AN ABSTRACT OF THE THESIS OF

Kathleen M. McGaughey for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on November 29, 2001.

Title: The Effects of Protein Associations on Pyrimidine Deoxyribonucleotide Biosynthesis.

Abstract approve

Christopher K. Mathews

The faithful replication of DNA depends on the appropriate balance of DNA precursors. From studies conducted in bacteriophage T4, models for deoxyribonucleotide biosynthesis producing pools appropriate for DNA replication have made it possible to understand more complex systems. A portion of that body of evidence supports the concept that deoxyribonucleotide biosynthesis for bacteriophage T4 is carried out by an association of enzymes and other cellular components in a complex called the dNTP synthetase complex. This dissertation explores potential direct protein-protein interactions within this complex for the preparation of pyrimidine deoxyribonucleotides.

Direct associations for enzymes involved in pyrimidine deoxyribonucleotide biosynthesis were examined by affinity chromatography. It was determined that there was a significant direct relationship between T4 thymidylate synthase and T4 dCMP deaminase, between T4 dCTPase/dUTPase and T4 dCMP deaminase as well. The

interaction between thymidylate synthase and dCMP deaminase was significantly influenced by the presence of dCTP, a positive effector of dCMP deaminase.

Furthermore, protein associations changed the kinetic character of pyrimidine deoxyribonucleotide production. T4 dCTPase/dUTPase, a member of the dNTP synthetase complex, significantly alters the kinetic nature of thymidylate synthase by working with thymidylate synthase in a reciprocal relationship. T4 single-stranded DNA binding protein, a member of the replication complex, alters the activity of thymidylate synthase as well. Attempts to isolate a kinetically coupled complex from two or more constituent proteins of the dNTP synthetase complex were frustrated by protein degradation to fragments under 10 kDa in size.

Pyrimidine deoxyribonucleotide synthesis is located between the significant energy investment of ribonucleotide reductase and phosphate attachments by kinases to prepare the deoxyribonucleotide molecules for DNA replication. In bacteriophage T4, intermediate reactions are driven by mass action but are modulated by subtleties including direct protein associations and the presence of small molecules that influence enzyme function. Through these and potentially similar controls, pools of deoxyribonucleotides are prepared and delivered in a timely, balanced manner to the DNA replication apparatus.

The Effects of Protein Associations

on

Pyrimidine Deoxyribonucleotide Biosynthesis

by

Kathleen M. Mc Gaughey

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APPROVED:

Redacted for Privacy

Major Professor, Representing Biochemistry and Biophysics

Redacted for Privacy

Chair of Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State libraries. My signature below authorizes release of my thesis to any reader upon request.

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Kathleen M. McGaughey, Author

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To my friends in Seattle for their endless support, I doubt I could have ever made it without you. Your support has meant so very much in very troubled times.

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DEDICATION

To Sister Rose Marie who taught me I had a mind.

The Effects of Protein Associations on Pyrimidine Deoxyribonucleotide Biosynthesis

CHAPTER ONE

INTRODUCTION

Survival strategies must give an organism advantage in an ever-changing environment. Bacteriophage T4 has accumulated and implemented a number of such strategies to the extent that it is very difficult to discriminate where one strategy ends and another begins, making the organism itself highly adaptable. In no arena is this more evident than the preparation of the template for the next generation. T4 has become a laboratory for investigation to understand how a virus can take over its host and create new progeny.

An Historical Perspective

It is difficult to remember that a number of the advances taken for granted which define how we think about inheritance and DNA did take place during the twentieth century. Many of these landmark investigations involved bacteriophage and are recounted in an account of early phage work by A. H. Doermann (1983) delineating contributions from both genetic and biochemical perspectives. Most of the work with bacteriophage T4 sprang from the one-step growth experiment of Ellis and Delbrück published in 1939, which identified reproducible latent periods for phages. Investigators using this one-step technique were able to grow only one kind of particle at a time and were forced to conclude that only one type of particle could grow in a host cell at a time until the breakthrough work of Hershey in 1946, whose work showed mottled plaques, demonstrating that two forms of T2 (wild-type and *r*-mutant) could grow simultaneously in a host. In the same year, Delbrück and Bailey reported that not only could related viruses live in the same host but they could

exchange information as well (any pair of T2, T4 or T6). In 1947 Luria reported an experiment in which he had exposed phage particles to small doses of radiation rendering them unable to grow, but following multiple infections they were able to produce viable progeny. Doermann himself characterized the processes of multiplication and recombination occur during a vegetative phase entrenching the growth cycle within the earlier identified latent periods. Utilizing the discovery by Anderson that the phage capsid was susceptible to osmotic shock and the development of a method for phage preparation making it possible to take electron micrographs, investigators were able to see that the phage attached to the host cell tail first. Following the "blender experiment" of Hershey and Chase, reported in 1952, it became clear that the capsid was a carrier of material and that material injected into the host was DNA from the phage. By 1953 at a Cold Spring Harbor Symposium organized by Max Delbrück, discussion of the phage life cycle was well under way, including a distantly related presentation by James Watson explaining the model developed by himself and Francis Crick of the double helical nature of DNA (Doermann, 1983).

Although bacteriophages were discovered as early as 1915 by Twort and in 1917 by d'Herelle, their role as biological model systems significantly changed and progress was expedited when early phage investigators were brought together in 1945 by Max Delbrück and asked to restrict their work to phages T1 through T7 (Doermann, 1983). Within this domain, T2, T4 and T6 were found to form a closely related subset to the extent that many of the proteins from individual phages were very similar in function and sequence. The group was referred to as the T-even phages. It was frequently possible to extrapolate the work for one of the T-even phages to another due to this similarity and, in some cases, the members of the subset are

considered interchangeable. There are at least two distinguishable strains of T4: T4B and T4D. All the proteins described in this thesis are from T4D.

The Carrier of Information

The model postulated by Watson and Crick put into sharp focus how it was possible to generate the phenotypes recognized by geneticists from the information transmitted to the host by a phage. By the late 1950's it was generally accepted that the DNA sequence was converted into a protein sequence. Without the well characterized system of Seymour Benzer for *r*IIA and *r*IIB, Crick and his Cambridge colleagues would have had difficulty conducting experiments using frameshift mutants that characterized the genetic code as read from a fixed starting point and later proved the code degenerate. To translate nucleotide triplets into the appropriate amino acid, Khorana's method employed the triplet binding procedure of Leder and Nirenberg, and by the Cold Spring Harbor Symposium of 1966, Khorana and his colleagues had identified each of the triplet codons with the exception of UGA, which was identified as a chain termination codon. Using the gene for T4 lysozyme, whose products could readily be isolated and characterized, Streisinger and his collaborators demonstrated the correctness of the triplet translation along with additional information that synthesis began on the left and that a functional mutant protein could be produced by combining the appropriate frameshifts. Conditional-lethal mutants of T4 were used by Epstein and his colleagues to systematically identify genes essential to replication of the virus. By 1976 Wood and Revel were able to compose a list of 135 genes identified from a DNA molecule composed of 166,000 nucleotide pairs. Since their original identification, the order and relationship of these genes have been refined on the T4 map (Figure I-1). From a practical point of view, Edgar and Wood generated an infectious phage from one that lacked a tail fiber *in vitro* using the conditional-

Figure I - 1. Genomic map of bacteriophage T4. The positions of the T4 genes are shown on the outside circle (Mosig and Eiserling, 1988). The numbering on the inside of this genomic map refers to the distance in kb from the *rII*A - *rII*B junction. The relative position of restriction fragments by *Bgl II* is shown (Yee and Marsh, 1981; Kim and Davidson, 1974). The capital letters (A - H) adjacent to arrowheads refer to the positions of various T4 origins of replication (Lin, 1988). (From the thesis of T.P. Gary [G. Mosig])

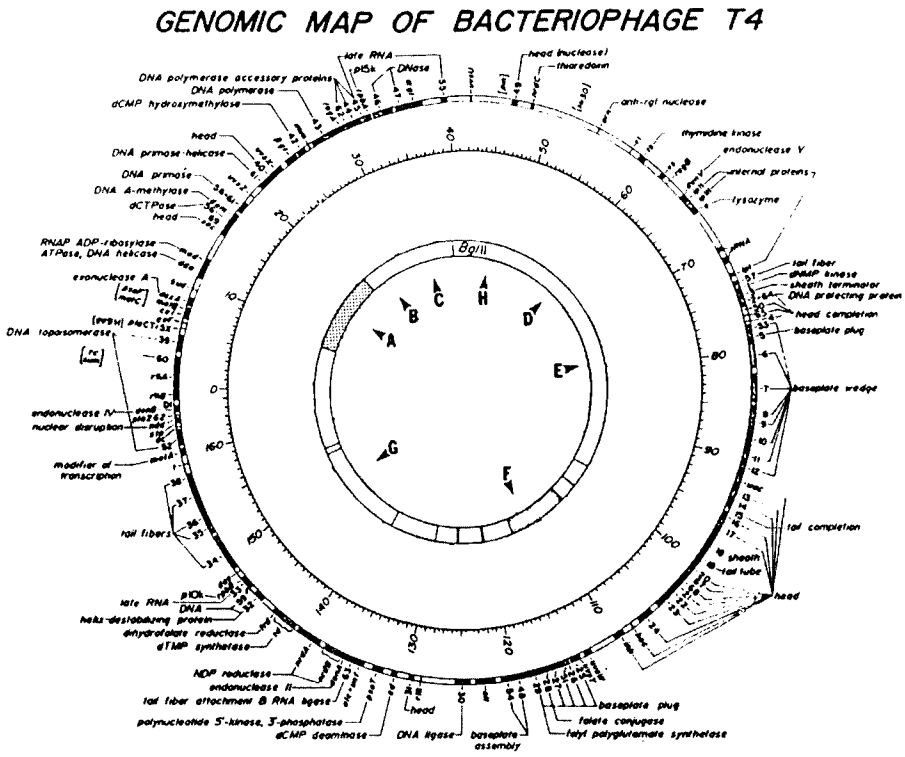

GENOMIC MAP OF BACTERIOPHAGE T4
BglII
A
B
C
H
D
E
F
G
late RNA
p15k
DNase
DNA polymerase accessory proteins
DNA polymerase
dCMP hydroxymethylase
head
DNA primase-helicase
DNA primase
DNA A-methylase
dCTPase
head
RNAP ADP-ribosylase
ATPase, DNA helicase
exonuclease A
DNA topoisomerase
endonuclease IV
nuclear disruption
modifier of transcription
tail fibers
late RNA
gp10k
DNA
helix-destabilizing protein
dihydrofolate reductase
dTMP synthetase
NDP reductase
endonuclease II
tail fiber attachment & RNA ligase
polynucleotide 5'-kinase, 3'-phosphatase
dCMP deaminase
DNA ligase
baseplate assembly
head
baseplate plug
folate conjugase
folyl polyglutamate synthetase
head (nuclease)
thioredoxin
anti-rgl nuclease
thymidine kinase
endonuclease V
internal proteins
lysozyme
tail fiber
dHMP kinase
sheath terminator
DNA protecting protein
head completion
baseplate plug
baseplate wedge
tail completion
sheath
tail tube
head

Figure I – 1.

lethal collection of Epstein. Using these and other experiments, it was possible to deduce the sequence of assembly of T4 particles.

Thanks to early organization of the work and an almost autonomous nature for T4, many discoveries we now take for granted about the role of genetic material and proteins were orchestrated in bacteriophages. Included in that list of discoveries are the role of mRNA, the nature of the genetic code itself, the process of replication, gene control and the diverse field of recombination.

Some Special Aspects of Bacteriophage T4 DNA

For a small organism, T4 contains some elements that are usually found in higher organisms. Many viruses use the deoxynucleotides available in the host but bacteriophage T4 contains an almost complete set of genes encoding the enzymes responsible for deoxyribonucleotide biosynthesis. Three genes found in bacteriophage T4's deoxyribonucleotide biosynthetic enzymes contain group I introns, which are usually reserved for eukaryotes. These T4 genes are *td*, thymidylate synthase; *nrd B*, the small subunit of aerobic ribonucleotide reductase; and *nrd D*, the anaerobic ribonucleotide reductase (Shub *et al.*, 1988). The role played by these self-splicing introns in the process of deoxynucleotide biosynthesis is still undergoing investigation.

Another unique aspect of bacteriophage T4's DNA definitely provides survival advantage. DNA, generally, has two unique characteristics: it contains the sugar deoxyribose and the pyrimidine base thymidine. Bacteriophage T4 uses the pyrimidine nucleoside 5-hydroxymethyl deoxycytidine as the nucleotide HM-dCMP instead of the nucleoside deoxycytidine as the nucleotide dCMP. Furthermore, the 5-hydroxymethyl deoxycytidine is glucose-protected in the DNA chain after

incorporation so some restriction enzymes do not attack the T4 DNA. Both HM-dCMP and dTMP are synthesized at the nucleotide level by bacteriophage T4, using reactions which transfer a single carbon unit to position 5 on the pyrimidine ring, releasing a hydrogen from this position rather than modification after incorporation into T4 DNA. Beginning with the discovery of deoxycytidylate hydroxymethylase responsible for the synthesis of 5-hydroxymethyl deoxycytidine monophosphate, described in 1957 by Flaks and Cohen, all the enzymes known to be involved in DNA precursor biosynthesis had been described by 1970 (Mathews and Allen, 1983). Even though thymidylate synthase and deoxycytidylate hydroxymethylase are synthesized early following infection of the host by phage T4, these enzymes do not begin synthesis until replication begins to take place, suggesting that other elements needed to be assembled before they could carry out their enzymatic function (Mathews, 1985). The unique nature of both pyrimidine replication precursors from T4 made it possible to systematically follow their production and suggested a coordinated approach for deoxynucleotide biosynthesis.

Enzymes of Deoxyribonucleotide Biosynthesis

Initially, the enzymes of deoxynucleotide biosynthesis were thought to exist as independent enzymes. Traditionally, intermediates between enzymes were thought to diffuse away from the producing enzyme into the surroundings from which they were rapidly reclaimed by another independent enzyme. Thymidylate synthase and the other enzymes in deoxynucleotide biosynthesis are soluble and found in the cytoplasm. Previous enzyme aggregates that exhibited isolated complexes were found to be insoluble (Agius and Sherratt, editors, 1997). Studies utilizing gentler methods for enzyme isolation have shown that many enzymes assumed to be in

solution independently are involved in more complex organizations of partially associated enzymes and other cell components (Mathews and Sinha, 1982; Chiu *et al.*, 1982; Mathews and Allen, 1983; Mathews, 1985; Moen *et al.*, 1988). In these systems, the intermediates are thought to be channeled between enzymes. In addition to catalyzing sequential reactions to each other including thymidylate synthase, it has been established that the enzymes in deoxyribonucleoside triphosphate synthesis are physically associated with one another as part of the deoxyribonucleoside triphosphate (dNTP) synthetase complex (Mathews *et al.*, 1979, McGaughey *et al.*, 1996, Wheeler *et al.*, 1996). The dNTP synthetase complex is thought to allow substrate channeling to enhance efficiency and control of dNTP synthesis and it has been suggested that disruption of this complex could be used as an effective drug strategy (Song *et al.*, 1999). The concept of channeling has arisen largely from observations of behavior that cannot be explained by independent enzymes, including the reduction of transient times from those of single enzymes. Moen *et al.* (1988), from the Mathews laboratory using a complex isolated by gel filtration and containing a number of enzymes known to participate in pyrimidine synthesis could make dTMP with a lower concentration of substrate than the K_m would suggest. Despite its importance, only suggestions from single enzyme structures are available for how the proteins are associated in the dNTP complex.

Much of the T4 replication process supports the concept that the involved enzymes are linked in the dNTP synthetase complex(Mathews and Sinha, 1982; Mathews and Allen, 1983; Mathews 1985; Moen *et al.*, 1988). One aspect is time required for replication. For T4, the DNA chain grows at about 850 nucleotides per second at 37°, which is faster than conventional enzyme function would support. Two independent groups using a partially purified dNTP multi-enzyme complex have

demonstrated multi-step reaction pathways in nucleotide precursor production (Chiu *et al.*, 1982; Moen *et al.*, 1988). Additional evidence has been obtained from cell-free enzyme aggregates (Reddy *et al.*, 1977; Reddy and Mathews, 1978; Allen *et al.*, 1983; and Chiu *et al.*, 1982) and direct studies (Chiu *et al.*, 1982; Allen *et al.*, 1983). The initiation of T4 replication is determined by the production of active ribonucleotide reductase as a member of the dNTP synthetase complex (Greenberg and Hilfinger, 1996). Thus, significant data have accumulated to support the existence of the dNTP synthetase complex which exhibits some of the characteristics associated with channeling.

The Deoxyribonucleoside Triphosphate (dNTP) Synthetase Complex

The isolated complex has a molecular weight of about 1300 kDa established by gel filtration and consists of at least eight phage enzymes and two *E. coli* host enzymes. The identified enzymes of the complex include aerobic ribonucleotide reductase (rnr) [composed of subunits R1 and R2 produced by T4 genes *nrdA* and *nrdB*], thymidylate synthase (ts) [T4 gene *td*], deoxycytidine 5'-monophosphate (dCMP) deaminase [T4 gene *cd*], deoxycytidine 5'-triphosphatase (dCTPase)/deoxyuridine 5'-triphosphatase (dUTPase) [T4 gene 56], deoxycytidine 5'-monophosphate (dCMP) hydroxymethylase [T4 gene 42], dihyrofolate reductase (frd) [T4 gene *frd*], deoxyguanosine 5'-monophosphate (dGMP)/deoxythymidine 5'-monophosphate (dTMP)/5-hydroxymethyl deoxycytidine 5'-monophosphate (HM-dCMP) kinase [T4 gene 1], deoxyadenosine 5'-monophosphate (dAMP) kinase (*E. coli*) and nucleoside diphosphokinase (ndk) (*E. coli*) (Greenberg and Hilfinger, 1996). The work of Moen *et al.* also includes RegA protein (translational repressor)

Figure I-2. A cartoon representation of the precursor synthesis of deoxynucleotides for bacteriophage T4. The heavy lines represent significant energy investment. The lighter lines represent portions of the synthesis driven by mass action rather than direct energy investment. The enzymes are labeled in italics and the substrate/products are in normal font. Two kinase enzymes are provided by the host (adenylate kinase and NDK) while the remainder are native to bacteriophage T4.

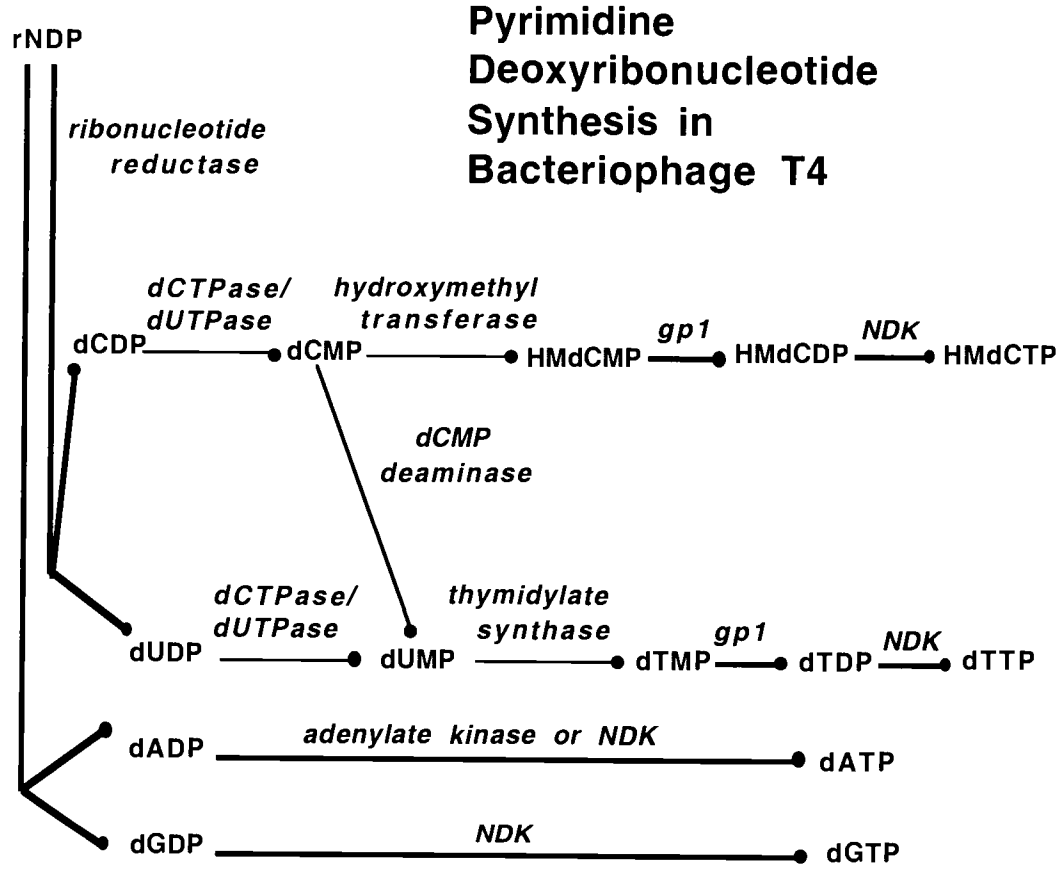

Pyrimidine
Deoxyribonucleotide
Synthesis in
Bacteriophage T4
rNDP
ribonucleotide
reductase
dCTPase/
dUTPase
hydroxymethyl
transferase
gp1
NDK
dCDP
dCMP
HMdCMP
HMdCDP
HMdCTP
dCMP
deaminase
dCTPase/
dUTPase
thymidylate
synthase
gp1
NDK
dUDP
dUMP
dTMP
dTDP
dTTP
dADP
adenylate kinase or NDK
dATP
dGDP
NDK
dGTP

Figure I – 2.

in this group. The overall catalytic relationship between these enzymes is outline in Figure I- 2. Significant energy is collected to produce the radical responsible for the removal of oxygen by ribonucleotide reductase. Energy is invested directly to produce the dNTPs needed for replication from mono- and di- phosphates. The reactions between these two stages apparently carry out their function by mass action.

Specifically, this thesis examines the interaction of some proteins that are involved in the early pyrimidine synthesis portion of dNTP synthetase complex for bacteriophage T4. Bacteriophage T4 is a unique vehicle in which to study the production of pyrimidine nucleotides. Like most other organisms it uses deoxythymidine but it also uses hydroxymethyl deoxycytidine, unique to T-even phages. Previous work established that hydroxymethyl deoxycytidine is synthesized prior to DNA synthesis rather than modification taking place after the nucleotide is incorporated into the DNA strands. Following hydroxymethyl dCMP incorporation, the strand of T4 DNA is further protected by glycosylation from restriction endonucleases causing degradation of unprotected DNA. Hydroxymethyl deoxycytidine is not the only fate for deoxycytidine. A portion of the deoxycytidine is also deaminated to produce the precursor for thymidylate synthase by dCMP deaminase. The relationship between these and other proteins works to maintain the ratio of the two pyrimidine precursors. The focus of this thesis is to characterize the relationship between thymidylate synthase, dCMP deaminase and dCTPase/dUTPase (gp 56), the phosphatase that prepares substrates for the other enzymes in the pyrimidine deoxynucleotide pathway.

Recognizing that the DNA precursors might be delivered to the replication apparatus directly rather than by diffusion, a connection has been sought between deoxyribonucleotide synthesis and replication. Evidence from a number of sources

suggests that T4 single-stranded DNA binding protein (gp 32) is a plausible candidate for the connection of deoxyribonucleotide synthesis and replication and is included in this study. Previous work from the Mathews' lab indicates that a connection exists between the synthesis of nucleotides and their efficient delivery to the replication apparatus. Furthermore, it has been demonstrated that if the pools of nucleotides are not in the correct balance, then mutations take place at a higher frequency (Mathews, 1985). The ratio of synthesis of dTMP to HMdCMP is regulated by precursor levels which reflect the levels needed for DNA production (Greenberg and Hilfinger, 1996). Most literature describes the production of deoxynucleotides and suggests that burgeoning pools await the initiation of replication. In T4 infection, the initial exponential growth in DNA replication is controlled by the formation of the dNTPs (Greenberg and Hilfinger, 1996). Since there is evidence of coordination between the systems, the influence of gp 32 is explored in this study.

Below is a brief description of each of the proteins in the pathway.

Thymidylate synthase (gp td) (ts) [T4 gene *td*] E.C. 2.1.1.45

Little available dUTP is found in the cell to prevent incorporation of dUMP into DNA. The substrate for thymidylate synthase, dUMP, is also found at low cellular concentrations and thymidylate synthase is inhibited by its product dTMP (Greenberg and Hilfinger, 1996). The overall reaction for thymidylate synthase is described in Figure I-3. During the reaction, a single carbon unit is transferred from the cofactor polyglutamated methylene tetrahydrofolate to position 5 of the pyrimidine ring of dUMP to produce dTMP and dihydrofolate. Methylene tetrahydrofolate is regenerated by dihydrofolate reductase and probably serine hydroxymethyl transferase found in *E. coli*. The ts monomeric protein unit has a

mass of 33 kDa but is not capable of enzyme function. An obligate homodimer must be formed creating an active site with contributions from each monomer making the enzyme mass about 67 kDa. The gene for this enzyme is found at about 140 on the bacteriophage T4 map, a region populated by other enzymes associated with dNTP synthesis found in Figure I-1. Following T4 infection, the ratio of intracellular flux rates for dTMP and HMdCMP synthesis is about 2.1:1, which reflects the ratio of the two pyrimidine deoxynucleotides in T4 DNA (Flanegan and Greenberg 1977; Greenberg and Hilfinger, 1996). Thymidylate synthase, because of its unique role in DNA synthesis and as a drug target, is very well studied. It is a protein that is highly conserved in all known organisms.

Deoxycytidine 5'-monophosphate (dCMP) deaminase [T4 gene *cd*] E.C. 3.3.4.12

The major role for this enzyme is demonstrated when T4 is infected by a mutant in the *cd* gene. The ratio of dTMP to HMdCMP synthesis *in vivo* drops to 0.6:1 (Greenberg and Hilfinger, 1996). This enzyme catalyzes the hydrolytic deamination of the amino group from position 4 of the pyrimidine ring of dCMP, producing ammonia and dUMP, the substrate for thymidylate synthase. The monomeric unit has a mass of 21 kDa but for enzymatic function to take place a homohexamer must be formed making the enzyme mass approximately 120 kDa. This enzyme is activated by HM-dCTP and dCTP (HM-dCTP is 80 times more effective than dCTP) and negatively regulated by dTTP (Fleming and Bessman, 1967; Maley *et al.*, 1967; Maley and Maley, 1982). The gene for this protein is found at about 130 on the map in Figure I - 1, not far from the region populated by other

Figure I - 3. The overall reaction of thymidylate synthase.

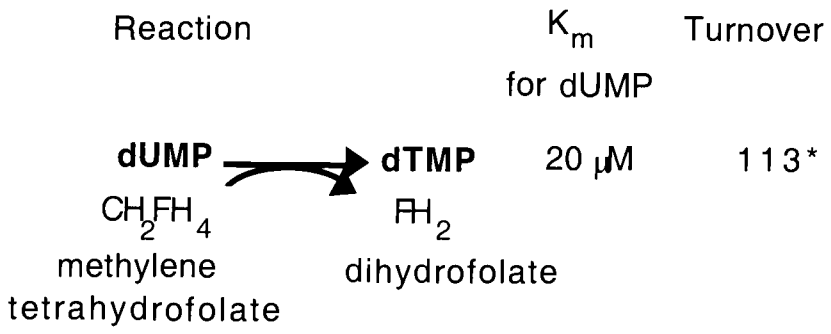

Reaction
K_m for dUMP
Turnover
dUMP
dTMP
20 μM
113*
CH_2FH_4
methylene tetrahydrofolate
FH_2
dihydrofolate
* moles product/moles enzyme/minute at 37 ° and pH7.0. (Capco et al., 1973)

Figure I – 3.

Figure I - 4. The overall reaction of dCMP deaminase.

Reaction	K_m	Turnover
dCMP + H_2O → dUMP + NH_3	100 μM	31,605*

* moles product/moles enzyme/minute at 37 ° and pH 8.0. (Scocca, et al., 1969)

Figure I – 4.

enzymes associated with dNTP synthesis. The *E. coli* host does not have a dCMP deaminase but does possess a dCTP deaminase which yields dUTP. Each mole of monomer dCMP deaminase contains two moles of zinc ions (Moore *et al.*, 1993a). One of the zinc ions is known to be involved in catalytic activity. The role of the second zinc ion was not known at the initiation of these studies. Mutant forms of dCMP deaminase are included in these studies to elucidate the function of the second zinc ion. Figure I - 4 provides an overview of this enzyme's function.

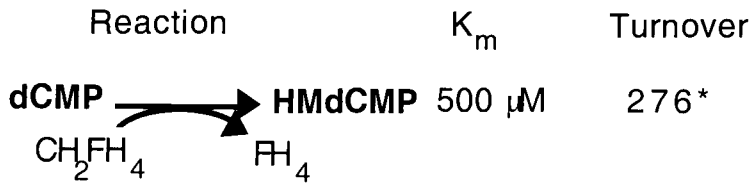
Deoxycytidine 5'-monophosphate (dCMP) hydroxymethylase [T4 gene 42] (gp 42) E.C. 2.1.2.b

This enzyme, although not part of the direct study of this thesis is an integral part of the relationship. It hydroxymethylates dCMP at position 5 of the pyrimidine ring using the cofactor polyglutamated methylene tetrahydrofolate in a process similar to thymidylate synthase but different in that the one-carbon functional group does not undergo reduction. Water present in the active site is responsible for the hydroxyl conversion. The monomeric unit has a mass of 28 kDa but for enzymatic function to take place an obligate homodimer must be formed to yield the active site, making the enzyme mass 56 kDa. The gene for this enzyme is found at about 25 on the map in Figure I - 1 in the region populated by other enzymes associated with some of the unique qualities of T4 replication. Figure I - 5 describes this enzyme.

Deoxycytidine 5'-triphosphatase (dCTPase)/deoxyuridine 5'-triphosphatase (dUTPase) [T4 gene 56] (gp 56) E.C. 3.6.1.12

This enzyme removes a phosphate from dUDP or dCDP and pyrophosphate from dUTP or dCTP. The monomeric unit has a mass of 18 kDa but a homotrimer

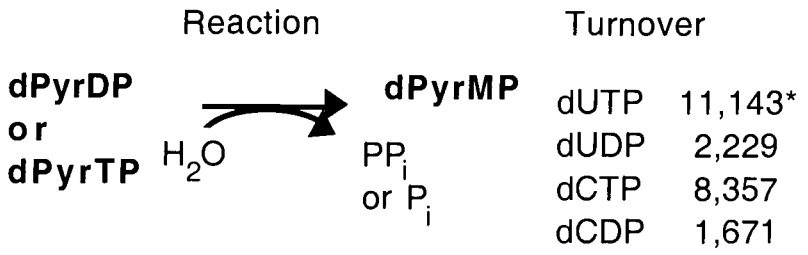
Figure I - 5. The overall reaction of dCMP hydroxymethylase.



* moles product/moles relative enzyme/minute at 37 ° and pH7.0. (North and Mathews, 1977)

Figure I – 5.

Figure I - 6. The overall reaction of dCTPase/dUTPase.



* moles product/moles relative enzyme/minute at 37 ° and pH8.4. (Price and Warner, 1969)

Figure I – 6.

must be formed for enzyme function making the enzyme mass about 55 kDa. The gene for this enzyme is found in the region of the map populated by other enzymes associated with the unique qualities of T4 replication. Initially, the dCTPase activity of the enzyme was detected. This enzyme was responsible for excluding cytosine from incorporation into DNA and providing a precursor for dCMP hydroxymethylase and dCMP deaminase. The dUTPase separately identified enzyme function excludes uracil from incorporation into DNA and provides a precursor for thymidylate synthase. It was determined that the same enzyme possessed both dCTPase and dUTPase functions. Figure I - 6 describes this enzyme.

The following four enzymes are known to be part of the dNTP synthetase complex but are not directly studied in this thesis.

Dihydrofolate Reductase (frd) [T4 gene *frd*] E.C. 1.5.1.3

The gene for dihydrofolate reductase is found at about 142 adjacent to thymidylate synthase on the bacteriophage T4 map in Figure I - 1. This region of the map is populated by other enzymes associated with dNTP synthesis. As described previously, this enzyme uses dihydrofolate to regenerate methylene tetrahydrofolate needed by thymidylate synthase. It has been examined as a drug target as well. Figure I - 7 outlines the enzyme's function.

Ribonucleotide Reductase (rnr) [composed of subunits R1 and R2 produced by T4 genes *nrdA* and *nrdB*] E.C. 1.17.4.

The genes for ribonucleotide reductase are found at about 135 adjacent to thymidylate synthase on the bacteriophage T4 map in Figure 1-3. This region of the map is populated by other enzymes associated with dNTP synthesis. This enzyme is

Figure I - 7. The overall reaction of dihydrofolate reductase.

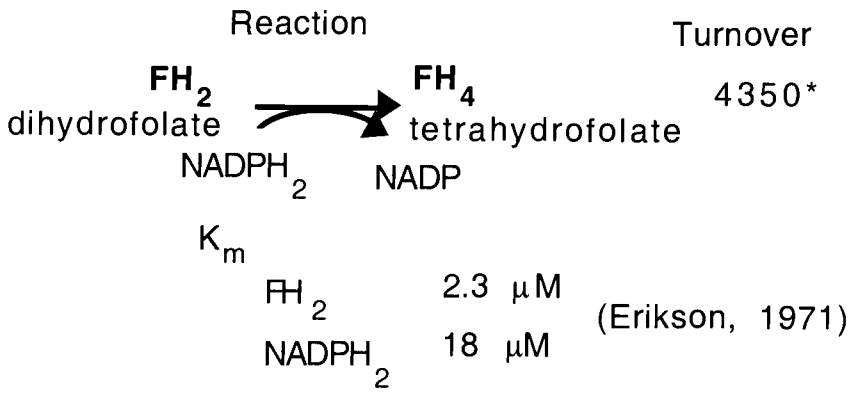

Reaction
Turnover
FH2
dihydrofolate
FH4
tetrahydrofolate
4350*
NADPH2
NADP
Km
FH2
2.3 μM
NADPH2
18 μM
(Erikson, 1971)
* moles product/moles enzyme/minute at
37 ° and pH 7.0. (Erikson and Mathews, 1971)

Figure I – 7.

Figure I - 8. The overall reaction of ribonucleotide reductase.

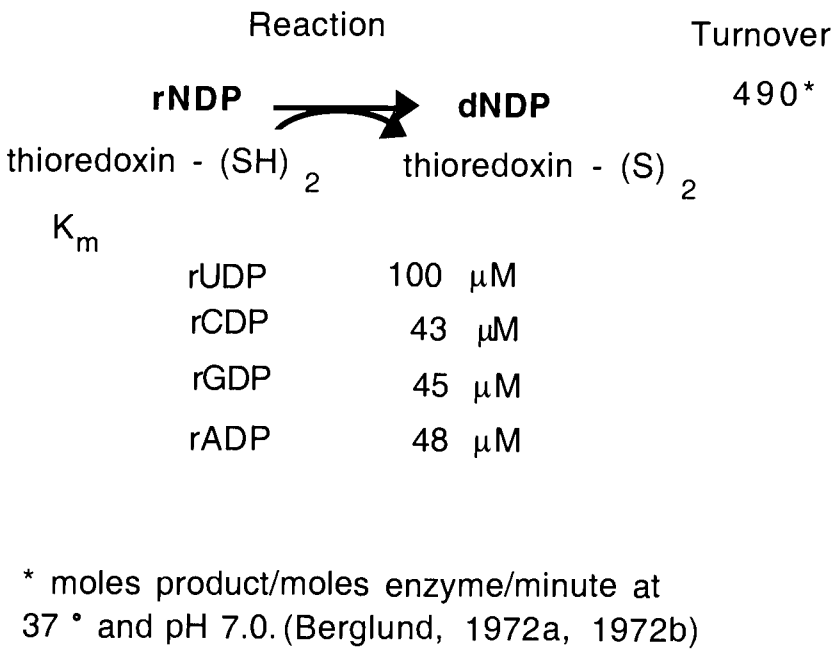

Reaction
Turnover
rNDP
dNDP
490*
thioredoxin - (SH) 2
thioredoxin - (S) 2
Km
rUDP 100 μM
rCDP 43 μM
rGDP 45 μM
rADP 48 μM
* moles product/moles enzyme/minute at
37 ° and pH 7.0. (Berglund, 1972a, 1972b)

Figure I – 8.

Figure I - 9. The overall reaction of gp 1.

Reaction

	K_m		Relative Rate
dTMP	300	μM	1.0
HMdCMP	56	μM	0.5
dGMP	85	μM	1.0

(Bello and Bessman, 1963)

Figure I – 9.

critical in the initiation of deoxynucleotide production. Figure I - 8 outlines the enzyme's function.

Deoxyguanosine 5'-Monophosphate (dGMP)/Deoxythymidine 5'-Monophosphate(dTMP)/5-Hydroxymethyl Deoxycytidine 5'-Monophosphate(HMdCMP) kinase (gp 1) [T4 gene 1] E.C. 2.7.4

The gene for dNMP kinase is found at about 75 on the bacteriophage T4 map in Figure I - 1. This region of the map is also populated by thymidine kinase. Figure I - 9 describes the enzyme's function and its relative specificity for its substrates.

Nucleoside Diphosphokinase (ndk) (*E. coli*)

This enzyme is provided by the host. It is capable of working on a variety of substrates. Since it is provided by the host, it is not included on the T4 map.

Single stranded DNA binding protein (gp 32)

This protein binds to single-stranded DNA to keep it from reforming duplex. It exists and operates as a monomeric unit of approximately 35 kDa but demonstrates significant cooperativity in a collective sense on a single strand of DNA. This protein is not part of the dNTP synthetase complex but is included in this introduction because evidence presented in the later portion of this chapter suggests that it might be the link that exists between deoxyribonucleotide production and DNA replication. The gene for this protein is found on the map in Figure I - 1 at about 145 in the region of the map populated by enzymes that are involved in dNTP synthesis.

Figure I - 10. The overall reaction of dNDP kinase.

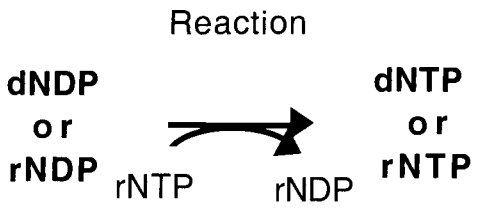


Figure I – 10.

Protein-Protein Interactions

Thus far in the discussion, proteins that have been identified as components of the dNTP synthetase complex have been described and their role outlined. That list is known to be short and incomplete. How these proteins relate to one another has not been described. One of the first steps in looking for linkage between potential components of a complex is to determine if protein-protein interactions are taking place. A number of methods are available to determine the nature of heterologous protein-protein interactions. Most of these techniques employ an avenue of investigation that dwell on the special nature of protein-protein interaction signifying that the proteins involved in the interaction have changed in some way *e.g.* the change in absorbance for a tyrosine reflecting a perturbation in its immediate environment. Paul Srere dedicated an issue of *Methods* (October, 1999) to exploring some of the more productive practices that support the concept that most proteins exist and function in bound form. Many of the methods have inherent limitations so it is common practice to employ more than one technique to substantiate that the site is involved in an interaction and increase the probability that the interaction may be characterized in its uncompromised form. Furthermore, some of the techniques are conducted under conditions that do not exist *in situ,* making it possible to question whether the interaction occurs under cellular conditions at all. It is also possible not to be able to detect an interaction that actually exists using these methods, due to lack of sensitivity, accessibility to the interaction or other similar constraints.

Chromatographic and electrophoretic methods have been used in the Mathews' lab to successfully elucidate protein-protein interactions in the dNTP synthetase complex. One of the most successful has been the use of affinity columns and two-dimensional gel electrophoresis (Formosa et al., 1983) to determine which

proteins associate with one another. The technique, described in more detail in the next chapter, involves binding a purified protein of interest as an affinity column and then passing an extract of radiolabeled proteins from the organism of interest over the column. Adventitious binding is reduced by washing the column in buffer. Increasing sodium chloride step concentrations are applied to elute the bound proteins. The proteins are precipitated and resuspended in a smaller volume so they can be separated by two-dimensional electrophoresis: the first dimension depends on the isoelectric point of each protein and the second on the mass. By using these different strategies, the possibility of any overlapping of proteins is significantly reduced. Proteins at specific positions on the gel have been identified by using deletion mutants and labeled proteins. The following figures include gels generated in this fashion providing insight into the associations of de *novo* T4 enzymes in the dNTP synthetase complex.

The first protein to be studied in the Mathews' lab using the two-dimensional gel system was dCMP hydroxymethylase (Wheeler *et al.*, 1992)(see Figure I-11). The results of this experiment significantly extended the list of candidates for inclusion in the dNTP synthetase complex. It not only included those proteins already identified as being part of the dNTP synthetase core but also contained a number of proteins known to be part of the replication machinery, including T4 single stranded DNA binding protein. The interactions that were deemed most significant were the proteins eluted at concentrations of NaCl between 0.2 M and 0.6 M as was seen in the work of Formosa with single stranded DNA binding protein but it was not possible to determine which proteins were directly associated with one another. Please note that dCMP deaminase does not appear in

Figure I - 11. Two-dimensional gel electrophoresis of [^{35}S]met-labeled 5 to 8 minutes after infection T4 proteins that bind to T4 dCMP hydroxymethylase (gp 42). The column buffer used 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.025 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound at 0.2 M NaCl but are eluted by 0.6 M NaCl. (Wheeler *et al.*, 1992)

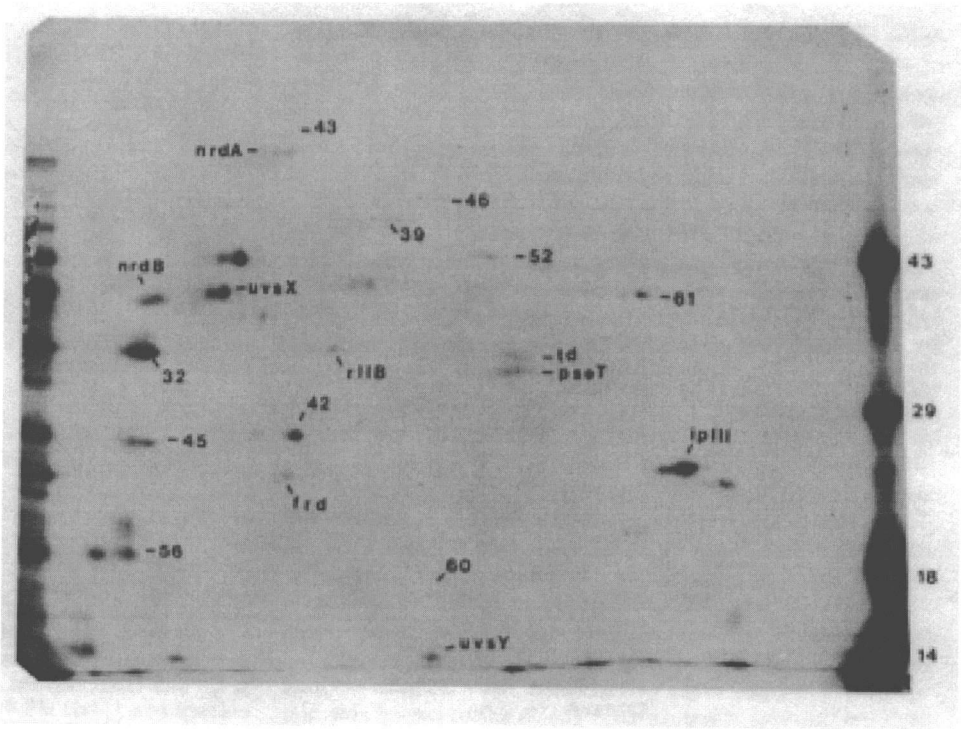

-43
nrdA-
-46
39
-52
nrdB
-uvsX
-61
43
-td
-pseT
32
rIIB
42
29
-45
ipIII
frd
-56
60
18
-uvsY
14

Figure I – 11.

this gel. The work of Thylen and Mathews (1989) provide strong support for the specificity of interactions within the dNTP complex.

Additional proteins thought to be part of the dNTP synthetase core were also bound to respective affinity columns and labeled extracts passed over them. The results for dCTPase/dUTPase (Wheeler *et al.*, 1996)(see Figure I-12) and thymidylate synthase (McGaughey *et al.*, 1996) (see Figure I-13) are included. Like bound hydroxymethylase these gels also showed the presence of proteins from both the dNTP synthetase complex and the replication process. As in the previous examples, dCMP deaminase was not detected on the two-dimensional gels. Since it was believed to be a necessary constituent of the dNTP synthetase complex, purified dCMP deaminase was used to make an affinity column and labeled extract was passed over it. These experiments were conducted over a period of time and the buffer used for each is included with the appropriate figure. For the deaminase investigation a new buffer was utilized to take advantage of the finding that potassium and glutamate were the major ionic constituents of *E. coli* cells (Richey *et al.*, 1987). From comparison with the Tris-HCl buffer, it was determined that the same proteins were eluted but by using the potassium glutamate buffer spots were easier to identify (Wheeler *et al.*, 1996). The proteins eluted by the NaCl step between 0.2 M and 0.6 M are displayed on the gel found in Figure I-14 (McGaughey *et al.*, 1996). This work was also done by Linda Wheeler. As with other proteins acting in the affinity role, a number of proteins were present that were other constituents of the dNTP synthetase complex as well as T4 single stranded DNA binding protein.

In fact, T4 single stranded DNA binding protein was found as part of the 0.6 M collection on two-dimensional gel systems run for eight T4 proteins. Two-dimensional gels have been run for T4 single stranded DNA binding protein showing

it to interact with a number of proteins from replication, recombination and repair as well (Formosa *et al.*, 1983; Krassa *et al.*, 1991; Hurley *et al.*, 1993). T4 single stranded DNA binding protein probably does not bind directly to all of these materials. Since there was evidence that it might bind to thymidylate synthase it was thought to be a good potential candidate for linkage of the dNTP synthetase complex to the replication system (Wheeler *et al.*, 1996).

From these two-dimensional gels there is no direct evidence that indicates which proteins are bound to one another. The goal of this thesis is, by using reaction relationships and proteins that appear together on two-dimensional gels, to select candidates to test for direct physical associations. For those relationships that demonstrate a relation that meets the criterion of 0.6 M NaCl for release from one another, some will be selected for further investigation. Further investigation will include the effect of small molecules on the stability of the relationship as well as kinetic implications.

Figure I - 12. Two-dimensional gel electrophoresis of [^{35}S]met-labeled 5 to 8 minutes after infection T4 proteins that bind to T4 dCTPase/dUTPase (gp 56). The column buffer used 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.025 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound at 0.2 M NaCl but are eluted by 0.6 M NaCl. Each superscript dot identifies a nonspecifically bound protein. Nonspecific is defined as a protein that also binds to immobilized bovine serum albumin. (Wheeler *et al.*, 1996)

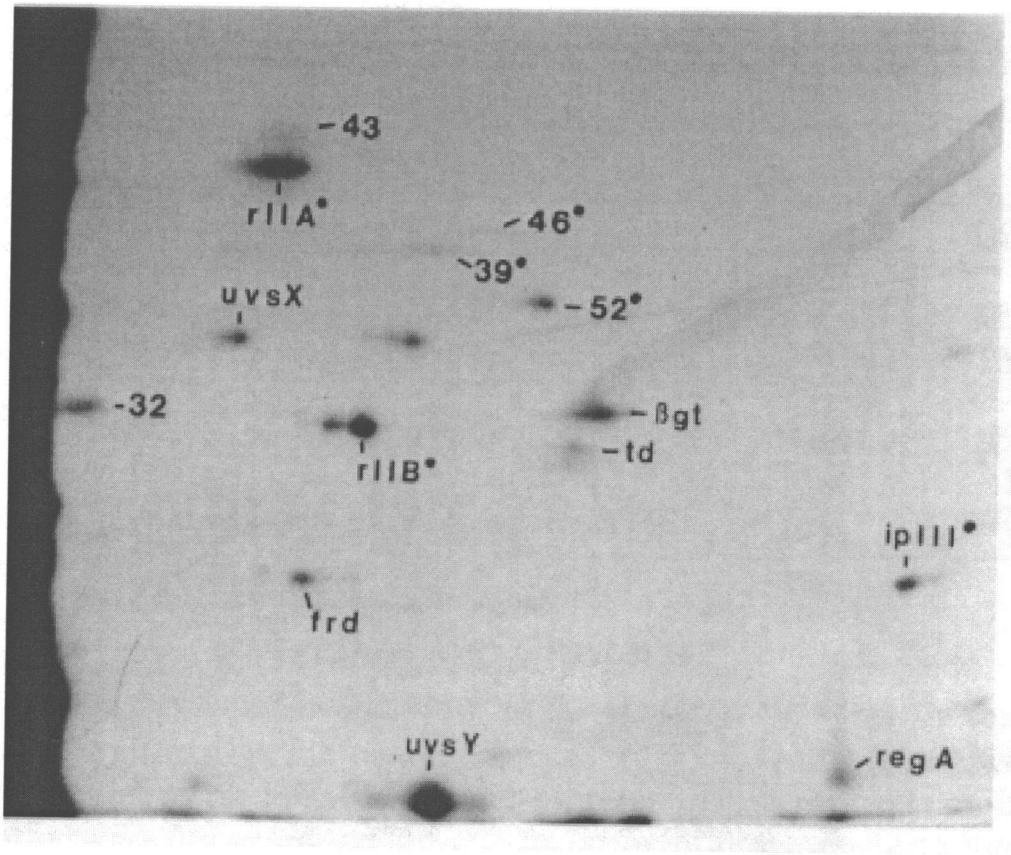


Figure I – 12.

Figure I - 13. Two-dimensional gel electrophoresis of [^{35}S]met-labeled 5 to 8 minutes after infection T4 proteins that bind to T4 thymidylate synthase by Linda Wheeler. The column buffer used 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.025 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound at 0.2 M NaCl but are eluted by 0.6 M NaCl. The numbers to the left identify molecular weights of marker proteins in kDa. (McGaughey *et al.*, 1996)

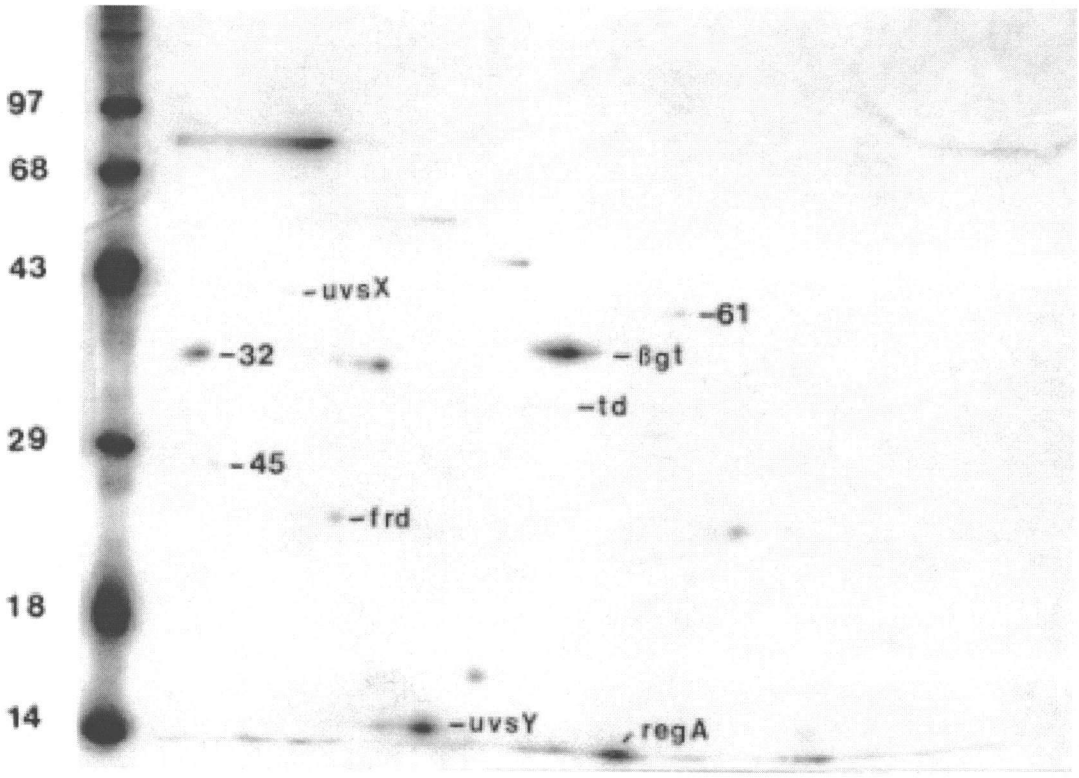


Figure I – 13.

Figure I - 14. Two-dimensional gel electrophoresis of [^{35}S]met-labeled 5 to 8 minutes after infection T4 proteins that bind to T4 dCMP deaminase by Linda Wheeler. The column buffer used 0.1 M potassium glutamate (pH 8.0),1mM β-mercaptoethanol, 0.5 mM magnesium acetate, 0.025 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound at 0.2 M NaCl but are eluted by 0.6 M NaCl. (McGaughey *et al.*, 1996)

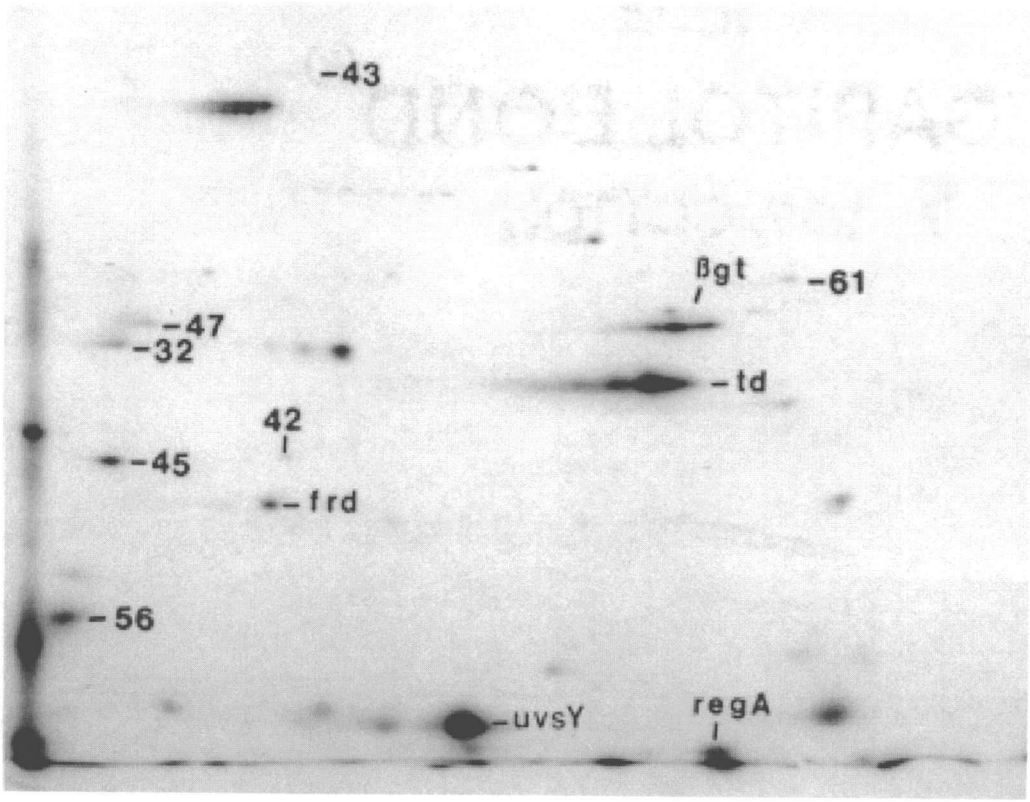


Figure I – 14.

CHAPTER TWO

NEAREST NEIGHBOR ASSOCIATIONS

Introduction

As stated previously, Dr. Mathews' lab has studied the dNTP synthetase complex for a significant period of time in an effort to model the complex. Deoxythymidylate monophosphate, a unique constituent of DNA, can be produced by thymidylate synthase from deoxyuridine monophosphate following the loss of phosphate(s) catalyzed by dCTPase/dUTPase or following the deamination of deoxycytidine monophosphate by dCMP deaminase in bacteriophage T4 (see Figure I - 2). T4 thymidylate synthase produces dTMP, which is further phosphorylated by gp1 (T4) and NDK (*E. coli*) and sent forward for incorporation into DNA. Two enzymes that are functionally closely associated and should be closely physically related as well are T4 deoxycytidylate deaminase (dCMP deaminase) and T4 thymidylate synthase. Even though both T4 thymidylate synthase and T4 dCMP deaminase are known parts of the dNTP synthetase complex, a functional relationship was the only connection established. The work of Moen *et al.* (1988) shows them to be kinetically linked. By looking at Figures I - 13 and I - 11 one sees that thymidylate synthase binds early proteins like dCMP hydroxymethylase but in neither gel were the investigators able to detect deoxycytidylate deaminase. To test the hypothesis that dCMP deaminase bound early proteins, Linda Wheeler prepared two-dimensional gels using dCMP deaminase as the affinity column (Figure I - 14), and detected thymidylate synthase as well as other early and replication proteins. Many proteins that eluted with 0.6 M NaCl for other affinity columns are present on the 0.6 M NaCl release two-dimensional gel of dCMP deaminase. It is probable that some

of these proteins represent direct associations while others are present due to partners (Greenberg and Hilfinger, 1996). The proteins are too numerous for each to be directly associated with dCMP deaminase which is bound on the affinity column. Formosa suggests testing the interaction directly but keeping in mind that the binding between two proteins *in vitro* should be taken as a starting point for study *in vivo* (Formosa et al., 1991; Wittmeyer and Formosa, 1995). To determine if an interaction is one of nearest neighbors, affinity columns were prepared with purified T4 thymidylate synthase, *E. coli* thymidylate synthase and T4 dCMP deaminase. Each column was exposed to the protein of interest and the elution protocol employed for the latest affinity column producing two-dimensional gels with radiolabeled proteins was used. The same elution time schedule and buffer discussed for the affinity column of bound dCMP deaminase with increasing sodium chloride was used for successive elutions of the retained material.

Methods of Investigation

Recombinant T4 thymidylate synthase was purified by Linda Wheeler using the procedure described by Young and Mathews (1992). Recombinant T4 dCMP deaminase was purified in the Maley lab as previously described by Moore *et al.* (1993a). Recombinant *E. coli* thymidylate synthase was also purified by Maley and Maley (1988).

Affinity Column

Affinity columns were prepared for T4 and *E. coli* thymidylate synthase and T4 dCMP deaminase using approximately 4 mg of material for each column. Each affinity column was prepared by coupling a purified single protein to Affi-Gel 10

(Bio-Rad), an N-hydroxysuccinimide ester of a derivative cross-linked agarose gel bead support containing a neutral ten-atom spacer arm. To carry out the coupling, the purified protein was dialyzed into 0.1 M MOPS buffer, pH 7.5, and the protein concentration was determined using the Bradford method. Affi-Gel 10 was activated by three washings with cold, purified water as per the manufacturer's instructions. The activated gel was combined with protein in buffer and rocked in the cold box overnight (Four hours is the minimum time of exposure). On the following day, a sample of the supernatant was removed. The Bradford method was used to determine the amount of protein remaining in solution and, by subtraction, how much had bound to the column. Remaining activated sites on the gel were blocked by mixing the gel with ethanolamine for at least 15 minutes as per manufacturer's instructions. The slurry was poured into a column, packed, washed in buffer and exposed to the most extreme salt concentration used to condition the column and to remove material that was not strongly bound. The high salt buffer was removed rapidly to facilitate bound protein integrity.

Elution Procedure

After the column was returned to neutral condition with copious washing with buffer, the protein of interest under the conditions being investigated was applied and circulated overnight at a low speed in a small volume of buffer to facilitate binding. The circulating solution was removed and the column was washed with column buffer using a series of washes with waiting periods to remove non-specifically bound protein. The bound material was eluted by a series of sodium chloride concentrations dissolved in potassium-glutamate column buffer applied in steps, and fractions were collected according to the following schedule:

- collect material that has been circulating overnight = o;
- wash with 25 mM potassium glutamate buffer, pH 8: 10 mls = ft1;
- stop pump for 30 minutes;
- wash with 25 mM potassium glutamate buffer, pH 8: 10 mls = ft2;
- wash with 25 mM potassium glutamate buffer, pH 8: 10 mls = ft3.

A similar procedure is followed for each salt wash:

- wash with 0.2 M NaCl in 25 mM potassium glutamate buffer, pH 8: 3 mls = a1;
- wash with 0.2 M NaCl in 25 mM potassium glutamate buffer, pH 8: 2 mls = a2;
- stop pump for 30 minutes;
- wash with 0.2 M NaCl in 25 mM potassium glutamate buffer, pH 8: 8 mls = a2;
- wash with 0.2 M NaCl in 25 mM potassium glutamate buffer, pH 8: 5 mls = a3.
- wash with 0.6 M NaCl in 25 mM potassium glutamate buffer, pH 8: 3 mls = b1;
- wash with 0.6 M NaCl in 25 mM potassium glutamate buffer, pH 8: 2 mls = b2;
- stop the pump for 30 minutes;
- wash with 0.6 M NaCl in 25 mM potassium glutamate buffer, pH 8: 8 mls = b2;
- wash with 0.6 M NaCl in 25 mM potassium glutamate buffer, pH 8: 5 mls = b3.
- wash with 2.0 M NaCl in 25 mM potassium glutamate buffer, pH 8:

3 mls = c1;

- wash with 2.0 M NaCl in 25 mM potassium glutamate buffer, pH 8:

2 mls = c2;

-stop the pump for 30 minutes;

- wash with 2.0 M NaCl in 25 mM potassium glutamate buffer, pH 8:

8 mls = c2;

- wash with 2.0 M NaCl in 25 mM potassium glutamate buffer, pH 8:

5 mls = c3.

The proteins for each salt concentration were precipitated and resuspended in a smaller volume using the same procedures that were employed for two-dimensional gel analysis. SDS-PAGE samples were prepared and run for the overnight collection (o), ft1, ft2, ft3, a2 (A), b2(B), and c2(C).

Two-Dimensional Gel Analysis

Affinity columns prepared for *E. coli* and T4 thymidylate synthases were used for comparison purposes. Two-dimensional gel analysis was not available for *E. coli* thymidylate synthase interacting with T4 early proteins, so this was carried out. Extracts of early T4D proteins (3 to 8 minutes), labeled with [^{35}S] methionine were prepared following infection at 37° as described previously (Wheeler *et al.*, 1992). An extract of *Escherichia coli* strain B (wild-type) infected with bacteriophage T4 was prepared by labeling the early proteins with ^{35}S-methionine. The *E. coli* culture was grown to a density of 5 x 10^8 cells per ml at 37° in M9 minimal media and infected with phage at a multiplicity of 10. Five aliquots of 0.1 mCi of ^{35}S-methionine (0.5 mCi total) were added at one minute intervals at 3 to 8 minutes post infection labeling early proteins. At 12 minutes post infection, cold methionine was added to a

concentration of 20 μg/ml to deplete the radio-labeled methionine pool so proteins would no longer be labeled. The cells were chilled on ice and harvested by centrifugation at 5,000 x g for 20 minutes. The supernatant was discarded and the pellet was stored in the –80° freezer. From this pellet, a cell extract was prepared by resuspending the pellet in a volume of cell extraction buffer that was three times the pellet mass and sonicating the resulting suspension. The cell debris was removed by centrifugation and, following the addition of $CaCl_2$ and $MgCl_2$ to a final concentration of 5 mM each, the extract was incubated for 30 minutes with pancreatic DNase and micrococcal nuclease. The supernatant was applied to the affinity column and was circulated overnight at a slow speed so that material in the extract had more than one opportunity to bind to the affinity column.

E. coli Two-Dimensional Gels

After the extract of radiolabeled T4 early proteins was applied to the *E. coli* thymidylate synthase affinity column, it was washed with potassium-glutamate buffer and eluted with increasing NaCl concentration in the same potassium-glutamate column buffer described for the experiments involving dCMP deaminase by Linda Wheeler (McGaughey et al., 1996) using the elution procedure described above for the affinity column. The results are presented in Figure II - 1, II - 2 and II -3. Proteins are identified by work from other investigators involving mutants and labels that have been accumulated (Cowan *et al.*, 1994). Varied approaches have led to an agreed assignment of protein identity by gel location with respect to other proteins. Some proteins that are native to *E. coli* are used by bacteriophage T4 so they would be expected to bind to *E. coli* thymidylate synthase with equal or increased strength. The results demonstrate that most of the T4 proteins were eluted with the 0.2 M NaCl

Figure II - 1. Two-dimensional gel electrophoresis of radiolabeled T4 proteins that bind to *E. coli* thymidylate synthase. The column buffer used 0.1 M potassium glutamate (pH 8.0),1 mM β-mercaptoethanol, 0.5 mM magnesium acetate, 0.025 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound after washing with potassium-glutamate buffer but are eluted by 0.2 M NaCl in the same buffer. The numbers to the left identify molecular weights of marker proteins in kDa.

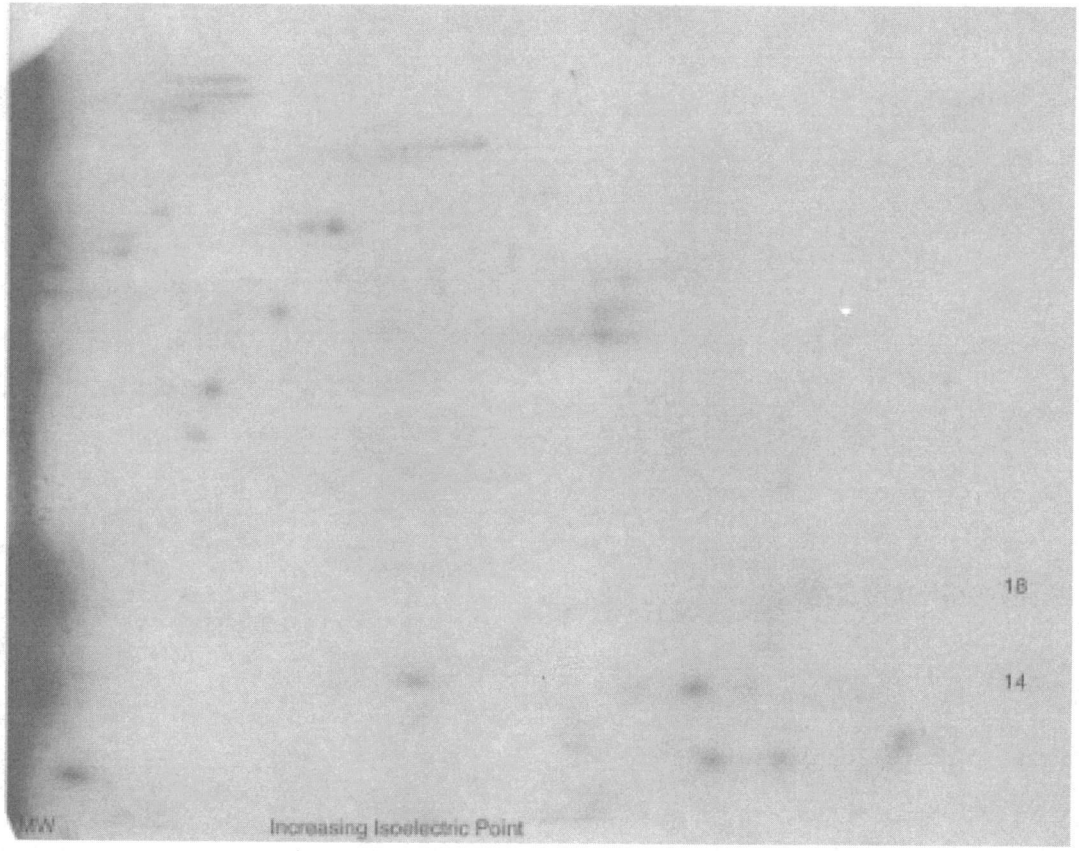


Figure II – 1.

Figure II - 2. Two-dimensional gel electrophoresis of radiolabeled T4 proteins that bind to *E. coli* thymidylate synthase. The column buffer used 0.1 M potassium glutamate (pH 8.0),1 mM β-mercaptoethanol, 0.5 mM magnesium acetate, 0.025 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound at 0.2 M NaCl in potassium glutamate buffer but are eluted by 0.6 M NaCl in potassium-glutamate buffer. The numbers to the left identify molecular weights of marker proteins in kDa.

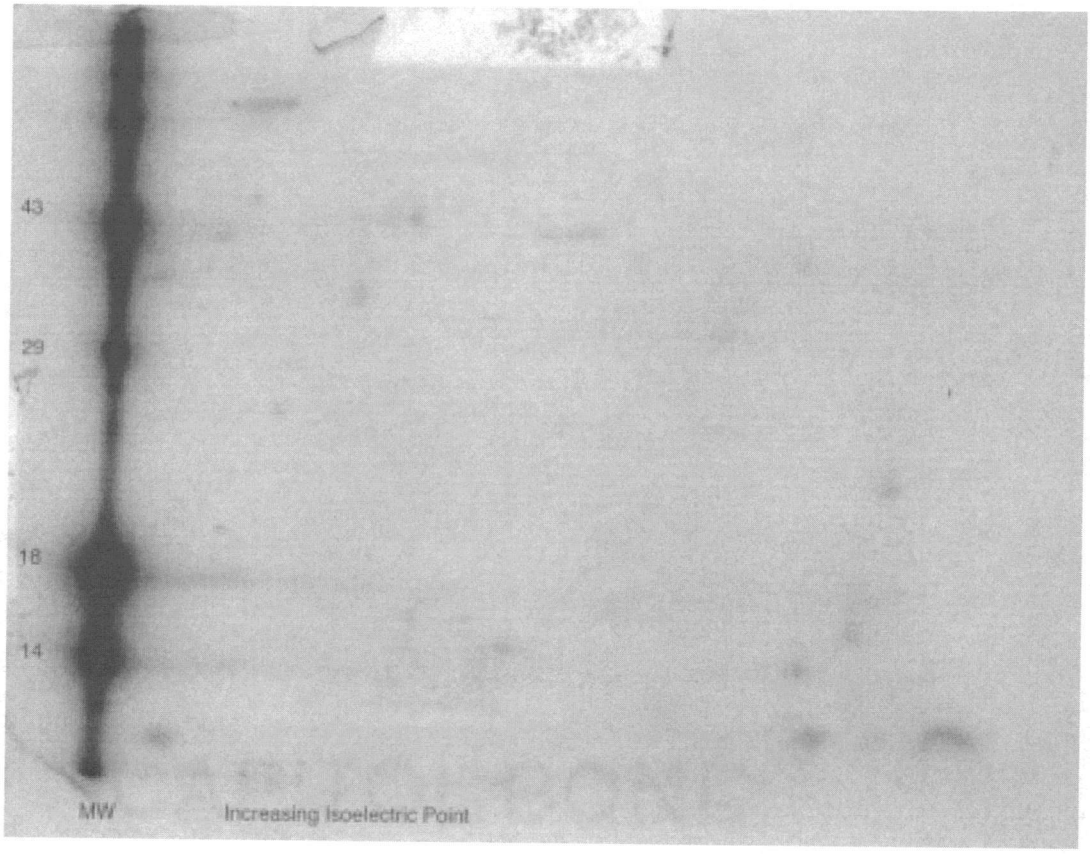


Figure II – 2.

Figure II - 3. Two-dimensional gel electrophoresis of radiolabeled T4 proteins that bind to *E. coli* thymidylate synthase. The column buffer used 0.1 M potassium glutamate (pH 8.0),1 mM β-mercaptoethanol, 0.5 mM magnesium acetate, 0.025 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound at 0.6 M NaCl in potassium-glutamate buffer but are eluted by 2.0 M NaCl in potassium-glutamate buffer. The numbers to the left identify molecular weights of marker proteins in kDa.

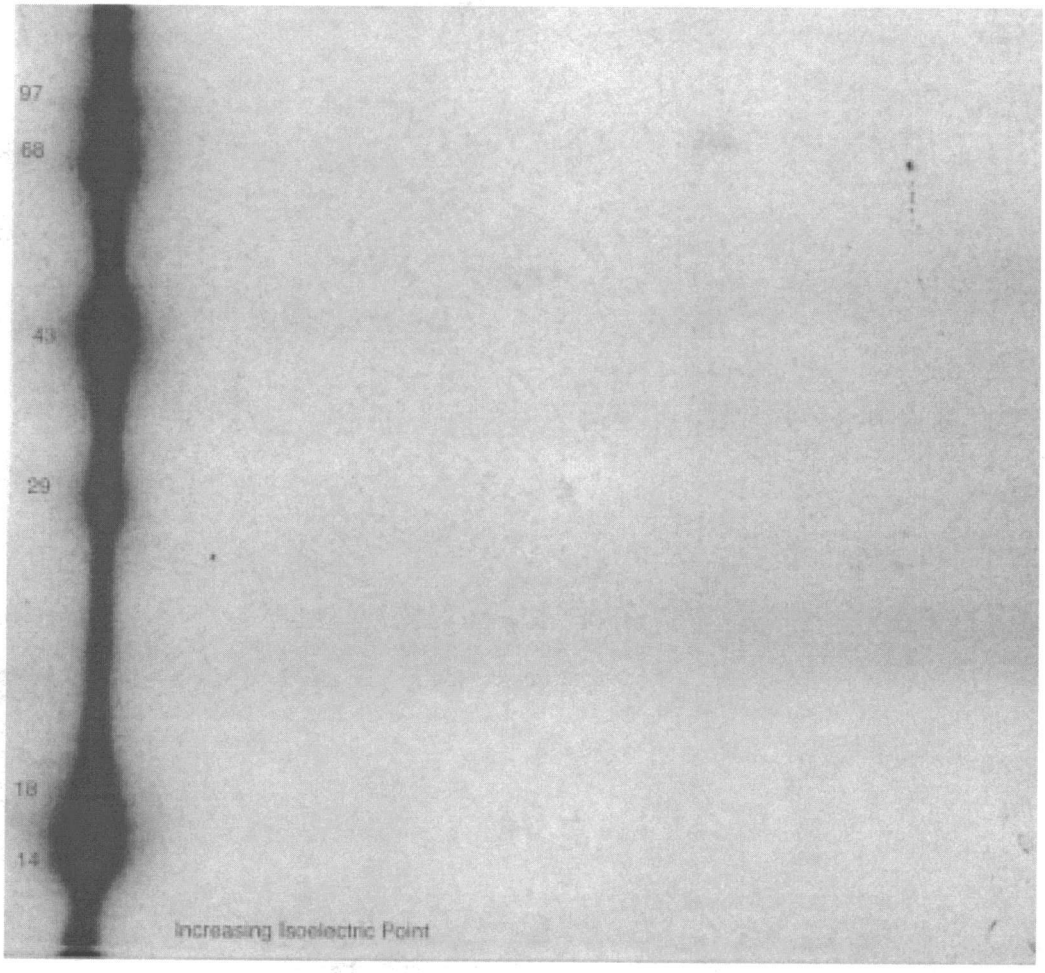


Figure II – 3.

wash. To see if these results were dependent on the experimenter, I repeated the work of Linda Wheeler for T4 thymidylate synthase. The results for those proteins remaining after the 0.2 M NaCl wash but released by the 0.6 M NaCl are comparable to Figure I - 13 and are presented in Figure II - 4. Both the *E. coli* and T4 proteins used the same preparation of tube gels for isoelectric separation. When one examines Figure I - 13 and II - 4, it is clear that a number of protein spots repeat themselves horizontally. This process is referred to as "stuttering" and results from the separating tube gel not being appropriately isoelectrically balanced. Linda Wheeler discovered from experiments with commercially prepared ampholine mixtures that the mixtures prepared in the lab were the source of the stuttering problem. Since both *E. coli* and T4 thymidylate synthase shared the same gel mixture for the first dimension separation, it is still possible to compare the results. Although some T4 proteins bound to the *E. coli* thymidylate synthase affinity column, they did not bind as strongly as those to T4 thymidylate synthase affinity column. Most relevant proteins were released at the 0.2 M NaCl addition for *E. coli* thymidylate synthase column and are of similar composition to those that are released by 0.6 M NaCl wash for T4 thymidylate synthase affinity column. Few T4 proteins remain bound to the *E. coli* thymidylate synthase column at the 0.6 M NaCl release and by the 2.0 M NaCl release no protein was detected on the gel (Figure II - 3). Many of the proteins released by 0.6 M NaCl are considered to be non-specific in their binding. From these observations, interactions with *E. coli* thymidylate synthase were used as a negative control for the experiments with T4 dCMP deaminase.

Figure II - 4. Two-dimensional gel electrophoresis of radiolabeled T4 proteins that bind to T4 thymidylate synthase. The column buffer used 0.1 M potassium glutamate (pH 8.0),1 mM β-mercaptoethanol, 0.5 mM magnesium acetate, 0.025 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound at 0.2 M NaCl in potassium glutamate buffer but are eluted by 0.6 M NaCl in potassium-glutamate buffer. The numbers to the left identify molecular weights of marker proteins in kDa.

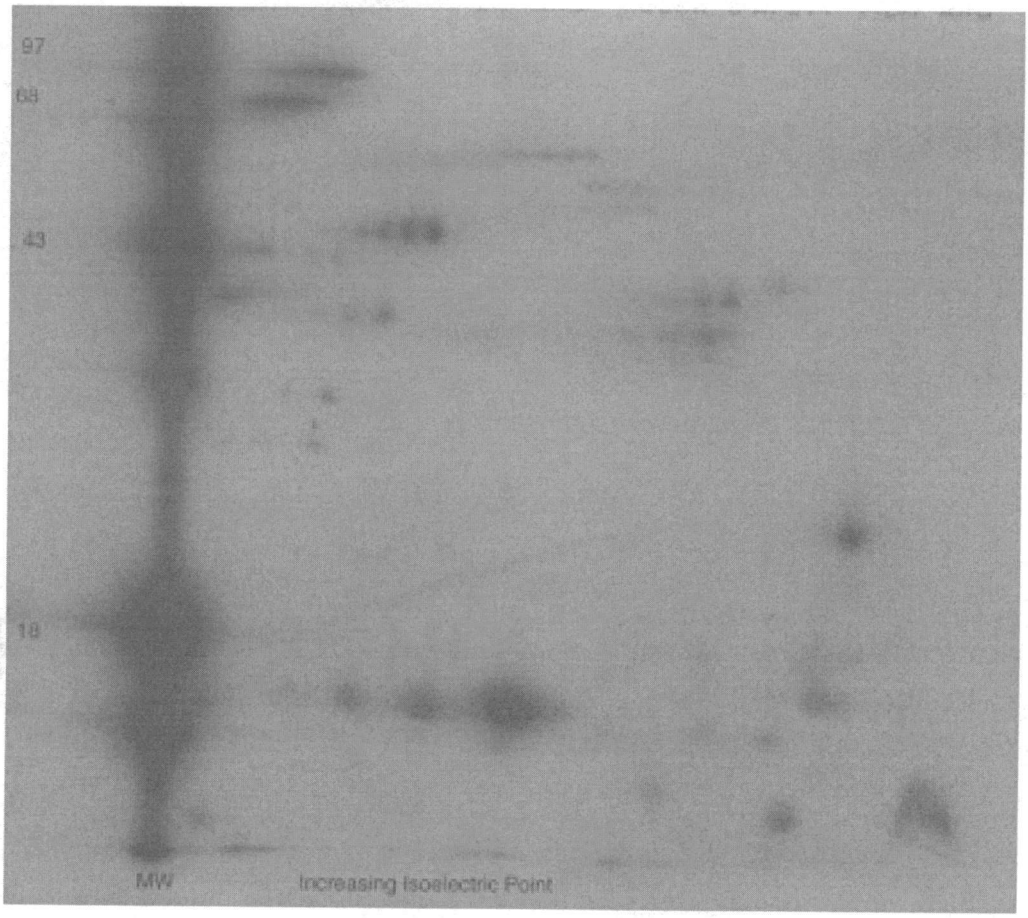


Figure II – 4.

Testing Direct Interactions

Beginning with T4 thymidylate synthase and T4 deoxycytidylate (dCMP) deaminase, pairs of proteins were tested for direct interaction using the affinity protocol. Deoxycytidylate deaminase bound to T4 thymidylate synthase under potassium-glutamate column buffer conditions and was eluted by 0.6 M NaCl wash. The gel in Figure II - 5 demonstrates the significant difference in binding between T4 and *E. coli* thymidylate synthases. It is clear that dCMP deaminase binds to T4 thymidylate synthase and not to *E. coli* thymidylate synthase under the same buffer conditions.

In earlier publications, dCTP had been identified as a positive allosteric effector for both T2 (Maley *et al.*, 1972) and T4 (Moore *et al.*, 1994) dCMP deaminases. To examine the influence of dCTP on the interaction between T4 thymidylate synthase and dCMP deaminase, dCTP was introduced at various times and concentrations thought to potentially influence binding. The gel in Figure II - 6 demonstrates a significant difference in the interaction between the two proteins for the two buffers containing dCTP compared to the buffer without dCTP. When dCTP is present, dCMP deaminase is not as susceptible to breakdown. The presence of the positive allosteric effector increases the durability of the interaction between T4 thymidylate synthase and dCMP deaminase. The interaction was stronger when 200 μM dCTP was applied (Figure II - 7). The timing of the application of the positive effector also played a role in the durability of the interaction. When 200 μM dCTP was added to the column buffer during the initial application of dCMP deaminase, binding was found to have increased significantly between T4 dCMP deaminase and T4 thymidylate synthase. Application of dCTP in lower concentration and at later times was not as effective.

Figure II - 5. Retention of purified T4 dCMP deaminase on an immobilized T4 thymidylate synthase column and an *E. coli* thymidylate synthase column. One mg of T4 dCMP deaminase was applied to each column. No positive effector was introduced. This gel is a 12.5% polyacrylamide gel stained with Coomassie Blue. Depicted in the lanes are marker, the deaminase initially applied to each column, the material collected from 0.8 ionic strength (0.6 M NaCl) elution of T4 thymidylate synthase column and the material collected from 0.8 ionic strength (0.6 M NaCl) elution of E. coli thymidylate synthase. The column buffer used 0.1 M potassium glutamate (pH 8.0),1 mM β-mercaptoethanol, 0.5 mM magnesium acetate, 0.025 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 10% glycerol.

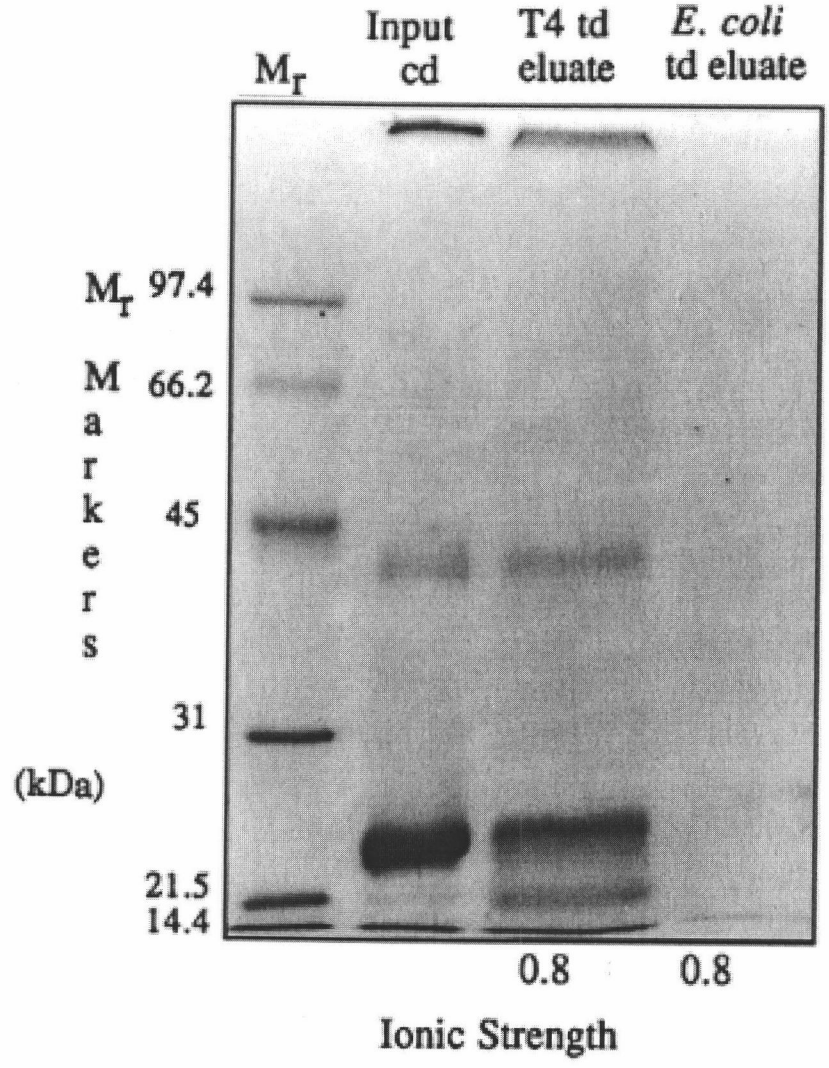

Input cd
T4 td eluate
E. coli td eluate
M_r
M_r Markers (kDa)
97.4
66.2
45
31
21.5
14.4
0.8
0.8
Ionic Strength

Figure II – 5.

Figure II - 6. Retention of purified T4 dCMP deaminase on an immobilized T4 thymidylate synthase column using 200 μM dCTP at different stages of application. This gel is a 12.5% polyacrylamide gel silver stained. Seven tenths of a mg of deaminase was applied to the column for each collection. Without the positive effector, the dCMP deaminase was eluted at 0.8 ionic strength level and exhibited protein degradation. With the positive effector, dCMP deaminase bound more tightly.

0.4 ionic strength = 0.2 M NaCl

0.8 ionic strength = 0.6 M NaCl

2.2 ionic strength = 2.0 M NaCl

4.2 ionic strength = 4.0 M NaCl

For each ionic strength, there are three lanes representing how the material was treated. The letters refer to different times that the dCTP was added to the experiment : A = no dCTP added to either the column or the eluting buffers; B = 200 μM dCTP was added to dCMP deaminase when applied to the column and not to the eluting buffers; C = 20 μM dCTP was added to dCMP deaminase when applied to the column and 200 μM dCTP was added to the eluting buffers.

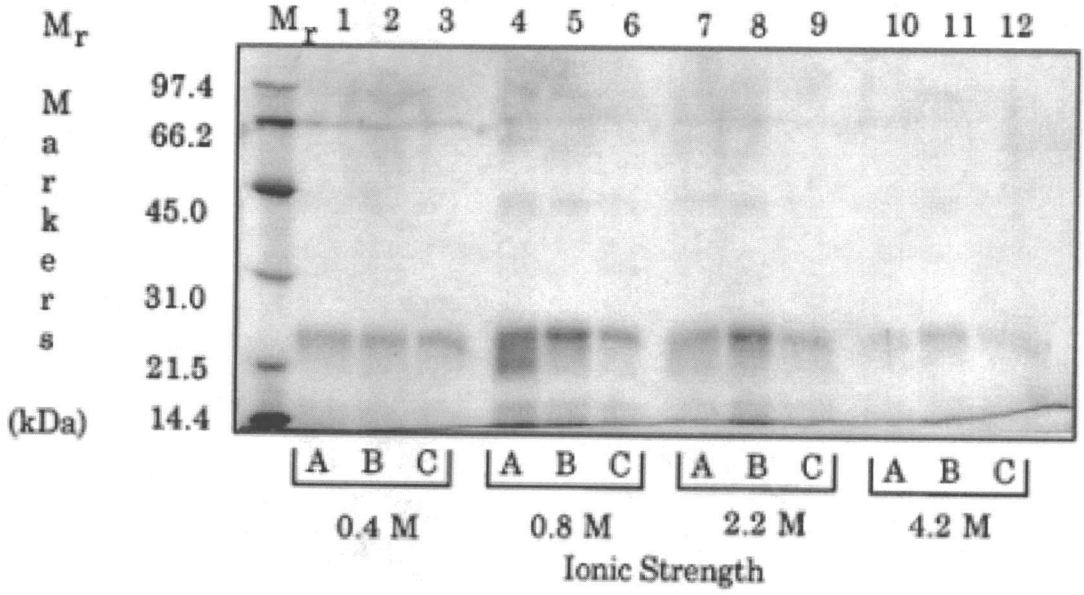

Mr
M
a
r
k
e
r
s
(kDa)
97.4
66.2
45.0
31.0
21.5
14.4
Mr 1 2 3 4 5 6 7 8 9 10 11 12
A B C
A B C
A B C
A B C
0.4 M
0.8 M
2.2 M
4.2 M
Ionic Strength

Figure II – 6.

Figure II - 7. Retention of purified T4 dCMP deaminase on an immobilized T4 thymidylate synthase column using 200 μM dCTP. This gel is a 12.5% polyacrylamide gel stained with Coomassie Blue. One mg of deaminase was applied to the thymidylate synthase column. Without the positive effector the deaminase only bound to 0.8 ionic strength level. With the positive effector it bound more tightly.

0.4 ionic strength = 0.2 M NaCl
0.8 ionic strength = 0.6 M NaCl
2.2 ionic strength = 2.0 M NaCl

For each ionic strength, there are three lanes representing how the material was treated.

Lane 1 is a sample from the 3 ml collection following a change in buffer concentration. Lane 2 is a continuation of that same strength buffer but includes a rest period after the collection begins to allows the salted buffer exposure time with the bound proteins. Two mls are collected and the column is allowed to stand in buffer for 30 minutes. Following the rest period, 8 mls more are collected making a total collection of 10 mls for lane 2. Lane 3 is the final collection of 5 mls with the same buffer. Please note that much of the deaminase is released under 0.6 M salt conditions but some remains and is released by 2.0 M NaCl. The column buffer used 0.1 M potassium glutamate (pH 8.0),1 mM β-mercaptoethanol, 0.5 mM magnesium acetate, 0.025 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 10% glycerol.

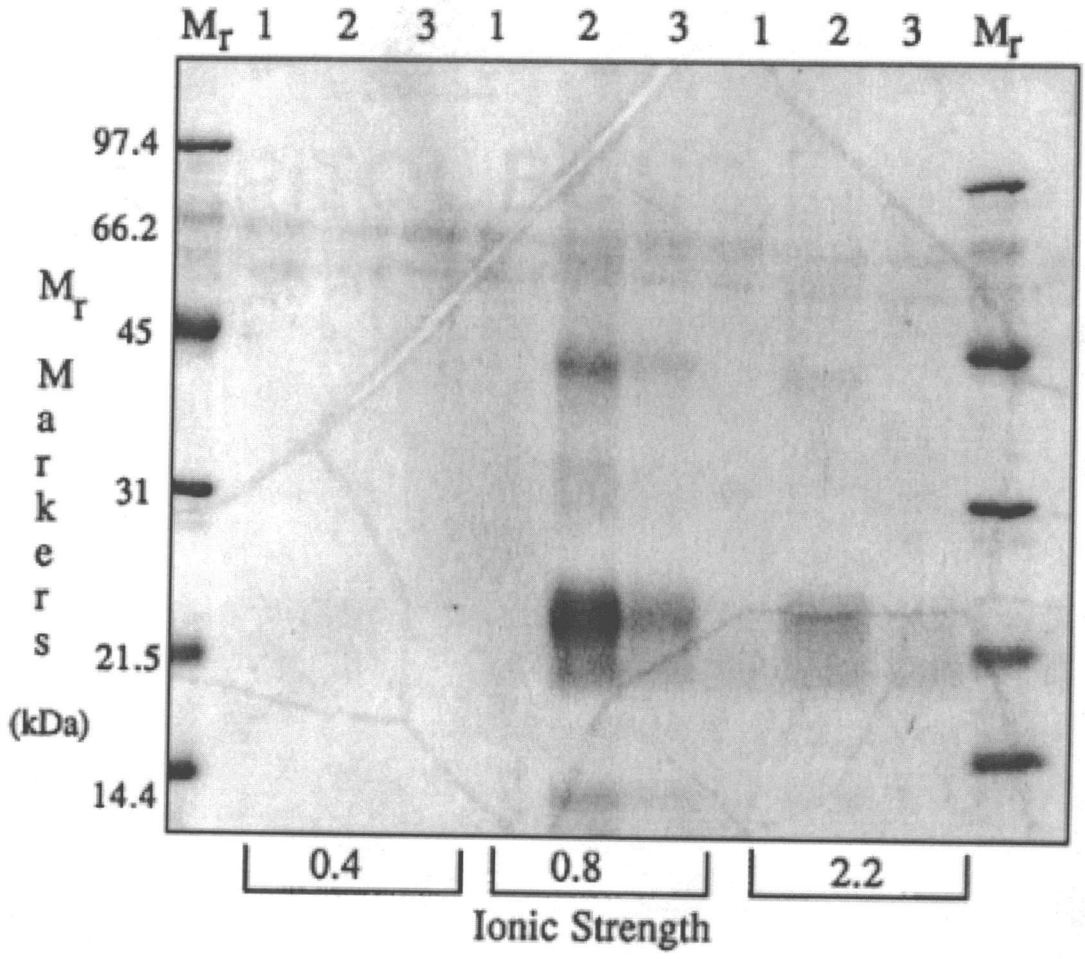

M_r 1 2 3 1 2 3 1 2 3 M_r
97.4
66.2
45
31
21.5
14.4
M_r Markers (kDa)
0.4
0.8
2.2
Ionic Strength

Figure II – 7.

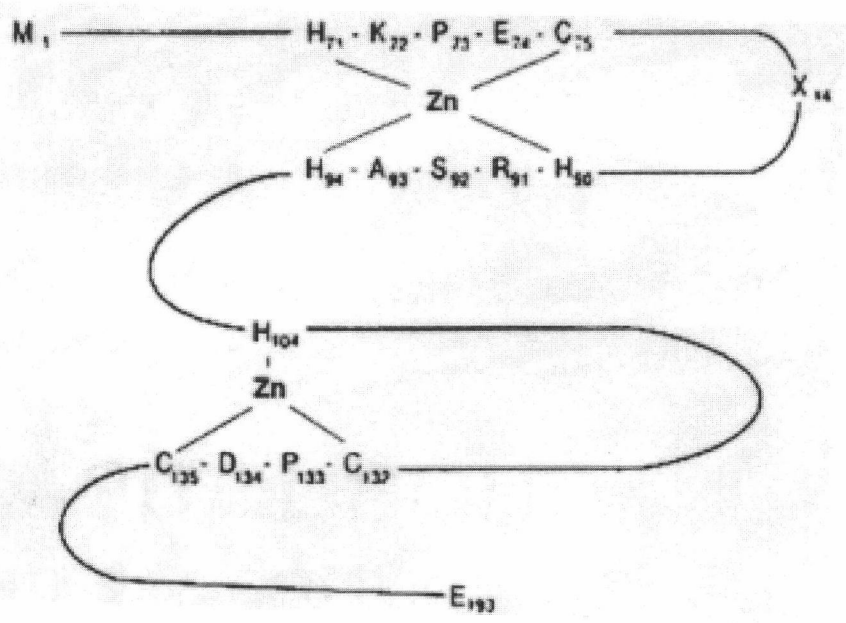
T4 Thymidylate Synthase and dCMP Deaminase Are Physically Related

T4 dCMP deaminase binds directly to T4 thymidylate synthase and not to *E. coli* thymidylate synthase at medium ionic strength. This further demonstrates the specificity of proteins in relevant interactions. Not only does the presence of dCTP strengthen binding in the T4 case, it also preserves the integrity of the dCMP deaminase protein as can be seen in the band definition for the deaminase protein at about 23 kDa in Figures II -5 and II -7 where it is possible to see that the protein is beginning to break down in the situation where there is no dCTP present. The addition of the positive effector dCTP at the time of initial binding is the most efficient means of application.

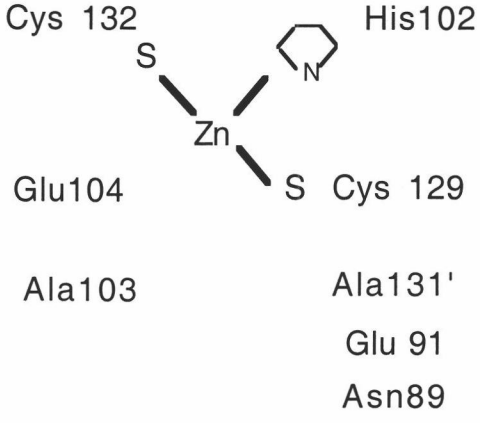
Exploring the Role of Zinc in dCMP Deaminase

T4 phage dCMP deaminase contains two moles of bound zinc per mole of deaminase monomer unit (Moore *et al.*, 1993a). At least one of the bound zinc ions is essential for catalytic activity but the role of the second bound zinc is uncertain. Figure II - 8 presents a proposed model for T4 dCMP deaminase and for the catalytically bound zinc found in *E. coli* cytidine deaminase (Betts *et al.*, 1994). One possible role for the other bound zinc in T4 involves the binding of allosteric effectors dTTP and 5-hydroxymethyl dCTP (Fleming and Bessman, 1967; Maley *et al.*, 1967; Maley and Maley, 1982). Five-hydroxymethyl dCTP, the effector used *in vivo*, is replaced by dCTP in all studies in this thesis. Although not as effective as HMdCTP, dCTP performs the same role. The negative allosteric effector dTTP was shown to bind to phenylalanine 112 in experiments conducted in the Maley lab (Moore *et al,* 1994). The binding site of the positive allosteric effector for deaminase function had not yet been determined but its necessity has been established (Keefe *et al.*, 2000).

Figure II - 8. Zinc Catalytic Site. A. Moore's model for zinc retention in T4 dCMP deaminase (Moore *et al.*, 1993a).B. Residues known to be bonded to the zinc ion in the catalytic site of *E. coli* cytidine deaminase. Other residues are known to stabilize the high energy intermediate. Residues indicated with an ' are found in the other monomer across the dimeric interface.



A.



B.

Figure II – 8.

When the work with dCMP deaminase began in the Mathews' lab, the model of T4 dCMP deaminase of Moore *et al.* (1993a) suggesting that His-90 and His-94 formed part of the same zinc binding site beginning at His-71 and extending through His-94 was used. Thus, even though slightly removed from one another, the mutations of H90 and H94 prepared by the Maley lab were expected to yield similar results for zinc binding and catalytic activity as well as to influence protein-protein interactions in a similar manner. Two mutant forms of dCMP deaminase (H90N and H94N) each involving a histidine thought to participate in zinc binding were produced by converting the histidine in question into an asparagine to try to elucidate their respective roles. Asparagine is sometimes used to mimic the activity that might be taking place on one side of histidine. The H90N mutant form was found to be fully catalytically active while the H94N mutant form was found to be inactive. The quality of bound zinc differed as well. H90N contained two tightly bound zinc ions but H94N contained one tightly bound zinc ion and one loosely bound zinc ion (McGaughey *et al.*, 1996). The Moore model could not explain the results so a new model began to evolve. Without a crystal structure for T4 dCMP deaminase, modeling was based on *E. coli* cytidine deaminase (Betts *et al.*, 1994). Using a comparison of amino acid sequences and consensus sequences representing the active site (Reizer *et al.*, 1994), a model was developed and is presented in Figure II - 9. Overall conservation between cytidine deaminase and dCMP deaminase is low but by using a PHD sequence alignment and secondary structure predictions (Sander and Schneider, 1991; Rost and Sander, 1993, 1994) critical residues were found to be aligned with one another. In the new model, His-94 is found in a short section of β-sheet near the zinc ion associated with the active site. In *E. coli* cytidine deaminase, the active site zinc is coordinated by His-102, Cys-129 and Cys-132 with similar

Figure II - 9. A proposed structural model of T4 dCMP deaminase. This model was generated based on the crystal structure *of E. coli* cytidine deaminase. Numbers refer to E. coli cytidine deaminase locations and numbers in parentheses refer to approximate T4 dCMP deaminase locations.

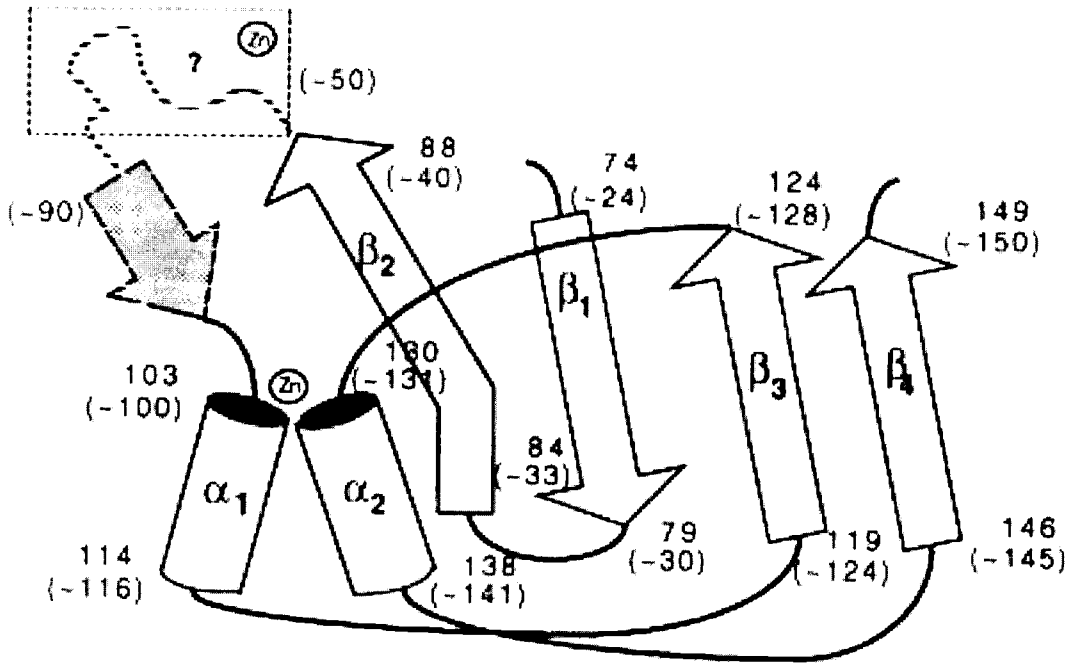

?
Zn
(-50)
88
(-40)
74
(-24)
124
(-128)
149
(-150)
(-90)
β2
β1
β3
β4
103
(-100)
Zn
180
(-131)
84
(-33)
α1
α2
114
(-116)
138
(-141)
79
(-30)
119
(-124)
146
(-145)

Figure II – 9.

residues used in T4 dCMP deaminase (see Figure II - 9). *E. coli* cytidine deaminase contains only one zinc ion which is involved in catalytic activity. The function of a major portion of the protein has a yet to be determined but may be used simply to insure structural integrity (Betts *et al.*, 1994).

His-90 is found in a region comprising about 50 amino acids from residue 46 to 90+. In an attempt to better characterize the binding region of the second zinc of dCMP deaminase a literature search was conducted since the model of *E. coli* cytidine deaminase could provide little additional insight. The only other deaminase structure available was for adenosine deaminase and there was little help there. In the search, I found a potential explanation in the two-zinc ion system of alcohol dehydrogenase. In this protein one of the zinc ions has a catalytically active site similar to the deaminase case and the other zinc ion is found proximal in a support role. The appropriate consensus sequence was also present in T4 dCMP deaminase. The region would stabilize the charge system developed around the active site in the catalytic process (Bergman *et al.*, 1992; Bergman *et al.*, 1993). Confirmation would best be provided by a model developed from a crystal structure of T4 dCMP deaminase, but that was not an available option. It was possible, however, to examine whether these mutations affect protein-protein interactions. Figure II - 10 shows the two-dimensional gel prepared by Linda Wheeler of the radiolabeled proteins retained by 0.2 M NaCl in the potassium-glutamate buffer but released by 0.6 M NaCl for the mutant protein H94N. Some proteins appear to be less strongly bound than in the wild type dCMP deaminase gel (Figure I - 14)(McGaughey *et al.*, 1996). The binding by H94N to thymidylate synthase seems comparable to the behavior of the wild type dCMP deaminase, whereas the binding of dCTPase/dUTPase (gp 56) appears significantly reduced in the H94N case. To clarify this observation, a study

comparing the binding of wild type and mutant forms of deaminase to thymidylate synthase and dCTPase/dUTPase was carried out for three purposes: 1.) to determine if the interaction between dCTPase/dUTPase and dCMP deaminase is a direct physical interaction; 2.) to determine if there is a discernible difference between the behavior exhibited by the wild type deaminase and the two mutant forms; and 3.) to address concerns regarding the buffer used in the original work.

Figure II - 10. Proteins moderately tightly bound to H94N mutant T4 dCMP deaminase. The figure contains a depiction of two-dimensional gel analysis done by Linda Wheeler of radiolabeled proteins from 3 to 8 minutes after infection with T4D retained at 0.4 M ionic strength but released at 0.8 M ionic strength. Each arrow marks a protein less strongly bound compared to wild type dCMP deaminase. The column buffer used 0.1 M potassium glutamate (pH 8.0),1 mM β-mercaptoethanol, 0.5 mM magnesium acetate, 0.025 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 10% glycerol. The gel represents the results obtained for proteins that remain bound at 0.2 M NaCl but are eluted by 0.6 M NaCl.

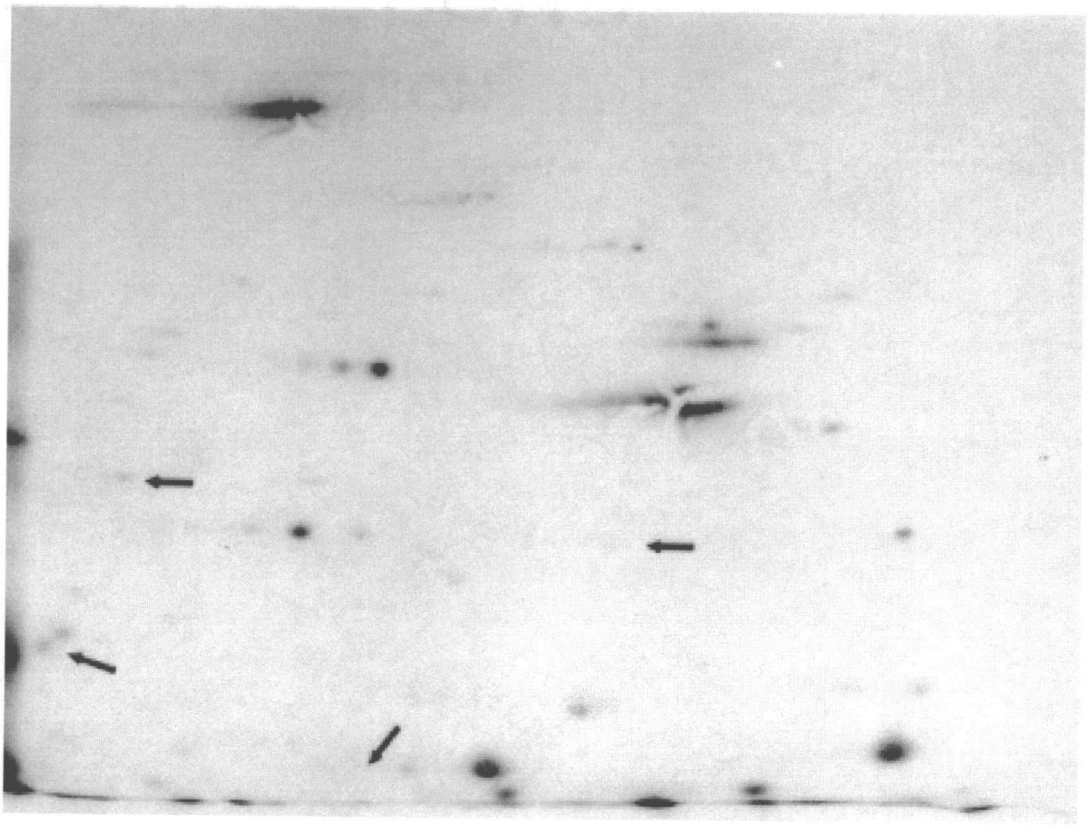


Figure II – 10.

Preparing Thymidylate Synthase and dCTPase/dUTPase Affinity Columns

Affinity columns were prepared using about 3 milligrams each of thymidylate synthase and dCTPase/dUTPase as described earlier in this chapter. Recombinant T4 thymidylate synthase was purified by the method of LaPat-Polasko *et al.* (1990). The frozen pellet for recombinant T4 dCTPase/dUTPase was resuspended in French press buffer, pressed and centrifuged to remove cell debris. Polymin P was added dropwise to a final concentration of 5%. The mixture was stirred for 15 minutes on ice and the precipitate collected by centrifugation. The resulting pellet was resuspended in extraction buffer and sufficient NaCl was added to reach a final salt concentration of 0.4 M. The suspension rested in the cold box overnight with periodic agitation and was spun down the following day. The supernatant collected was desalted and applied to a DEAE cellulose column and eluted with a continuously increasing sodium chloride gradient to 1 M. Samples of the fractions containing protein were run on an SDS - PAGE and those fractions with the appropriately sized protein were desalted, concentrated and run again on a DEAE column with a shallower gradient. The fractions with protein were sampled once again and those fractions with a single appropriately sized band and catalytic activity were used for experimentation.

A More Appropriate Buffer

One concern expressed about the initial two-dimensional gel work with T4 dCMP deaminase was that there was no buffering agent to maintain the pH of the system. To address that concern, a buffer was sought that retained the qualities of the potassium-glutamate system as discussed below, but possessed a discernible buffering agent that was known to maintain pH. The initial work by Record and his

colleagues dealt with the effect of ion concentration regarding protein-DNA interaction and gene expression (Richey *et al.*, 1987). In subsequent investigations, they determined that by replacing potassium chloride with potassium glutamate they were able to dramatically enhance those same interactions *in vitro* that had been studied *in vivo* (Leinmo et al., 1987). Zori and Richardson (1991) demonstrated similar effects with protein-protein interactions. They concluded that salts that demonstrated high termination efficiencies in studying rho binding had two properties: 1.) anions were zwitterions and 2.) weak chelators of Mg^{2+} ion. Work conducted by Lohman *et al.* (1989) during Rep helicase protein studies demonstrated a dependence of single stranded DNA-dependent ATPase activity on both cations and anions found in monovalent salts. The activity was greater in glutamate than in acetate than in chloride (Lohman *et al.*, 1989). From these collected observations, it became evident that the same buffer system used to examine protein-protein interactions should be used in kinetic studies as well. After examining papers from investigators that studied T4 replication or closely associated activities, the following buffer was selected (Latham *et al.*, 1999): 25 mM Tris-acetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. This buffer met the criterion of a buffering agent but the chelating effects of glutamate would not have to be titrated away. Since this was not the same buffer as the one in which the initial investigations with T4 dCMP deaminase were conducted, the interaction with wild type dCMP deaminase was repeated for comparison and new interactions of mutants were investigated as well. In ths buffer system, there were differences in pH, anions present and concentration which would make comparison to previous work questionable. The pH moved from 8.0 to 7.5, closer to *in vivo* expectations. The anion is now acetate instead of glutamate. In addition to the presence of Tris, the

concentration of potassium has changed from 100 to 150 mM; the concentration of β-mercaptoethanol has moved from 1 to 10 mM and the concentration of magnesium ion has changed from 0.5 to 10 mM. More dCMP deaminase was applied in the overnight circulation than in the previous invesitgation. The gels were visualized by silver staining because although the same interactions were detected, the bands would possibly be less discernible by Coomassie blue in this new buffer system. The results are found in Figures II - 11 through II - 16.

Figure II - 11. Retention of purified wild type T4 dCMP deaminase on an immobilized T4 thymidylate synthase column using 200 μM dCTP during protein application. This gel is a 12.5% polyacrylamide gel visualized by silver staining. Over one mg of deaminase was applied to the column. The column buffer is 25 mM Tris-acetate (pH 7.5),150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. MW = molecular weight marker; o = overnight; ft = flow through (wash until no material is detected); A = column buffer plus 0.2 M NaCl; B = column buffer plus 0.6 M NaCl and C = column buffer plus 2.0 M NaCl.

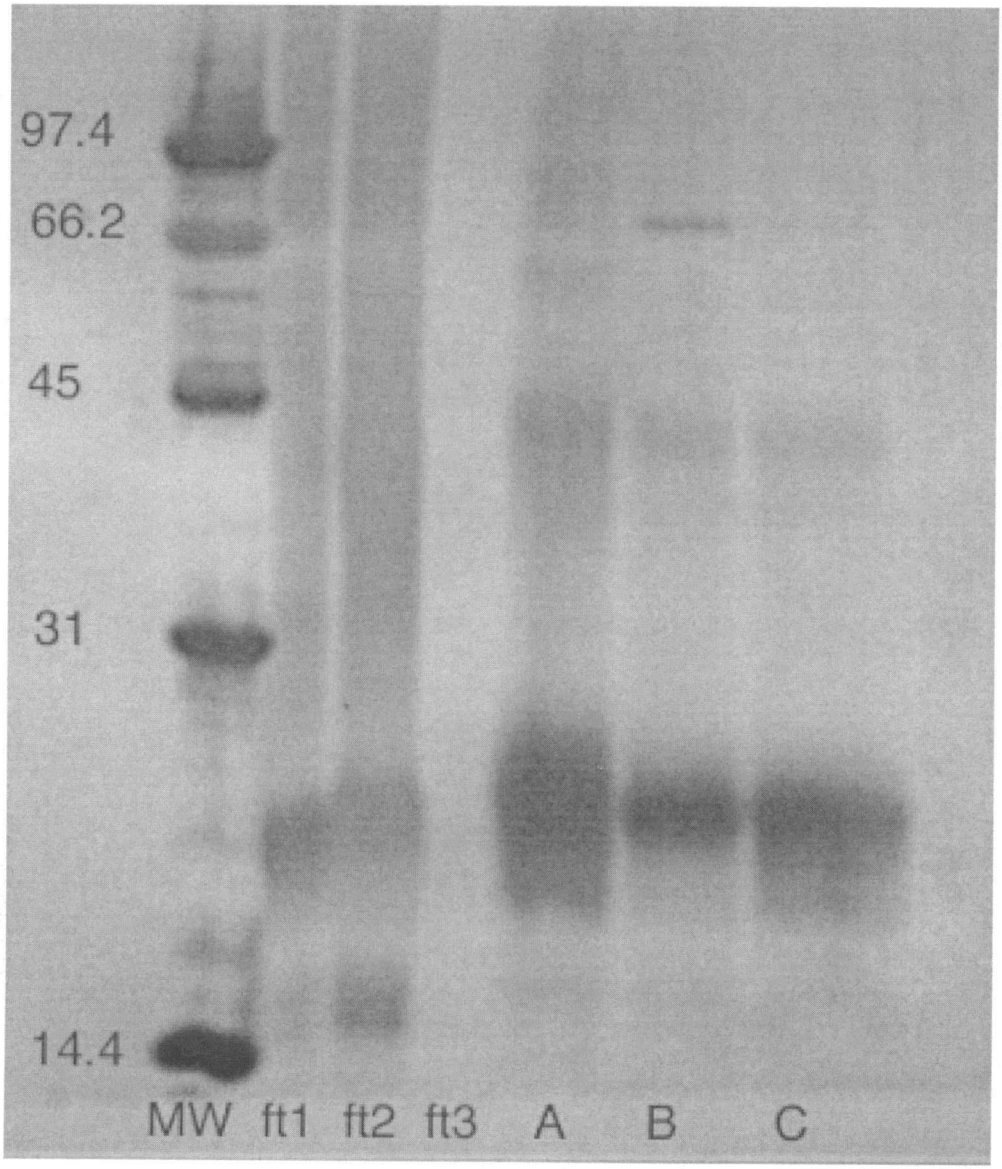


Figure II – 11.

Figure II - 12. Retention of purified wild type T4 dCMP deaminase on an immobilized T4 dCTPase/dUTPase column using 200 μM dCTP during protein application. This gel is a 12.5% polyacrylamide gel visualized by silver staining. Over one mg of deaminase was applied to the column. The column buffer is 25 mM Tris-acetate (pH 7.5),150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. MW = molecular weight marker; o = overnight; ft = flow through (wash until no material is detected); A = column buffer plus 0.2 M NaCl; B = column buffer plus 0.6 M NaCl and C = column buffer plus 2.0 M NaCl.

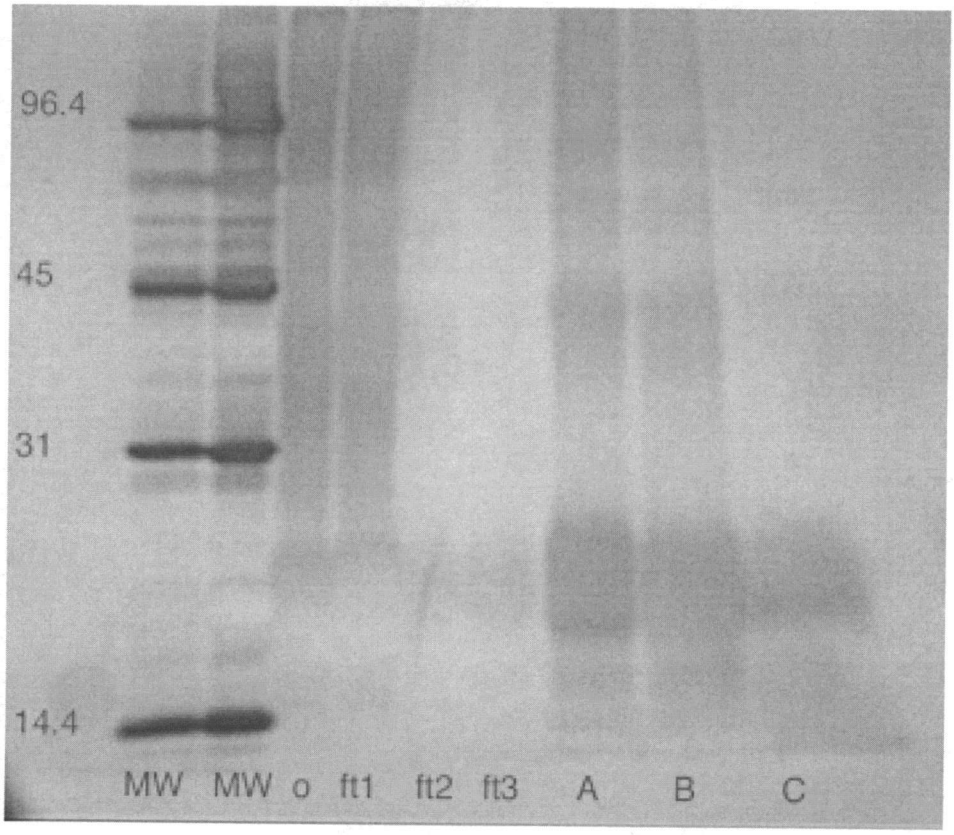


Figure II – 12.

Figure II - 13. Retention of purified H90N T4 dCMP deaminase on an immobilized T4 thymidylate synthase column using 200 μM dCTP during protein application. This gel is a 12.5% polyacrylamide gel visualized by silver staining. One mg of deaminase was applied to the column. The column buffer is 25 mM Tris-acetate (pH 7.5),150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. MW = molecular weight marker; o = overnight; ft = flow through (wash until no material is detected); A = column buffer plus 0.2 M NaCl; B = column buffer plus 0.6 M NaCl and C = column buffer plus 2.0 M NaCl.

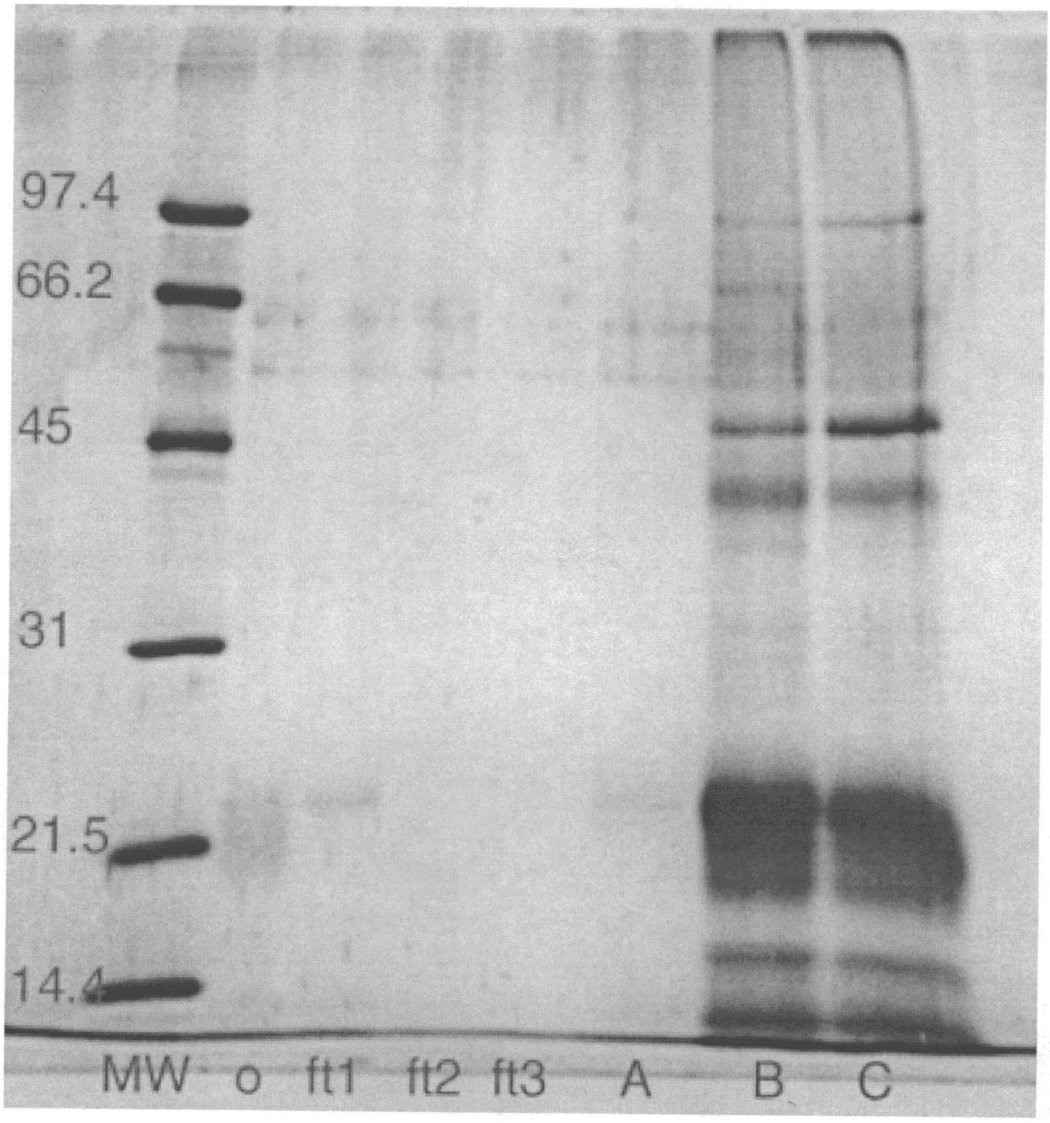


Figure II – 13.

Figure II - 14. Retention of purified H90N T4 dCMP deaminase on an immobilized T4 dCTPase/dUTPase column using 200 μM dCTP during protein application. This gel is a 12.5% polyacrylamide gel visualized by silver staining. One mg of deaminase was applied to the column. The column buffer is 25 mM Tris-actetate (pH 7.5),150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. MW = molecular weight marker; o = overnight; ft = flow through (wash until no material is detected); A = column buffer plus 0.2 M NaCl; B = column buffer plus 0.6 M NaCl and C = column buffer plus 2.0 M NaCl.

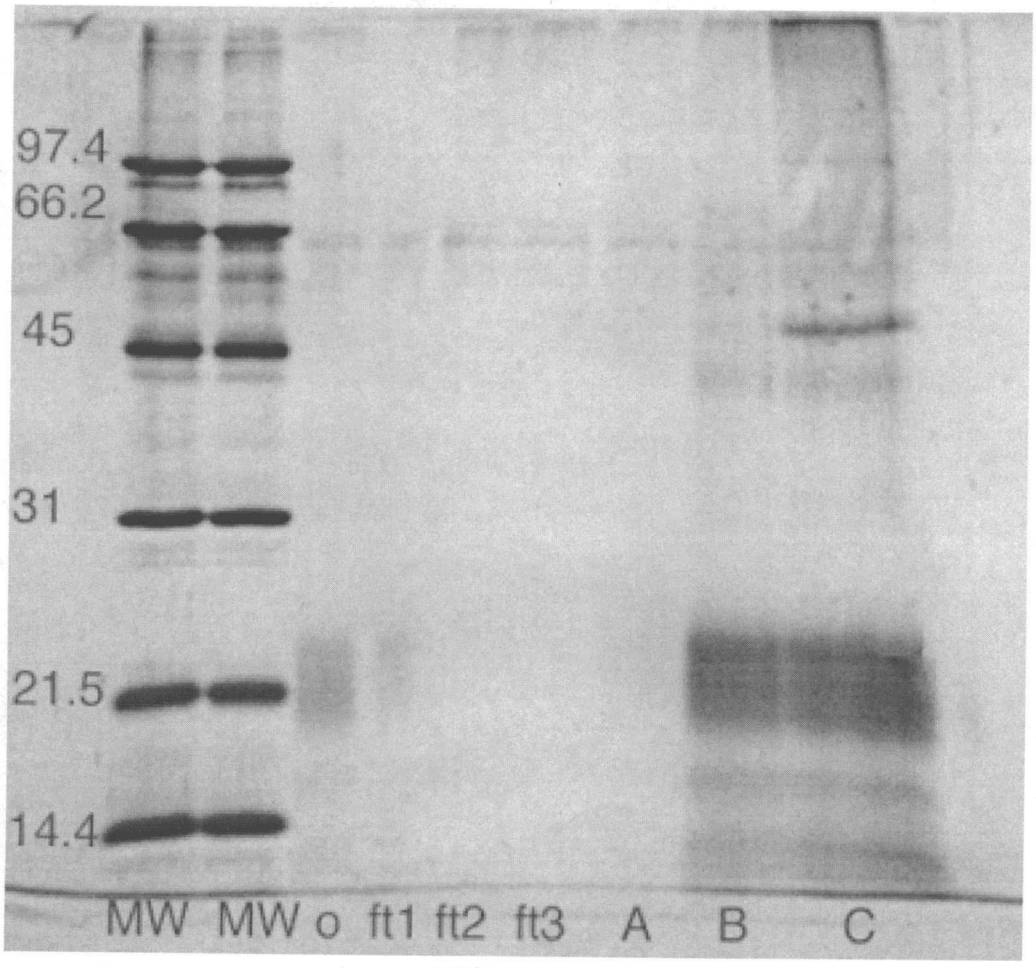


Figure II – 14.

Figure II - 15. Retention of purified H94N T4 dCMP deaminase on an immobilized T4 thymidylate synthase column using 200 μM δ dCTP during protein application. This gel is a 12.5% polyacrylamide gel visualized by silver staining. One mg of deaminase was applied to the column. The column buffer is 25 mM Tris-actetate (pH 7.5),150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. MW = molecular weight marker; o = overnight; ft = flow through (wash until no material is detected); A = column buffer plus 0.2 M NaCl; B = column buffer plus 0.6 M NaCl and C = column buffer plus 2.0 M NaCl.

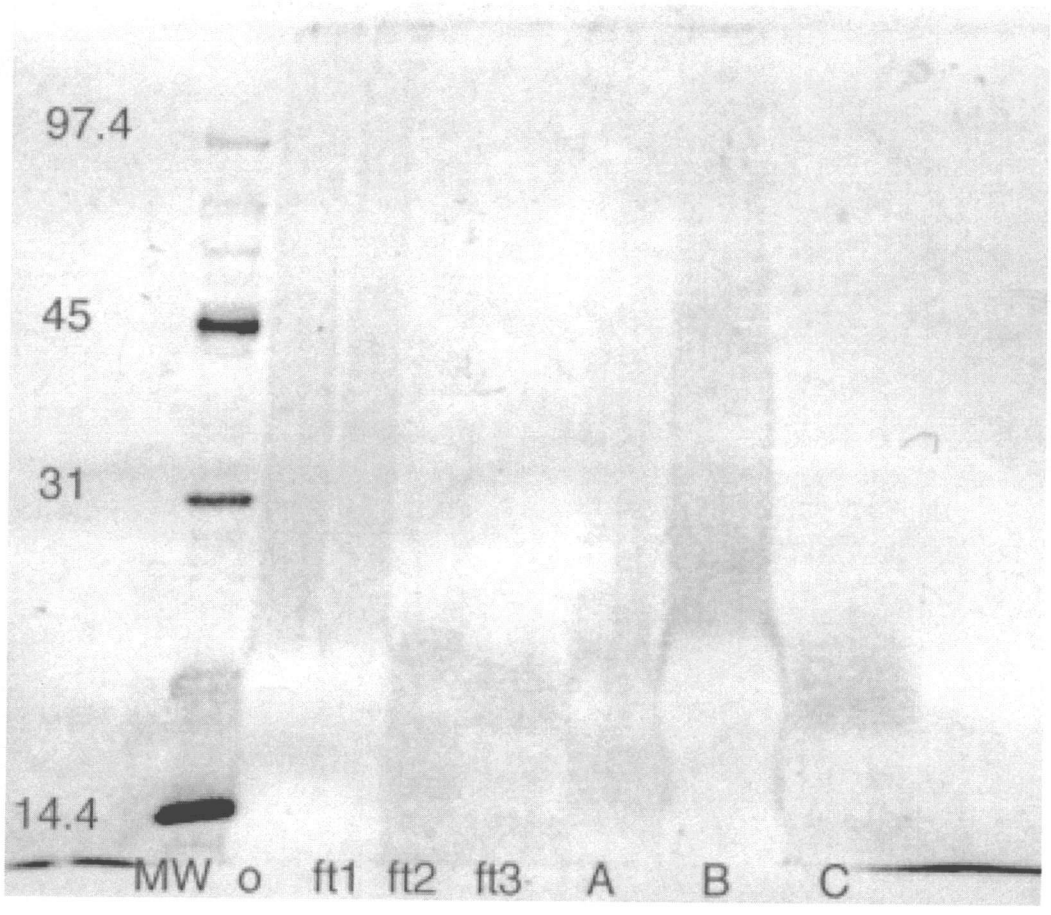


Figure II – 15.

Figure II - 16. Retention of purified H94N T4 dCMP deaminase on an immobilized T4 dCTPase/dUTPase column using 200 μM dCTP during protein application. This gel is a 12.5% polyacrylamide gel visualized by silver staining. One mg of deaminase was applied to the column. The column buffer is 25 mM Tris-actetate (pH 7.5),150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. MW = molecular weight marker; o = overnight; ft = flow through (wash until no material is detected); A = column buffer plus 0.2 M NaCl; B = column buffer plus 0.6 M NaCl and C = column buffer plus 2.0 M NaCl.

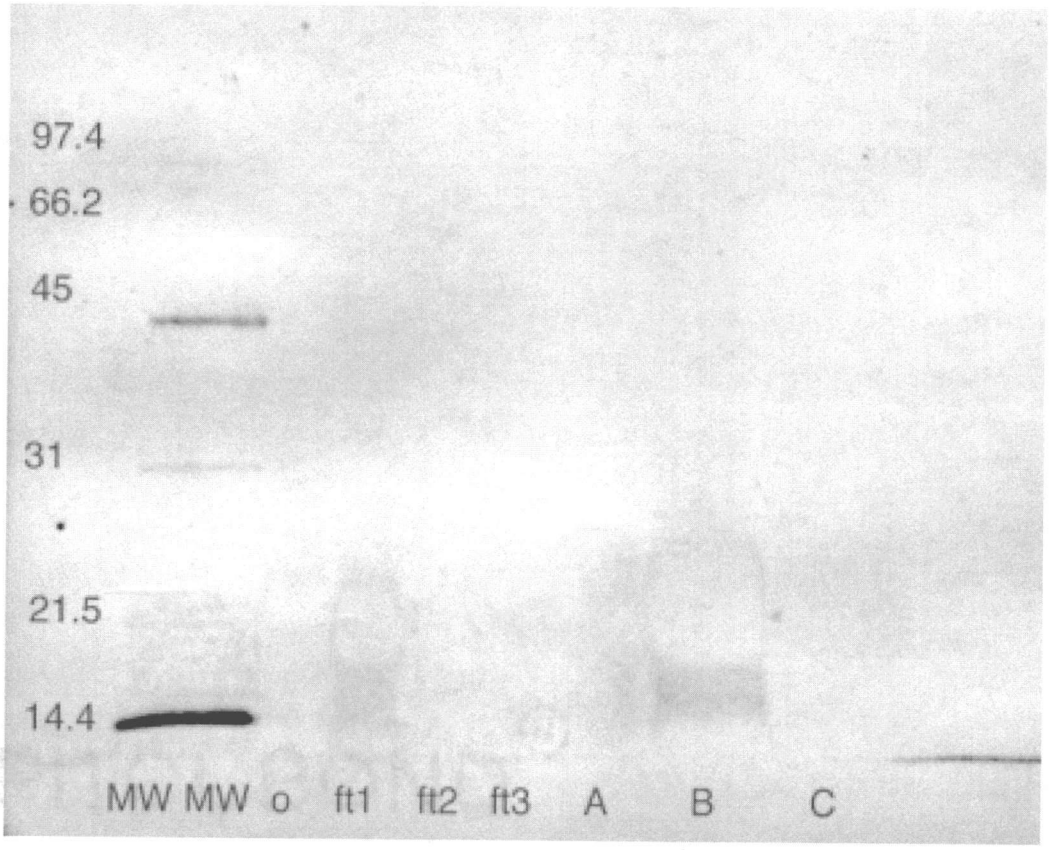


Figure II – 16.

Results of Specific Interaction Between dCMP Deaminase, Thymidylate Synthase and dCTPase/dUTPase

From these gels it is clear that dCMP deaminase has a strong, specific physical relationship with both T4 thymidylate synthase and dCTPase/dUTPase. The difference in pH for the new column buffer from pH 8.0 to 7.5 does not seem to have markedly changed the binding for the interactions between thymidylate synthase and dCMP deaminase as well as between dCTPase/dUTPase and dCMP deaminase. The change in anion selection as well as constituent concentration does not seem to have influenced the nature of the interaction. With additional wild type deaminase, the interactions are more informative about the breakdown of deaminase in the absence of dCTP evident in broad less-defined bands. The release of wild type dCMP deaminase takes place by 0.6 M NaCl as is evident by the well-defined bands.

What is confusing about these gels is the nature of the binding behavior of mutant forms H90N and H94N from one another. The bands are located at the same salt concentrations but exhibit a different banding pattern. Like the catalytic activity and zinc ion content, the two mutants are different from one another. The behavior of H90N is similar to wild type. There is a distinction in the band clarity of the mutant form from the wild type protein as well as longer retention on the thymidylate synthase affinity column demonstrating tighter binding than the wild-type. Since both of these fomrs contain two tightly bound zinc ions, it is possible that the positive allosteric effector might reside in an area influenced by the mutation. The band clarity and the presence of the second band in the 0.6 M sample for both wild type and H90N may reflect that both samples are more stable due to two strongly bound zinc ions. If one examines lane 2 of the 0.8 M ionic strength elution of Figure II - 7, one notes that there are two bands for the protein: one at about 23 kDa and the other just

under 45 kDa. These are SDS-PAGE gels and the protein samples were boiled in loading buffer containing fresh β-mercaptoethanol as well as SDS which should denature the protein and produce the monomeric form. The two band behavior for purified dCMP deaminase could be repeated and only was present in samples that contained the positive effector dCTP. I repeatedly checked it and someone else in the lab ran a gel and found the same behavior.

To determine if the band at about 45 kDa was a contaminant I performed two experiments: 1.) from an SDS gel the 23 kDa band was cut out and used to prepare an antibody; and 2.) using a similarly prepared gel, the bands at 23 kDa and 45 kDa were isolated and sequenced. Following a western transfer, both bands lit up with the antibody prepared from the 23 kDa band and sequencing done at the Central Services Lab returned the first thirteen amino acids for each band as identical. The bands were from dCMP deaminase in monomeric and dimeric forms. If one examines the comparable lane of the wild type dCMP deaminase interaction with thymidylate synthase in this collection, one sees something at about 23 kDa and 45 kDa. H90N has bands in the medium salt release that fit the presence of a 23 kDa and 45 kDa proteins as well and for the high salt release there is an additional band at about 65 kDa. The antibody was not used to confirm the identity of these bands. The actual enzyme composition of dCMP deaminase is of 6 monomeric units but it is very intriguing that the presence of the small molecule dCTP stabilizes the protein dimer so much that it can run on an SDS-PAGE gel as a sharp band like the monomeric unit when it retains both zinc ions. To be active, the enzyme must have these dimeric units. Alcohol dehydrogenase also has dimeric units (see Figure II - 17). Additionally, it contains two zinc ions in proximity to one another with a shared sequence to dCMP deaminase.

Figure II - 17. Structure of alcohol dehyrogenase. (Eklund *et al.*, 1994)

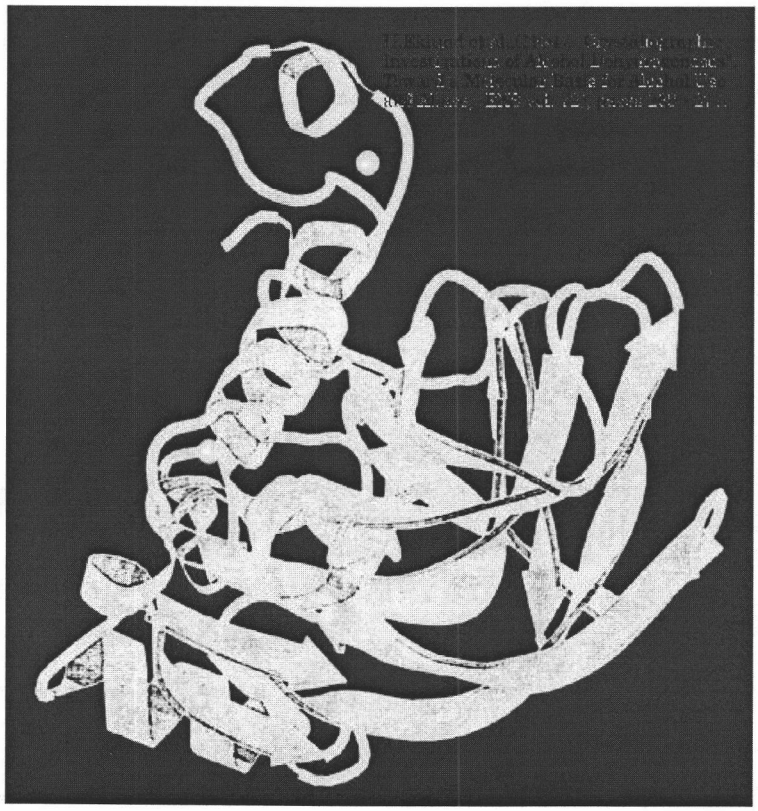


Figure II – 17.

An Alternative Path

Deoxycytidine 5'monophosphate (dCMP) has two potential fates when it is released by T4 dCTPase/dUTPase. One fate is as a substrate for dCMP deaminase. The interactions necessary for this portion of the pathway have been addressed in previous sections. Another fate for dCMP is as a substrate for dCMP hydroxymethylase to produce HMdCMP which, following phosphorylation, is incorporated into T4 DNA. To determine if dCTPase/dUTPase binds directly to dCMP hydroxymethylase, dCMP hydroxymethylase purified to a single band on a Coomassie stained gel using the procedure of Graves *et al.*, (1992) up to the MonoQ column was circulated over the dCTPase/dUTPase column used in the investigations described previously. Less than one milligram of dCMP hydroxymethylase was applied to the dCTPase/dUTPase column and circulated overnight. Following removal of the circulating material, the column was washed in Tris-acetate buffer and sodium chloride was applied in steps following the protocol for affinity chromatography mentioned earlier in this chapter. Figure II – 18 shows the resulting gel. Hydroxymethylase has a monomeric unit mass of 28 kDa. A protein about that size is released in the flow though wash with actetate buffer. There is a trace of the material in the 0.6 M NaCl wash but the data are not sufficiently definitive to make a judgment. At about 32 kDa there is a band in the 0.6 M NaCl wash whose identity remains a mystery. The bands present at about 20 kDa are probably dCTPase/dUTPase. This was the first use of the column and even though it had been exposed to high salt, more material is released with first use.

No conclusions can be drawn about the binding of dCMP hydroxymethylase to dCTPase/dUTPase.

Figure II - 18. Retention of purifiedT4 dCMP hydroxymethylase on an immobilized T4 dCTPase/dUTPase column. This gel is a 12.5% polyacrylamide gel visualized by silver staining. Less than one mg of hydroxymethylase was applied to the column. The column buffer is 25 mM Tris-actetate (pH 7.5),150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. MW = molecular weight marker; o = overnight; ft = flow through (wash until no material is detected); A = column buffer plus 0.2 M NaCl; B = column buffer plus 0.6 M NaCl and C = column buffer plus 2.0 M NaCl.

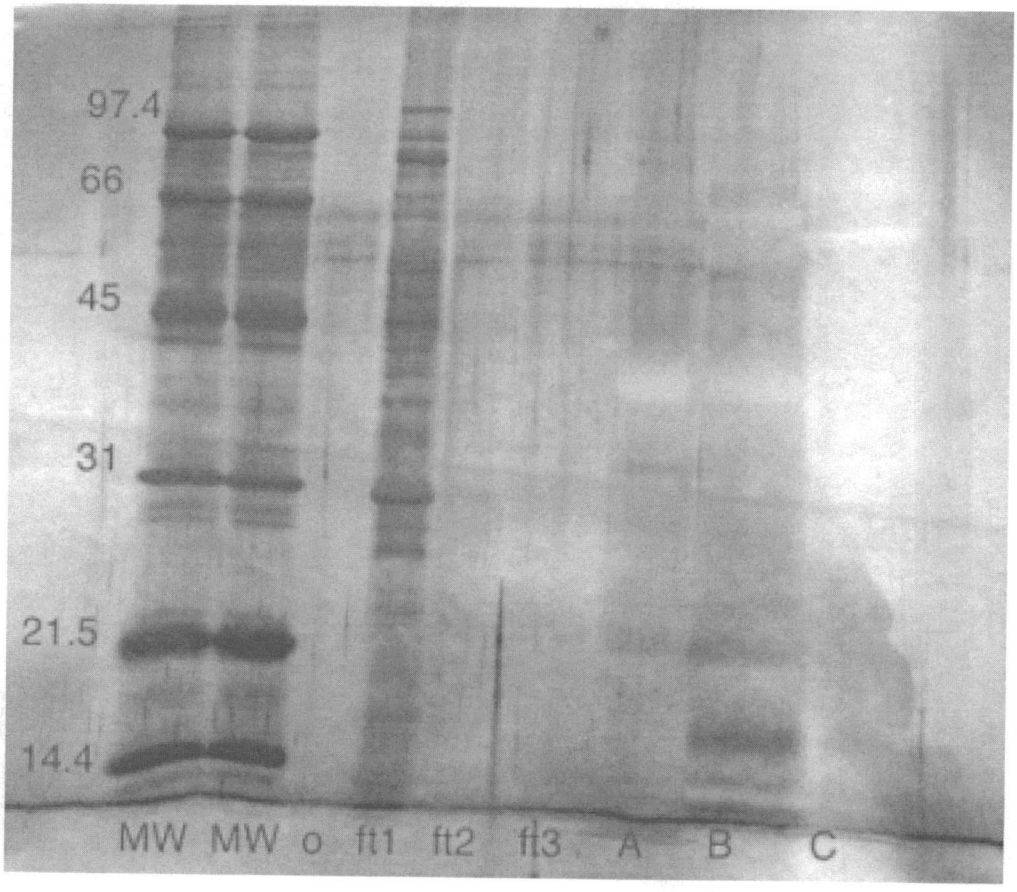


Figure II – 18.

The Importance of Structure

Because of its relevance to DNA production, the sequence of thymidylate synthase has been determined from numerous species. Thymidylate synthase is a highly conserved enzyme over a very broad range from viruses to prokaryotes to eukaryotes including plants. The abundance of examples makes it possible to identify many of the conserved amino acids considered essential. Furthermore, many examples of thymidylate synthase have been crystallized and are available in the database. In order to learn if it was possible to see those regions that might lend themselves to analysis of protein-protein interactions the structures of *E. coli* and T4 thymidylate synthase were examined. Blaine Mooers of Dr. Ho's lab overlaid the structures of T4 and *E. coli* thymidylate synthases using a modeling program he generated to make it possible to visualize the loop-out regions of *E. coli* thymidylate synthase that are distinct from the T4 enzyme (Figure II – 19). It was believed that these regions might hold the key to the differences in protein-protein interactions for *E. coli* and T4. Other investigators pursuing this line of reasoning identified six regions that were considered unique to T4. By making insertions and deletions in these regions they hoped to gain insight into protein-protein interaction. The results were mixed in that the investigators found that regions of hydrophobicity were favored but specificity was not demonstrated (Hazzebrouck *et al.*, 1999).

To see how closely the structures of these enzymes related to one another the backbone of thymidylate synthase from T4, *E. coli* and *Bacillus subtilis* were aligned with a program created by Dr. Karplus and used by Saavas Savides. The *Bacillus* structure was selected as a result of studies conducted in a class on ancestral relationships taught by Dr. Steven Giovannoni. The *Bacillus subtilis* thymidylate

Figure II – 19. Two views of the overlay of structures from *E. coli* thymidylate synthase and bacteriophage T4 thymidylate synthase. Of interest are the regions that are not common to T4 and E. coli thymidylate synthases. In the upper diagram, there are two loops from T4 that do not overlay on comparable regions of *E. coli* thymidylate synthase. In the lower diagram, there is a loop from *E. coli* thymidylate synthase that is not shared by the structure from bacteriophage T4.

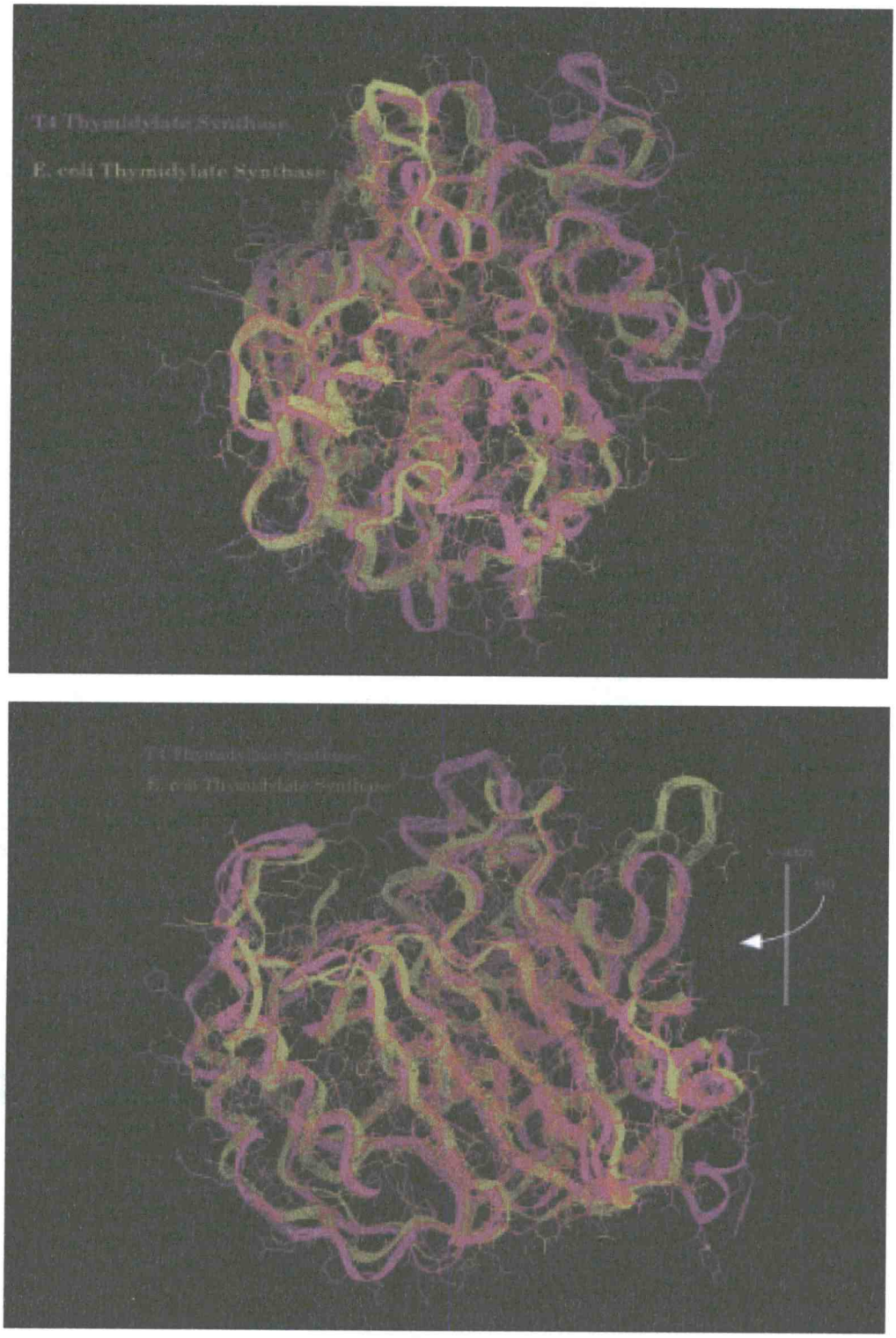


Figure II – 19.

synthase was found to be T4's nearest neighbor even though it is the smallest of the known sequences. *Bacillus subtilis* has two different thymidylate synthases which are induced under different conditions. The smaller one may be the contribution of a virus. It has a faster turnover than that the larger version native to *Bacillus subtilis* making it possible to generated DNA precursors more rapidly from the smaller thymidylate synthase. The backbone from each of the three enzymes aligned very closely supporting the observation that there is little basic difference in the backbone structure for any of the thymidylate synthases. A similar observation can be made for most highly conserved proteins. What seems most important for protein-protein interactions are the contours generated by the side chains and backbone of the amino acids available on the surface.

By examining the amino acids that constitute the surface of the protein, pockets and regions of difference can be observed. To better characterize those regions most important for interaction, I examined the alignment of seventeen thymidylate synthases for amino acids that seemed conserved but were not associated directly with the active site of the enzyme. Next the location of potential candidates was determined from known structures. Daniel Santi and his co-workers addressed one of these regions in *E. coli* and found that mutating the amino acid of interest did not affect the enzyme's performance necessarily (Variath, P. *et al.*, 2000). In addition, investigators have been able to produce a functional chimera *in vivo* from thymidylate synthase from different species (Greene *et al.*, 1993) and mutants from the same species which suggests a great tolerance for the synthesis process (Saxl *et al.*, 2001). Many residues play more than role in a protein. The difficulty in determining which residues might be critical for this line of reasoning led to leaving investigations of this nature for others in the future.

Attempting to Reconstruct a Part of the Complex

In the work done by Moen *et al.*, the dNTP synthetase complex was isolated from a T4 infection of *E. coli* by using a series of columns. The buffers used in these isolations contained Tris-HCl at 25 mM and 5 mM at pH 7.0 and culminated in 10 mM phosphate at pH 7.2. I chose to use the acetate buffer previously described in this chapter for affinity chromatography to maintain consistency and because phosphate buffer would probably interfere with the effects of small molecules like dCTP. The sizing column was chosen because of the work of Mike O'Donnell's group in reconstructing the *E. coli* DNA polymerase III beta clamp (Onrust *et al.*, 1995) and because of previous work by another member of Dr. Mathews' lab. The enzymes were combined in ratios and sequence used in kinetic studies (described in the next chapter) at room temperature but tetrahydrofolate was not included in the reaction mixture. Ten microliters of each enzyme sample included was added to an Eppendorf tube with 100 µl of either 10 mM dUTP or dCTP appropriate to the reaction under study, 500 µl of the tetrahydrofolate stock solution minus tetrahydrofolate containing the acetate buffer and allowed to rest at room temperature on the bench for 15 minutes. Two hundred microliters of the sample was injected onto a Superose 6HR sizing column that had been previously conditioned with the acetate buffer. The elution program ran for 90 minutes at 0.3 mls/ min at room temperature on an FPLC. One-milliliter fractions were collected. Those samples that contained protein were concentrated and run on an SDS-PAGE gel in Figure II - 21. Two hundred microliters of each enzyme was also injected to identify peaks and determine if the material remained in its enzymatically active form.

The previous investigator found that if the enzymes were not combined in an appropriate ratio, an aggregate formed that was released in the void volume. Figure II

– 20 shows the most successful of these attempts to reconstruct that portion of the dNTP synthetase complex involving thymidylate synthase, dCMP deaminase and dCTPase/dUTPase. Little material was released in the void volume, which was taken as an indication that the stoichiometric relation and addition order was appropriate. Thymidylate synthase maintained its dimeric composition alone and when combined with dCMP deaminase and dCTPase/dUTPase in this system with dCTP. The column evidence suggests that both dCTPase/dUTPase and dCMP deaminase did not maintain their multimeric state when run individually but became monomers. In future investigations conditions which maintain the multimeric nature of the individual enzymes should be determined. This behavior may be a result of insufficient crowding or the nature of the buffer. It is relevant to observe that the materials were kinetically active in the same buffer.

From information in protocols for the growth of these enzymes from recombinant systems, it is clear that these materials or their products are toxic to their hosts. To see if it was possible to determine if the complex was detectable after enzymatic function for a significant duration, the procedure was repeated with tetrahydrofolate included in the mixture under the conditions used for a kinetic assay. A kinetic assay to check activity was performed and a sample immediately applied to the Superose 6 HR column without benchtop incubation. One mixture was prepared that included thymidylate synthase, dCMP deaminase, dCTPase/dUTPase and dCTP and another was prepared that included thymidylate synthase, dCTPase/dUTPase and dUTP. The results are presented in Figure II - 22. It is clear that the proteins are no

Figure II – 20. Results from partial complex reconstruction. Thymidylate synthase, dCMP deaminase and dCTPase/dUTPase were each run separately and then in combination without folate in the buffer. Only thymidylate synthase maintained its native composition while dCMP deaminase and dCTPase/dUTPase returned to monomeric form.

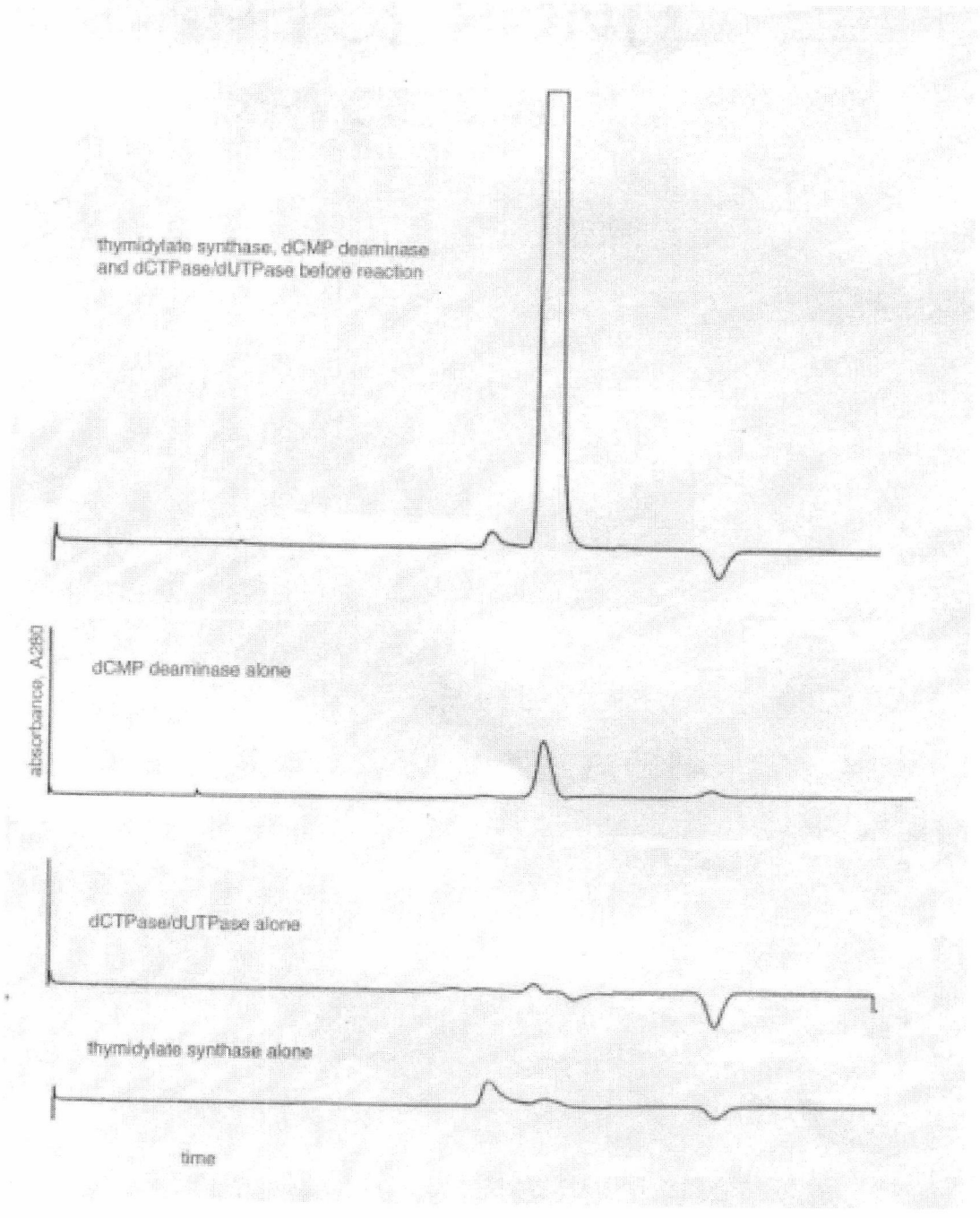

thymidylate synthase, dCMP deaminase and dCTPase/dUTPase before reaction
absorbance, A280
dCMP deaminase alone
dCTPase/dUTPase alone
thymidylate synthase alone
time

Figure II – 20.

Figure II –21. SDS-PAGE gel of concentrated fractions after attempt to reconstruct the dNTP synthetase complex. Lane 1: molecular weight markers; lane 2: mixture of thymidylate synthase, dCMP deaminase and dCTPase/dUTPase; lane 3: fraction 16; lane 4: fractions 17 and 18; lane 5: fraction 19; lane 6: original dCMP deaminase; lane 7: original thymidylate synthase; lane 8: original dCTPase/dUTPase.

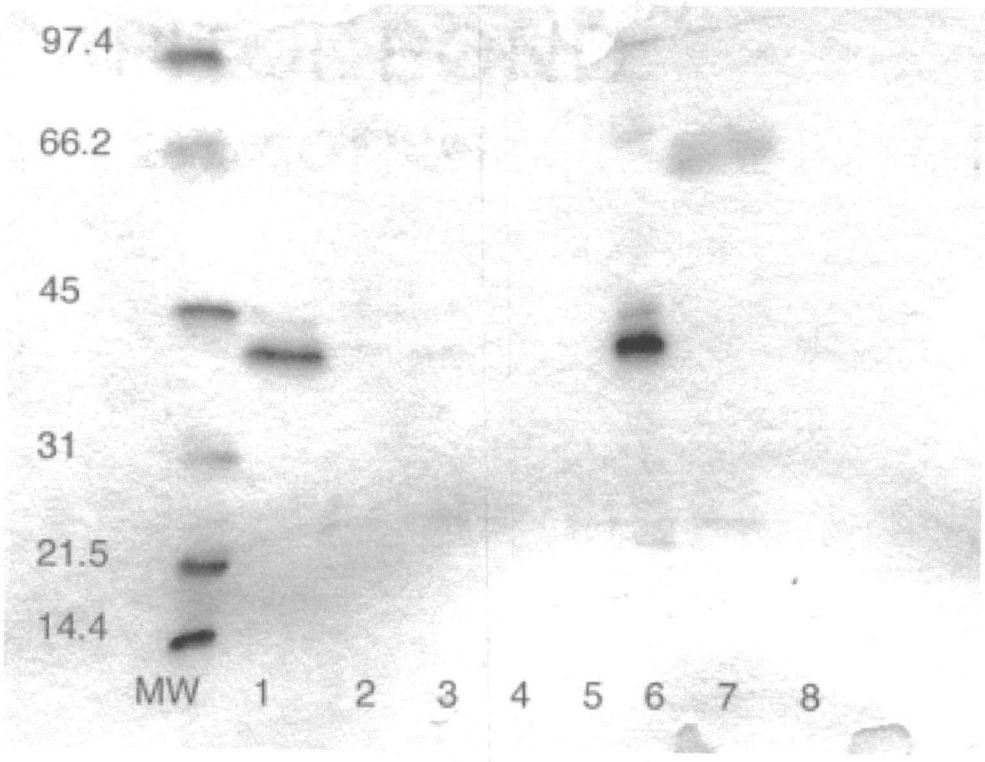


Figure II – 21.

Figure II – 22. Separation of partial complex reconstruction mixture following kinetic assay. The assay measures the production of dihydrofolate produced as a result of thymidylate synthase activity. Thymidylate synthase, dCMP deaminase, dCTPase/dUTPase (middle) and dCTP were separated on a Superose 6 column following a kinetic assay. Thymidylate synthase, dCTPase/dUTPase (bottom) and dUTP were separated on a Superose 6 column following a kinetic assay as well. In both cases, the proteins present in Figure II – 20 are no longer present. The cobalamin standard is included for approximate size determination.

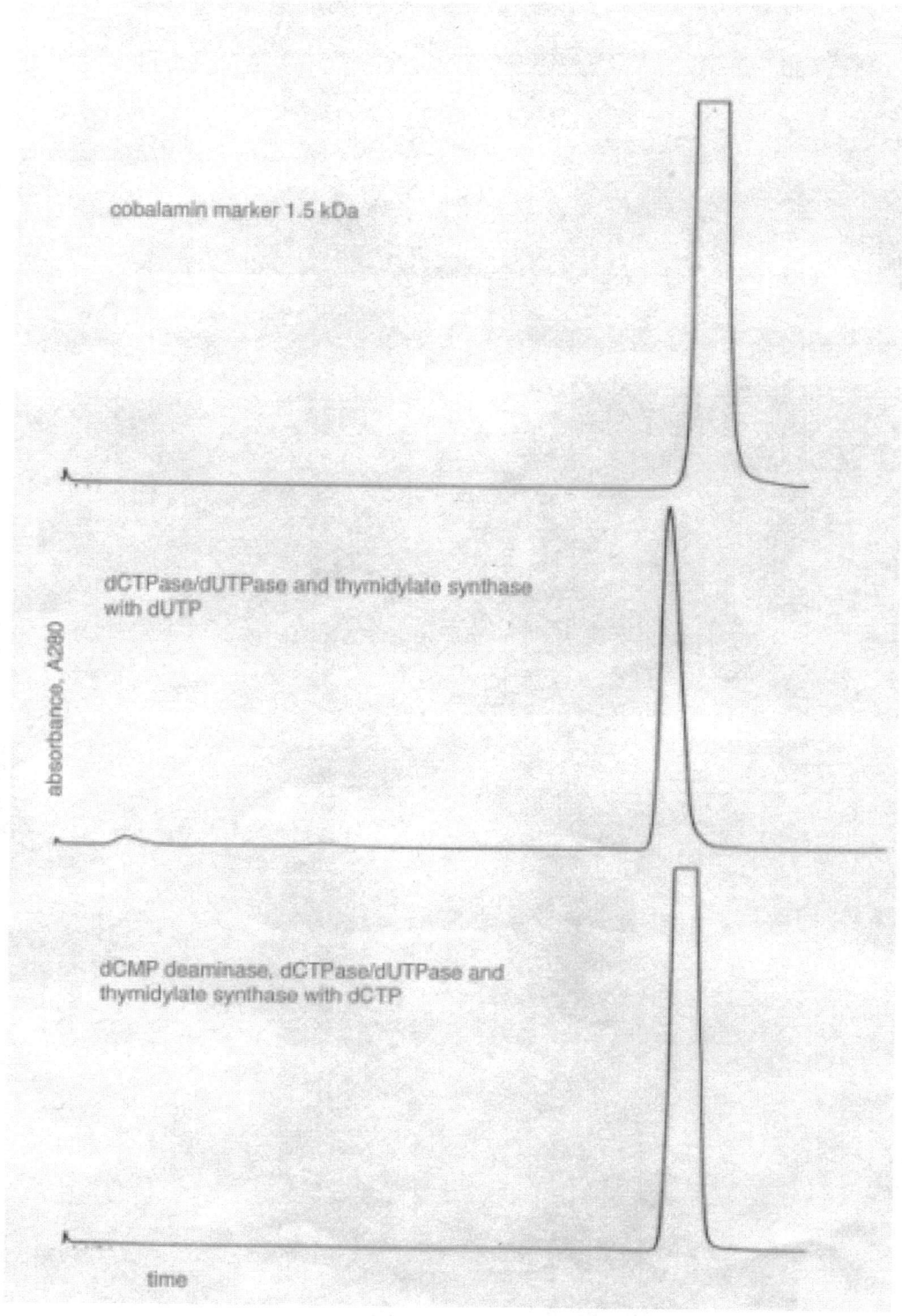

cobalamin marker 1.5 kDa
dCTPase/dUTPase and thymidylate synthase with dUTP
absorbance, A280
dCMP deaminase, dCTPase/dUTPase and thymidylate synthase with dCTP
time

Figure II – 22.

longer in their multimeric or monomeric states. Only an approximate size can be inferred for the fragments of about 5 kDa.The results explained another observation. Following a kinetic assay comparable samples were filtered with a 10 micron nanocep filter to remove protein and the resulting supernatant applied to an HPLC apparatus Mary Lynn Tassotto was using to separate nucleotides. Standards for the expected products of the reaction were prepared and run as well. When the filtered kinetic mixtures were run, numerous additional peaks appeared in retention times appropriate to larger molecules. From both of these observations, it is clear that the proteins break down into smaller fragments whose exact size cannot be precisely determined from this data.

No further attempts were possible because the Superose column collapsed and now runs at about 0.1 ml/min. Prior to the experiments described above, the column was allowed to run dry. Faced with either repacking or trying to resolubilize the system, I chose the latter. To determine the quality of the column, standards were run and compared to manufacturer's literature values. Colored markers also made it possible to visualize flaws in the column as well as the location of the void volume and the end of the separation phase. Attempts to determine the size of the protein fragments on a gel were not successful either. The protein fragments continued to run with the gel marker against standards that included 7.5 kDa as the smallest marker. Determination of actual fragments' size is left to others.

In future studies, investigators must address concentration issues. The amount of material inoculated on the column was far too sparse. The concentration of material used must be greater than what I applied to facilitate the survival of an interaction. In addition, agents that might facilitate crowding or small molecules that are part of the complex might be included to facilitate complex survival.

There is a close physical association between T4 thymidylate synthase and T4 dCMP deaminase that is facilitated by the presence of dCTP. From the mutant deaminase - thymidylate synthase affinity studies, it is possible that one of the zinc ions in dCMP deaminase plays a role in the effectiveness of dCTP. Furthermore, dCMP deaminase has a close physical association with dCTPase/dUTPase. To determine the role of dCTP in that interaction is left to others. From these experiments, it is clear that the small molecule dCTP influences the binding and stability of materials involved in the dNTP synthetase complex. Limited success in an attempt to reconstitute the pyrimidine portion of the complex was achieved. Addressing issues of complex integrity are left to future investigators.

CHAPTER THREE

THE INFLUENCE OF NEAREST NEIGHBORS ON KINETICS

Introduction

The initiation and continuation of DNA replication depends on a dependable supply of the four deoxyribonucleotides. That a connection exists between the replication apparatus and the dNTP synthetase complex will be examined more closely in the next chapter but previous work suggests there is one. In T4, the initial exponential increase in DNA replication is controlled by the kinetics of formation of the dNTPs, apparently at the level of assembly of the dNTP synthetase complex (Greenberg and Hilfinger, 1996). Following infection by T4, a number of events take place very rapidly, including phage mRNA synthesis and protein production. T4 DNA replication is not detected for about five minutes following infection at 30°. Tomich *et al.* conducted an experiment to measure both deoxyribonucleotide synthesis and DNA replication. These investigators introduced a mixture of [5-^{3}H] uridine and [6-^{3}H]uridine into an *E. coli* culture shortly after T4 infection. The labeled uridine became part of the ribonucleotide pool responsible for the production of both pyrimidine ribonucleotides. Eventually, the material was converted into the ribonucleotides UTP and CTP and, following ribonucleotide reductase catalysis, became deoxyribonucleotides which were available to thymidylate synthase and dCMP hyroxymethylase for deoxyribonucleotide precursor synthesis (Tomich *et al.*, 1974). The substrates [5-^{3}H] dUMP and [5-^{3}H] dCMP both were converted to dTMP and HMdCMP respectively releasing 5-^{3}H into the water phase assayed as part of the conversion process. The [6-^{3}H] products retain their radio-label and

proceed to DNA replication. Figure III - 1 shows the results obtained from simultaneous measure of deoxyribonucleotide synthesis and DNA replication *in vivo* (Greenberg and Hilfinger, 1996). Ribonucleotide reductase is the limiting assembler of the dNTP synthetase complex (Greenberg and Hilfinger, 1996). The release of 5-^{3}H into the water is considered to be a tracking device for the formation of the dNTP synthetase complex as governed by ribonucleotide reductase. The experimental findings of these investigators were that the rate of deoxyribonucleotide synthesis and the rate of DNA replication were equal and that curves of initially exponential growth for both began at about five minutes following infection (Greenberg and Hilfinger, 1996). In a separate experiment using chloramphenicol to reduce enzyme concentrations, 4.8 minutes was found to be the lag time before the process began at 30° (see Figure III-2). Clearly, replication and dNTP synthesis are intimately related.

A burst of activity following a delay of about 5 minutes suggests coordinated activity between the enzymes involved. Furthermore, the burst of activity implies rates different from ordinary function. To ascertain if protein associations might influence kinetic behavior, a series of kinetic experiments using proteins known to directly associate with one another in the pyrimidine pathway were investigated in an attempt to gain insight into the workings of the dNTP synthetase complex. These proteins were combined in pairs or small groups and initially studied in a buffer common to the enzyme test for thymidylate synthase. Later, modifications were made so that the same buffer used for the study of protein association was used to study these kinetic reactions.

Figure III-1. Simultaneous initiation *in vivo* of tritium release and incorporation into water from pyrimidine deoxynucleotide synthesis and DNA replication following T4 infection. The curves of the two processes are superimposable. The insert shows the ^{3}H release plotted on a semi-log scale. From Tomich, P., Chiu, C.-C., Wovcha, M.G. and Greenberg, G.R. (1974) *J. Biol. Chem.* **249**:2196 - 2206 [Greenberg and Hilfinger, 1996]).

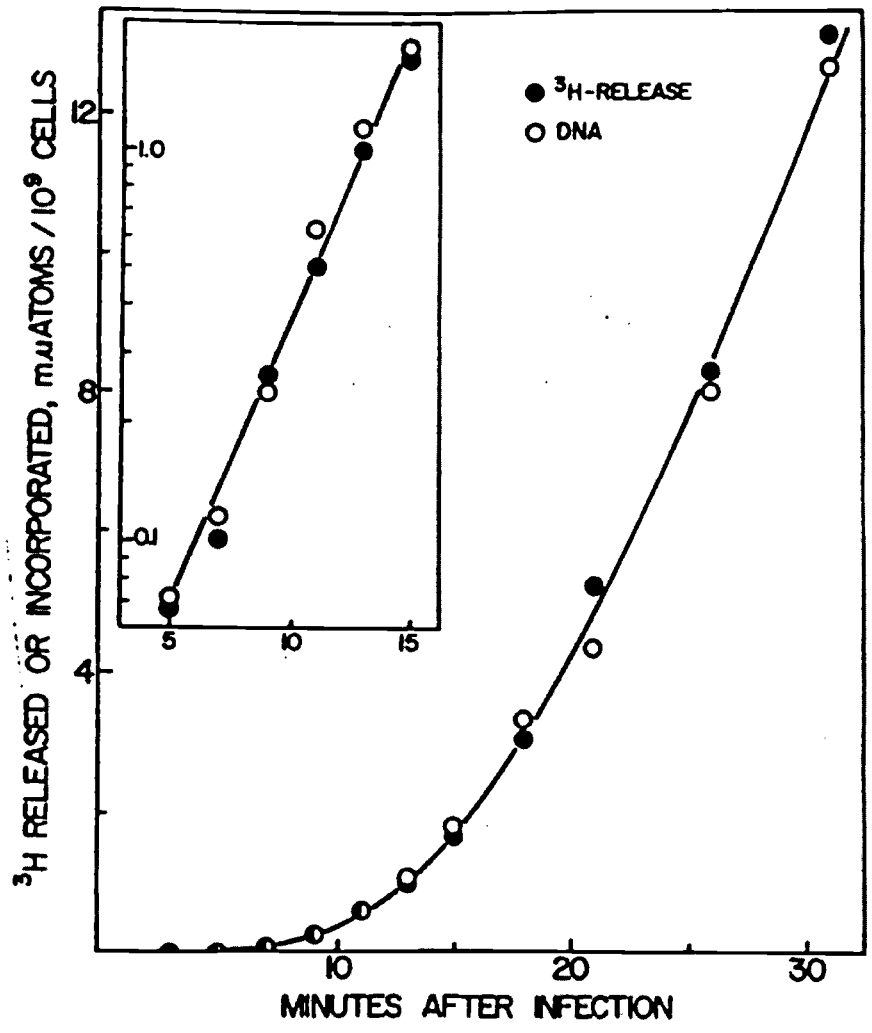

^{3}H-RELEASE
DNA
^{3}H RELEASED OR INCORPORATED, mμATOMS / 10^9 CELLS
MINUTES AFTER INFECTION
1.0
.1
5
10
15
4
8
12
10
20
30

Figure III – 1.

Figure III-2. Time of initiation of T4 DNA replication. At the indicated times following T4 infection of an *E. coli* culture at 30°, chloramphenicol (CM) was added. The insert shows the time of initiation of tritium release (HMdCMP and dTMP synthesis) run in the previous Figure. The time of initiation for the two processes is 4.8 minutes ± 0.5 minutes. From Chiu, C.-S., Tomich, P.K. and Greenberg, G.R.(1976) *Proc. Natl. Acad. Sci., U.S.A.* **73**:757 – 763 [Greenberg and Hilfinger, 1996]).

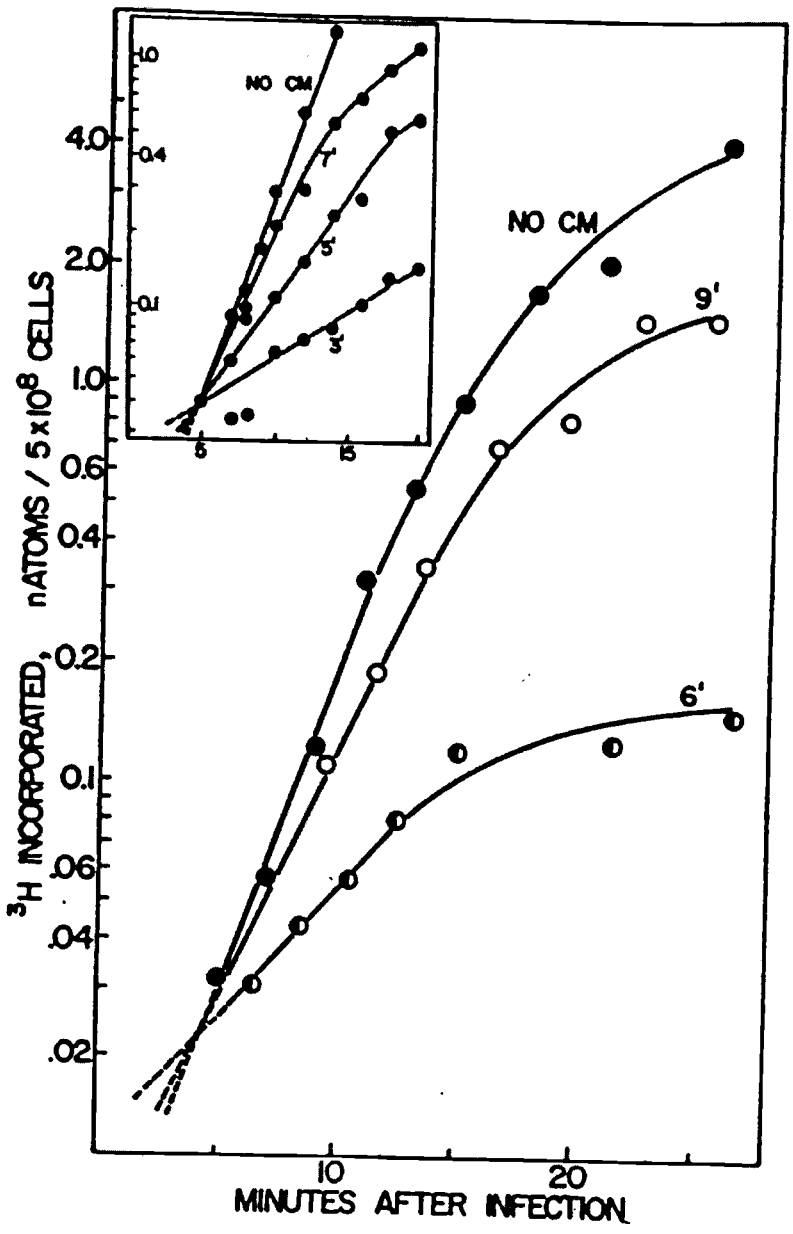

NO CM
9'
6'
NO CM
T'
5'
3'
^{3}H INCORPORATED, nATOMS / 5x10^8 CELLS
MINUTES AFTER INFECTION
4.0
2.0
1.0
0.6
0.4
0.2
0.1
.06
.04
.02
10
20
5
15

Figure III – 2.

Physical Association and Function

The following experiments were undertaken to determine if those enzymes that participate in the pyrimidine pathway and have demonstrated strong physical associations might also demonstrate kinetic influence on one another. The small molecule dCTP is included not only for its importance in influencing the stability of dCMP deaminase but it may also influence the kinetic behavior of other enzymes in the protein complex. The turnover of enzymes involved in the dNTP synthetase complex vary a great deal. In Greenberg and Hilfinger (1996), the authors cite dCTPase/dUTPase as the most rapid in the group but the turnover number for dCMP deaminase is a factor of ten greater than that of dCTPase/dUTPase (see Chapter One). I am intrigued by the notion that 'slow enzymes' like thymidylate synthase are physically associated with dCMP deaminase that exhibits a turnover number two orders of magnitude faster. In addition, thymidylate synthase as an obligate dimer apparently only uses one active site at a time which might also contribute to its apparent slowness. This portion of the thesis will focus on thymidylate synthase and how it is influenced by potential associations with other enzymes in the pyrimidine pathway.

Method of Investigation

The enzymes were purified as described in the preceding chapter and when possible, all the experiments used material from the same preparation for continuity with those preceding experiments. In the process of purification, enzymes are usually tested for activity. Since thymidylate synthase was the central focus of this work, the enzyme assay for thymidylate synthase used in the Mathews' lab became the basis for kinetic comparison. Traditionally, the assay was based on monitoring the production

of dihydrofolate at 338 nm released from methylene tetrahydrofolate. The assay used a buffer composed of freshly prepared 0.5 mM tetrahydrofolate in a stock solution of 0.2 M Tris-Cl (pH 7.4), 0.2 M KCl, 50 mM $MgCl_2$ with 0.2 M beta-mercaptoethanol and 30 mM formaldehyde added to produce methylene tetrahydrofolate. The tetrahydrofolate originally purchased from Sigma was in stock powder aliquoted in small capped vials and stored in the freezer. Previously, the samples were stored in sealed vessels. The new system did not preserve the material as well. In order to make comparisons, all samples were run from a single batch of freshly prepared tetrahydrofolate solution. Comparisons are descriptive. The substrate for thymidylate synthase, dUMP, was a 1.0 mM stock solution prepared from material purchased from Sigma as were the other deoxyribonucleotides. Some of the nucleotides demonstrated contamination from decomposition products following storage. Thymidylate synthase does not react with dUTP when the sample is freshly prepared. Many of these reactions were initially monitored in Tris-Cl buffer because this buffer was the basis for the thymidylate synthase assay. In an effort to examine kinetics under the same conditions as protein-protein interactions, the stock tetrahydrofolate buffer was changed to the Tris-acetate buffer used to test protein-protein interactions in the previous chapter (25 mM Tris-acetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol). The tetrahydrofolate solution also included formaldehyde and additional beta mercaptoethanol as described previously.

Each reaction mixture was composed of water, tetrahydrofolate solution, enzyme(s) and appropriate deoxyribonucleotides to a volume of 1 milliliter. A sample reaction was composed as follows:

390 μL H_20

10 μL enzyme preparation

500 μL tetrahydrofolate solution

100 μL of 1.0 mM deoxyribonucleotide stock solution.

The amount of water and enzyme were varied to attain the volume of 1 mL but the tetrahydrofolate and deoxyribonucleotide volumes remained constant to maintain their concentration in the system. The amount of thymidylate synthase reacting alone with its substrate was selected to be that which produced a linear relationship as seen in Figure III - 3. The same amount of thymidylate synthase was used in subsequent investigations unless otherwise stated. This reaction was used as a basis in all assays for comparison.

Thymidylate Synthase and Deoxynucleotides

Thymidylate synthase has been shown to exhibit Michelis-Menten kinetics when dUMP is used as its substrate. When thymidylate synthase is combined with fresh dUTP, no reaction takes place. Thymidylate synthase is only known to react with its substrate dUMP. Figure III – 3 shows a typical reaction trace for thymidylate synthase in Tris-Cl buffer with its substrate dUMP. The blank at the bottom of the graph does not contain dUMP. Figure III – 4 compares the kinetics of thymidylate synthase in the presence of other deoxyribonucleotides used in this study. Table III –1 presents this data in tabular form.

When dUMP is not available, thymidylate synthase does not enlist another deoxyribonucleotide as substrate. There was some activity detected with dUTP as the substrate but that is most likely from decomposition of the material due to freeze-thaw and contamination of the original substrate because the activity was short-lived.

Figure III - 3. Thymidylate synthase catalysis monitored in Tris - Cl stock buffer. The substrate for thymidylate synthase is dUMP. The assay exhibits a linear segment which culminates in a plateau. The concentration of thymidylate synthase is 5×10^{-8} M for this assay.

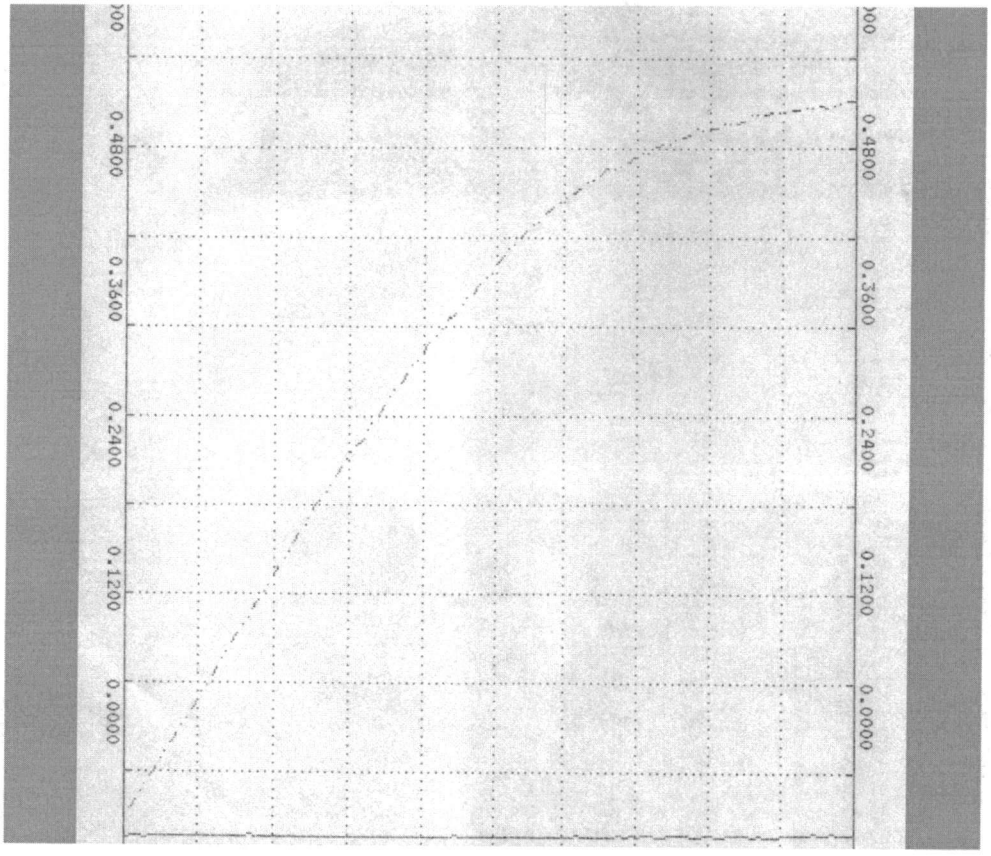


Figure III – 3.

Figure III - 4. Thymidylate synthase catalysis in Tris - acetate stock buffer in the presence of different deoxyribonucleotides. The stock buffer is 25 mM Tris-acetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. The concentration of thymidylate synthase is 5×10^{-8} M.

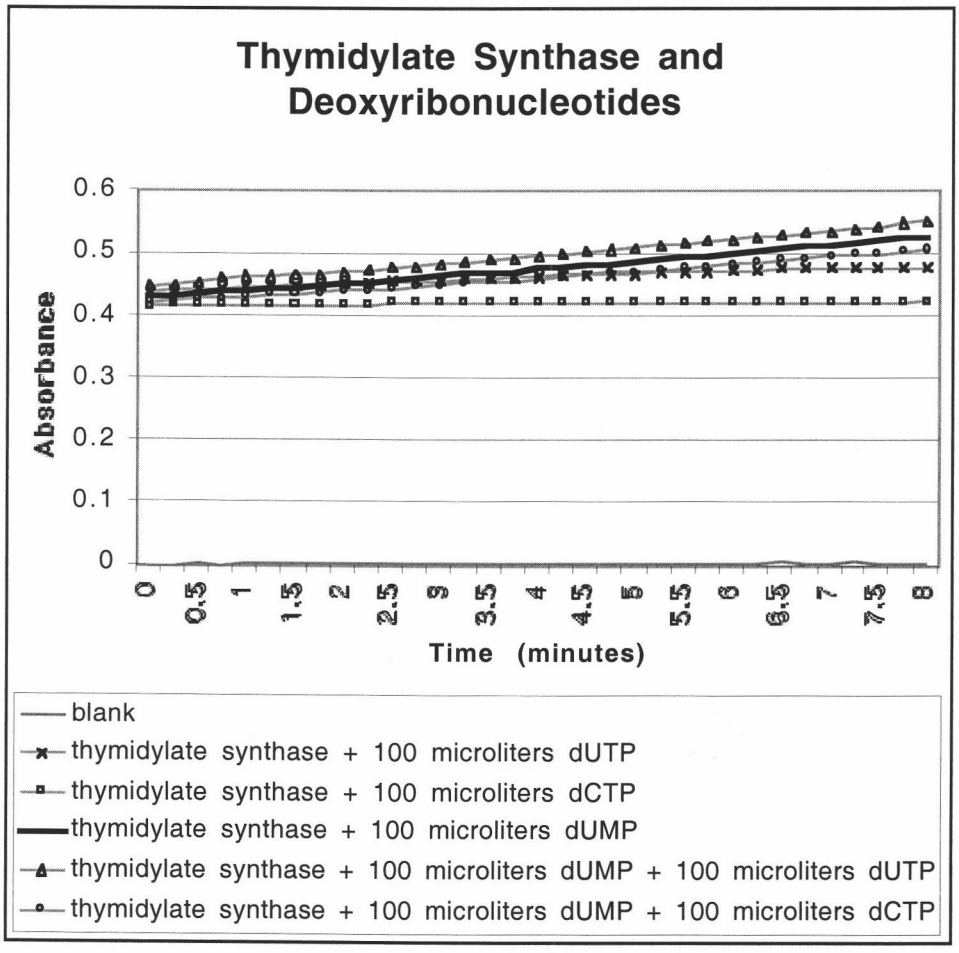

Thymidylate Synthase and Deoxyribonucleotides
Absorbance
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
Time (minutes)
blank
thymidylate synthase + 100 microliters dUTP
thymidylate synthase + 100 microliters dCTP
thymidylate synthase + 100 microliters dUMP
thymidylate synthase + 100 microliters dUMP + 100 microliters dUTP
thymidylate synthase + 100 microliters dUMP + 100 microliters dCTP

Figure III – 4.

Table III – 1. A kinetic comparison of deoxyribonucleotide use by thymidylate synthase. This table summarizes the data presented in the preceding graph by calculating the absorbance change, concentration change and activity for each reaction.

reaction	1	2	3	4	5	6
Δ A/min	0	0	0	.054	.053	.047
Δc μM/min	0	0	0	8.18	8.03	7.12
Activity units/min	0	0	0	114	112	99

1: blank
2: thymidylate synthase and 100 μL dUTP
3: thymidylate synthase and 100 μL dCTP
4: thymidylate synthase and 100 μL dUMP
5: thymidylate synthase and 100 μL dUMP and 100 μL dUTP
6: thymidylate synthase and 100 μL dUTP and 100 μL dCTP

Table III – 1.

No reaction was detected with dCTP alone but the vessel containing it in addition to the substrate dUMP seemed to persist in activity after the others began to slow (data not shown). Thymidylate synthase is known to be negatively controlled by its own product. From these assays, thymidylate synthase uses only dUMP as a substrate. The presence of dUTP does not seem to affect enzymatic function but the presence of dCTP does slightly depress function but it eventually occurs.

Thymidylate Synthase and dCTPase/dUTPase

Thymidylate synthase is influenced by dCTPase/dUTPase in two ways: one is the direct production of dUMP by dCTPase/dUTPase and the other is the release of dCMP, which is used by dCMP deaminase to produce dUMP. Each pathway requires a different substrate for dCTPase/dUTPase but it most likely uses the same site for both functions.

I began to investigate the relationship between thymidylate synthase and dCTPase/dUTPase using the original Tris-Cl buffer. The two enzymes were purified as previously described and the substrate used was dUTP. A previous paper had suggested an assay that used thymidylate synthase as a reporter for dUTPase function as a means for testing the activity without the use of tritium. Figure III –5 shows a typical assay conducted in the Tris-Cl buffer when either thymidylate synthase or dCTPase/dUTPase is in relatively low concentration. It is clear that Michelis-Menten kinetics is not governing the kinetic behavior in this situation due to the lack of a single linear portion of the graph culminating in a plateau. There is an apparent burst at a rapid rate that slows to a slower rate. Table III –2 summarize a number of experiments conducted examining this relationship with the amount of dCTPase/dUTPase held constant. The burst is controlled by thymidylate synthase.

Figure III - 5. Thymidylate synthase catalysis in the presence of varying concentrations of dCTPase/dUTPase in Tris - Cl stock buffer using dUTP as the substrate. The concentration of thymidylate synthase is 5. x 10^{-8} M and the concentration of dCTPase/dUTPase is 0.9 x 10^{-11} M for this particular assay.

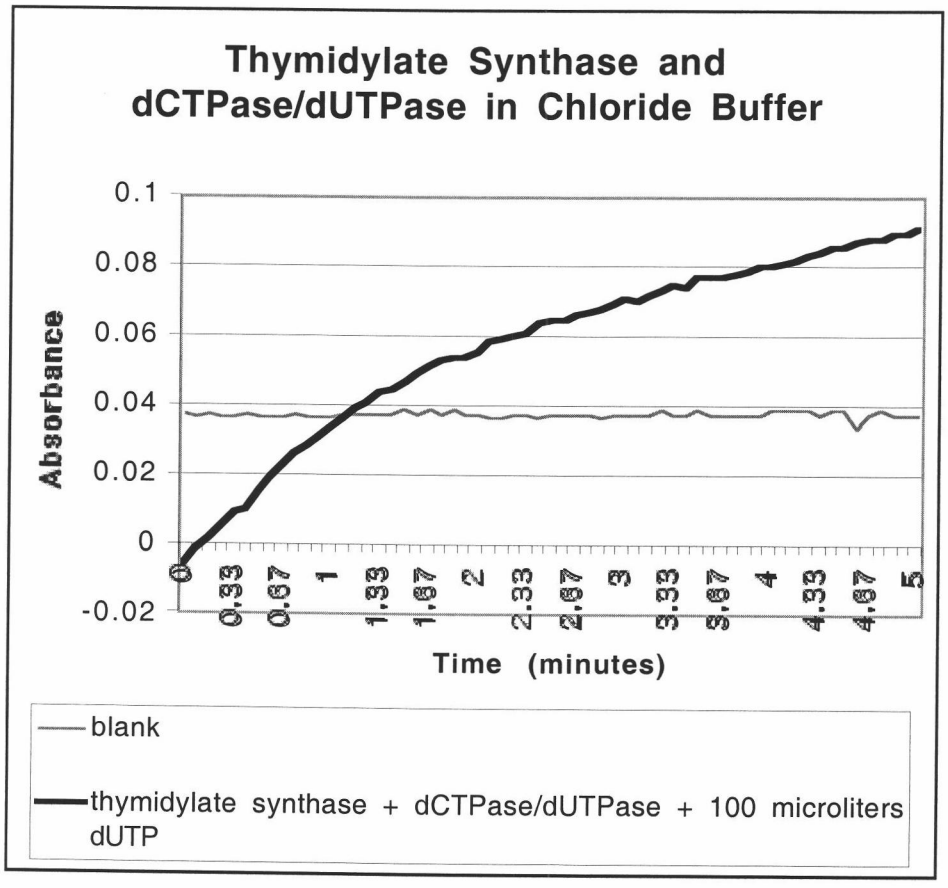

Thymidylate Synthase and dCTPase/dUTPase in Chloride Buffer
Absorbance
0.1
0.08
0.06
0.04
0.02
0
-0.02
0
0.33
0.67
1
1.33
1.67
2
2.33
2.67
3
3.33
3.67
4
4.33
4.67
5
Time (minutes)
blank
thymidylate synthase + dCTPase/dUTPase + 100 microliters dUTP

Figure III – 5.

Table III – 2. A summary of data collected from experiments that behaved like the data presented in Figure III – 5. In these reactions, the amount of dCTPase/dUTPase is held constant at 1.8×10^{-8} M and thymidylate synthase is allowed to vary as described. The slopes are handled separately for each reaction. The activity is reported in terms of thymidylate synthase (ts) and dCTPase/dUTPase (gp56).

Thymidylate synthase	Δ A/ min	Δ c μM/min	Activity (ts) units/min	Activity (gp56) units/min
2.5 x 10^{-8} initial slope	0.046	6.97	91	387
2.5 x 10^{-8} secondary slope	0.032	4.85	63	269
5.1 x 10^{-8} initial slope	0.052	7.88	77	438
5.1 x 10^{-8} secondary slope	0.029	4.39	43	244
7.65 x 10^{-8} initial slope	0.070	10.61	83	589
7.65 x 10^{-8} secondary slope	0.015	2.27	18	126

Table III – 2.

Table III – 3. A summary of data collected from experiments related to the data presented in Figure III – 5. In these reactions, the amount of dCTPase/dUTPase is varied and thymidylate synthase is held constant at 5 x 10^{-8} M. The slopes examined are what were characterized as the secondary slopes in Table III - 2. The activity is reported in terms of thymidylate synthase (ts) and dCTPase/dUTPase (gp56).

dCTPase/ dUTPase M	Δ A/min	Δ c μM/min	Activity (ts) units/min	Activity (gp56) units/min	Activity product
0.9×10^{-8}	0.0115	1.67	33	185	6105
1.8×10^{-8}	0.013	1.97	39	109	4251
2.7×10^{-8}	0.021	3.18	62	118	7316
3.6×10^{-8}	0.0255	3.86	75	107	8025
4.5×10^{-8}	0.029	4.39	86	97	8342
5.4×10^{-8}	0.0325	4.92	96	92	8832
6.3×10^{-8}	0.0375	5.68	111	90	9990
7.2×10^{-8}	0.03	4.54	89	63	5607

Table III – 3.

The secondary phase appears to be controlled by dCTPase/dUTPase. Further data is needed for a more definitive understanding of this second slope. In the next set of experiments the amount of thymidylate synthase was held constant and the amount of dCTPase/dUTPase was varied. The results are reported in Table III – 3. They confirm that dCTPase/dUTPase controlled the second slope of the graph. Single turnover experiments would provide a broader opportunity for understanding this relationship but the commercial availability of dUTP was the limiting factor and these experiments were not performed.

To do a better job of characterizing this interaction, I removed the magnesium ion from the stock buffer and introduced it when the substrate was added. The reasoning behind this decision was that the magnesium ion was essential for the reaction to begin and would allow further control of the initial stages. The magnesium ion and substrate were introduced with a stirring spoon to the cuvette. I was unable to coordinate the activity to obtain good mixing. I returned to my previous method. From the relationship between dCTPase/dUTPase and thymidylate synthase in the chloride buffer, stoichiometry used in other experiments was generally determined. The kinetics for thymidylate synthase is definitely influenced by its association with dCTPase/dUTPase. When either thymidylate synthase or dCTPase/dUTPase are in short supply, a consistent burst phase is seen that exhibits a rate of reaction that is different from thymidylate synthase alone. When the proteins are present in approximately equivalent amounts, the rate is different from that experienced in the rapid burst phase. Experiments were not conducted to see if this relationship persisted in the new acetate buffer system. The potential effects of the presence of other nucleotides is explored in Figure III – 6 and tabulated in Table III – 4. The relationship between thymidylate synthase and dCTPase/dUTPase is slightly

inhibited by dCTP. The relationship between the two proteins appears to be one of reciprocity.

Within the pyrimidine deoxynucleotide preparation, synthesis is modulated to produce dTMP and HMdCMP in a ratio of 2.1:1 which reflects the average occurrence of these deoxynucleotides in T4 DNA. The ratio seems to be an intrinsic property of the enzymes associated in the dNTP synthetase complex (Greenberg and Hilfinger, 1996). The ratio also seems to be influenced by the rate of formation of the substrates for thymidylate synthase and dCMP hydroxymethylase, dUMP and dCMP respectively which could be accomplished by dCMP deaminase. It is known that dCMP deaminase is negatively effected by dTTP and positively effected by HMdCTP. The major role of dCMP deaminase can be demonstrated on infection by using a mutant in the *cd* gene. The ratio reported earlier drops to 0.6:1 and the rates change as in the study in Figure III - 7. dCTPase/dUTPase has a very high turnover number but as the above studies indicate, it has a significant role in the interaction with thymidylate synthase. To independently determine if the physical interaction of dCMP deaminase and thymidylate synthase influenced their kinetic behavior, their relationship is examined next.

Figure III - 6. Thymidylate synthase catalysis in the presence of dCTPase/dUTPase in Tris - acetate buffer using dUTP as the substrate. The stock buffer is 25 mM Tris-actetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. The concentration of thymidylate synthase is 5×10^{-8} M and the amount of dCTPase/dUTPase varies around a similar concentration.

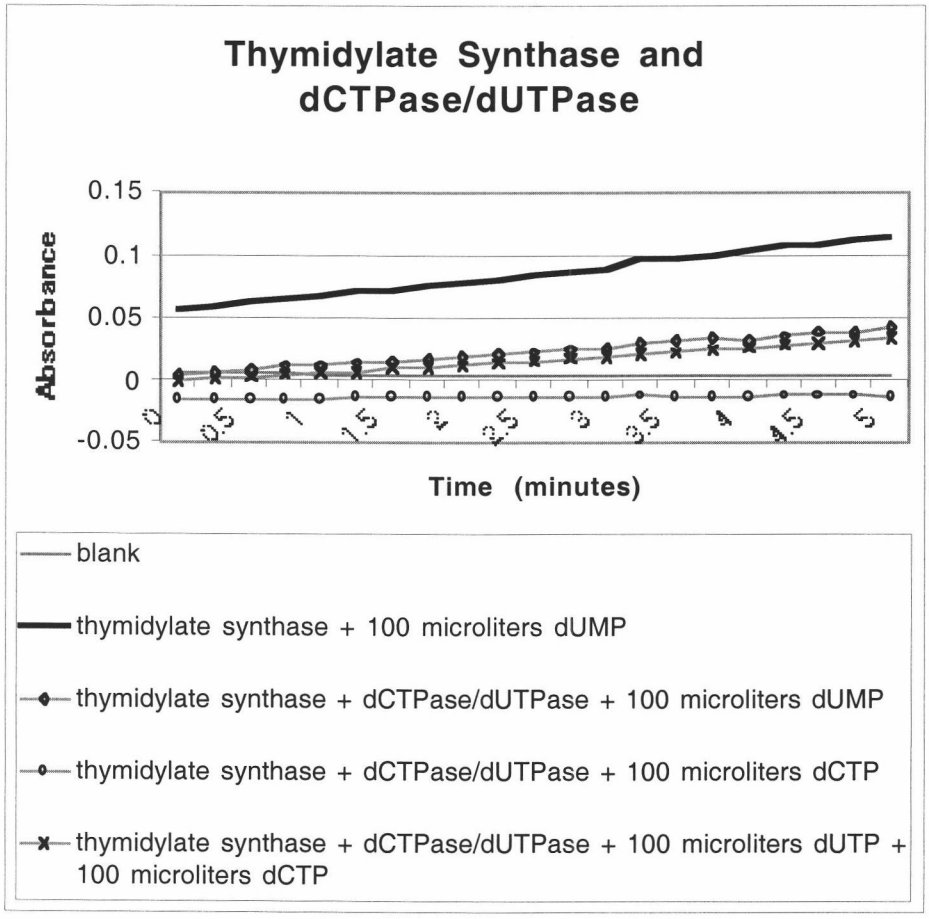

Thymidylate Synthase and dCTPase/dUTPase
Absorbance
0.15
0.1
0.05
0
-0.05
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Time (minutes)
blank
thymidylate synthase + 100 microliters dUMP
thymidylate synthase + dCTPase/dUTPase + 100 microliters dUMP
thymidylate synthase + dCTPase/dUTPase + 100 microliters dCTP
thymidylate synthase + dCTPase/dUTPase + 100 microliters dUTP + 100 microliters dCTP

Figure III – 6.

Table III – 4. A kinetic comparison of deoxyribonucleotide use by thymidylate synthase and dCTPase/dUTPase. This table summarizes the data presented in the preceding graph by calculating the absorbance change, concentration change and activity for each reaction.

reaction	1	2	3	4	5
Δ A/min	0	0.011	0.0075	0.001	0.0065
Δc μM/min	0	1.67	1.14	0.15	0.98
Activity (ts) units/min	0	23	16	2	13.6
Relative Activity (ts)	-	1	0.72	0.08	0.59

1: blank
2: thymidylate synthase and 100 μL dUMP
3: thymidylate synthase, dCTPase/dUTPase and 100 μL dUTP
4: thymidylate synthase, dCTPase/dUTPase and 100 μL dCTP
5: thymidylate synthase, dCTPase/dUTPase and 100 μL dUTP and 100 μL dCTP

Table III – 4.

Figure III - 7. Synthesis of pyrimidine deoxynucleotides from UDP and CDP along with replication of DNA following infection with bacteriophage T4. The labeled precursor is [5 - ^{3}H]uridine. The figure shows the sites of 3H release into the aqueous phase. The numbers are calculated relative rates at each step of the wild - type infection. Figures in parentheses are the values obtained following infection with phage containing mutant *cd* so is lacking dCMP deaminase. The ratios of dTMP to HMdCMP were determined by simultaneous labeling with [6- ^{3}H]uracil which give identical results to uridine label (Greenberg and Hilfinger, 1996).

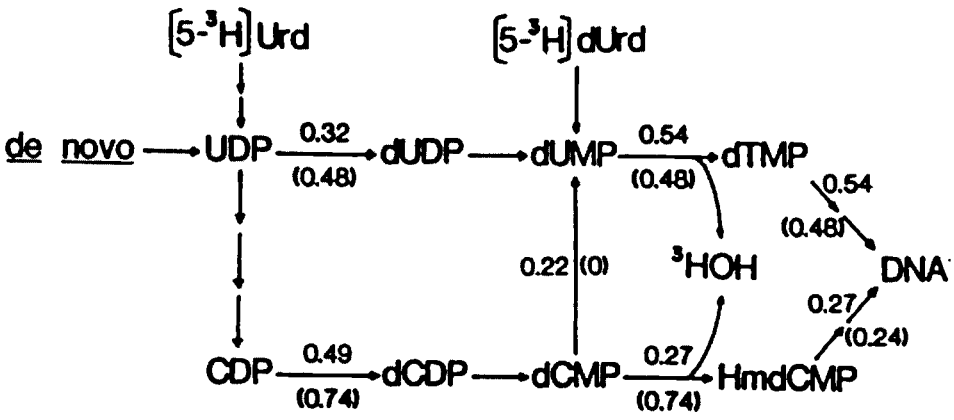

[5-3H]Urd
[5-3H]dUrd
de novo
UDP
0.32
(0.48)
dUDP
dUMP
0.54
(0.48)
dTMP
0.54
(0.48)
0.22 (0)
3HOH
DNA
0.27
(0.24)
CDP
0.49
(0.74)
dCDP
dCMP
0.27
(0.74)
HmdCMP

Figure III – 7.

Thymidylate Synthase and dCMP deaminase

Figure III – 8 reflects a different type of relationship than that between thymidylate synthase and dCTPase/dUTPase previously described because the dCMP deaminase must remove an amino group rather than phosphates. The curve's general shape follows that usually exhibited by thymidylate synthase alone. The data is tabulated in Table III – 5. All of these cuvettes might have completed the early stage of their reaction before the system began to record. The turnover number for dCMP deaminase is two orders of magnitude greater than thymidylate synthase. I was not successful in diluting either material sufficiently to observe other than approximately linear behavior. It seemed that is was possible for thymidylate synthase to react with its usual substrate in the presence of dCMP deaminase. From their behavior there is no way to determine if the proteins are associating. It is apparently possible for the substrate dUMP to reach thymidylate synthase in the presence of dCMP deaminase and react with a rate slightly faster than thymidylate synthase alone. Even with duplication, I cannot tell if the difference is significant or not. It is clear, however, that the presence of dCTP supports the kinetics of this reaction and might have exhibited an earlier burst phase because of the crossing over behavior and curvature at the beginning of some recordings. Without information about the stoichiometry of these two reactants, I was not able to make plans for the single turnover experiments that could describe if channeling was taking place. Dilution experiments should be done for both dCMP deaminase and thymidylate synthase to see if other than linear behavior is observed. Some of the rates are different from one another sufficiently that further experimentation might yield non-parallel data. I went on to test larger combinations to see if the effect seen on thymidylate synthase by dCTPase/dUTPase could be detected.

Figure III - 8. Thymidylate synthase catalysis when combined with dCMP deaminase in Tris - acetate buffer using substrates as indicated. The stock buffer is 25 mM Tris-actetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. Approximately equivalent amounts are used for each enzyme.

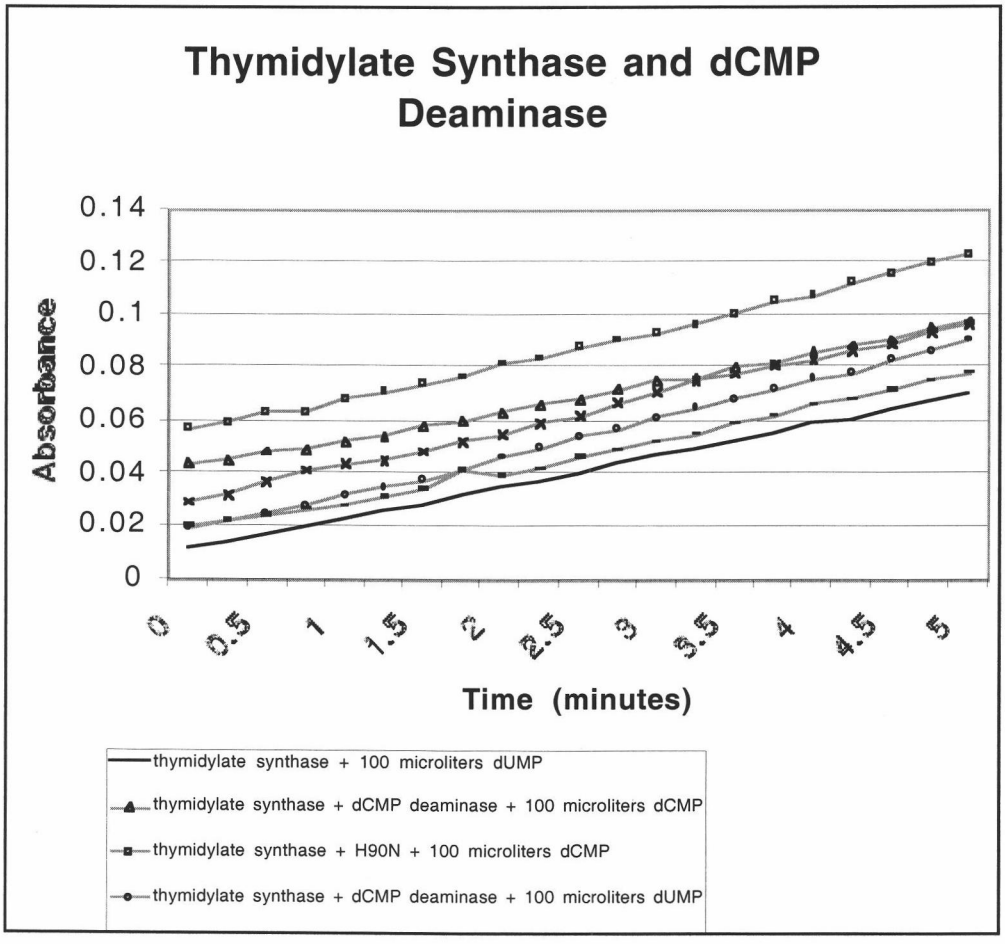

Thymidylate Synthase and dCMP Deaminase
Absorbance
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Time (minutes)
thymidylate synthase + 100 microliters dUMP
thymidylate synthase + dCMP deaminase + 100 microliters dCMP
thymidylate synthase + H90N + 100 microliters dCMP
thymidylate synthase + dCMP deaminase + 100 microliters dUMP

Figure III – 8.

Table III – 5. A kinetic comparison with thymidylate synthase and dCMP deaminase. This table summarizes the data presented in the preceding graph by calculating the absorbance change, concentration change and activity for each reaction.

reaction	1	2	3	4	5	6
Δ A/min	0.012	0.011	0.014	0.014	0.012	0.011
Δc μM/min	1.82	1.67	2.12	2.12	1.82	1.67
Activity (ts) units/min	25	23	29	29	25	23
Relative Activity (ts)	1	0.92	1.16	1.16	1	0.92

1: thymidylate synthase and 100 μL dUMP
2: thymidylate synthase, dCMP deaminase and 100 μL dCMP
3: thymidylate synthase, H90N and 100 μL dCMP
4: thymidylate synthase, dCMP deaminase and 100 μL dUMP
5: thymidylate synthase, dCMP deaminase and 100 μL dCMP and 100 μL dCTP
6: thymidylate synthase, dCMP deaminase and 100 μL dCMP and 100 μL dUTP

Table III – 5.

Thymidylate Synthase, dCMP Deaminase and dCTPase/dUTPase

Thymidylate synthase, dCMP deaminase and dCTPase/dUTPase were combined to see if any information could be obtained about the relationship of these proteins to one another in terms of the relative amount of dTMP that is generated. Figure III – 9 and Table III –6 contain the data and it well might be the case that the data began to be collected after the initial phases of the reaction were complete. The change in absrobance is small and the trace for the vessel containing dCTP looks like a plateau behavior for the three enzymes together. The crossing over behavior of the vessel containing dUTP could also be taken as behavior indicative of a plateau. The turnover numbers for both dCMP deaminase and dCTPase/dUTPase are significantly greater than thymidylate synthase alone. The graphs are taken as affirmation that, together, these enzymes behave differently than thymidylate synthase alone. The reaction appears to be inhibited slightly in the presence of dUTP reflecting the preference of dCTPase/dUTPase for dUTP over dCTP in attempting to process both substrates at the same time. There is curvature at the beginning of both traces suggesting that a burst phase might have taken place and not have been recorded. It is certainly an indication that a change is taking place. This is clear evidence that an interaction is taking place and influencing the kinetics of the system.

Figure III - 9. Thymidylate synthase catalysis when combined with dCMP deaminase and dCTPase/dUTPase in Tris - acetate buffer using substrates as indicated. The stock buffer is 25 mM Tris-actetate (pH 7.5),150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol.

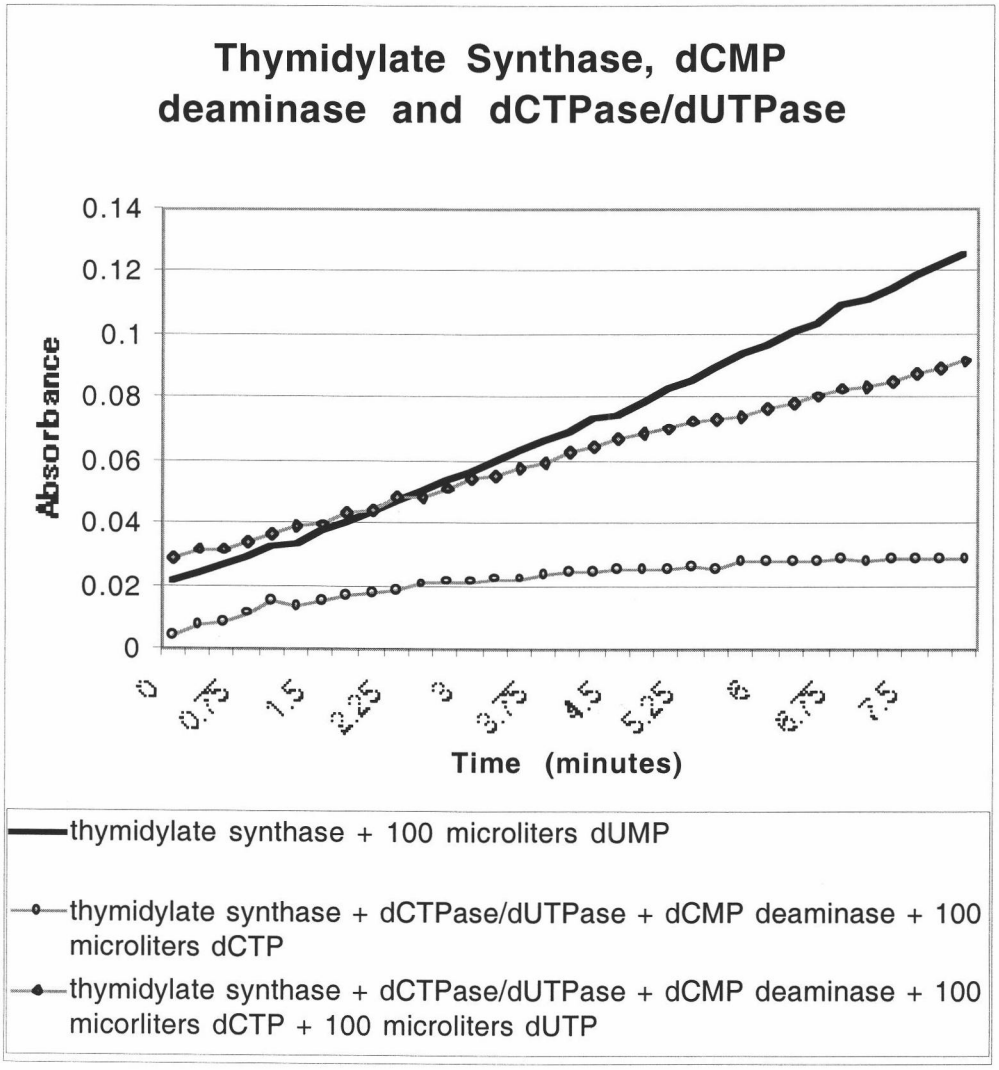

Thymidylate Synthase, dCMP deaminase and dCTPase/dUTPase
Absorbance
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Time (minutes)
thymidylate synthase + 100 microliters dUMP
thymidylate synthase + dCTPase/dUTPase + dCMP deaminase + 100 microliters dCTP
thymidylate synthase + dCTPase/dUTPase + dCMP deaminase + 100 micorliters dCTP + 100 microliters dUTP

Figure III – 9.

Table III – 6. A kinetic comparison with thymidylate synthase, dCMP deaminase and dCTPase/dUTPase. This table summarizes the data presented in the preceding graph by calculating the absorbance change, concentration change and activity for each reaction.

reaction	1 initial	1 later	2 initial	2 later	3 initial	3 later
Δ A/min	0.05	0.003	0.06	0.002	.0.08	0..004
Δc μM/min	7.58	0.45	9.09	0.30	12.12	0.61
Activity (ts) units/min	105	6.3	126	4.2	168	8.4
Relative Activity (ts)	1	1	1.2	.67	1.6	1.3

1: thymidylate synthase and 100 μL dUMP
2: thymidylate synthase, dCMP deaminase, dCTPase/dUTPase and 100 μL dCTP
3: thymidylate synthase, dCMP deaminase, dCTPase/dUTPase, 100 μL dCTP and 100 μL dUTP

Table III – 6.

Thymidylate Synthase, dCMP Deaminase, dCTPase/dUTPase and dCMP Hydroxymethylase

In this series of experiments thymidylate synthase, dCMP deaminase, dCTPase/dUTPase and dCMP hydroxymethylase are combined representing the collection of enzymes that might be associated with one another reflecting the fate of dCTP. Figure III – 10 shows the graphs for these kinetic interactions and Table III – 7 tabulates the data. The relative rate closer to the origin is greater than that for a later time but there is so little data an approximation cannot be made. It appears that the system with only dCTP has already reached or it near its plateau. The system that contains dUTP in addition to dCTP is somewhat behind indicated by the crossing over behavior of the traces. This behavior would support the premise that dUTP has a priority over dCTP with dCTPase/dUTPase. From these reactions one can see that the combined systems are near plateau behavior for the same amount or greater substrate than thymidylate synthase. Further experiments are definitely required. Finding concentrations that might indicate the relation of the enzymes must be determined so the range of protein concentration for exploration in single turnover experiments can be ascertained.

Figure III - 10. Thymidylate synthase catalysis when combined with dCMP deaminase, dCTPase/dUTPase and dCMP hydroxymethylase in Tris - acetate buffer using substrates as indicated. The stock buffer is 25 mM Tris-actetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol.

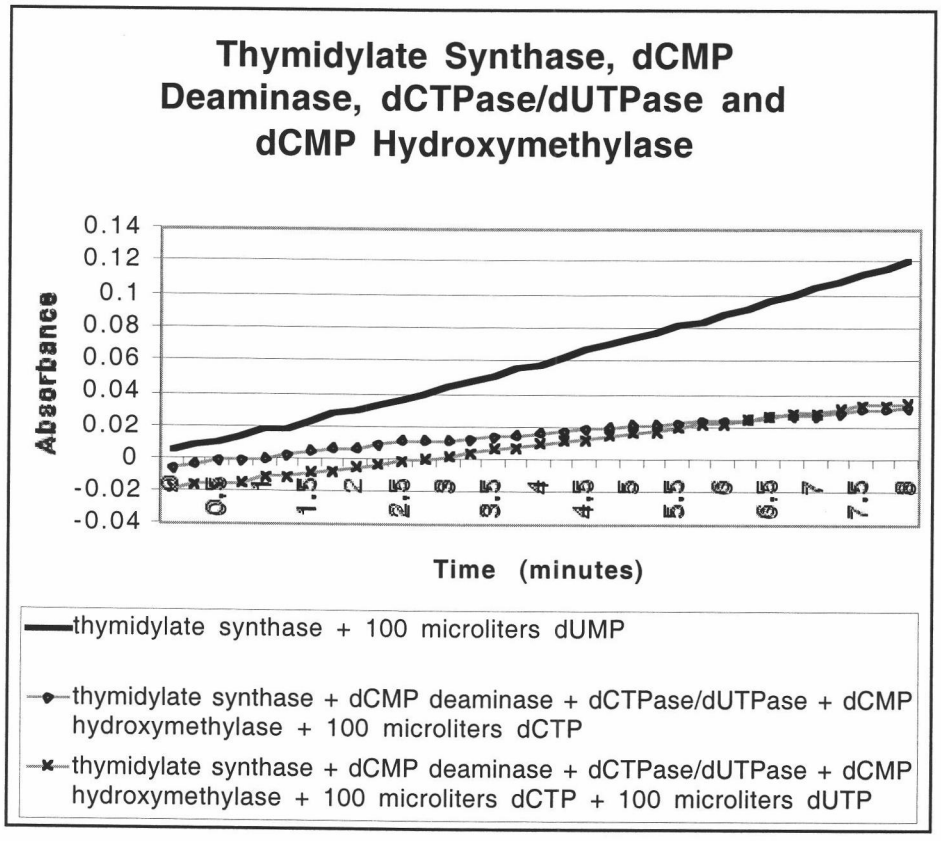


Figure III – 10.

Table III – 7. A kinetic comparison with thymidylate synthase, dCMP deaminase, dCTPase/dUTPase and dCMP hydroxymethylase. This table summarizes the data presented in the preceding graph by calculating the absorbance change, concentration change and activity for each reaction.

reaction	1	2	3
Δ A/min	0.014	0.005	0.007
Δc μM/min	2.12	0.75	1.06
Activity (ts) units/min	29	10.5	14.7
Relative Activity (ts)	1	0.36	0.59

1: thymidylate synthase and 100 μL dUMP
2: thymidylate synthase, dCMP deaminase, dCTPase/dUTPase, dCMP hydroxymethylase and 100 μL dCTP
3: thymidylate synthase, dCMP deaminase, dCTPase/dUTPase, dCMP hydroxymethylase and 100 μL dCTP and 100 μL dUTP

Table III – 7.

Although it can be said that the close physical association of enzymes influences their kinetics as well, the actual relationship remains elusive. The kinetics respond to changes in substrate as well as changes in enzymes being associated. There are at least two factors influencing the kinetics of these reaction: 1.) the presence of other enzymes and 2.) the presence of dCTP. Neither of these factors are obligatory to enzyme function like the presence of magnesium ion is obligatory to enzyme function. Both factors might change the conformation of the enzyme making conferring a different rate. The influence of dCTP is not unknown in the T4 system. HMdCTP is known to act as a positive effector for T4 ribonucleotide reductase at a concentration of 100 μM (Berglund, O., 1972 a, 1972 b) and its influence on dCMP deaminase is well documented. It might play an organizing role in the dNTP synthetase complex as well.

CHAPTER FOUR

A CONNECTION TO REPLICATION

Previous Work

In the first chapter, there was a brief discussion concerning the relationship between the dNTP synthetase complex and the replication process. In summary, of the eight enzymes thought to be part of the T4 dNTP synthetase complex tested in the Mathews' lab using two-dimensional gel analysis, each included T4 single-stranded DNA binding protein in the collection of proteins eluted with medium salt concentration from the affinity column under consideration. Furthermore, when a similar process was carried out using T4 single-stranded DNA binding protein as the affinity ligand, a number of these same proteins were eluted by the same medium salt concentration. With this in mind, T4 single-stranded DNA binding protein was applied to T4 thymidylate synthase in the potassium glutamate buffer system and was eluted at the 0.6 M NaCl level (Wheeler *et al.*, 1999) demonstrating a strong positive relationship.

In 1993, Hurley and his co-workers suggested that the portion of the T4 single-stranded DNA binding protein experiencing all protein - protein interactions were the last approximately 80 residues on the carboxy terminus of the protein. These researchers demonstrated a number of interactions using antibodies and affinity column chromatography with this region but only a sample in the 0.2 M salt elution was potentially attributed to thymidylate synthase. To understand the relationship of thymidylate synthase to the carboxy terminus of T4 single-stranded DNA binding protein further investigations were required.

Further Investigation

Recombinant T4 single-stranded DNA binding protein was purified as reported previously (Wheeler *et al.*, 1999). The recombinant form for the carboxy terminal fragment of T4 single-stranded DNA binding protein was purified according to the method of Hurley *et al.* (1993) with the modification that following the clarification of the protein extract after cell lysis, the extract was brought to a boil and reclarified. The resulting extract was then sized to isolate the carboxy terminal fragment.

Figure IV - 1 and IV -2 show the interaction with for the intact wild type T4 single-stranded DNA binding protein and the carboxy terminal fragment of the same protein with thymidylate synthase respectively in the Tris - acetate buffer system. These interactions had been studied earlier in other buffer systems and in order to compare them with the current work they were studied. The full - length protein was released in the 0.2 M to 0.6 M salt fraction but the bulk of the carboxy terminal fragment was eluted in the column washes. Two potential explanations include that the carboxy terminal fragment was not able to fold correctly or stay folded for the interaction to take place. The fragment is under 10-kDa in size even though it runs as a larger protein during electrophoresis suggesting a non-globular structure. The second possibility is that the carboxy terminal portion is not responsible for the interaction with thymidylate synthase. For all the proteins that are known to associate with T4 single-stranded DNA binding protein to bind to the carboxy terminal portion of the protein, it would be necessary for the region to be significantly larger. In the Tiris-acetate buffer system, the previously observed interaction between T4 single-stranded DNA binding protein and thymidylate synthase persisted.

Figure IV - 1. Retention of purified T4 single-stranded DNA binding protein on an immobilized T4 thymidylate synthase column. This gel is a 12.5% polyacrylamide gel visualized by silver staining. One mg of single-stranded DNA binding protein was applied to the column. The column buffer is 25 mM Tris-actetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. Lane 1: molecular weight marker (MW); Lane 2: after removal of the circulating suspension, column buffer wash is applied (ft1); Lane 3: column buffer wash (ft2); Lane 4: column buffer wash (ft3); Lane 5: column wash buffer plus 0.2 M NaCl (A); Lane 6: column buffer plus 0.6 M NaCl (B) and Lane 7: column buffer plus 2.0 M NaCl (C).

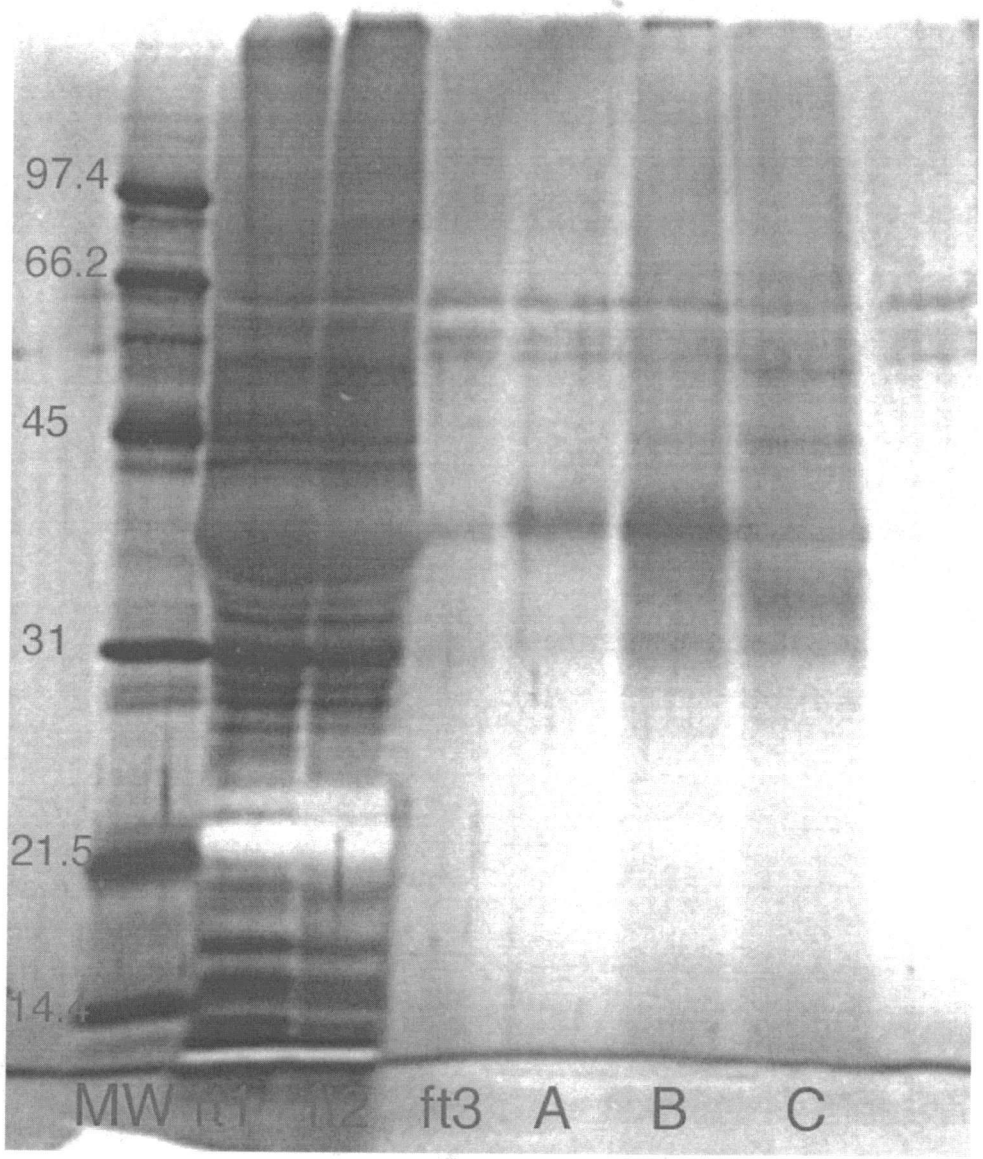


Figure IV – 1.

Figure IV - 2. Retention of purified carboxy terminal portion of T4 single-stranded DNA binding protein on an immobilized T4 thymidylate synthase column. This gel is a 12.5% polyacrylamide gel visualized by silver staining. One mg of sample was applied to the column. The column buffer is 25 mM Tris-actetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol. Lane 1: molecular weight marker; Lane 2: overnight (applied material) (o); Lane 3: column buffer wash (ft1); Lane 4: column wash buffer (ft2); Lane 5: column buffer wash (ft3); Lane 6: column buffer plus 0.2 M NaCl (A); Lane 7: column buffer plus 0.6 M NaCl (B); Lane 8: column buffer plus 2.0 M NaCl (C).

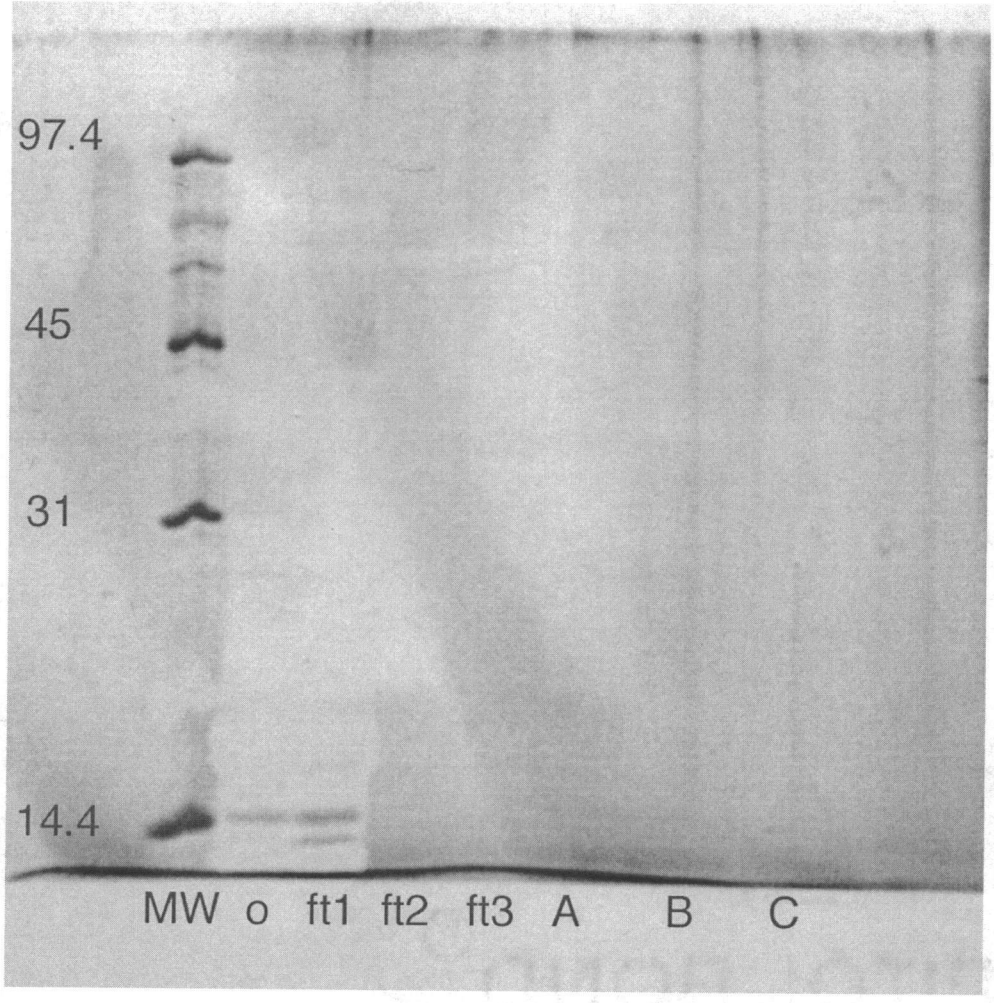


Figure IV – 2.

When a strong physical association was observed between T4 thymidylate synthase and T4 single-stranded DNA binding protein, it was included in the initial kinetic studies in Tris-Cl buffer. The experiments monitored the interaction under conditions of varying thymidylate synthase concentrations and constant single-stranded DNA biding protein concentrations as well as constant thymidylate synthase concentrations and varying single-stranded DNA binding protein concentrations. Table IV – 1 summarizes the data obtained. It was clear that the kinetics of thymidylate synthase were not influenced by the presence of T4 single-stranded DNA binding protein.

Since a strong physical interaction persists for the full length single-stranded DNA binding protein in Tris-acetate buffer, the kinetics of thymidylate synthase and T4 single-stranded DNA binding protein were monitored in the presence of the full length protein. Figure IV - 3 shows the reaction of thymidylate synthase with and without full length single-stranded DNA binding protein present in the Tris-acetate buffer system. Additional experiment monitors the effect of dCTP on the interaction. These results are summarized in Table IV – 2 and are in significant conflict with the previous observations made in Tris-Cl buffer.

The reaction rates for thymidylate synthase in the presence of single-stranded DNA binding protein in Tris-acetate are consistently different from that for thymidylate synthase alone. The presence of dCTP may reduce the rate because it could act as a competitive inhibitor for the dUMP site of thymidylate synthase. The effect is much more pronounced than for thymidylate synthase alone. Small molecules like these have few potential attachment sites within the enzyme and many of these sites are likely in common for dUMP and dCTP.

Table IV – 1. A summary of data collected during experiments between T4 thymidylate synthase and T4 single-stranded DNA binding protein in Tris-Cl buffer. In reactions summarized in the top of the table, the amount of thymidylate synthase is varied and the amount of single-stranded DNA binding protein is held constant at 7 x 10^{-8} M. In reactions summarized in the lower portion of the table, the amount of thymidylate synthase is held constant at 5 x 10^{-8} M and the amount of single-stranded DNA binding protein is varied.

Single-stranded DNA binding protein M	Δ A/min	Δ c μM/min	Activity (ts) units/min
3.5×10^{-8}	0.069	10.45	205
7×10^{-8}	0.0685	10.38	203
10.5×10^{-8}	0.074	11.21	220
17.5×10^{-8}	0.073	11.06	217
4.5×10^{-8}	0.085	12,88	252
7.5×10^{-8}	0.103	15.61	204
10×10^{-8}	0.128	19.39	190
12.5×10^{-8}	0.160	24.24	190

Table IV – 1.

Figure IV - 3. Kinetics of T4 thymidylate synthase in the presence of T4 single-stranded DNA binding protein in Tris-acetate buffer. The reaction blank is found along the bottom of the graph. The stock buffer is 25 mM Tris-actetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 10 mM β-mercaptoethanol.

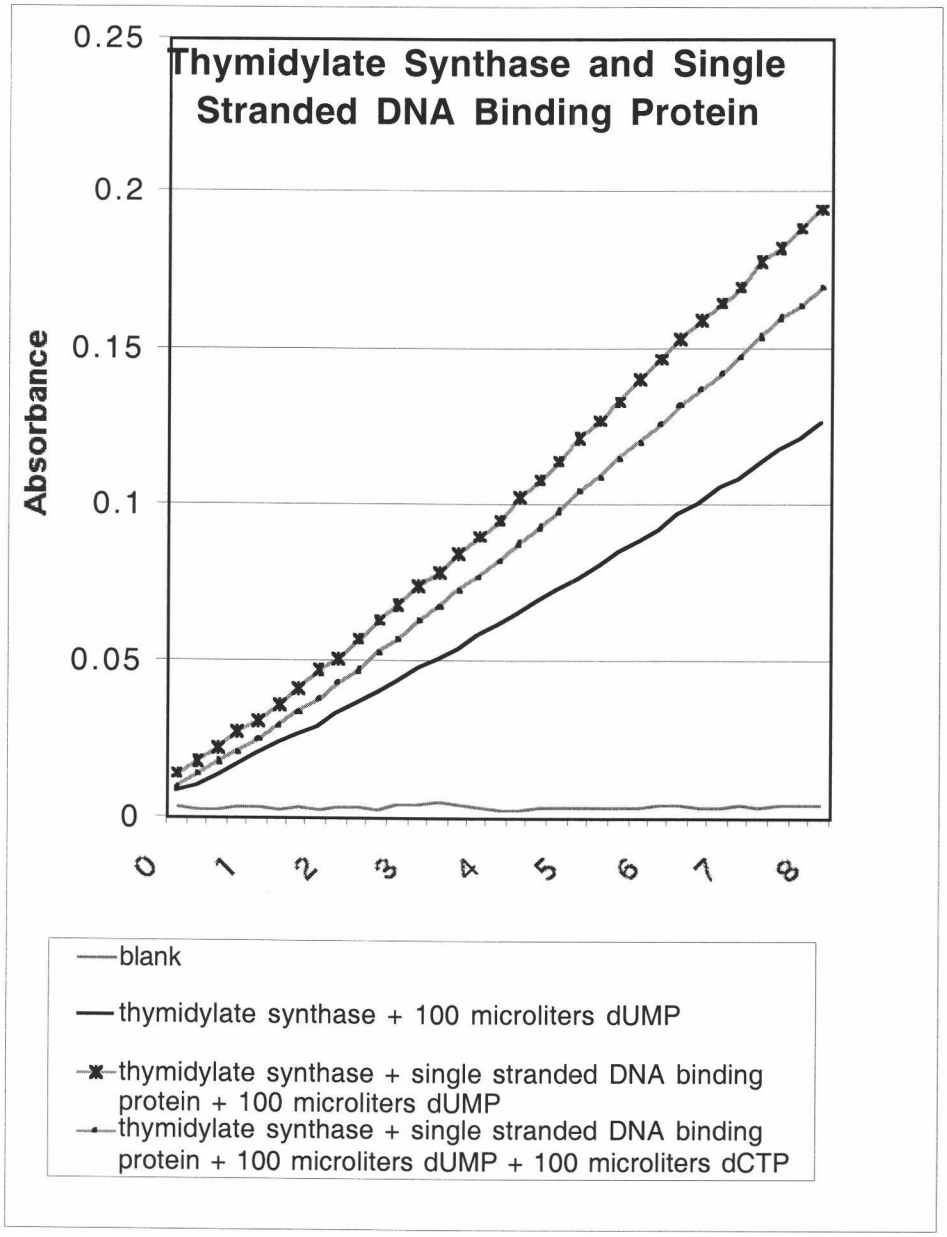

Thymidylate Synthase and Single Stranded DNA Binding Protein
Absorbance
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
6
7
8
blank
thymidylate synthase + 100 microliters dUMP
thymidylate synthase + single stranded DNA binding protein + 100 microliters dUMP
thymidylate synthase + single stranded DNA binding protein + 100 microliters dUMP + 100 microliters dCTP

Figure IV – 3.

Table IV – 2. A summary of data collected during experiments between T4 thymidylate synthase and T4 single-stranded DNA binding protein in Tris-acetate buffer. The interaction is also monitored in the presence of dCTP in the system.

	Δ A/min	Δ c μM/min	Activity (ts) units/min	Relative Activity (ts)
1	0	0	0	0
2	0.042	6.36	88	1
3	0.066	10	139	1.58
4	0.058	8.79	122	1.39

1: blank
2: thymidylate synthase and 100μL dUMP
3: thymidylate synthase, single-stranded DNA binding protein and 100μL dUMP
4: thymidylate synthase, single-stranded DNA binding protein, 100μL dCTP and 100μL dUMP

Table IV – 2.

Clearly, the rate of T4 thymidylate synthase is affected by the presence of T4 single-stranded DNA binding protein. It should be determined if T4 single-stranded DNA binding protein is bound to any single-stranded DNA under these circumstances. The binding assays that characterized T4 single-stranded DNA binding protein initially would be relevant. The protein could have acquired single-stranded DNA in the purification process because the medium used for purification is known to leach single-stranded DNA. Another support that uses single-stranded DNA is cross-linked so that the DNA would not be released with the protein in the purification process. If there is bound DNA, it may act as an activator to change the conformation of single-stranded DNA binding protein so that the conformation changes thymidylate synthase when they interact. It is also well documented that the chloride buffer may have interfered with the binding of single-stranded DNA. The concentration dependence would need to be determined. These concerns are left for future investigators.

Close physical associations have been determined within the pyrimindine portion of the dNTP synthetase complex and between thymidylate synthase of that complex and single-stranded DNA binding protein of the replication complex. Small molecules like dCTP affect these associations and the resulting enzymatic activity.

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