by deletion or amber mutants of T4. With this pattern at hand, it is possible to identify a specific T4 protein according to its location on the two-dimensional radioautogram (Wheeler et al., 1992). The proteins are eluted from the affinity column in three steps (0.2, 0.6, 2.0 M NaCl). The 0.6 M fraction is most important, because the proteins in this fraction represent moderate to stronger protein-protein interactions. In addition, the 2.0 M fraction provides information about strong ionic interactions with the immobilized protein. The elution of labeled T4 proteins from an affinity column shows two overlapping effects: (i) the direct interaction with the immobilized protein, and (ii) the indirect interaction of the proteins that are "piggybacked" by an interacting protein. The analysis can provide information about the amount of protein retained by ionic interactions, but is unable to distinguish between types of interaction. Several proteins were eluted from the column at 0.2-0.6 M NaCl (Figure II-4 and for comparison Figure II-3). In the following analysis we excluded proteins that were previously identified to bind to bovine serum albumin (BSA), i.e. the products of the genes *ipIII*, *riIB*, 39, 52, and 60 (Wheeler et al., 1992). Still, several proteins were specifically retained by the dCTPase-dUTPase column. These proteins fall into two categories: (i) the dNTP precursor synthesis enzymes thymidylate synthase (*td*) and dihydrofolate reductase (*frd*), and (ii) the proteins associated with DNA metabolism, such as the eluted single-stranded binding protein (*gp32*), a polymerase accessory protein (*gp62*), two DNA repair proteins (*uvrY* and *X*), the DNA  $\beta$ -glucosyltransferase, or the DNA kinase/phosphatase (*pseT*). Through the final 2.0 M salt elution step all

remaining strongly bound proteins are washed off. Dihydrofolate reductase and thymidylate synthase were detected in this step.

Affinity chromatography gave some indication about specific protein-protein interactions, however, the identification of direct interactions was dependent on additional analysis. Therefore, I selected the generation of antiidiotypic antibodies to further characterize specific interactions involving dCTPase-dUTPase.

*The antiidiotypic antibodies to dCTPase-dUTPase are specific for gp1—* Antibodies were raised against the Protein A-purified dCTPase-dUTPase antibodies. We hypothesized that the small portion of antibodies to *E.coli* proteins can be neglected compared to the large amount of specific dCTPase-dUTPase antibodies. In fact, the induced antibodies represent up to 10% of the total IgG immunoglobulins (Harlow et al., 1988). Therefore, the amount of injected antibodies needed to stimulate a response was much higher than the amount of primary antigen. The injected antibodies were specific to dCTPase-dUTPase, as shown by a competition experiment in which pure gp56 was added to the antibodies before the addition of the labeled T4 proteins. An additional T4 protein was not precipitated (Figure II-5, lanes 3-5). After the third injection of anti-dCTPase-dUTPase antibodies, one of the two rabbits showed a response to the antigen. A single T4 protein of 26 kDa was immunoprecipitated by the antiserum. This band was not detected in the preimmune serum (Figure II-5, lanes 6-8). Only one protein of the dNTP synthetase complex corresponds to this molecular weight, the deoxynucleoside monophosphokinase (gp1). It was possible to dilute out the 26-kDa band by adding 20 µg of pure gp1 to the reaction mixture.

In a previous attempt to raise antibodies to dCTPase-dUTPase, the antibodies brought down a second protein during an immunoprecipitation experiment with labeled T4 proteins. This second response was detected after the second injection with the antigen (six weeks). Again, the molecular weight of 26 kDa suggested an antibody to gp1. These antibodies were not specific to

dCTPase-dUTPase, because a dilution of the band with purified gp56 eliminated only the gp56 specific band (Figure II-6, lane 2). To our own surprise, pure gp1 competed away the second band. The injected antigen, dCTPase-dUTPase, appears to have been so antigenic to this animal that it stimulated an antiidiotypic response immediately after the attempted antibody response.

*Native gel electrophoresis demonstrates specific interaction of dCMP deaminase with dCTPase-dUTPase and dCMP hydroxymethylase* — To test for *in vitro* interactions of enzymes in the dNTP precursor synthesis, we applied native gel electrophoresis (Hoffmann et al., 1992). This method is based on an acidic protein's ability to migrate into the gel while carrying along a basic, specifically interacting protein. The enzyme dCTPase-dUTPase is fairly acidic with an isoelectric point (pI) of 4.76. Following the hypothesis that proteins catalyzing sequential reactions might interact in order to allow efficient catalysis to happen, dCMP deaminase was selected (pI=7.4) for interaction with gp56. As seen in Figure II-7, the dCMP deaminase takes the dCMP, which has been hydrolyzed by dCTPase-dUTPase, and deaminates it to dUMP. The dCMP can serve as a substrate to dCMP hydroxymethylase, which adds a hydroxymethyl group to the C-5 on the pyrimidine ring. When dCMP deaminase was preincubated with either dCTPase-dUTPase or with dCMP hydroxymethylase, it was shifted into the gel (Figure II-7, lane 2,3). However, it remained at the top of the gel when applied pure (lane 1). The protein was detected by immunoblotting, as described in Materials and Methods. The dCTPase-dUTPase and dCMP hydroxymethylase did not interact in this *in vitro* approach.

*Antibody columns to test the interactions involving dCMP deaminase* — To further characterize the interaction observed in the native gel, I used a different approach through which I intended to test the retention of the dNTP synthetase complex with immobilized antibodies. More specifically, I wanted to know which of the antibodies would be able to interact with the surface of the dNTP synthetase complex, i.e. which of the antigens would be exposed to the

surface. Thus, we were interested to know which of the immobilized antibodies retained more than its own antigen on the column. This experiment is similar to the affinity chromatography described before, with the exception that the antibodies were expected to retain a whole complex if the antigen would be surface exposed. In contrast, affinity chromatography considers primarily the single protein-protein interactions and secondly the reformation of the complex around an immobilized protein.

We found several proteins of the dNTP complex that were retained on the antibody column. Figure II-8 shows that both the dCTPase-dUTPase and dCMP hydroxymethylase antibody column retained most of the dNTP synthesis proteins. Furthermore, these antibodies columns were the only ones to retain dCMP deaminase.

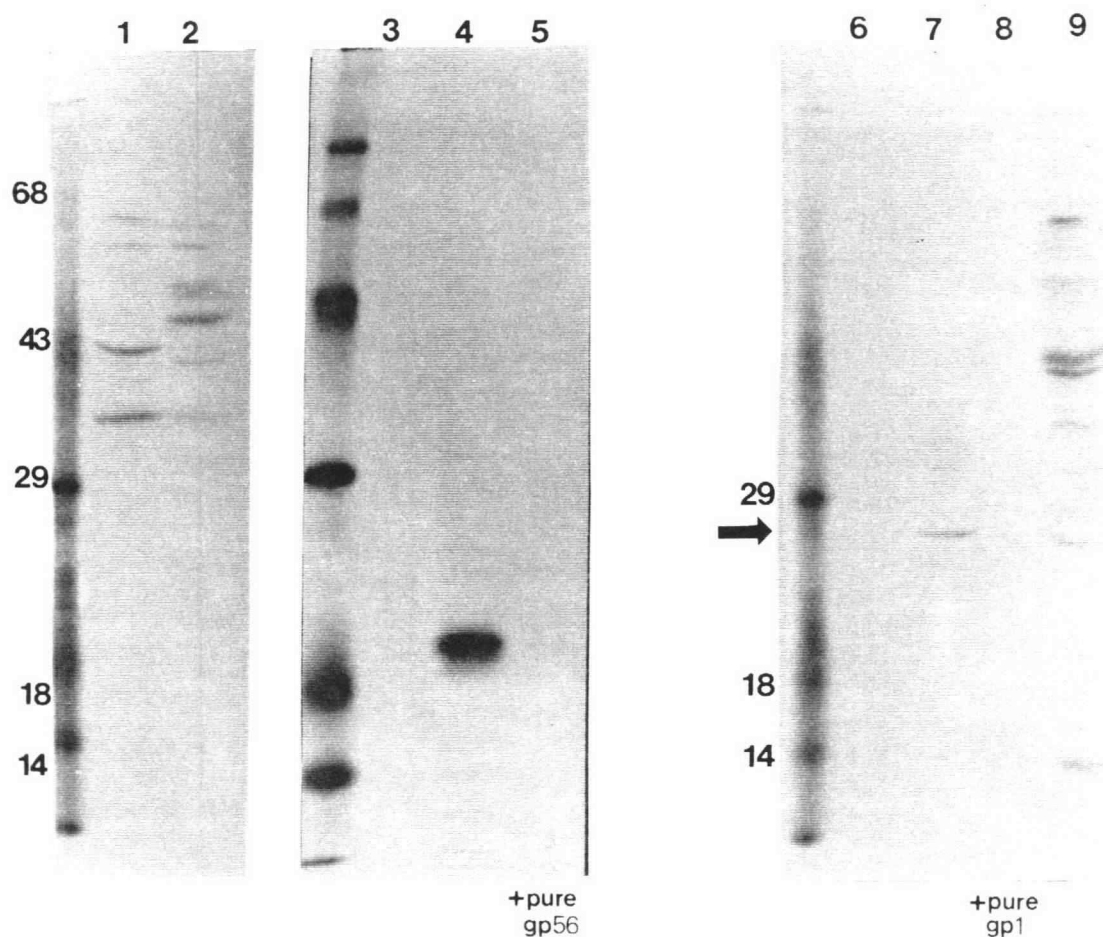


Figure II-5. Antiidiotypic antibodies to dCTPase-dUTPase are specific for gp1. Immunoprecipitations with [ $^{35}$ S]-labeled *E.coli* extract: Lane 1, preimmune serum of rabbit used for antibody generation to dCTPase-dUTPase (rabbit 1); lane 2, antiserum to dCTPase-dUTPase; lane 9, antiserum of rabbit 2; Immunoprecipitations of [ $^{35}$ S]-labeled T4-proteins: Lane 3, preimmune serum of rabbit 1; lane 4, antiserum to dCTPase-dUTPase; lane 5, antiserum rabbit 1 after preincubation with 20  $\mu$ g of cold dCTPase-dUTPase; lane 6, preimmune serum of rabbit used for the generation of antiidiotypic antibodies (rabbit 2); lane 7, antiidiotypic antibodies (rabbit 2); lane 8, antiserum of rabbit 2 after preincubation with 20  $\mu$ g of pure gp1;

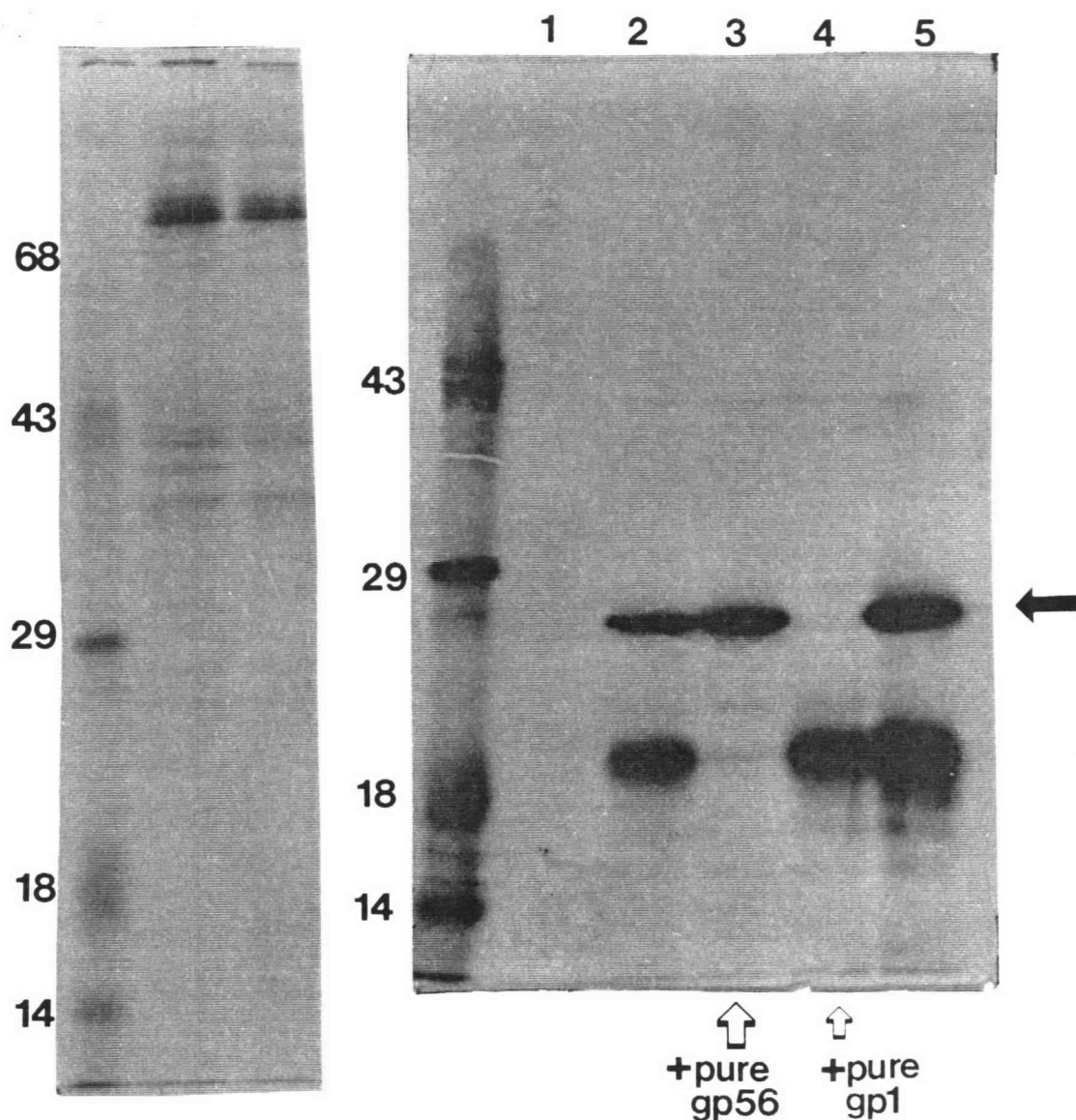


Figure II-6. Antibodies to dCTPase-dUTPase do contain an antiidiotypic antibody to gp1 as analyzed by immunoprecipitation with  $^{35}\text{S}$ -labeled *E.coli* proteins (lanes a,b) and T4-proteins (lanes 1-5). Lane a, antiserum to dCTPase-dUTPase; lane b, after preincubation of the antiserum with 20  $\mu\text{g}$  of pure gp1; lane 1, preimmune serum; lane 2 and 5, antiserum to dCTPase-dUTPase; lane 3, after preincubation of the antibodies with 20  $\mu\text{g}$  of pure dCTPase-dUTPase; lane 4, after preincubation of the antiserum with 20  $\mu\text{g}$  of pure gp1.

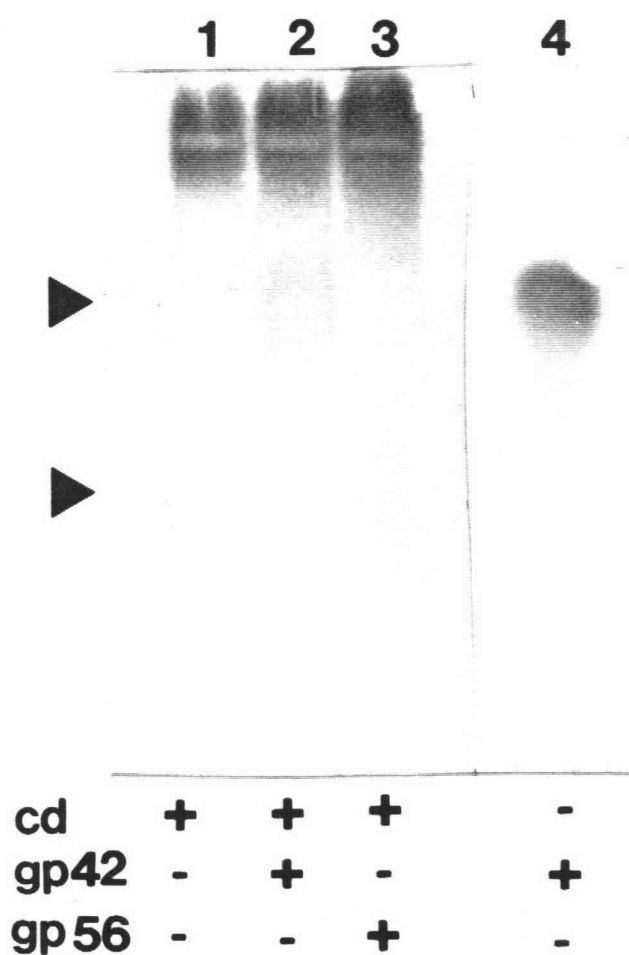
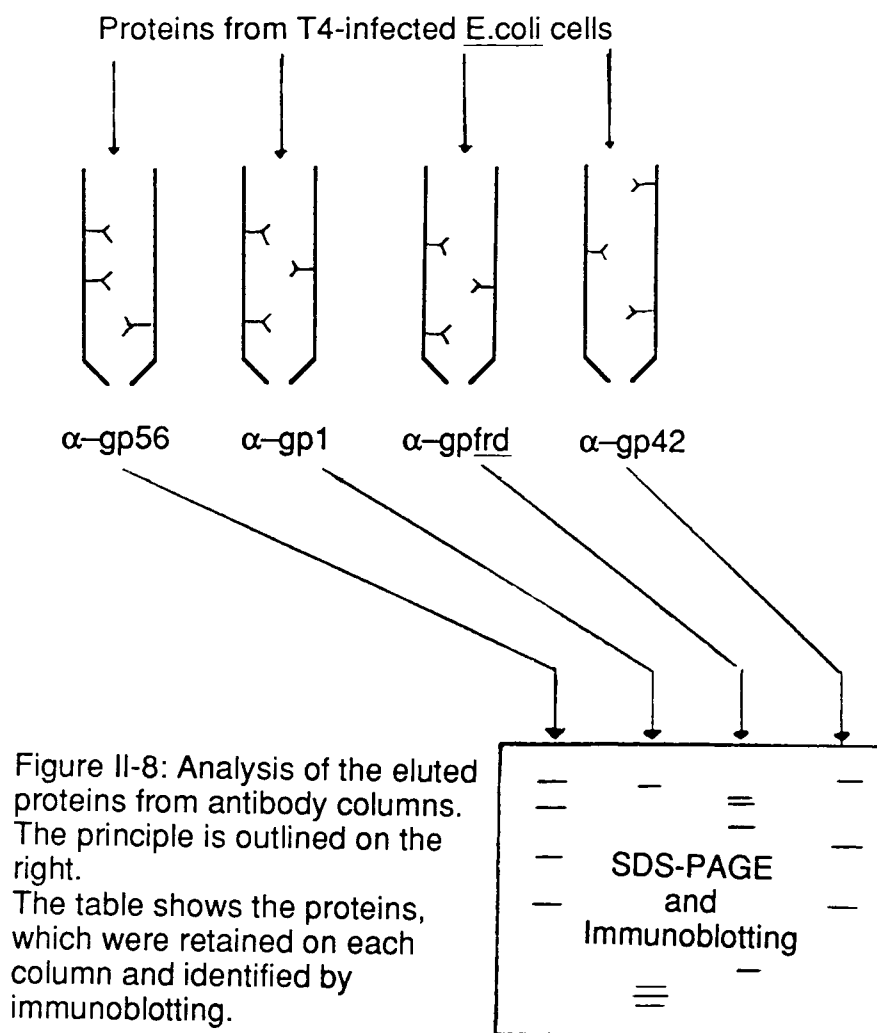


Figure II-7. Native gel electrophoresis demonstrates the interaction of dCMP deaminase with dCTPase-dUTPase and dCMP hydroxymethylase as visualized by Western blotting. Lane 1-3 were probed with an antibody to dCMP deaminase. Lane 4 was probed with an antibody to dCMP hydroxymethylase.



| <u>probed with</u> | <u>eluted from</u><br>antibody column |               |                 |                |
|--------------------|---------------------------------------|---------------|-----------------|----------------|
|                    | $\alpha$ -gp56                        | $\alpha$ -gp1 | $\alpha$ -gpfrd | $\alpha$ -gp42 |
| $\alpha$ -gp56     | +                                     |               |                 |                |
| $\alpha$ -gp1      | +                                     | +             | +               | +              |
| $\alpha$ -gpfrd    | +                                     |               |                 |                |
| $\alpha$ -gp42     | +                                     |               | +               | +              |



## DISCUSSION

Before the current study, little information was available about protein-protein interactions involving the dCTPase-dUTPase of the bacteriophage T4. Certainly, this enzyme has a key function in the dNTP synthetase complex, as it lowers the dUTP and dCTP pools in the infected cell. Furthermore, inactivation of the corresponding gene inactivates the phage (Munroe et al., 1968). The central role of dCTPase-dUTPase in the complex is supported by affinity chromatography. When the enzyme was immobilized as an affinity ligand the protein retains two enzymes of the complex and several DNA replication proteins. If we consider the retained dihydrofolate reductase and the thymidylate synthase — both of which are detected in the final elution step — it is appealing to propose that these proteins might have assembled into a complex that may be present *in vivo*. This is conceivable, since these proteins catalyze sequential reactions (Mathews et al., 1979). Preliminary experiments showed that the migration of dihydrofolate reductase in a native polyacrylamide gel changed in the presence of dCTPase-dUTPase.

The dCTPase-dUTPase antibody column retained more enzymes than did the gp56 itself. The result of this column analysis does not agree completely with the results obtained with the affinity chromatography. The dCMP deaminase found on the antibody column has not been identified yet on the two dimensional gel pattern. Actually, the labeling with [<sup>35</sup>S] methionine is disadvantageous for the identification of this protein, because it has only two methionine residues per polypeptide subunit, as compared to ,e.g., nine in the dCTPase-dUTPase. The deoxynucleoside monophosphokinase is retained on the antibody column, but was not detected in affinity chromatography analysis. This protein is only loosely associated with the complex. A complete sonication, as done in the affinity analysis, might disrupt the whole complex. If the gp1 is bound to the complex in a cooperative way or depends on the organization of the enzymes to bind in a stable

### Association of T4 Proteins with Immobilized Enzymes

| Gene | Gene product                      | Retained by Immobilized |                  |                     |                   |                              |
|------|-----------------------------------|-------------------------|------------------|---------------------|-------------------|------------------------------|
|      |                                   | dCMP<br>HMase           | dTMP<br>synthase | dCTPase-<br>dUTPase | dCMP<br>deaminase | <i>E. coli</i><br>NDP kinase |
| 42   | dCMP<br>HMase                     | +                       |                  |                     |                   | +                            |
| td   | dTMP<br>synthase                  | +                       | +                | +                   | +                 |                              |
| frd  | DHF<br>reductase                  | +                       | +                | +                   | +                 | +                            |
| 56   | dCTPase-<br>dUTPase               | +                       |                  |                     |                   |                              |
| nrdA | rNDP reduc-<br>tase R1            | +                       |                  |                     |                   | +                            |
| nrdB | rNDP reduc-<br>tase R2            | +                       |                  |                     |                   |                              |
| 32   | ssDNA-bind-<br>ing protein        | +                       | +                | +                   | +                 | +                            |
| 44   | polymerase<br>accessory           |                         |                  |                     |                   | +                            |
| 45   | polymerase<br>accessory           | +                       | +                |                     |                   | +                            |
| 61   | DNA<br>primase                    | +                       | +                |                     |                   | +                            |
| 62   | polymerase<br>accessory           |                         |                  | +                   |                   |                              |
| uvrX | repair, re-<br>combination        | +                       | +                | +                   | +                 |                              |
| uvrY | repair, re-<br>combination        | +                       | +                | +                   |                   | +                            |
| βgt  | DNA/glucos-<br>yltransfer-<br>ase |                         | +                | +                   | +                 |                              |
| pseT | DNA kinase-<br>phosphatase        | +                       |                  | +                   |                   |                              |
| regA | translation-<br>al control        |                         | +                |                     | +                 | +                            |

Table II-1: Overview of proteins retained by immobilized enzymes. Experiments were done with dCMP HMase and dTMP synthase (Linda Wheeler), NDP kinase (Nancy Ray), dCMP deaminase (Sonja Gerrard) and dCTPase-dUTPase.

conformation, then the disruption of the complex during sonication might disable the protein to rebind to a newly formed aggregate. In contrast, if the protein was associated with the complex, the interaction could be strong enough for the protein to be detected in the eluate of the antibody column. This hypothesis, however, remains to be tested.

An overview of interactions between T4 proteins and immobilized enzymes is given in Table II-1. This table sums up the research on immobilized enzymes carried out in the laboratory of C.K. Mathews. It stresses the possibilities, but also the limits of affinity chromatography. The dCTPase-dUTPase plays a pivotal role in the dNTP synthetase complex, but as of yet we are not sure whether or not the interactions with the replication proteins indicate a physical linkage of the dNTP synthetase complex to the replication machinery. This aspect of the problem is not further discussed in this article. One criticism to the present approach is the exclusive focus on ionic interactions when the affinity column is eluted. Hydrophobic interactions cannot be detected, because the strength of these interactions increases with an increase of ionic strength. Thus, our observations may be incomplete.

In a second approach to detect protein-protein interactions we intended to raise antiidiotypic antibodies to the dCTPase-dUTPase. Through the use of antiidiotypic antibodies, specific interactions between *gptd* and *gp42* in the dNTP synthetase complex have been demonstrated (Young and Mathews, 1992). In the present study, the purification of the antibodies over Protein A beads proved to be adequate to raise an antiidiotypic antibody. The same approach was successful in showing the interaction of a vaccinia virus protein with the vaccinia virus ribonucleotide reductase (Davis and Mathews, 1993). However, the more interesting result, as regarded from an immunological perspective, is the detection of the same antiidiotypic antibody in the primary antiserum of the first set of rabbits. We exclude a contamination in this experiment because of (i) the very strong band seen in the immunoprecipitation, (ii) the application of an artificial adjuvant along with the injected antigen, (iii)

the minor amount of *E.coli* proteins immunoprecipitated by the same antibodies, and (iv) the failure to separate the antibodies on a dCTPase-dUTPase affinity column. Although pure gp1 dilutes out a minor band in the labeled *E.coli* extract (Figure II-6, lane a and b), this appears to be caused by crossreactivity of the gp1-specific antibody with an *E. coli* protein. In the case of a crossreactivity of an antibody to an *E.coli* protein — as a result of a contamination —, one would have expected to see a strong immunoprecipitation of one *E.coli* protein (Figure II-6, lane a). This was not observed.

It is surprising that we were able to see the antiidiotypic response both in the primary and in the secondary response. The antigenicity of the protein may have been a critical parameter contributing to the first strong reaction. The antigen was purified for each of the two immunizations the same way (see Materials and Methods) and was electrophoretically homogeneous when it was injected. The antigenicity, however, might vary with each preparation, thereby contributing to the difference. The detection of antiidiotypic antibodies during repeated immunization with the same antigen has been observed before (Somerville et al., 1990). Furthermore, the antiidiotypic antibodies are thought to regulate the idiotype response in the mammalian immune system (Jerne, 1974). If the idiotype and the antiidiotypic response is followed over time, an alternation of these two antibody types can be seen. Therefore, the surprising result is not the detection, but the short time that was necessary to get the antiidiotypic response, in this case about six weeks. The selected artificial adjuvant might have contributed to the fast response. Because I detected an antiidiotypic antibody to the same protein in the "classic" immunization protocol, the interpretation given may be justified.

The dCTPase-dUTPase and the deoxynucleoside monophosphokinase do not carry out consecutive reactions. The gp1 is a very specific enzyme for hmdCMP, dTMP, and dGMP (see Introduction). The interaction probably represents an example of a three dimensional arrangement of these proteins in the dNTP synthetase complex. The influence of one protein on the others'

kinetic behavior has not yet been tested. These experiments are in progress in our laboratory.

Native gel electrophoresis, however, was informative in demonstrating the interaction of proteins carrying out sequential reactions. Since we found that dCMP deaminase was also retained on the gp56- and dCMP hydroxymethylase-antibody column, it is appealing to postulate that this aggregate of proteins is also present *in vivo*. Some of the protein-protein interactions inside of the complex are difficult to detect in a native gel. The cooperative binding of proteins, the molecular crowding effect through the high protein concentration (about 300 mg/ml) inside infected cells, and the selection of the buffer systems are parameters that influence protein-protein interactions. However, with purified enzymes of the dNTP synthetase complex in hand, we will attempt a reconstitution of the complex, possibly with the support of bifunctional crosslinkers.

## CHAPTER 3

**Attempt To Purify The dNTP Synthetase Complex With The Histidine-Tagged dCMP Hydroxymethylase**

## INTRODUCTION

The bacteriophage T4 forms a dNTP synthetase complex during the first minutes of the infection cycle on its host *E.coli*. The complex sequesters the deoxynucleotides and their precursors, which are set free during the nuclease-mediated breakdown of the host DNA or present from *de novo* synthesis, to provide dNTPs for the phage's own DNA synthesis. To accomplish an efficient supply of dNTPs to the replication sites, the enzymes constituting the complex have been proposed to interact directly.

Our laboratory has successfully purified the dNTP-synthetase complex before, but encountered stability problems during the course of the purification (Moen et al., 1988). Unfortunately, the complex is held together by relatively weak protein-protein interactions. Therefore, we decided to study the importance of regulatory interactions within the complex by focussing on individual protein-protein interactions. Recently, our laboratory was able to identify some specific interactions between complex enzymes (Young et al., 1992, Wheeler et al., 1992). It may be possible to answer some questions about the regulatory interactions and kinetic linkage through reconstitution with purified proteins, but the original protein content and the possible linkage to the replication machinery is still dependent on the purification of the whole complex.

For a new purification attempt, I tried to minimize the dissociation of complex-proteins during purification. I attached an affinity tag consisting of six consecutive histidines to one of the complex enzymes, dCMP hydroxymethylase. The addition of six histidines to the protein is a very mild modification that does — in most cases — not impact the activity of modified protein. The modification enables the protein to interact specifically with a  $\text{Ni}^{2+}$

column. In addition, the protein can be eluted under mild conditions by applying an imidazole gradient to the column. I attempted to purify the dNTP synthetase complex through infection of the gp42-overexpressing bacteria with a gp42-deficient strain of T4. The modified protein was supposed to complement for the missing activity in the mutant phage, thereby allowing the dNTP synthetase complex to interact with the  $\text{Ni}^{2+}$  column when the affinity tag would be accessible. In this section I will show that it was possible to clone this protein, but that a purification of the complex failed.

## EXPERIMENTAL PROCEDURES

*Materials* -- The  $\text{Ni}^{2+}$  NTA resin was purchased from Qiagen, CA. Restriction enzymes were purchased from Promega. The Centricon 100 devices were purchased from Amicon.

*Construction of the histidine tagged dCMP-hydroxymethylase* — Polymerase chain reaction (PCR) on T4 DNA (provided by Eric Hanson) was carried out with two primers. These introduced the additional bases coding for the histidines on both the 3' and the 5' end, along with two convenient restriction sites: *Nde*I on the 5' end, and *Bam*HI on the 3' end. The PCR fragment was cut with the two restriction endonucleases and purified over a Centricon-100 device (Amicon). It was ligated into a pET 11a expression vector with a controllable T7 promoter. Finally, the ligated construct was electroporated into *E.coli* BL21DE3 cells, which contain a plasmid carrying an IPTG inducible promoter with the T7 RNA polymerase gene downstream. The clone was termed *E.coli* His42.

*Overproduction and test for interaction with the  $\text{Ni}^{2+}$ -column* — To test the overproduction of *E.coli* His42, the bacteria were grown to an OD=0.8 and induced with 0.001M IPTG for 4 hrs. Three ml were pelleted and taken up into 200  $\mu\text{l}$  of sonication buffer containing 0.2 M Tris-HCl, pH 7.4, 0.002 M  $\beta$ -mercaptoethanol. After sonication on ice, the cell lysate was incubated with 30  $\mu\text{l}$  of  $\text{Ni}^{2+}$  NTA resin for 30 min. The solution was centrifuged briefly and the resin was washed twice with 1 ml of 0.2 M Tris-HCl, pH 6.3. Finally, the protein was eluted with 20  $\mu\text{l}$  washing-buffer containing 0.1 M EDTA, and the supernatant was analyzed by SDS-PAGE.

*Induction of *E.coli* His42 followed by infection of T4 am42(N55)* — *E.coli* His42-bacteria were grown to an OD=0.5 and induced with IPTG. After 3 min the bacteriophage was added at a ratio of 10 phages to one bacterium. The infection was stopped after 15 min, and the cells were chilled and pelleted. The pellet was taken up into 0.5 M potassium glutamate buffer, pH 7.4, containing



5% glycerol, and subjected to gentle sonication with a Branson sonicator (Moen et al., 1988). The lysed cells were centrifuged for 10 min at 10,000 x g. The supernatant was applied to a 3-ml  $\text{Ni}^{2+}$  NTA-column, which was equilibrated with 10 volumes of potassium glutamate buffer, and circulated for 1 h at 0.1 cm/min. The column was washed with the same buffer, and the proteins were eluted within an imidazole gradient from 0 to 0.5 M. One-ml fractions were collected and subjected to a SDS-PAGE analysis.

*Activity tests and immunoblotting* — The dCMP hydroxymethylase activity assay was performed as described previously (Pizer and Cohen, 1962). To test for the presence of complex enzymes in the eluted fractions, an assay was carried out, and the conversion of dCTP to dTMP was followed spectrophotometrically. This assay tests for the activities provided by dCTPase-dUTPase, dCMP deaminase, and thymidylate synthase (Moen et al., 1988). In addition, the fractions were tested for the presence of additional complex proteins by immunoblotting.

*Plaque assay* — *E. coli* bacteria of different strains were grown at 37°C to an OD=0.5 and infected with phage T4am42(N55) at a ratio of 1 phage to 10 bacteria. After 4 hrs of shaking, the lysate was chilled and centrifuged at 5000 x g for 10 min. The supernatant was saved and titered for phages on *E.coli* CR 63, an amber suppressor strain.

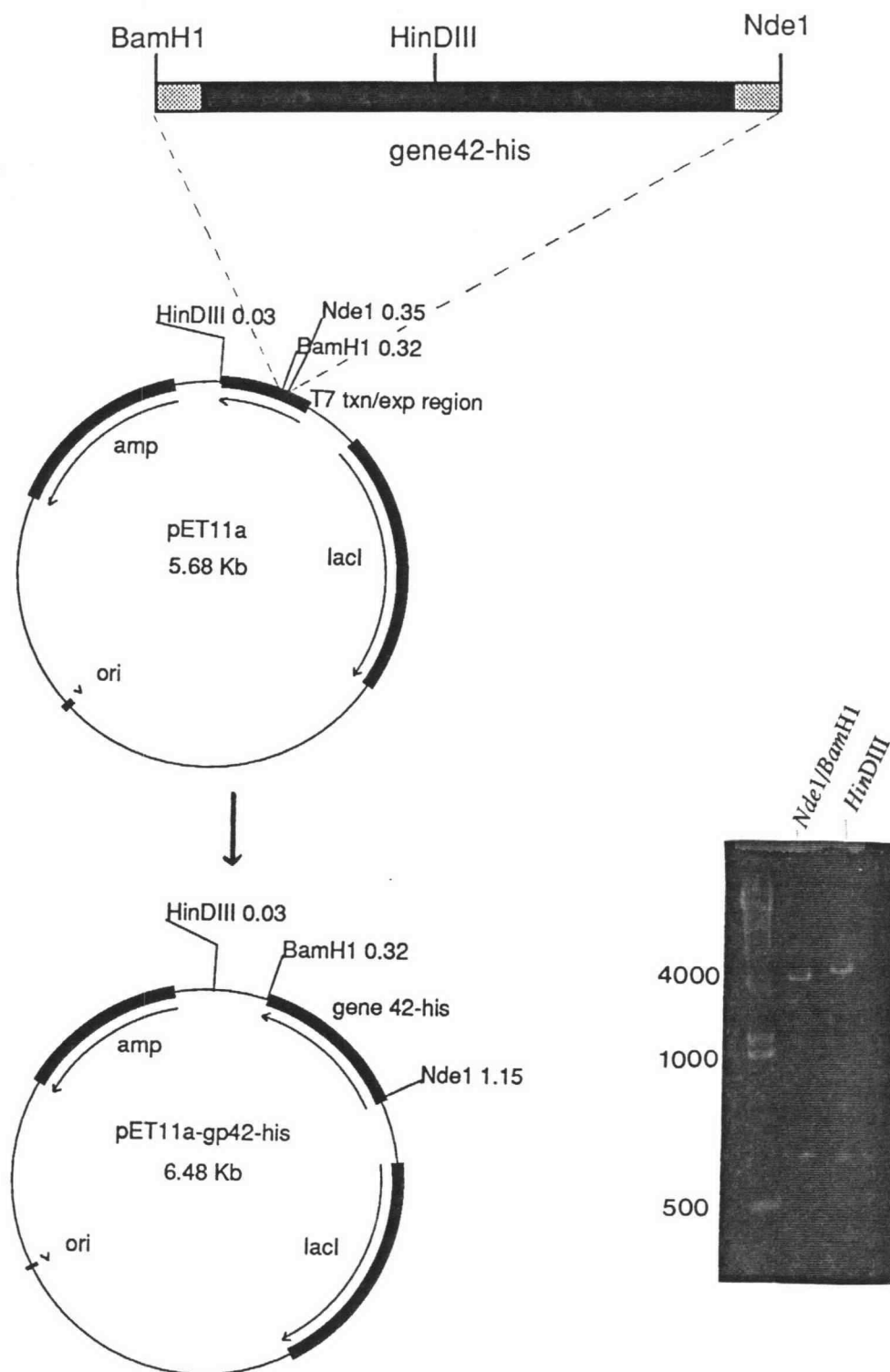


Figure III-1: Cloning strategy to modify gene 42. The modified gene 42 was generated by polymerase chain reaction and cloned into the pET11a plasmid. The selected construct was analyzed by cutting with restriction enzymes.

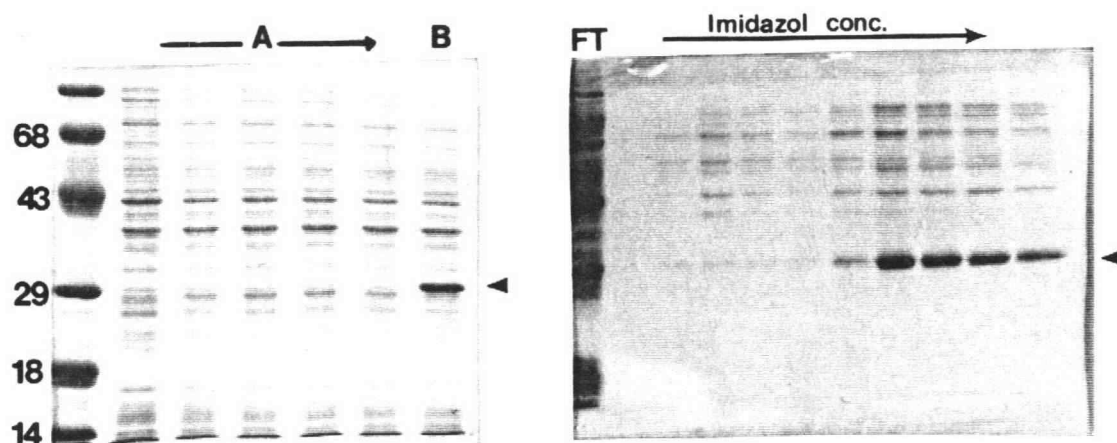


Figure III-2: Overexpression of his-tagged gp42. Two clones (A,B) were induced and run on a SDS polyacrylamide gel (right). Only B showed significant induction and was selected for the further work. The left SDS polyacrylamide gel shows the purification of the selected clone on the Ni<sup>2+</sup>-column. The protein was eluted in an imidazole-gradient from 0-0.5 M. FT is the flow-through fraction.

| Growth bacteria                   | Number of plaques<br>at phage dilution |                  |
|-----------------------------------|--|------------------|
|                                   | 10 <sup>-7</sup>                       | 10 <sup>-9</sup> |
| <i>E.coli</i> BL21DE3             | 0                                      | 0                |
| Wild-type dCMP-HMase (uninduced)  | 20                                     | 0                |
| His-tagged dCMP-HMase (uninduced) | 0                                      | 0                |
| Wild-type dCMP-HMase (induced)    | >10 <sup>3</sup>                       | 22               |
| His-tagged dCMP-HMase (induced)   | 188                                    | 2                |

Table III-1: Plaque assay to test for gp42-complementation. Various growth bacteria were infected with a gp42-deficient strain of T4. The lysate was tested on an amber suppressor strain that allows the mutant T4 strain to grow.

## RESULTS AND DISCUSSION

Because the T4-encoded dCMP hydroxymethylase (gp 42) is one of the central enzymes of the complex it was selected for the modification. Six consecutive histidines were added to the N- and C-terminus of the enzyme (Figure III-1). The overproducing bacteria were infected with a dCMP hydroxymethylase-deficient strain of T4, shortly after induction of gp42. This enzyme was supposed to complement for the enzyme missing in T4 and to participate in the formation of the complex.

As seen in Figure III-2, active dCMP hydroxymethylase with the attached histidines was produced after induction with IPTG. This protein interacts with the  $\text{Ni}^{2+}$ -NTA resin and is almost pure after the column purification. In addition, the activity of the histidine-tagged gp42 is comparable to the wild-type activity.

I considered the possibility that a large amount of gp42 would destroy the complex formation. Therefore, I tested the activity of the gp42 in T4D-infected cells, as compared to the induced activity in the His42 cells. I decided to infect as early as 3 min after induction with the mutant phage. After infection of the bacteria with the mutant phage-strain T4am(N55), the sonicated protein extract was passed over the column. No additional proteins were detected on a SDS gel in comparison to the uninfected cells. Most of the proteins of the dNTP synthetase complex have a molecular weight around 20-30 kDa, like the dCTPase-dUTPase (21 kDa) and the deoxynucleoside monophosphokinase (26 kDa). Therefore, we expected to see some additional bands in the low molecular weight region of the gel. Additionally, an immunoblot with antibodies to deoxynucleoside monophosphokinase and dCTPase-dUTPase did not reveal a signal (data not shown).

Thymidylate synthase can be spectrophotometrically assayed through the dUMP-dependent increase in absorbance at 338 nm, as 5,10-methylenetetrahydrofolate is oxidized to dihydrofolate. When substituting dCTP for dUMP, one can also test for the presence of dCTPase-dUTPase and dCMP

deaminase in the extract (Allen et al., 1980). This kinetic test failed to detect the complex.

Two interpretations for this failure are conceivable: (i) The histidine tag is not accessible, because the complete enzymes are associated with the gp42. (ii) The phages are not viable on the induced bacteria. To test whether the induced bacteria facilitate the proliferation of the T4am42(N55), a plaque assay was performed. As seen in Table III-1 the induced bacterial strain led to a high titer with both the histidine-tagged and the wild-type gp42 produced after induction. Virtually no phages were produced when the bacteria were not induced or did not carry the plasmid. Therefore, the activity provided by the host cell was able to allow proliferation of the phage. This finding establishes that the induced clone can substitute for the hydroxymethylase activity, which the phage cannot code for. It is not possible to distinguish whether the host-encoded enzyme takes part in the dNTP synthetase complex or provides its activity in an uncomplexed form. However, there is no support for the second possibility. When bacteria were infected with phages coding for the full length hydroxymethylase, a functional dNTP synthetase complex was isolated. However, truncation of the hydroxymethylase led to a dissociation of the complex and prevented a purification of the dNTP synthetase complex (Thylén and Mathews, 1989). In addition, the previously described activity assay failed, although the dCMP hydroxymethylase does not take part in it. Therefore, I conclude that the new approach to isolate the complex was unsuccessful because the histidine tag is not accessible. The failure of this experiment does not mean that the approach is wrong. The dCMP hydroxymethylase is a central enzyme in the complex as demonstrated before, and probably located in the core of the complex (Wheeler et al., 1992; Thylén and Mathews, 1989). Therefore, it is essential to attempt the same modification with proteins of the complex that are more likely to be on the outside. A good choice would be the nucleoside diphosphokinase from *E.coli*, one of the bacterial proteins participating in the complex, or the deoxynucleoside monophosphokinase from

T4. The latter has previously been found to be loosely associated to the complex. This would make the isolation of the complex more difficult in terms of stability, but might ensure the proper accessibility of the histidine tag.

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## APPENDIX

### **Purification of dCTPase-dUTPase from T4 from the heat inducible strain M5219 plam\*71**

To perform the experiments outlined in Chapter 1 the T4 dCTPase-dUTPase was purified to homogeneity from a clone provided by Gisela Mosig, Vanderbilt University. The corresponding gene is located downstream of a heat inducible  $\lambda$ -promoter.

#### Purification of dCTPase-dUTPase

*Activity assay*– The enzyme dCTPase-dUTPase is tested with a coupled spectrophotometric assay. The substrate dUTP is hydrolysed by dCTPase-dUTPase to dUMP, which serves as a substrate for thymidylate synthase. The dUMP-dependent increase in absorbance at 338 nm by the oxidation of 5,10-methylenetetrahydrofolate to dihydrofolate is therefore dependent on the activity of dCTPase-dUTPase.

1. A 20 ml culture of SLBH medium containing 0.05 mg/ml ampicillin is inoculated with a single colony from a plate. The culture is grown at room temperature with gentle bubbling through an air bubbling device. The temperature needs to remain below 25°C, because the strain is heat sensitive.

2. Three liters of growth medium are inoculated with 7 ml of the overnight stock and grown to an OD=0.5. Two ml of 25 mg/ml ampicillin are added per 1 liter culture. Then the cultures are transferred to a waterbath of 42°C and induced for 30 min. After 30 min of induction, the activity of the dCTPase-dUTPase decreases again. The dCTPase activity of the enzyme is extremely toxic to the cells. The cells are chilled and pelleted at 7,000 x g for 10 min. The pellet is collected, weighed and frozen at -20°C.

3. The pellet is redissolved in (three times mass in g) ml sonication buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM  $\beta$ -mercaptoethanol) and

sonicated on ice 5 times 2 min each on the small sonicator. Thirty sec are sufficient for each sonication with the large Branson sonicator. The lysate is centrifuged at 10,000 x g for 15 min. The supernatant is treated further. 100 ml of the supernatant is saved and tested for activity.(A)

4. The volume of the supernatant is measured and 0.3 volumes of 7% streptomycin sulfate are slowly added to the solution. The precipitate is centrifuged in Corex glas tubes at 13,000 x g for 10 min.(100 ml supernatant = B)

5. The supernatant is saved. The pellet is redissolved in 0.2 M potassium phosphate buffer, pH 6.9, containing 0.002 M  $\beta$ -mercaptoethanol. Use a small glas tube for redissolving the pellet. The solution is centrifuged again (15,000 x g for 15 min) and the supernatant is saved for further purification. ( 100 ml = C) All three samples are tested for increasing specific activity.

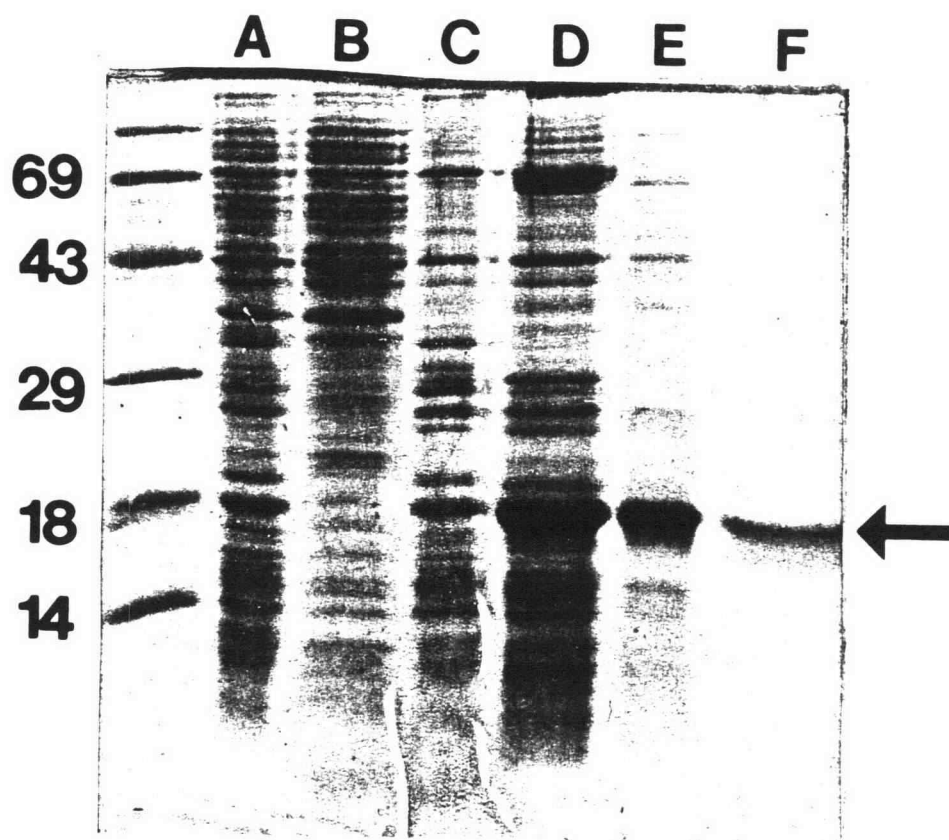
6. The supernatant is applied to a DEAE-cellulose column (anion exchange) which has been equilibrated with 0.2 M potassium phosphate buffer, pH 6.9, 0.002 M  $\beta$ -mercaptoethanol. The column is washed until baseline is achieved on the spectrophotometer. Then a salt gradient from 0 to 5 M NaCl in phosphate buffer is started. The elution profile is monitored on a spectrophotometer. The dCTPase-dUTPase comes off in the middle of the gradient. A SDS-polyacrylamide gel of the fractions is essential to determine the protein containing fractions. The corresponding fractions are concentrated on an Amicon ultrafiltration device to a volume of 4 ml.(D)

7. The Superose 12 column (Pharmacia), a gel exclusion column operated on a FPLC machine, is equilibrated with 0.2 M potassium phosphate buffer, pH 6.9, 0.002 M  $\beta$ -mercaptoethanol, 0.1 M NaCl. Samples of 500  $\mu$ l are applied to the column and 1 ml fractions are collected (flow rate 0.5 ml/min, 30 min time span). The protein comes off in the 14<sup>th</sup> and 15<sup>th</sup> fraction. These fractions are concentrated as before to about 1 mg/ml. The concentrate is tested for purify and activity.(E)

8. The last step is a hydrophobic interaction column. The Phenyl Superose FPLC column (Pharmacia) is equilibrated with a 0.2 M phosphate buffer containing 5 M NaCl. One ml of the sample is diluted in 1 ml of 5 M salt and the total of 2 ml are applied to the column. The proteins are eluted within a linear gradient from high to low salt. The protein elutes at the end of the gradient from the column. The fractions are pooled and concentrated.(F) The pure protein is tested for its activity. A gel is run to demonstrate the purification steps.



Purification of dCTPase-dUTPase of  
T4 from an overproducing E.coli -  
strain



- A sonicated cells
- B Streptomycin sulfate cut : supernatant
- C Streptomycin sulfate cut : redissolved pellet
- D DEAE column fraction (anion-exchange column)
- E Superose 12 (gel-exclusion column)
- F Phenyl sepharose column (hydrophobic-interaction column)

## Native Gel electrophoresis

This protocol is a modification of the published protocol by Echols et al. (1992). The basis of this method is the increase in acidity of a basic protein when it interacts specifically with an acidic protein. Therefore, one can detect the interaction by native gel electrophoresis. Two options are available:

(i) Polyacrylamide gels are used regularly with the denaturant SDS gel to separate proteins according to their mass. The native polyacrylamide gel can be run without a stacking gel. This has the advantage that one can follow the protein from the top to the inside of the gel after visualizing the protein in the immunoblot. The primary disadvantage is the narrow pore size of the gel. Proteins as large as 150 kDa do not or move only slightly into the gel. None of the T4 proteins that belong to the dNTP synthetase complex has a monomeric form. If two of these proteins do interact specifically, the molecular weight of the final multimer is easily over 150 kDa. Because of the relatively weak protein-protein interactions, the protein complex disintegrates through interaction with the lattice of the polyacrylamide .

(ii) In contrast, the agarose gel has larger pores and does not hinder a protein complex of this size. The interaction with the lattice is therefore minimal and interactions are more stable. Normal immunoblotting can be performed as easily as with the polyacrylamide gel. The buffer conditions can be designed to fit the isoelectric point (pI) of proteins of interest.

In sum, the protocol looks as follows:

1. Knowledge of the isoelectric points of the proteins is necessary to design the buffer conditions selected for the experiment. Generally, a protein is considered acidic when it is in the range of  $pI < 5.7$ . Basic proteins should be at least one unit above the pI of the basic protein to see a difference in the movement. Therefore, the buffer conditions are usually selected to insure optimal movement of the acidic protein (in general around pH 7.5).

2. The proteins of interest are mixed in a small volume to facilitate

interaction. A small amount of appropriate buffer and about 2 ml of glycerol are added, too. The proteins are incubated for at least 30 min at room temperature or for longer periods at 4°C. Shaking on the Orbitron rotor might facilitate the interaction.

3. A regular 0.7% agarose gel is prepared in the selected buffer and the same buffer is used for the electrophoresis.

4. The mixed proteins are loaded into the wells of the gel. The glycerol brings the proteins to the bottom of the well.

5. The gel is run for 1.5 h at 100 V. Then, the gel is soaked in running buffer containing 0.04% SDS for 30 min.

6. The gel is Western transferred to nitrocellulose for 1 h at 200 mA.

7. The gel is blocked with 1% gelatin solution and immunoblotting is carried out with antibodies specific to each of the selected proteins. If the interaction has taken place, one finds the single basic protein at the top, but after the interaction with the second acidic protein some distance inside of the gel. Because the migration in the gel is primarily dependent on the charge of the proteins, the position of the complexed protein should correspond to the position of the acidic protein.