

AN ABSTRACT OF THE THESIS OF

Rainer K. Warth for the degree of Master of Science in Biochemistry and Biophysics presented on August 31, 1993

Title: Large Subunit of Vaccinia Virus Ribonucleotide Reductase: Affinity Chromatography-based Purification and Photoaffinity Labeling

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

Ribonucleoside diphosphate reductase (RR) from vaccinia virus was recently cloned and overexpressed in *Escherichia coli*. The amino acid sequence identities of the small and large subunits between the mouse and the vaccinia virus reductase are approximately 80 and 72 percent, respectively. In addition, vaccinia virus RR displays similar complex allosteric regulation to the mouse enzyme and other eukaryotic reductases.

The overall activity of the enzyme, which has two subunits (R1 and R2), is regulated through binding to ATP, which activates the enzyme, and dATP which serves as an inhibitor. Both nucleotides bind to the same allosteric site, called the activity site, on the large subunit of RR. The specificity of the enzyme towards the four ribonucleoside diphosphate substrates is regulated by the binding of ATP, dATP, dTTP and GTP. Each of these nucleotides affects the reduction of a specific nucleoside diphosphate.

Although this enzyme's allosteric regulation is kinetically well understood it has not been possible so far to gain further structural information about the location of the activity site and specificity site. The use of deletion mutants and photoaffinity labeling of the large subunit to identify the location of the binding sites is the incentive for this thesis.

With the introduction of 6xHis/Nickel Nitrilo-tri-acetic acid (Ni-NTA) chromatography, the purification of the large subunit was improved in the *E. coli* and vaccinia virus/T7 RNA polymerase hybrid system. The purification of several deletion mutant forms of the large subunit was also attempted, but it was not possible to purify any of them from either of the expression systems.

The purified full-length large subunit obtained with the Ni-NTA-chromatography system was used for a photoaffinity labeling experiment with [ $^{32}\text{P}$ ]dATP and [ $^{32}\text{P}$ ]dTTP. The labeled proteins were proteolytically digested to find out about the specificity of the labeling experiment and also to map the binding site of the nucleotide. It was found that labeling of dATP yielded few discrete bands indicating specific binding, while a comparable experiment with dTTP indicated less specific binding, based on a larger number of labeled bands. In competition experiments with non-radioactive nucleotides, vaccinia virus R1 featured the same properties as the mouse and *E. coli* counterparts. This is consistent with data from kinetic experiments, which also establish the same kinetic properties between vaccinia virus RR with those of mouse and *E. coli* (RR).

To identify the sequence of the fragments carrying the label the digests were subjected to mass spectrometric analysis. However, it was

not possible to determine the sequence of the labeled fragment by mass spectrometry due to poor spectral resolution.

**Large Subunit of Vaccinia Virus Ribonucleotide Reductase:  
Affinity Chromatography-based Purification and Photoaffinity  
Labeling**

by

Rainer K. Warth

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APPROVED:

Redacted for Privacy

Professor of Biochemistry and Biophysics in charge of major

Redacted for Privacy

Head of department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School.

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Typed by Rainer K. Warth for Rainer K. Warth

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**Large Subunit of Vaccinia Virus Ribonucleotide Reductase:  
Affinity Chromatography-based Purification and Photoaffinity  
Labeling**

**I. Introduction**

The replication of a living cell's genome is the essential task of this smallest unit of life. This event occurs in all prokaryotic, eukaryotic and viral organisms and involves the time-dependent interplay of several mechanisms to ensure the successful replication of the genome. The synthesis of the daughter DNA strands is remarkable since it yields an exact copy of a macromolecule in a defined amount of time. To make this possible a wide variety of enzymes and proteins must work together.

These enzymes can be divided into two sets. One concerned with the actual synthesis of the DNA and the other providing the four deoxyribonucleoside 5'-triphosphates (dNTPs) precursors for DNA synthesis. The DNA-synthesizing enzymes depend on the supply of a balanced pool of dNTPs sufficient to accommodate the needs for DNA synthesis. To provide such a pool, a *de novo* biosynthetic pathway is in place as well as a salvage pathway, utilizing material from the degradation of RNA and DNA. The combination of these pathways must meet the needs of the cell cycle-dependent replication process.

The rate-limiting enzyme in the *de novo* biosynthesis of deoxynucleotides is ribonucleotide reductase (RR)(EC 1.17.4.1) (Thelander and Reichard, 1979). This enzyme catalyzes the reduction of the 2'-hydroxyl group on the ribose moiety of nucleoside diphosphates

(or triphosphates) to form the corresponding 2'-deoxynucleoside 5'-diphosphates (or triphosphates). The enzyme irreversibly reduces the four common nucleotides: adenosine diphosphate, guanosine diphosphate, uridine diphosphate and cytidine diphosphate. RR therefore catalyzes the first committed step in deoxynucleoside triphosphate synthesis.

Ribonucleotide reductases from different species have been studied for almost 30 years. With the characterization of RR from vaccinia virus, another system became available to address questions related to this complex enzyme. The vaccinia virus RR consists of a large homodimeric subunit (R1) and a small homodimeric subunit (R2). The sequence homology to other eukaryotic RR is high, and the same allosteric properties have been detected. In addition the viral RR suits particularly the needs for the study of eukaryotic-like RR since the virus is a large DNA virus encoding the enzymes necessary for its own replication. The site of replication is the cytoplasm of the host cell. Further, it has been shown that the host cell nuclear DNA is made unavailable to the virus (Parkhurst *et al.*, 1973), significantly reducing the salvage pathways. For these reasons, and because the virus is well defined genetically and provides a good expression system for manipulated genes, vaccinia is an ideal system to study RR.

This thesis is an attempt to further characterize the large subunit of ribonucleotide reductase coded by a large eukaryotic DNA virus. The use of deletion mutants will be discussed along with the results of a photoaffinity labeling experiment with deoxythymidine triphosphate (dTTP) and deoxyadenosine triphosphate (dATP). Both experiments should provide more information about the location of allosteric sites to

which dTTP, dATP, deoxyguanosine triphosphate (dGTP) and adenosine triphosphate (ATP) bind to regulate the enzymes activity and specificity towards the four ribonucleotides.

### **I.1. Vaccinia Virus Infection**

The vaccinia virus particle has a brick-shaped body composed of an outer lipoprotein envelope, a single duplex DNA genome, and two lateral protein bodies with unknown function. The virus replicates in the cytoplasm, as it is common for viruses from the family Poxviridae.

Vaccinia virus was originally identified as an immunizing agent against cowpox in 1798 by Edward Jenner (Jenner, 1959). The World Health Organization administered vaccinia in a campaign to eradicate smallpox (World Health Organization, 1988) and actually succeeded. Vaccinia continues to be used as a vector for developing recombinant viruses for use as live vaccines (reviewed by Fenner, 1988; Moss, 1991) as well as a cloning and expression vector for eukaryotic genes (Fuerst *et al.*, 1987).

#### *Viral Life-cycle*

The viral life-cycle is defined by the expression of early, intermediate and late genes. It takes about 8-12 hours to complete one cycle. Immediately after infection the transcription of the early genes begins. All enzymes necessary to make immediate transcription possible are packaged within the virion core of the infectious particle (Nevins and Joklik, 1977). The early gene products include proteins involved in DNA

replication, formation of virion core, and intermediate transcription. After about 2 hours DNA replication begins and the intermediate transcription factors turn on a cascade for expression of the late genes. The intermediate genes mainly encode for late transcription factors. The late transcription factors activate the genes for the synthesis of structural coat proteins, other virion proteins, early transcription factors, and the early transcription enzymes destined for encapsidation.

This interesting display of regulation of gene expression requires differences in the promoter consensus sequence for the three classes of genes. In addition, there is a difference in 5'-capping and 3'-polyadenylation of the various mRNAs.

Eventually a complex series of morphogenic events starts, resulting in the formation of virus particle. Many of the viral core and membrane polypeptides contain post-translational modifications, like proteolytic cleavage, phosphorylation, glycosylation, and ADP-ribosylation (Van Slyke and Hruby, 1990). These modifications appear to be necessary to regulate the events in viral morphogenesis.

This sophisticated life-cycle and the extensive protein synthesis requires a very efficient mechanism to take over the host cell. Shortly after entry into the host cell the virus suppresses the metabolic activities of its host. Vaccinia virus essentially inhibits host cell DNA synthesis (Jungwirth and Launer, 1969), cell protein synthesis (Moss, 1968), transfer of nuclear RNA into the cytoplasm, and eventually RNA synthesis (Becker and Joklik, 1964). It is believed that upon infection, host cell DNA synthesis is interrupted by the release of a host-specific deoxyribonuclease from the virus core. Subsequently cellular DNA is

degraded into large DNA fragments of discrete sizes (Parkhurst *et al.*, 1973). The degraded cellular DNA is not made available to the virus for utilization under normal conditions (Kit and Dubbs, 1962).

### *Genome Organization and Replication*

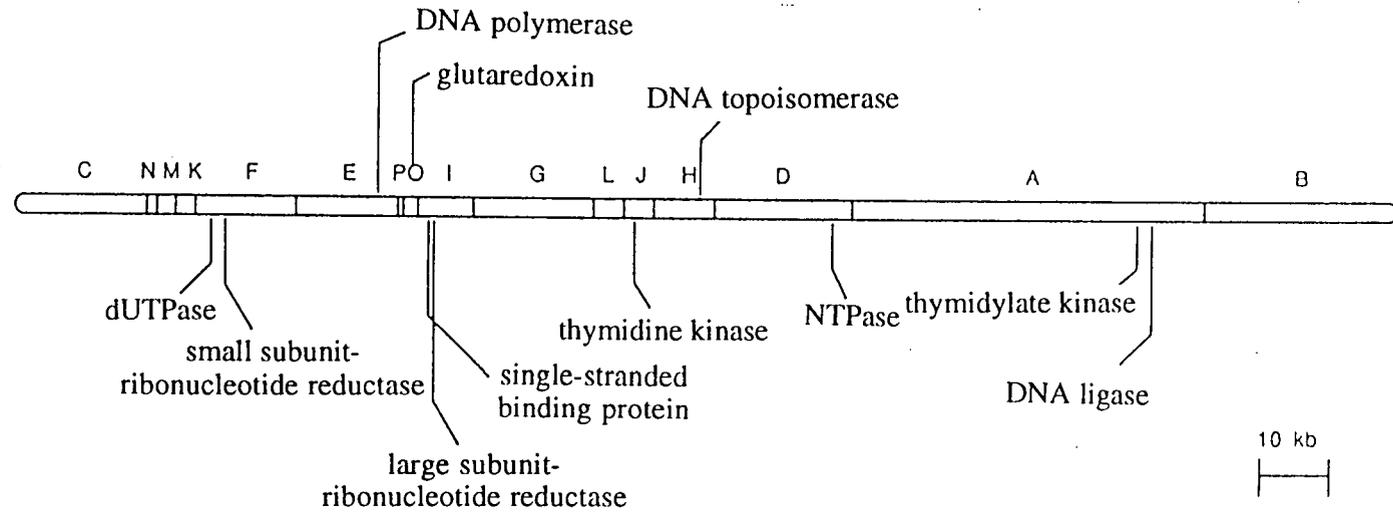
The vaccinia virus genome is a covalently closed linear duplex DNA molecule. The 190-kilobase-pair viral DNA has been sequenced and found to contain two 10-kilobase regions of AT-rich inverted terminal repeats (reviewed by Traktman, 1990) and contains 198 open reading frames (ORF's) that encode at least 60 amino acids each (Goebel *et al.*, 1990). High sequence homology between the mammalian protein counterparts and viral genes with known function was observed (reviewed by Moss, 1990). Through the use of temperature sensitive mutants and genetic analyses it was possible to map the essential genes in the genome (Condit and Motyczka, 1981; Condit *et al.*, 1983).

The locations of the genes encoding many of the enzymes involved in replication and deoxynucleotide metabolism have been determined and are shown in Figure I.1. It is remarkable that the genes that encode the two viral ribonucleotide reductase proteins are separated by approximately 35 kilobases.

Viral DNA replication takes place in cytoplasmic inclusion bodies called virosomes (Dahl and Kates, 1970). Immunological studies showed the presence of ribonucleotide reductase (Davis, 1992) and other replication enzymes at these viral DNA factories. Early studies demonstrated that 30% of all virally replicated genomes are packaged into virions after 24 hours of infection (Joklik and Becker, 1964). It has

also been shown that the rate of viral DNA replication is several times that of dividing eukaryotic cells. Quantitative filter hybridization studies following the accumulation of viral DNA led to the conclusion that DNA replication starts as early as 3 hours after the virus enters the cell and continues until 10-12 hours after infection (Ensinger, 1987; Rempel *et al.*, 1990).

The mechanism of viral DNA replication has been thoroughly studied (reviewed by Moss *et al.*, 1983; Moyer and Graves, 1981). First, DNA nicks are introduced near either end of the parental genome, then DNA synthesis starts at these sites initiated by a self-priming mechanism. DNA synthesis eventually yields viral concatemers containing the genomes joined head-to-head and tail-to-tail. Cleavage at each concatemeric junction results in the formation of two full length genomes.



**Fig.I.1** Location of vaccinia virus genes encoding replication and nucleotide metabolism proteins. Each lettered segment represents a *Hind*III restriction fragment

## I.2. Ribonucleotide Reductase

Nucleotide metabolism is essential for the successful replication of genomic DNA. Not only are sufficient amounts of nucleotides required, it is also critical to maintain balanced pools to ensure high fidelity of replication. This difficult task depends widely on the proper performance of ribonucleotide reductase, which has a central role in deoxynucleotide biosynthesis (Fig.II.2). This enzyme as studied in *Escherichia coli* features not only complex allosteric regulation, it also catalyzes a difficult chemical reaction, resulting in the reduction of 2'-hydroxyl group at the sugar moiety of nucleotide substrate. To catalyze such a reaction the enzyme generates a free radical. The reductant in this reaction is a dithiol functionality of thioredoxin or glutaredoxin (Holmgren, 1989). A mechanism formulated by Stubbe (1989) shows that the protein radical causes the abstraction of a hydrogen from the C-3' of the ribose. This facilitates the leaving of the hydroxyl group from the neighboring C-2', which is reduced subsequently by the dithiol functionality. The reaction mechanism is more thoroughly presented in Fig.I.3.

The investigated RRs have been grouped in three classes based on difference in the generation of their free radical. Class I enzymes represent RR from eukaryotic organisms; although the prototype of this class is the *E. coli* RR. A small protein subunit is involved in radical generation in these enzymes. Class II enzymes use adenosylcobalamin (AdoCbl) as the radical generator. To this class belong many prokaryotic RRs with *Lactobacillus leichmannii* as the best studied enzyme. S-

adenosylmethionine (AdoMet) is the radical generator for the Class III enzymes, to which the anaerobic *E. coli* RR belongs.

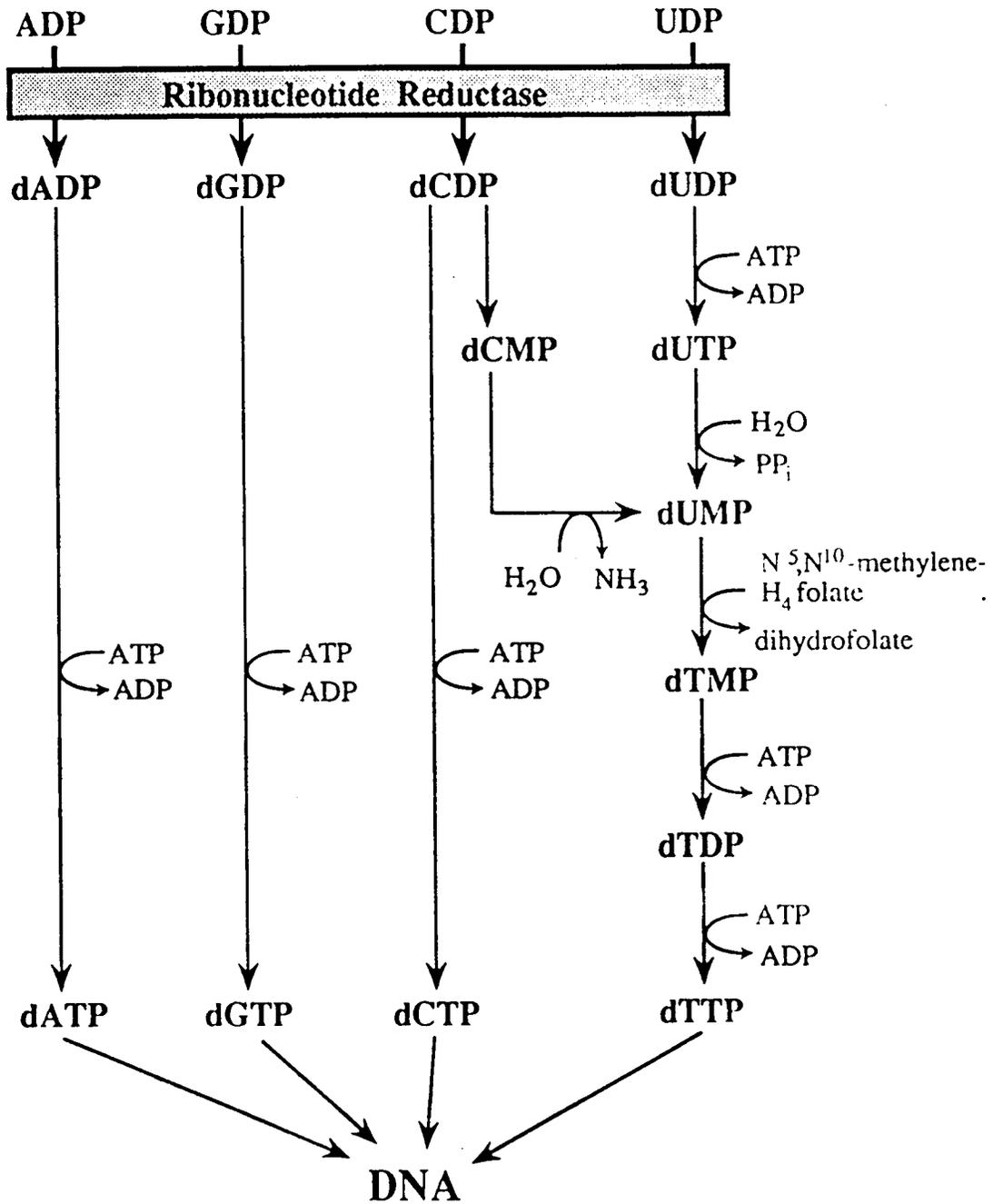


Fig.I.2 Deoxynucleotide Biosynthesis  
(Mathews and van Holde, 1990)

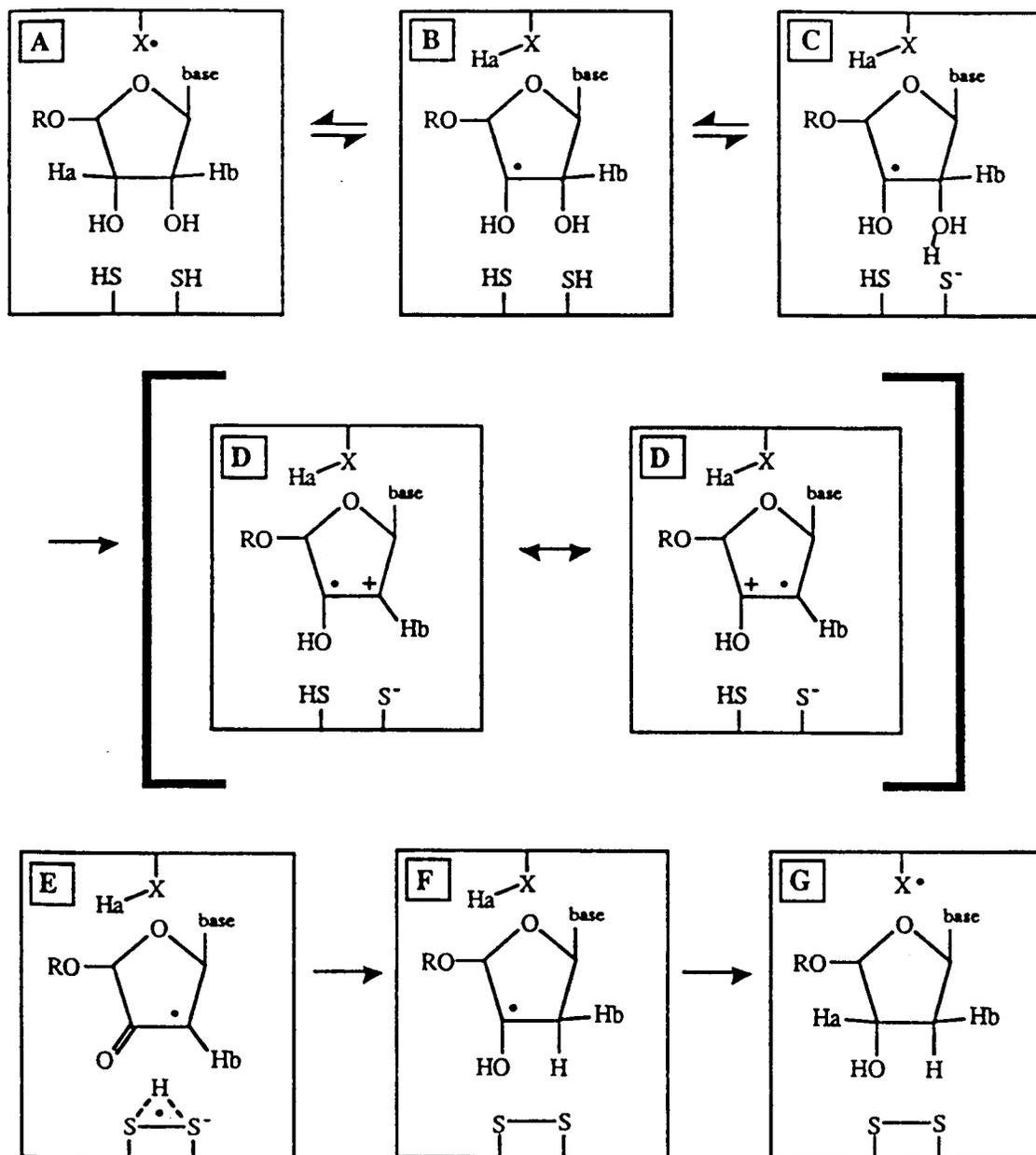


Fig.I.3 Proposed Reaction Mechanism for Ribonucleotide Reductase (Stubbe *et al.*, 1989)

### I.2.a. Class I

The most thoroughly studied of all ribonucleotide reductases is from *E. coli* (Fontecave *et al.*, 1992). This enzyme also serves as the prototype of the Class I enzymes, to which the mammalian, T4 phage, and herpes simplex virus enzymes belong. It has a molecular weight of 258 kD and an  $\alpha_2\beta_2$  protein structure (with  $\alpha_2$  usually referred to as R1 and  $\beta_2$  as R2). Each R1 subunit contains one substrate binding site and two separate allosteric sites. One of the allosteric sites serves as a switch for the regulation for balanced nucleotide pools and the other for the overall activity.

R2 features a tyrosyl radical Tyr<sup>122</sup> and an oxygen-linked iron [Fe(III)] center. All of the Class I enzymes known so far take rNDPs as substrate and reduce them to dNDPs. It was first assumed that the Tyr<sup>122</sup> radical would be involved in the abstraction of hydrogen from C-3'. From the X-ray crystal structure of R2 (Nordlund *et al.*, 1990), however, it is now known that Tyr<sup>122</sup> cannot interact with R1 since it is buried in the core of R2 about 10Å away from the surface of the protein. It is, therefore, thought that by long-range electron transfer Tyr<sup>122</sup> initiates the formation of thiyl radical at Cys<sup>439</sup> from R1 (Stubbe *et al.*, 1992). To satisfy the requirements of the reaction mechanism proposed by Stubbe, two electron donors are needed. These are apparently provided by the reduced forms of Cys<sup>225</sup> and Cys<sup>462</sup>.

The enzyme's activity can be inhibited by generating forms of R2 lacking the tyrosyl radical. Two forms of R2 lack the tyrosyl radical. One

is apoR2 and is formed by treatment of R2 with iron-chelating reagents, which causes the loss of iron and the radical (Reichard *et. al.*, 1980). The other is metR2, and it arises from treatment of R2 with radical scavengers, such as hydroxyurea, that leave the Fe(III) center intact (Reichard *et. al.*, 1987). The activity can be restored from both apoR2 and metR2 in presence of Fe(II) and oxygen. The simultaneous oxidation of Fe(II) and Tyr<sup>122</sup> reforms both the Fe(III) center and the tyrosyl radical.

#### *Allosteric control of Class I enzymes*

As already pointed out, RR is a tightly regulated enzyme containing two allosteric sites on the large subunit, both of which bind to nucleoside triphosphates. One of these sites is called the specificity site and the other the activity site. The two sites bind both dATP and ATP. Based on their affinity to dATP the activity site is also called l-site (l for low) and the specificity site is called h-site (h-high). When dATP binds to the activity site the enzyme's catalytic activity is inhibited. Binding of ATP to the same site activates the enzyme.

Substrate specificity is regulated by the specificity site. Upon binding of dATP or ATP the reduction of cytidine diphosphate (CDP) and uridine diphosphate (UDP) is activated. dTTP binding activates the reduction of GDP. Upon binding of dGTP the reduction of adenosine diphosphate (ADP) is activated.

### I.2.b. Class II

This class depends on adenosylcobalamin, which duplicates the function of the R2 subunit of the Class I enzymes. Through the cleavage of the bond between cobalt and C-5' of deoxyadenosine a putative deoxyadenosinyl radical is created. This radical then helps to create a protein radical (probably a thioyl radical) which is involved in the abstraction of the hydrogen atom from C-3' (Ashley and Stubbe, 1987).

*Lactobacillus* is a thoroughly studied representative of this class of enzymes. Sequence comparison of the enzyme with *E. coli* RR shows similarities only at the COOH-terminus. The Cys-Glu-Gly-Gly-Ala-Cys-Pro-Ile-Lys from *Lactobacillus* was found to be homologous to *E. coli* sequence Cys-Glu-Ser-Gly-Ala-Cys-Lys-Ile. The two redox-active cysteines in each sequence are thought to transfer electrons to the active site of the enzyme that directly reduces the substrate.

The enzyme's substrates are nucleoside triphosphates, with the exception of the RR from *Rhizobium meliloti*, which prefers ribonucleoside diphosphates and also belongs to the group of Class II enzymes. The mechanism of reduction was studied by Ashley and Stubbe (1987) and is similar to the one from *E. coli*.

#### *Allosteric regulation of Lactobacillus enzyme*

The *Lactobacillus* enzyme has only one allosteric site that binds to dNTP's. The affinity of this site for deoxyribonucleotides is several orders of magnitude lower than the ones observed for the *E. coli*

enzyme. Nevertheless, kinetic experiments show that reduction of each of the NTPs is increased by one specific effector: CTP reduction by dATP (and to a lesser extent ATP), UTP reduction by dCTP, ATP reduction by dGTP, GTP reduction by dTTP. These effects are slightly different as compared to the *E. coli* RR.

### I.2.c. Class III

Extracts from anaerobically grown *E. coli* showed ribonucleotide reductase activity (Fontecave et al., 1989), even though it is known that the Class I *E. coli* enzyme requires oxygen for the formation of the tyrosyl radical. Further immunological test and subsequent purification established the assumption that an anaerobic ribonucleotide reductase is present, different from the aerobic reductase already discussed as a Class I enzyme.

To obtain activity after purification it was necessary to incubate the enzyme with NADPH and AdoMet. The substrate was found to be the four nucleoside triphosphates.

Further investigation led to the conclusion that the anaerobic reductase is an FeS protein, which in its active form contains an oxygen-sensitive protein radical. Presumably this radical is a glycine radical close to the C-terminal end, based on sequence comparison with other anaerobic enzymes.

It is assumed that the iron cluster is reduced by a flavodoxin system that transfers electrons from NADPH. This cluster then transfers

to AdoMet to generate the 5'-deoxyadenosyl radical. This radical is supposed to help form the Gly<sup>681</sup> to generate the protein radical which is required for ribonucleotide reduction.

#### *Allosteric regulation of the anaerobic E. coli enzyme*

The allosteric properties of the enzyme are known only by kinetic experiments. dATP caused inhibition of the activity. Addition of the appropriate effector increased enzyme activity by about tenfold: ATP increased activity for CTP and UTP reduction, dTTP for GTP reduction, dGTP for ATP reduction. This indicates remarkably similar allosteric properties to the Class I enzyme.

### **I.3. Vaccinia Virus Ribonucleotide Reductase**

With the characterization and subsequent cloning of ribonucleotide reductase from vaccinia virus by Slabaugh and Mathews (1984) (Slabaugh *et al.*, 1988; Tengelson *et al.* 1988; Schmitt and Stunnenberg, 1988), another enzyme of this interesting class became available for study.

The vaccinia virus RR is a Class I enzyme with a  $\alpha_2\beta_2$  protein structure. The gene for the large subunit encodes a polypeptide of a molecular weight of 87 kD and the gene for the small subunit for a 37 kD polypeptide. Comparison of the amino acid sequence of ribonucleotide reductase from several species reveals a high degree of homology between the mammalian and vaccinia virus polypeptides. The amino acid sequence identities of the small and large subunits between the mouse and the vaccinia virus reductase are approximately 80 and 72 percent,

respectively. This high degree of homology is not observed for the *E. coli*, T4 phage and herpes simplex virus. The phylogenetic tree in Figure I.4. yields more information about the relationship of the RR from different species.

Initial kinetic studies of the vaccinia virus RR also demonstrate similarities to allosteric mechanisms observed for the mammalian enzyme (Slabaugh and Mathews, 1984). This is unusual since the viral enzymes in the case of T4 and herpes simplex virus are relatively insensitive to allosteric control by triphosphates *in vitro* (Berglund, 1972; Averett *et al.*, 1983). Contrary to these enzymes, RR encoded by vaccinia virus features an overall activation by ATP, inhibition by dATP, and differential activation toward its four substrates by specific dNTP effectors (Slabaugh *et al.*, 1984; Slabaugh *et al.*, 1991)

From the similarities in sequence and allosteric properties of the vaccinia enzyme to the mammalian enzyme, it is reasonable to assume that vaccinia virus ribonucleotide reductase can serve as a good model for eukaryotic reductase. Taking into account how easy it is to genetically manipulate the vaccinia virus model system over other eukaryotic model systems, the study of ribonucleotide reductase from vaccinia virus should contribute to our understanding of many unresolved question in this field.

One area where we lack a good understanding of ribonucleotide reductase is how the activity site, specificity site and the catalytic site are distributed on the large subunit. Knowledge of this structural information might open the door to address questions related to understanding of mechanisms involved in the complex allosteric regulation of the enzyme.

Information currently available about the locations of these sites was obtained from mutant mouse T-lymphoma RR (Caras and Martin, 1988) and photoaffinity labeling experiments with *E. coli* RR (Eriksson and Sjöberg, 1985). The cloned mutant mouse T-lymphoma R1 gene was sequenced and revealed alteration of codon 57, converting aspartic acid to asparagine. This mutation resulted in the loss of inhibitory effects of dATP on RR. The location of the mutation at the N-terminus led to the assumption that this region might be involved in the activity site of the enzyme.

Photoaffinity labeling of *E. coli* R1 with [<sup>32</sup>P] dTTP and subsequent sequencing of the tryptic peptides indicated that residues 288-298 are involved in the formation of the specificity site. Although other photoaffinity labeling experiments have been done with mouse RR and *E. coli* RR, no further information was gathered regarding the binding sites, leaving initial results about the location of the activity site and specificity unconfirmed.

With this thesis the author hopes to contribute to this effort by investigating the possibilities of using deletion mutations to map functional domains. Furthermore, photoaffinity labeling was used in an attempt to localize the binding site for dATP and dTTP and to establish the close relationship between the mouse and vaccinia virus ribonucleotide reductases.

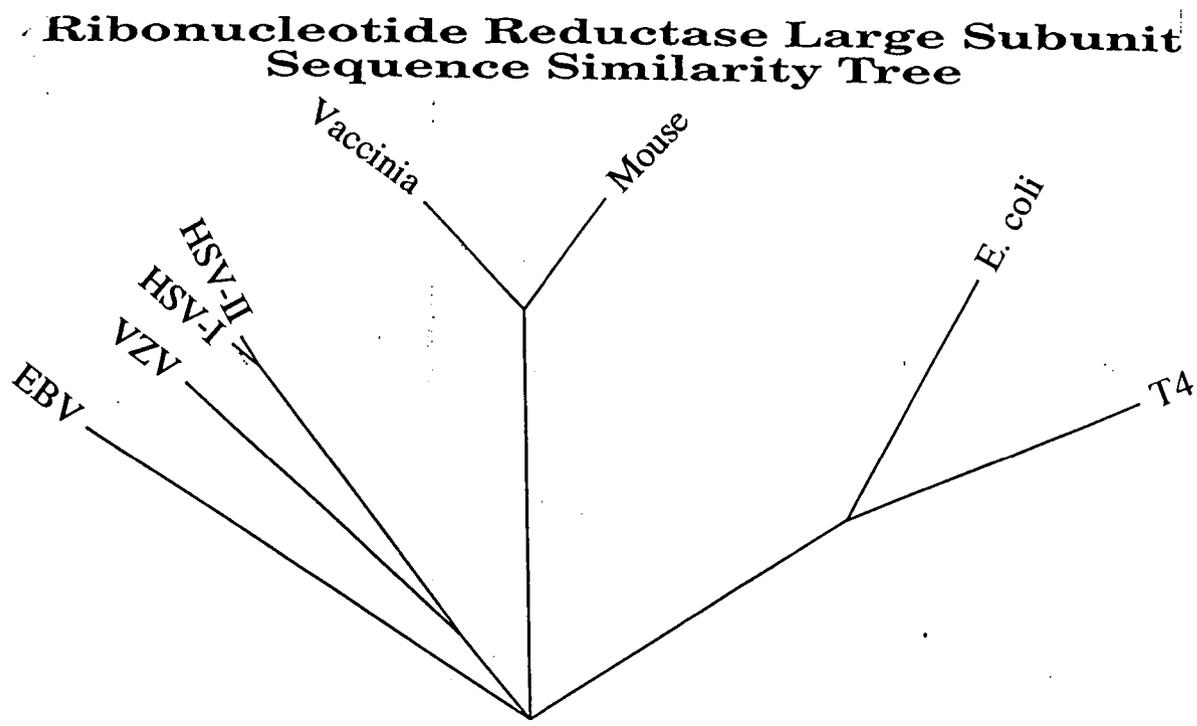


Fig. I.4: Phylogenetic tree of the large and small subunit of RR  
(Nilsson and Sjöberg, 1993)

**Ribonucleotide Reductase Small Subunit  
Sequence Similarity Tree**

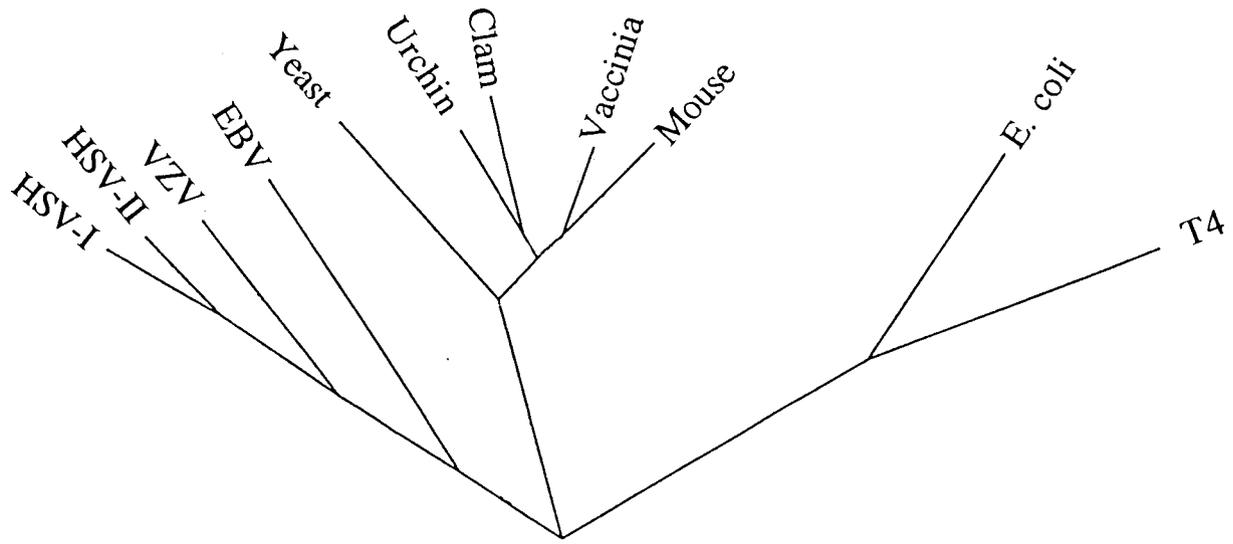


Fig. I.4, Continued

## II. Overexpression of R1 and R1 Deletion Mutant Genes

### II.1. Introduction

With the cloning and successful overexpression of R1 in *E. coli* (Slabaugh *et. al.*, 1993) functional studies can be undertaken by altering sequence of the R1 gene. Using this approach I investigated the properties of C-terminal deleted forms of R1. An *in vitro* transcription/translation system was chosen since it does not require further cloning steps. Different run-off transcripts could be synthesized from template DNA which was cut within the R1 gene. These transcripts could then be loaded into a rabbit reticulate lysate to express C-terminal deleted R1 labeled with [<sup>35</sup>S]-methionine.

As an alternative approach, the *E. coli* and the vaccinia virus/T7 RNA polymerase hybrid system was chosen. This required several cloning steps to delete the 3'-end of the R1 gene. Further a histidine affinity-tag was added to the 5'-end of the gene, which later could be used to purify the deletion mutants by means of Ni<sup>2+</sup>NTA-affinity chromatography (Janknecht *et. al.*, 1991).

For all three different expression systems, the goal was to obtain deletion mutants, which then could be tested for their ability to bind to dATP by using dATP-Sepharose. Since dATP binds to the activity site and specificity site, the assay could yield information for both sites. To probe for activity, the assay would be done in the presence of dGTP,

which binds specifically to the specificity site and therefore would inhibit binding of dATP to this site.

## **II.2. *In vitro* Transcription/Translation of R1**

### **II.2.a. Introduction**

The expression of proteins *in vitro* in rabbit reticulate lysate has become a commonly used technique to obtain structural information. Once the plasmid with the gene of interest is available, RNA transcripts can be synthesized, which are loaded into the rabbit reticulate lysate. Further, it is possible to obtain deletion mutants of the gene if convenient restriction sites are located within the gene. These linearized plasmids can then be used in an *in vitro* transcription reaction to synthesize run-off transcripts. After a quick purification by phenol/chloroform extraction the RNA can be loaded into the translation mix. Incorporation of [<sup>35</sup>S] methionine in the newly synthesized protein makes easy detection by autoradiography possible.

### **II.2.b. Materials and Methods**

#### *Enzyme and Translation mix*

T7 RNA polymerase (Biolabs Inc.); Rabbit reticulate lysate (BRL Inc.); Restriction Enzymes *Sa*II, *Pst*I, *Bam*HI, *Kpn*I, and *Nco*I were all purchased from Biolabs Inc.

### *CsCl gradient purification*

The protocol from Maniatis (1989) was followed. A 1-liter culture of *E. coli* strain BL21(DE3) strain harboring pET-11c-R1 was grown overnight at 37°C. After harvest cells were lysed by alkali lysis. The supernatant of the lysis was subjected to isopropanol precipitation (Appendix A.1). The DNA pellet was resuspended in 8 ml T.E. (10 mM Tris-HCl, 1 mM EDTA). To this solution 800 mg CsCl (100 mg/ml) was added and dissolved at room temperature (25°C). After dissolving 800 µl of 10 mg/ml ethidium bromide (EthBr) solution was added, resulting in a final volume of about 10 ml, which could be filled into two 5-ml centrifuge tubes. Mineral oil was layered on top of the CsCl/EthBr solution until the level reached the neck of the centrifuge tubes. The tubes were quickly sealed and placed in a vTi 60 rotor (Beckman Instruments, Inc.) After centrifugation at 45000 rpm for 12 hours at 25°C the tubes were removed. The resulting plasmid bands were identified under UV light (germicidal lamp, 200 to 320 nm) and the lower band was evacuated with a syringe (20 needle). Extraction with isoamyl alcohol (Appendix A.2) was used to remove ethidium bromide. To desalt, the solution was loaded in a Centricon 100 device and washed with T.E. by centrifugation at 3000 rpm in a SS32 rotor (Beckman Instruments, Inc.). After running 10 times the original volume through, the solution was concentrated to 1.5 g/ml.

### *In vitro transcription/translation*

The plasmid pET-11c-R1 containing the R1 gene (Slabaugh *et al.*, 1993) was purified by CsCl gradient purification (Maniatis, 1989). Subsequently

the plasmid was cut with the restriction enzymes *SalI*, *PstI*, *BamHI*, *KpnI*, and *NcoI* (Appendix A.3.) Each digestion mix was then purified by phenol/chloroform extraction and ethanol precipitation (0.3 M final concentration of ammonium acetate). Cleaved DNA (0.5  $\mu$ g) was transcribed (Appendix A.4) with T7 RNA polymerase in an RNAase free environment at 37°C for 1 hour. The synthesized RNA was analyzed electrophoretically with 1% agarose gel stained in 250 ml 1 mM ethidium bromide solution for 20 min.

The transcripts (0.5  $\mu$ g) were loaded in a rabbit reticulate lysate (15.5  $\mu$ l reaction volume) and incubated at 30°C. After one hour 10  $\mu$ l of the reaction mix was precipitated by 10% trichloroacetic acid (TCA) (30 min, 25°C) and 5.5  $\mu$ l were loaded on a 10% SDS-PAGE gel. After 45 min the gel was stopped, dried, and subjected to autoradiography (two days at 70°C with intensifying screen). One set of translation experiments was done in the presence of generic ssDNA (salmon sperm, Sigma Chemical Co.).

To compare the quality of the expression system chloramphenicol acetyltransferase (CAT) protein and a structural protein from yellow tulip mosaic virus called  $\Delta$ NBNS (Dreher *et al.*) was also synthesized. The same protocol was followed as for the R1 gene beginning with CsCl gradient purified template DNA for  $\Delta$ NBNS provided by Dr. T. Dreher, Department of Agricultural Chemistry, O.S.U., Corvallis, OR. CAT mRNA was provided by BRL and was loaded in the translation kit with no further treatment.

### II.2.c. Results

The *in vitro* transcription of the linearized DNA yielded the run-off transcripts of the expected size (Table II.1). Only the full-length DNA template for R1 and the *Pst*I- and *Bam*HI-cut R1 genes yielded an additional band of approximately 1400 bp on the agarose gel (Fig.II.1.B lane 3,4).

*In vitro* translation of R1 was very low and featured many early terminated peptides as judged by autoradiography (Fig.II.A). Incorporation of trichloroacetic acid precipitable counts was less than two-fold over background.

The control proteins appeared to be synthesized in higher levels and of the expected size. Synthesis of  $\Delta$ NBNS also yielded a considerable amount of early-terminated peptides.

**Table II.1 :**

Deletion mutants produced from run-off transcripts of pET-11c-R1

The data were calculated based on sequence information

pET-11c-R1 cut with	mRNA	Peptide length	M.W.
<i>Sal</i> II (full-length enzyme)	2313 bp	771 aa	86 kD
<i>Pst</i> I	2085 bp	695 aa	76 kD
<i>Kpn</i> I	792 bp	264 aa	29 kD
<i>Nco</i> I	366 bp	122 aa	13 kD

**Fig. II.1:** *In vitro* translation of R1 compared to CAT and  $\Delta$ NBNS

(A) Autoradiograph of translation products from rabbit reticulate lysate loaded with 1. CAT, 2. CAT + 0.04 mg ssDNA, 3.  $\Delta$ NBNS, 4.  $\Delta$ NBNS + 0.04 mg ssDNA, 5. R1, 6. R1 + 0.04 mg ssDNA, 7. R1 0.02mg ssDNA, M. Marker lane

(B) *In vitro* synthesized RNA analysed with 1% agarose gel stained with ethidium bromide. 1.  $\Delta$ NBNS, 2. unknown construct from Dr. R. Hoopes, Department of Agricultural Chemistry, O.S.U., Corvallis, OR 3. full-length R1 (linearized plasmid), 4. full-length R1 (uncut plasmid), M. Marker lane

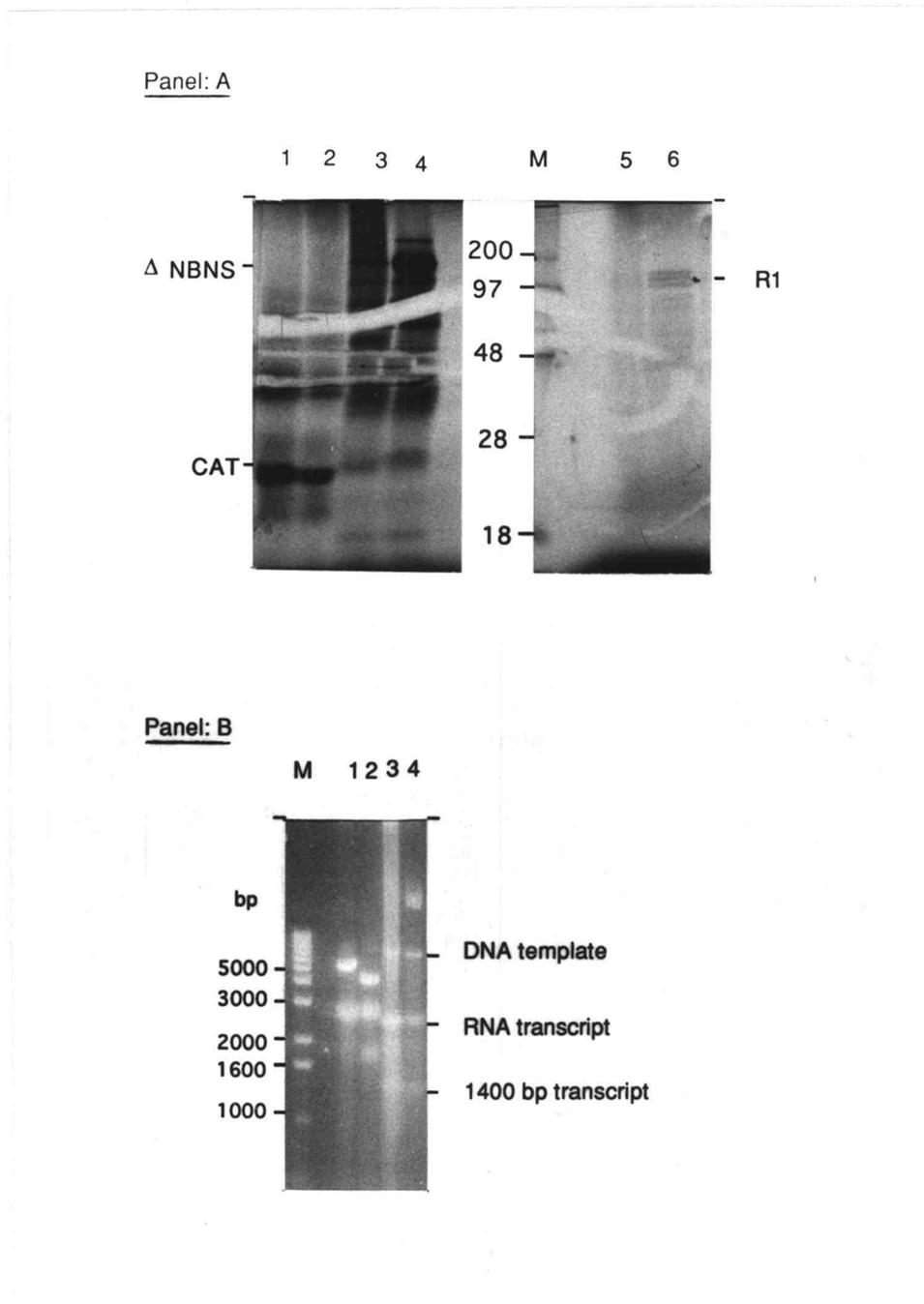


Fig.II.1

### II.2.d. Discussion

Very low amounts of protein could be synthesized by using the *in vitro* transcription/translation system. While the transcription yielded sufficient amounts of RNA, it was not possible to adjust the conditions for the translation reaction so that the R1 protein could be expressed at high levels. This observation was made for the full-length R1 protein and its deletion mutants. It appears that R1 was not suitable for *in vitro* translation under the conditions used in this experiment. The conditions, however, were appropriate for the control proteins  $\Delta$ NBNS and CAT. It was of special interest to see that  $\Delta$ NBNS, which underwent the same treatment as R1 showed a significant higher level of expression based on the autograph. This observation also excludes the possibility that the synthesized RNA carried along a contamination, which might have been inhibitory for the translation reaction.

The effect of the presence of ssDNA in the translation mix was also observed to be somewhat helpful for the expression of R1. The rationale for this measurement was that R1, which features high affinity for nucleotides, might tend to bind to mRNA non-specifically. The presence of ssDNA should help to compete R1 away from mRNA and free the RNA for translation.

An additional transcript obtained from the full-length gene was also synthesized in the transcription reaction with the pET-11c-R1 plasmid linearized with *Pst*I. This 1400-bp transcript was observed in the transcription reaction with plasmids cut either with *Kpn*I or *Nco*I (data not shown). This indicates that the 1400-bp transcript is the result of

early transcription termination caused by a cryptic T7 RNA polymerase termination site within the R1 gene.

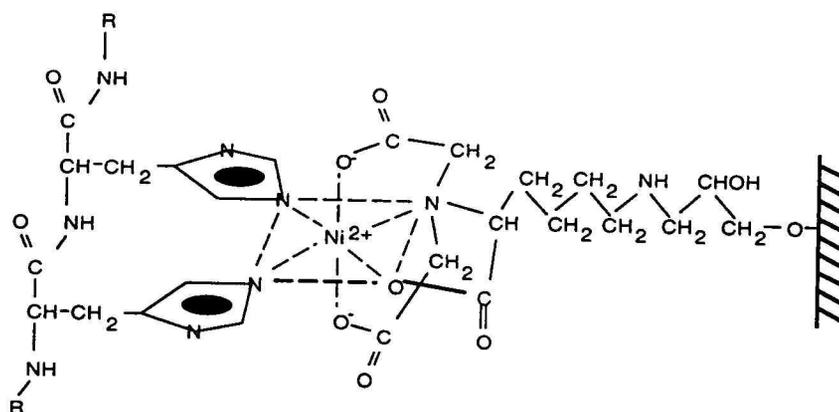
## **II.3. *E. coli* Expression System**

### **II.3.a. Introduction**

The overexpression of proteins in *E. coli* is the expression system of choice since it is inexpensive and simple compared to other systems. Its main disadvantage is that it is not always a good host for eukaryotic proteins. One of the problems is insoluble protein as a result of overexpression. This was also encountered with R1. Although R1 could be induced and successfully overexpressed in *E. coli*, most of the protein remained insoluble. With the protocol developed by M. Slabaugh and R. Davis it became possible to increase the amount of soluble R1 by growing the bacteria at 15°C overnight, which made the purification of R1 from *E. coli* possible (Slabaugh et al., 1993).

To map the binding domains of R1 by synthesizing deletion mutants the *E. coli* system would have been too cumbersome to deal with since the purification conditions for each of the deletion mutant proteins would have to be adjusted. An alternative to this was to simplify purification by using a fusion protein. In fusion protein constructs the added polypeptide has high affinity to a ligand immobilized on a resin. After looking at maltose binding protein and glutathione transferase, we concluded the 6xHis/Ni-NTA affinity chromatography system would best suit our needs.

6xHis/Ni-NTA affinity chromatography has now been used successfully to purify about 150 different proteins. It is based on the high affinity of proteins containing the 6xHis tag to  $\text{Ni}^{2+}$  interchelating with nitrilo-tri-acetic acid (NTA). NTA is attached to Sepharose CL-6B and has four chelating sites which can interact with metal ions. Thus, NTA occupies four of the six ligand binding sites in the coordination sphere of the  $\text{Ni}^{2+}$  ion, leaving two sites free to interact with the 6xHis tag of the protein (Fig.II.2). After washing the protein can be eluted by lowering the pH or by adding imidazole, which binds to Ni-NTA and therefore displaces the tagged protein (Hochuli et. al., 1990)



**Fig. II.2:** Interaction between 6xHis tag of a protein and Ni-NTA resin (QIAexpressionist Edition. 2).

### **II.3.b. Materials and Methods**

#### *Enzymes*

T4 DNA Ligase (BioLabs Inc.), Restriction enzymes *Nde* I, *Kpn* I, *Sal* I, *Xho* I, and *BamH* I were all purchased from BioLabs. Inc.

#### *Bacterial strains and vectors*

Vector pET-15c was purchased from Novagen Inc. Vector pET-11c-R1 was provided by M. Slabaugh. The bacterial *E. coli* strain BL21 (DE3) was used for cloning and overexpression.

#### *Buffer and media*

Sonication buffer: 50 mM sodium-phosphate pH 7.8, 300 mM NaCl (pH adjusted with NaOH). Wash buffer: 50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, (pH 6.0). LB medium: 10 g bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl. 5x SDS-PAGE Sample Buffer: 15%  $\beta$ -ME, 15% SDS, 1.5% bromophenol blue, 50% (v/v) glycerol.

#### *Cloning of pET-15c-R1 and its deletion mutants*

The pET-11c-R1 plasmid containing the R1 gene was used to subclone into plasmid pET-15b from Novagen Inc. First, the *Nde*I fragment was inserted in the right orientation into pET-15b at the *Nde*I site of the polylinker. This plasmid was called pET-15b-R1*Nde*I. To confirm the success of the subcloning procedure the 5' end was sequenced. To obtain the full-length protein the *Kpn*I-*Sal*I fragment was

cut out of pET-11c-R1 and ligated into pET-15b-R1*Nde*I, which was cut with *Kpn*I and *Xho*I. This yielded the full-length R1 gene and the plasmid was named pET-15b-R1. From pET-15b-R1*Nde*I and pET-15b-R1 the deletion mutant plasmids pET-15b-R1*Bam*HI, pET-15b-R1*Xho*I, and pET-15b-R1*Kpn*I could be derived following the strategy outlined in Fig.II.2. In Table II.2 the deletion mutants are summarized.

Through subcloning into pET-15b the full-length gene and the deletion mutants received a sequence at the N-terminal end, which coded for six histidine residues and a thrombin cleavage site. The sequence is composed of: Met Gly Ser Ser His His His His His Ser Ser Gly Leu Val Pro Arg Gly Ser His Met .....R1 sequence. The underlined amino acids indicate the cleavage site for the protease thrombin.

Table II.2: Deletion mutants of R1-His cloned from pET-15b-R1

<u>Plasmid</u>	<u>Length of peptide</u>	<u>name of peptide</u>	<u>M.W.</u>
pET-15b-R1	791 aa	R1-His	89 kDa
pET-15b-R1 <i>Bam</i> HI	664 aa	R1-His-C664	72 kDa
pET-15b-R1 <i>Nde</i> I	554 aa	R1-His-C554	60 kDa
pET-15b-R1 <i>Xho</i> I	386 aa	R1-His-C386	42 kDa
pET-15b-R1 <i>Kpn</i> I	264 aa	R1-His-C264	28 kDa

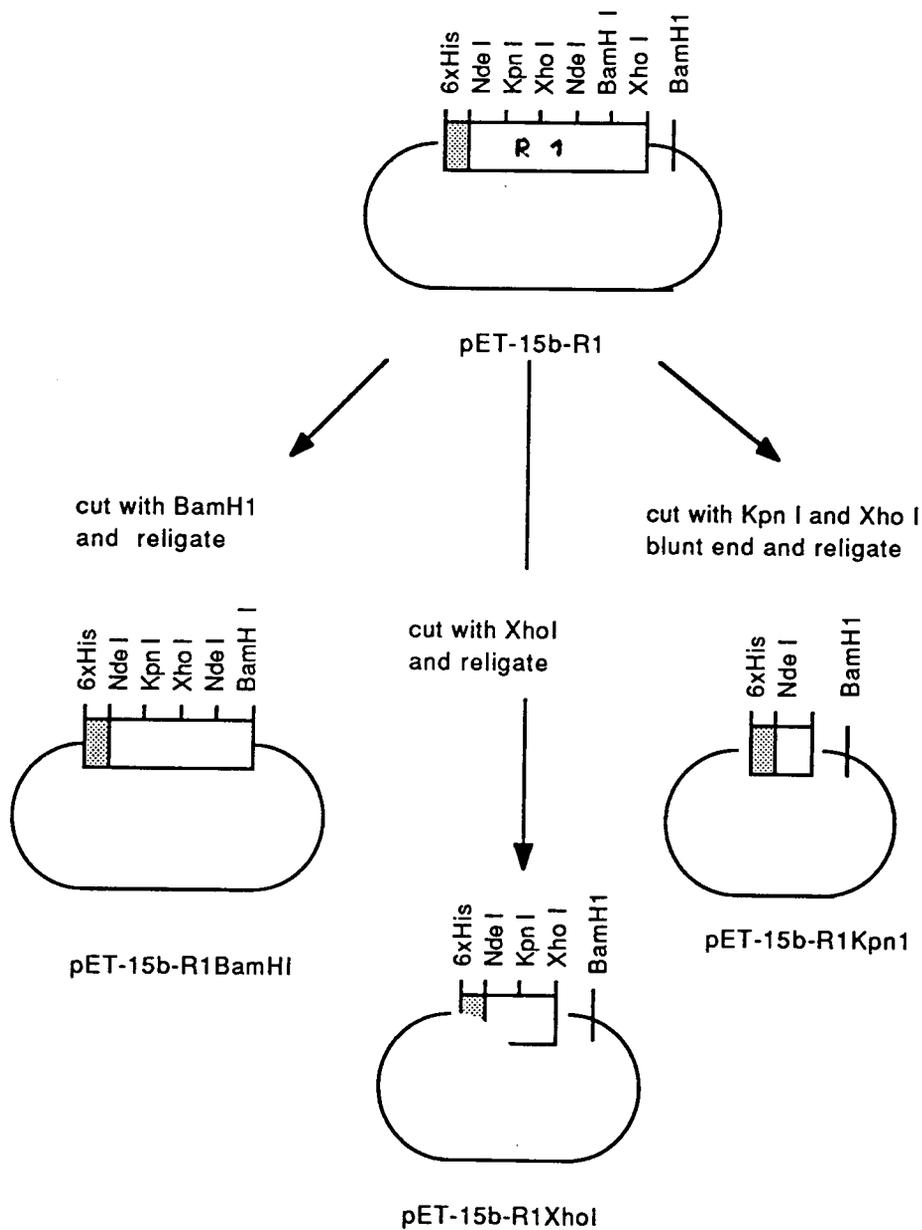


Fig. II.3: Cloning procedure for pET-15b-R1*Bam*HI, pET-15b-R1*Kpn*I and pET-15b-R1*Xho*I

### *Overexpression of R1 and R1 deletion mutants and purification*

#### a) Overexpression at 15°C:

R1 and the R1 deletion mutants were overexpressed in the *E. coli* strain BL21(DE3) at 15°C after induction with 0.05 mM isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) and addition of 5 mM hydroxyurea. The bacteria were grown in LB medium. After 18 hours the 1-liter culture was harvested by centrifugation at 4000xg for 20 min. The pellet was resuspended in 10 ml of Sonication Buffer and frozen. After thawing in cold water, the cells were lysed by sonication (1 min bursts/ 1 min cooling/ 200-300 Watt). The soluble fraction was run over a 4 ml Ni<sup>2+</sup>-NTA-column (resin was purchased from Quiagen Inc.) with a flow rate of 10 ml/h. The column was washed with 60 ml Sonication Buffer (flow rate 0.5 ml/min) followed by a wash with Wash Buffer until A<sub>280</sub> was less than 0.01. The protein was eluted with a 30 ml gradient of 0-0.5 M imidazole in Wash Buffer. One-ml fractions were collected and analyzed on SDS-PAGE (samples were heated for 10 min at 37°C). The fractions, which appeared to contain most of the protein, were selected for further purification. To verify that the purified protein is actually R1 a Western blot was performed with antibodies to R1 raised by Ralph Davis (1992) numbered 4089 (fusion protein) and 7581 (full-length) (Fig.II.6).

To further purify, the concentrated sample (10 mg/ml) was run in Wash Buffer over a Superose 12 column from Pharmacia Inc. to obtain purification based on differences in molecular weight. After loading the sample 1-ml fractions were immediately collected until no more protein

was eluted (35 fractions). Fractions 10 to 14 were selected for the photoaffinity labeling experiment in Chapter III.

b) Overexpression at 37°C:

R1 and R1 deletion mutants were induced with 0.4 mM IPTG at 37°C. After harvest, a soluble fraction, an insoluble fraction, and a fraction containing protein from the periplasmic space were prepared following the protocol from Quiagen, Inc. (1992). Samples were analyzed by 12% SDS-PAGE gel electrophoresis after boiling for 5 min in sample loading buffer (Laemmli, 1970).

### **II.3.c. Results**

#### *Overexpression and Purification*

The overexpression of R1 and R1 deletion mutants at 37°C yielded considerable amounts of protein in the insoluble fraction (Fig.II.4). To obtain soluble protein, the protocol of M. Slabaugh was adapted, which involves overexpression at 15°C. Although this reduced overexpression significantly, the amount of soluble protein was increased.

Only the full length protein was successfully purified with the 6xHis/Ni-NTA-chromatography system (Fig.II.5.A). The R1 deletion mutants showed very low quantities and higher impurities (data not shown). A two-liter culture yielded approximately 3 mg of full-length R1 after NTA-Chromatography.

The full length protein could be further purified with a Superose 12 column (Fig.II.5.B). This step did not yield pure R1-His, but did split

up the contaminants in different fractions. Fraction 10 and 11 contained a second band representing an approximately 70,000 molecular weight protein. To determine if the second protein is a derivative of R1 with a C-terminal deletion, a Western blot (Fig.II.6) was performed and the protein was probed for the 6xHis-tag by precipitation with Ni-NTA-resin. Both tests confirmed that the second band represents a R1-His C-terminal-deleted protein of approximately 70 kD which was, therefore, named R1-His-70kD.

### **II.3.d. Discussion**

The observation made by M. Slabaugh that R1 was difficult to overexpress as a soluble protein in *E. coli* at 37°C was also observed with the His-tagged R1. The majority of R1-His and its deletion mutants were in the insoluble fraction.

Overexpression at 15°C yielded small amounts of soluble R1 and its deletion mutants. The usefulness of the NTA-chromatography was limited to the full-length R1. It was not possible to purify substantial amounts of any of the deletion mutant proteins. The speculation can be made that the deletion of the C-terminal end caused the N-terminal end to be buried in the protein, therefore hiding the 6xHis-tag, which subsequently prohibited any purification attempts by NTA-chromatography.

The power of 6xHis/Ni-NTA-chromatography becomes obvious in the purification of the full-length protein. In a one-step purification it was possible to purify R1-His, from *E. coli* with few contaminants. One

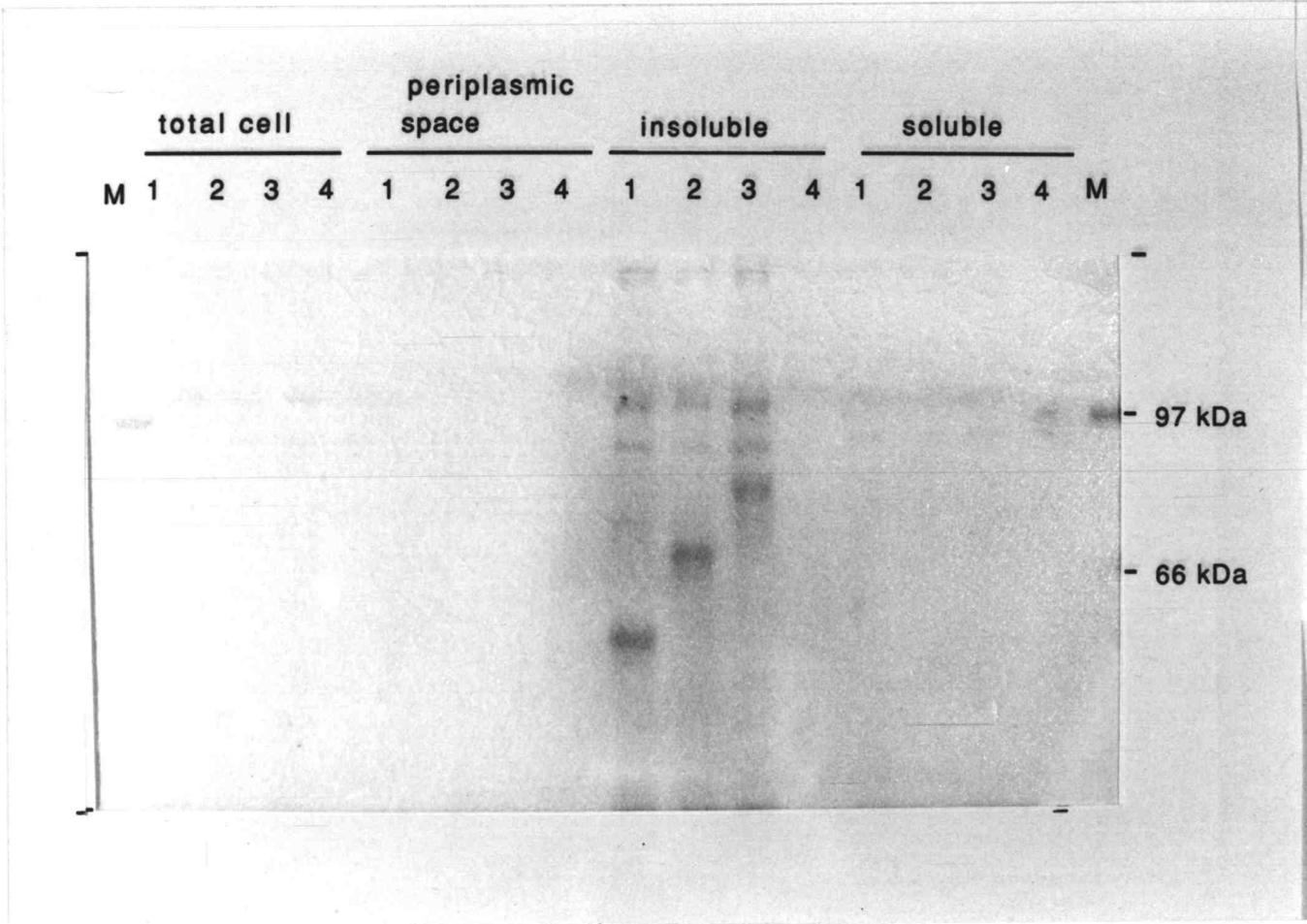
of the major contaminants was a 70-kD protein. It was found that this protein was a C-terminal deleted R1-His, based on the observation that it has the 6xHis tag and its affinity to R1 antibodies. This protein appears to be the result of proteolytic cleavage, which should be possible to reduce by using protease inhibitors and reducing freeze-thaw cycles. Separation was attempted with a run over a Superose 12 column but yielded only moderate separation of R1-His and R1-His-70kD. In any case Ni-NTA-chromatography-based purification is an important improvement to the method developed by Slabaugh. If the R1-His proves to feature the same properties as the native vaccinia virus R1 this system is a convenient way to isolate large quantities.

The option to cleave off the His-tag with thrombin should also be considered. This would leave the enzyme only with GlySerHis as additional amino acids which should not interfere with the natural properties of the protein.

**Fig.II.4:** Overexpression of R1-His in *E. coli* at 37°C

- (A) 8% SDS-PAGE gel, 1. No induction, 2. R1-His, 3. R1-His-C664, 4. R1-His-C554, M. Marker lane: Phosphorylase B (rabbit muscle) 97400 Da; Serum albumin (bovine) 66200 Da
- (B) 15% SDS-PAGE gel, 1. No induction, 2. R1-His-C386, 3. R1-His-C264, 4. R1-His-C554, M. Marker lane: Phosphorylase B (rabbit muscle) 97400 Da; Serum albumin (bovine) 66200 Da; Carbonic anhydrase (bovine) 31000 Da; Trypsin inhibitor (soybean) 21500 Da; Lysozyme (hen egg white) 14400 Da

Fig. II.4



Panel A

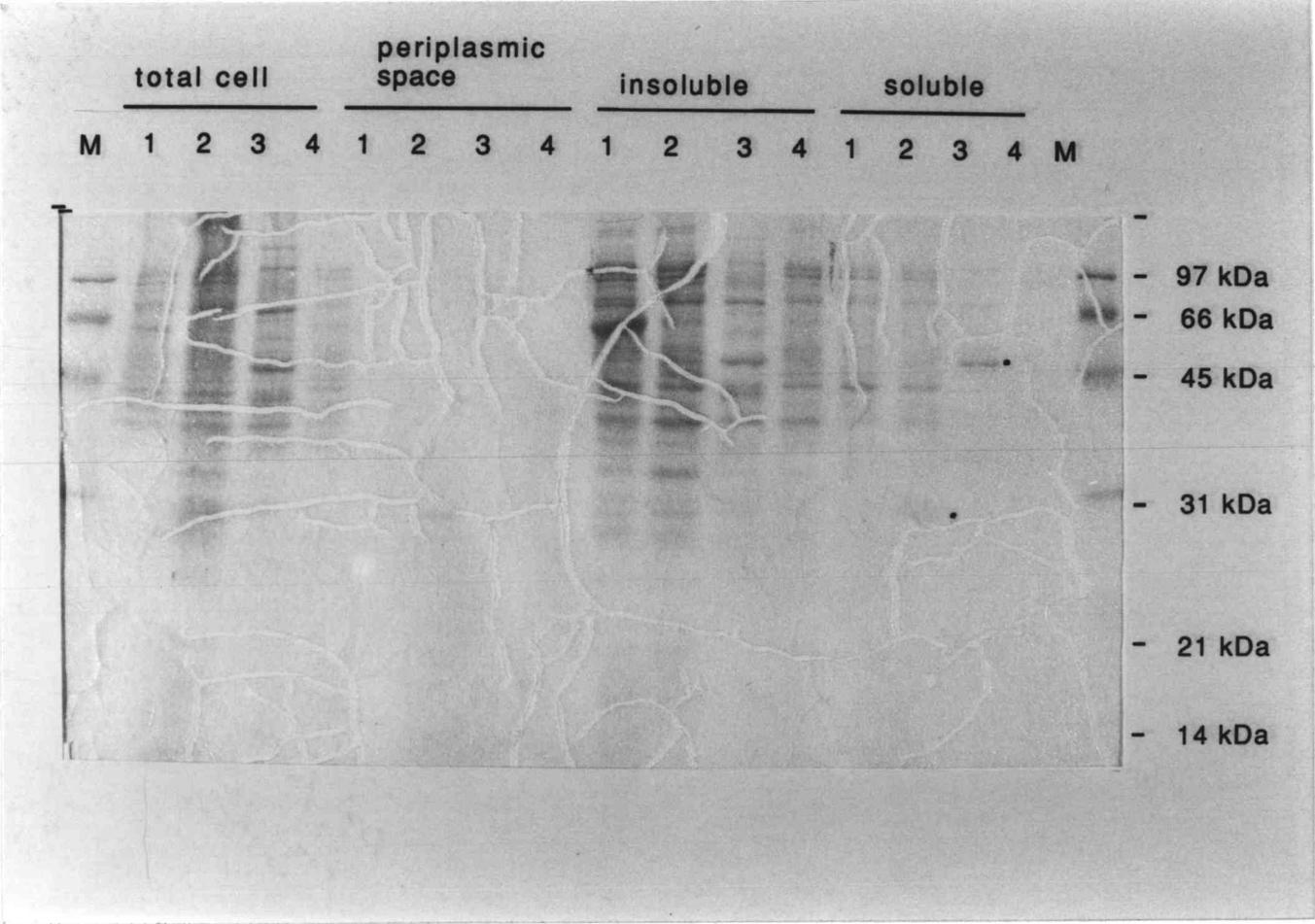


Fig. II.4, Continued

**Fig.II.5:** Elution profile of R1-His from Ni-NTA-chromatography and Superose 12 elution profile

- (A) 12% SDS-PAGE gel of fraction collected after elution with imidazole from Ni-NTA-column; every second fraction was loaded on the gel.
- (B) 12% SDS-PAGE gel of fraction collected after Superose 12 column run.

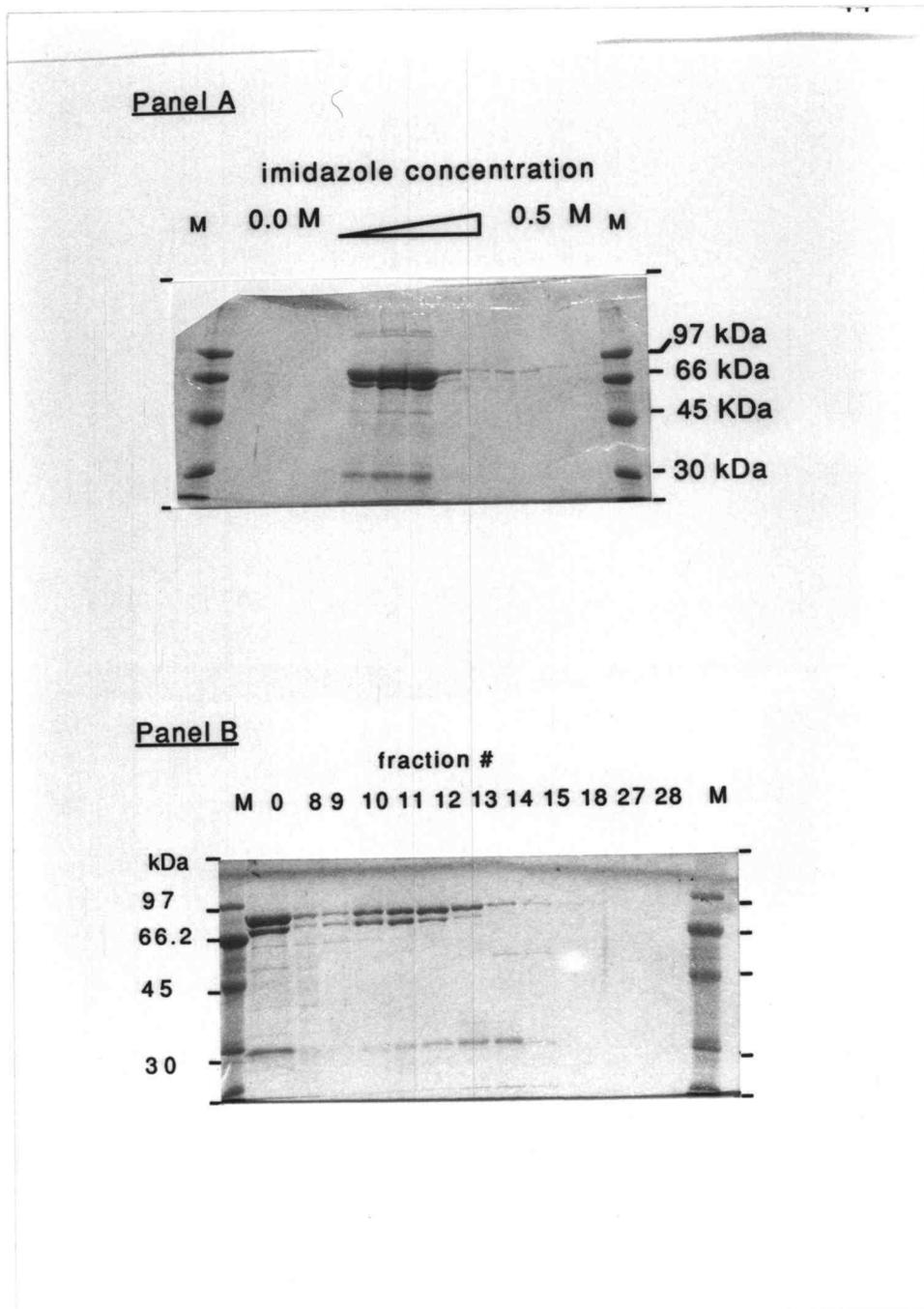
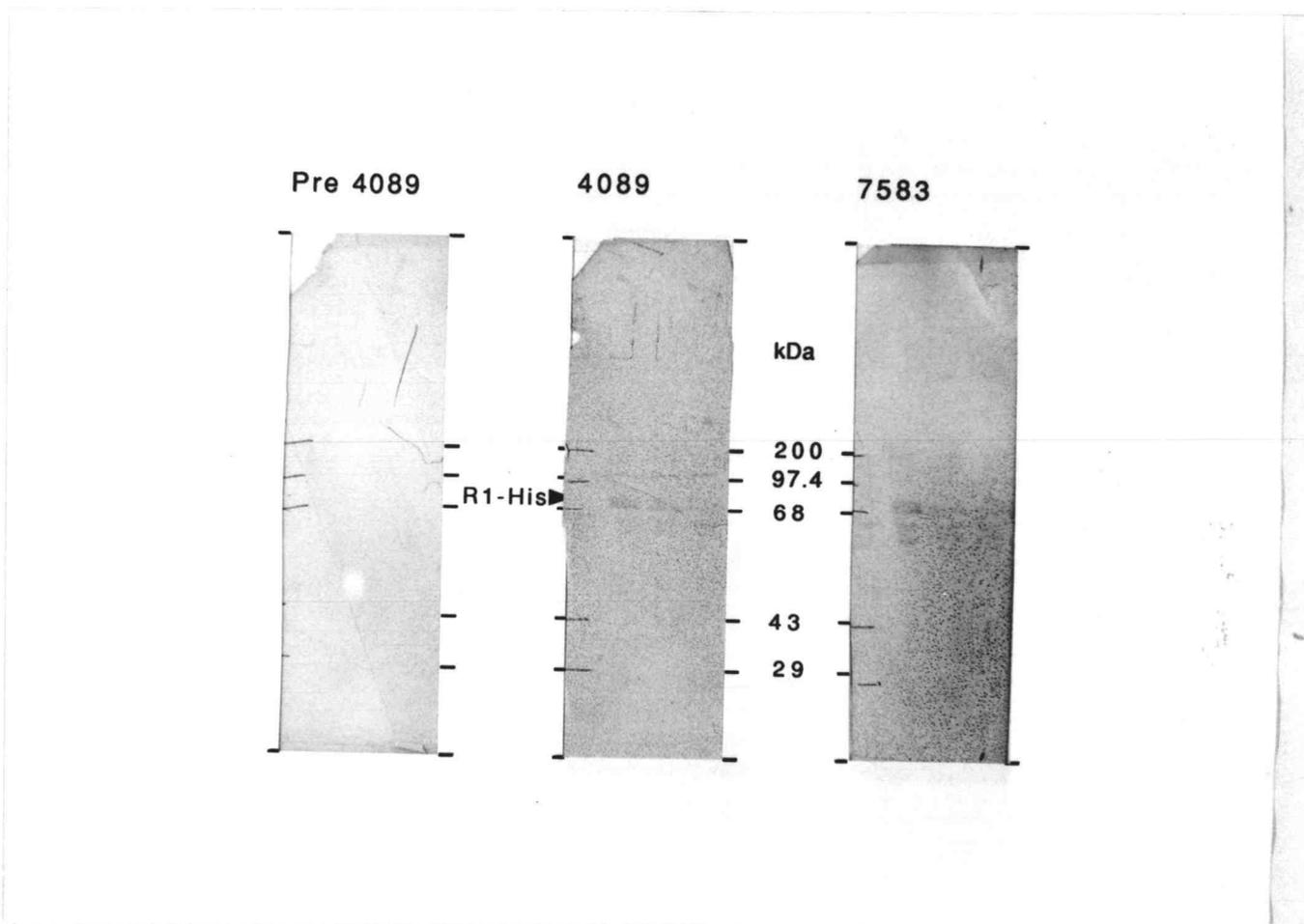


Fig.II.5

**Fig.II.6:** Western Blot of R1-His with antibody #4089 and #7583

8  $\mu\text{g}$ , 4  $\mu\text{g}$ , and 2  $\mu\text{g}$  of R1-His protein were loaded in each lane. #4089 is an antibody to lac-fusion protein lacking the N-terminal end (50 amino acid residues). #7583 is an antibody to the full-length R1 protein.

Fig. II.6



## II.4. Vaccinia Virus/T7 RNA Polymerase Hybrid System

### II.4.a. Introduction

The vaccinia virus/T7RNA polymerase hybrid system (Fuerst *et al.*, 1986) was developed to overexpress eukaryotic genes in a eukaryotic environment. By taking advantage of the strong T7 promoter high levels of RNA can be synthesized by the T7 RNA polymerase, which is integrated into the genome of vTF7-3 strain of vaccinia virus, once the plasmid containing the gene of interest is transfected into the infected cell. This RNA can then be translated into the desired protein.

This system is an alternative approach for obtaining the deletion mutants, which could not be isolated with the *E. coli* system. It accepts the same plasmid constructs as the ones used for the *E. coli* system. Further it yields RR overexpressed in its native environment.

### II.4.b. Materials and Methods

#### *Buffers*

Lysis buffer was 100 mM Tris-Cl (pH 8.0 at 25°C), 100 mM NaCl, 0.5 % Triton X-100, 0.2 mM phenylmethylsulfonylfluoride (PMSF). SDS loading buffer was 15%  $\beta$ -ME, 15% SDS, 1.5% bromophenol blue, 50% (v/v) glycerol.

#### *Virus and cells*

Vaccinia virus strain vTF7-3 was generously provided by B. Moss.

BSC-40 cells are monkey kidney cells, a clonal derivative BSC-1 cells selected for growth at 40°C.

BSC-40 cells were grown overnight in MEM medium (5% CO<sub>2</sub>) at 37°C to confluency in wells of a 6-well tissue culture dishplate (5x10<sup>5</sup> cells/well). The plasmids pET-15b-R1His and pET-15b-R1His-C554 were purified by CsCl gradient centrifugation. By means of liposome-mediated transfection (BRL, Inc.), 5 mg of plasmid was introduced to the cell (Appendix A.5). The transfected cells were then infected at a multiplicity of infection (MOI) of ten with the vaccinia virus vTF7-3 strain, which carries the gene for T7-RNA polymerase. After 24 hours the medium was aspirated off and 1 ml of PBS was added. After scraping the cells from the flask surface the cells were centrifuged at 3000 rpm for 1 min. The pellet was then resuspended and lysed in 200 ml lysis buffer by freezing and thawing three times. The overexpressed RI-His protein could then be precipitated by adding 10 ml of 50% NI-NTA-resin equilibrated in lysis buffer. After washing three times in lysis buffer, SDS loading buffer was added containing 0.1 M EDTA. The gels were stained with colloidal Coomassie stain (Neuhoff *et al.*, 1988) for 12 hours.

### **II.4.c. Results**

The Coomassie-stained gel and the Western blot of R1-His and R1-His-C554 show a band corresponding to approximately 87 kDa (Fig.II.7). This band is not visible in the control experiment with BSC40 cells infected with vTF7-3 and no plasmid. The deletion mutant R1-His-C554 could not be purified or overexpressed.

**Fig.II.7:** Western Blot and colloidal Coomassie-stained gel of R1-His purified from Vaccinia Virus /T7 RNA polymerase hybrid system

- (A) 12% SDS-PAGE gel stained with colloidal Coomassie stain. 1. R1-His-C554, 2. R1-His, 3. no plasmid
- (B) Western Blot (5% MeOH transfer buffer); 1. R1-His-C554, 2. R1-His, 3. no plasmid

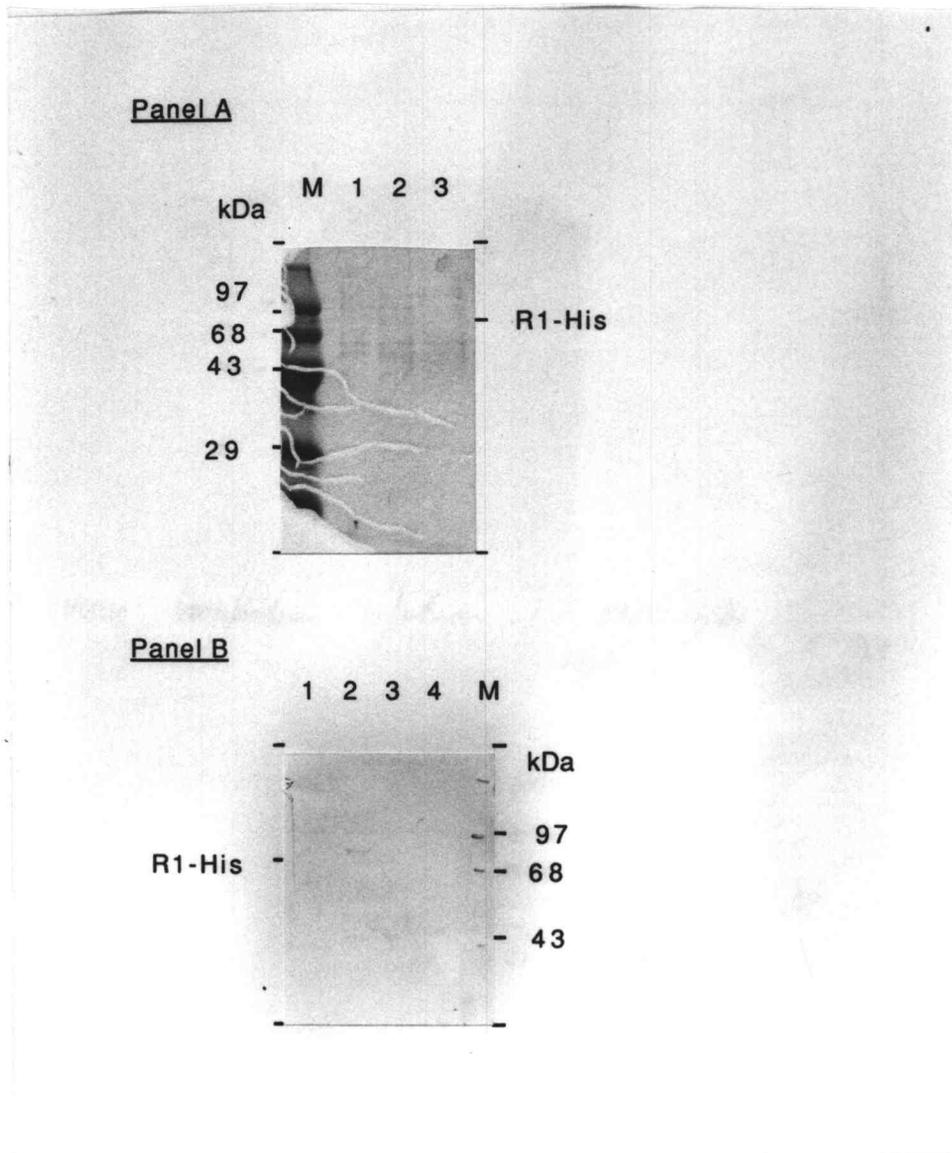


Fig.II.7

#### **II.4.d. Discussion**

The T7 RNA polymerase vaccinia virus/T7 RNA polymerase hybrid system yielded overexpressed full-length R1 protein, which could be purified with Ni-NTA-resin. This was not possible with the deletion mutants. Whether the overexpression or the purification was responsible for this inability to obtain the deletion mutant R1-His-C554, cannot be determined from this experimental set up. It is interesting to note that overexpression and purification of R1-His-C554 was neither possible in *E. coli* or the vaccinia virus/T7 RNA hybrid system.

The Coomassie-stained gel of the Ni-NTA-resin purified proteins showed significant impurities, which could possibly be reduced by more stringent washing conditions. On the other hand these impurities might also point to possible protein-protein interactions with the R1-His. This assumption does, however, not hold considering that the control experiment showed a similar pattern of bands. This indicates high nonspecific binding of the Ni-NTA-resin to proteins from the lysate.

Since the deletion mutant R1-His-C554 could not be synthesized or purified using the vaccinia virus overexpression system with subsequent Ni-NTA chromatography purification, it was concluded that the system could not be used to produce deletion mutants which could be used to test for their binding to dATP-resin. This would have been the system of choice since the gene studied would have been overexpressed in its native environment.

It would also be interesting to see if the overexpressed R1 has any effect upon nucleotide pools and DNA synthesis. Very high levels of R1

as observed in the expression system might affect the control mechanism involved in the regulation of balanced nucleotide pools for DNA synthesis.

## **II.5. Conclusion**

The use of deletion mutants to gain structural information about vaccinia virus R1 was not possible. It appears that the overexpression of C-terminal deleted R1 is not feasible. The deletion mutant protein which copurified with R1-His in the *E. coli* expression system is most likely a product of proteolytic cleavage of the full-length R1-His. With the introduction of the His-tag the purification of the deletion mutant should have been possible without further modifications to the purification procedure for each of the constructs.

The Ni-NTA affinity chromatography purification system increased the yield twofold of full-length R1-His from *E. coli* and simplified the purification scheme. The availability of large quantities of R1-His makes it now possible to use a different approach to map nucleotide binding sites on R1.

It should be kept in mind that R1-His has not been examined for its enzymatic activity. The additional sequence at the N-terminal end might interfere with assembly of the holoenzyme and therefore prohibit enzyme activity. This, however, might not necessarily mean that the nucleotide binding sites of the R1-His monomers are affected by the 6xHis tag. It should be noted that about 150 proteins purified with Ni-NTA

affinity chromatography showed identical biological properties as compared to their non-His-tagged counterparts.

### III. Photoaffinity Labeling of Full-length R1-His

#### III.1. Introduction

With the availability of large amounts of R1-His through Ni-NTA-chromatography it should be possible to do photoaffinity labeling to map nucleotide binding sites on R1-His. In this study the focus was on the dTTP and dATP binding sites. These nucleotides have been used in similar photoaffinity labeling experiments with mouse RR (Eriksson *et al.*, 1982; Caras and Martin, 1982). Choosing these two nucleotides should therefore make it possible to compare the mouse with vaccinia virus RR in terms of their binding properties to dTTP and dATP. Both nucleotides bind to the specificity site with high affinity. dATP in addition binds to the activity site with low affinity. After labeling, a proteolytic digestion was performed to identify fragments carrying the label. I also attempted to obtain the sequence of these fragments by mass spectrometry and therefore map the location of the nucleotide binding sites.

#### III.2. Material and Methods

##### *Buffers*

R1-His solution buffer was 20 mM Tris-Cl (pH 8.1 at 25°C), 1 mM KCl, 5.0 mM MgCl<sub>2</sub>, 1.0 mM EDTA, 0.5 mM DTT, 20% v/v glycerol, 1 μM of [<sup>32</sup>P] labeled nucleotide (spec. activity 650 Ci/mmol, purchased from

ICN Biomedicals, Inc.). Protease buffer was HEPES-NaOH (pH 8.0 at 25°C). 5x SDS Sample Buffer was 15%  $\beta$ -ME, 15% SDS, 1.5% bromophenol blue, 50% glycerol.

*Photoaffinity labeling of R1 with [ $^{32}$ P]dATP and [ $^{32}$ P]dTTP*

R1-His was purified from *E. coli* as described in Chapter II of this thesis. Fractions 10 and 11 were pooled and used for the labeling competition experiment of R1-His and BSA. Fractions 13 and 14 were pooled and used for the labeling experiment of R1-His with subsequent proteolytic digest.

The partially purified protein was brought to a concentration of 1.0  $\mu$ g/ $\mu$ l in R1 storage buffer. A volume of 15  $\mu$ l of this solution containing the appropriate nucleotide was then placed in the inside of the lid of a 1.5-ml Eppendorf tube. The lid was cut from the Eppendorf tube, then placed on a metal block cooled to 4°C sitting in an ice bath. Subsequently the samples were then exposed to UV light (gemicidal lamp, 50 W) at a 10-cm distance for 20 min. After exposure to UV light the lid was snapped on the top of the Eppendorf tube, which was spun in a microcentrifuge for 2 min.

The samples for the mass spectrometric analysis were treated the same but contained non-labeled nucleotides instead of [ $^{32}$ P]-labeled.

The labeling experiments with [ $^{32}$ P]-dATP for the proteolytic digestion were done in the presence of dGTP in a concentration that was ten-fold higher than [ $^{32}$ P]-dATP.

### *Proteolytic digestion*

After the photolabeling reaction, the solution was divided into four tubes. The first served as undigested control and the second, third and fourth for digestion by trypsin, chymotrypsin and V8 protease, respectively. The digestion was stopped after 20 min in experiment I and 90 min in experiment II. Trypsin and V8 were added in a 1:10 ratio (weight) of protease to R1-His, and chymotrypsin in a ratio of 1:100. The reaction was stopped by adding 5x SDS-PAGE sample buffer and heating for 2 min at 80°C. The samples were then loaded on a 12% acrylamide gel. After the electrophoresis the gels were dried and exposed to film.

After the digestion the non-radioactive samples were given to the Mass Spectrometry Lab of the Environmental Health Science Center at O.S.U for MALDI mass spectrometric analysis.

### **III.3. Results**

#### *Photoaffinity labeling of R1-His with [<sup>32</sup>P]dTTP*

The photoaffinity labeling of R1-His with dTTP yielded two bands corresponding to full-length R1-His and R1-His-70kD (Fig III.1.A). In competition experiments with cold dTTP and dGTP the label was significantly reduced for the full-length R1-His but not for the R1-His-70kD.

*Photoaffinity labeling of R1-His with [<sup>32</sup>P] dATP*

Photoaffinity labeling of R1-His with dATP yielded a band corresponding to R1-His (Fig. III.1.B). No labeling of the R1-His-70kD protein was observed. The competition experiment with cold dATP and cold dGTP yielded bands with much reduced intensities. It was not possible to discriminate the intensity of these bands. This experiment was carried out at both 4°C and -60°C to compare labeling efficiencies. It appeared that the different temperatures did not affect the labeling significantly.

*Photoaffinity labeling of bovine serum albumin with [<sup>32</sup>P]dTTP and [<sup>32</sup>P]dATP*

To test for specific labeling, bovine serum albumin (BSA) was chosen as a control. The experimental conditions were the same for BSA and R1-His. BSA was labeled with [<sup>32</sup>P]dTTP and [<sup>32</sup>P]dATP (Fig.III.2). For both experiments the labeling pattern was the same as for the corresponding R1-His labeling experiment. The labeled BSA could be competed away with the cold nucleotide as well as with dGTP.

*Proteolytic digest of dTTP labeled R1-His*

The digestions were carried out for 20 min in Experiment I (Fig.III.3) and 90 min in Experiment II (Fig. IV.) Only when compared to the control it was possible to identify peptides with specific label. The ones thought to carry a label are numbered. In Experiment I band 1 and 3

appear to be specifically labeled. Band 2 in the V8 digest shows almost the same intensity as the control experiment. The first two lanes show the non-digested R1-His. Bands 4 and 5 are impurities carrying a label. Interestingly enough only band 5 could be competed away in the control experiment.

In Experiment II, six bands appeared to be labeled. However, it should be noted that several bands were detected, making it difficult to determine which bands were specifically labeled.

#### *Proteolytic digestion of dATP-labeled R1-His*

The interpretation of the 90-min. digest with dATP-labeled R1-His was simplified by the fact that fewer fragments carried label (Fig.III.4). By comparison with the control lane it was possible to identify four bands (numbered 1,2,3, and 4) which appear to be specifically labeled. The tryptic digest also featured a band #5 which showed the same intensity in the control lane. The 20-min. digest was difficult to interpret since the autograph featured only weak labeling making it difficult to see over the very high background. The weak bands were found to correspond to 1 and 3 in the 90-min. digest.

#### *Mass spectrometric analysis of proteolytically cleaved R1-His*

Out of the 12 samples from Experiment I and II only the V8 digest yielded peaks whose masses were above 40 kDa. The other sample yielded peaks mostly below 40 kDa. Furthermore, it was difficult to determine the exact mass of those peaks showing up on the spectra. The peaks were often very wide and therefore it was difficult to obtain exact masses.

The spectra of V8-digested (90 min.) dATP-labeled R1-His is shown in Fig.III.6a as an example. Fig. III.6.b shows peak #3 scaled to show how wide the peak is.

Similar spectra were obtained for the other samples. An attempt to assign the labeled bands from the autograph into peaks of the mass spectrum was made based on similar molecular weight, but it became obvious that the assignments were too questionable to be used for further analysis.

#### *Sequence determining of labeled fragments*

Due to the difficulties in relating the peaks from the mass spectra to bands from the gel and inexact molecular weights of the protein fragments, we did not attempt to determine the sequence of the protein fragments.

**Fig.III.1: Photoaffinity labeling of R1-His with dTTP and dATP**

- (A) Autoradiograph of 8% SDS-PAGE gel. R1-His was labeled with [<sup>32</sup>P]dTTP; 1. No non-radioactive nucleotide added, 2. + non-radioactive dTTP, 3. + non-radioactive dGTP
- (B) Autoradiograph of 8% SDS-PAGE gel. R1-His was labeled with [<sup>32</sup>P]dATP; 1. No non-radioactive nucleotide added, 2. + non-radioactive dATP, 3. + non-radioactive dGTP

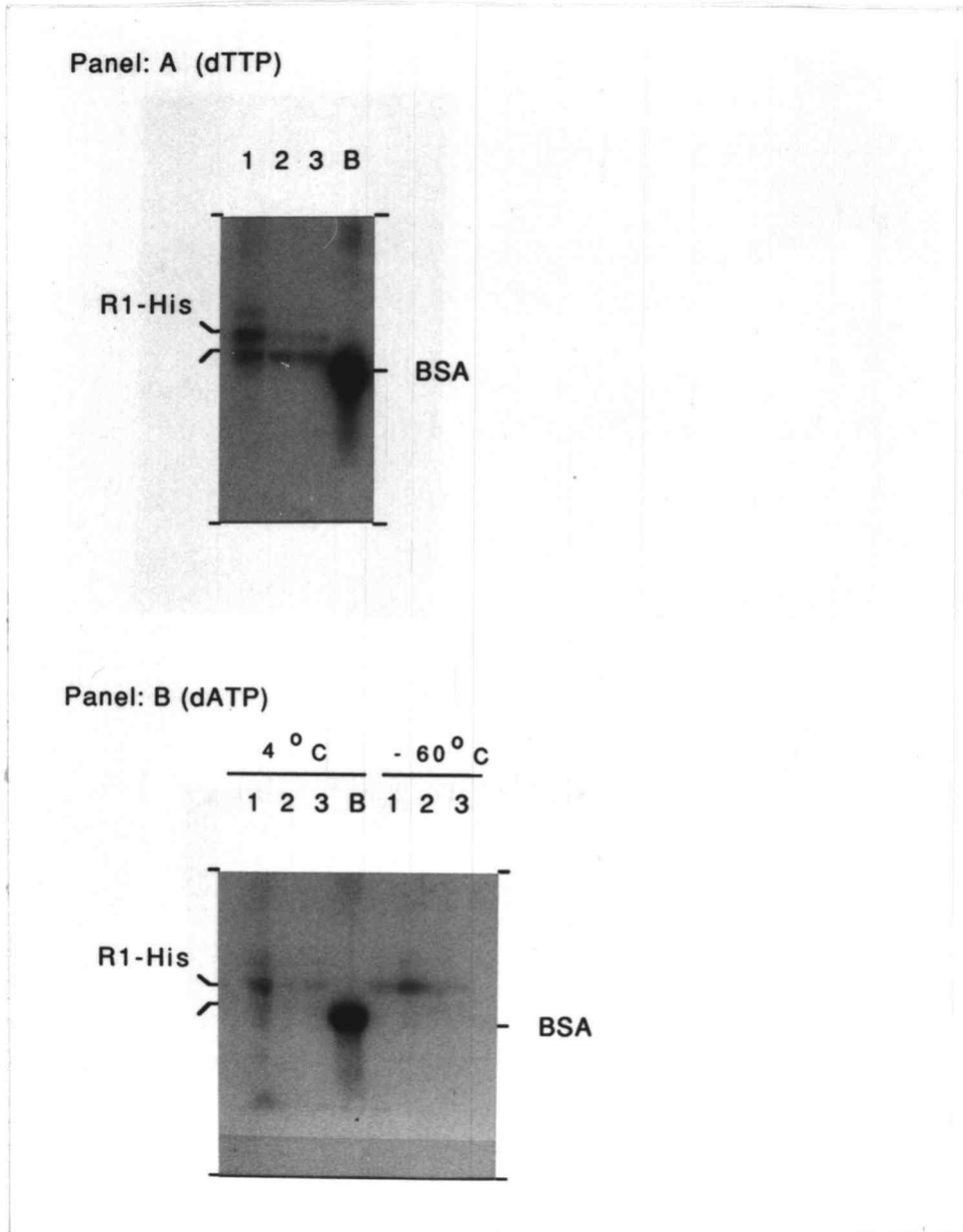


Fig.III.1

**Fig.III.2** Photoaffinity labeling of BSA with dTTP and dATP

Autoradiograph of 12% SDS-PAGE gel. 5  $\mu$ g BSA was labeled with [ $^{32}$ P]dTTP or [ $^{32}$ P]dATP; 1. no non-radioactive nucleotide added, 2. + non-radioactive dTTP or dATP, 3. + non-radioactive dGTP.

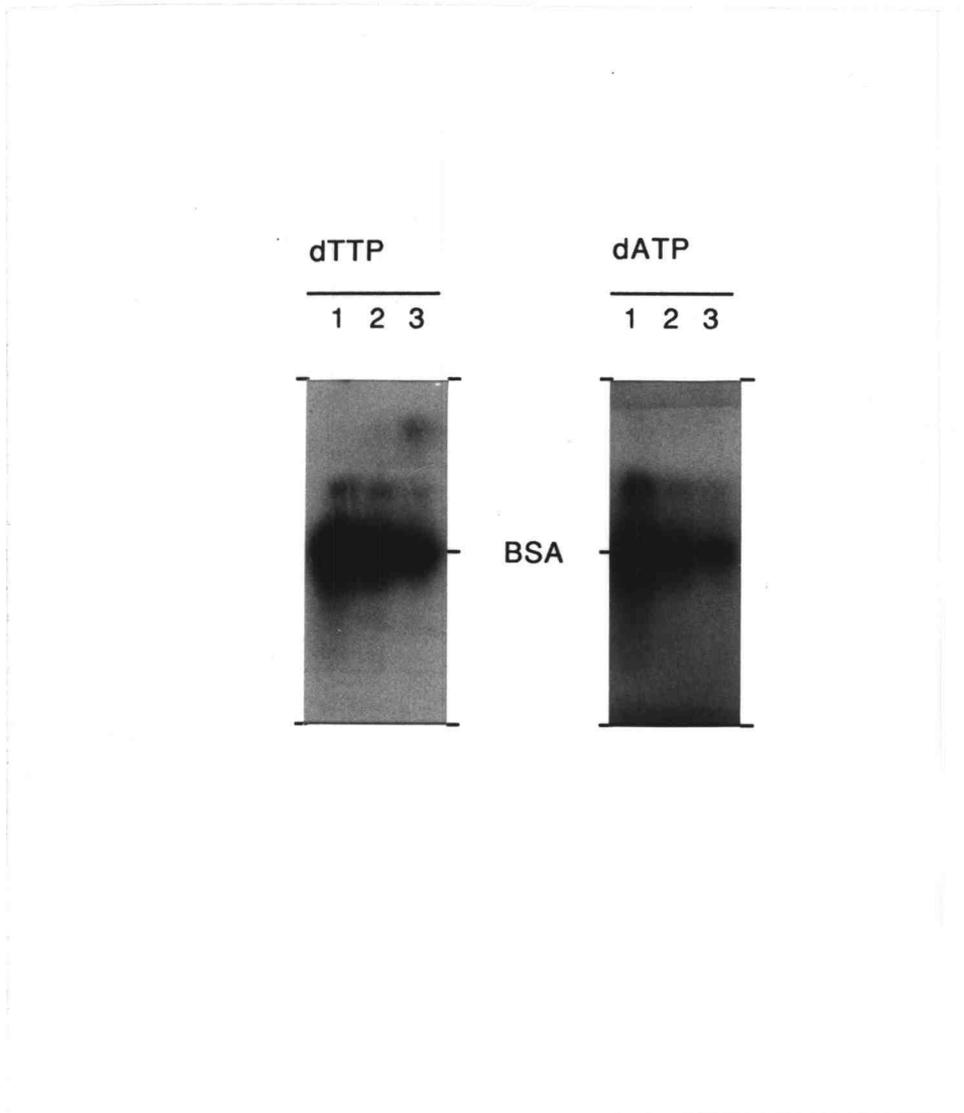
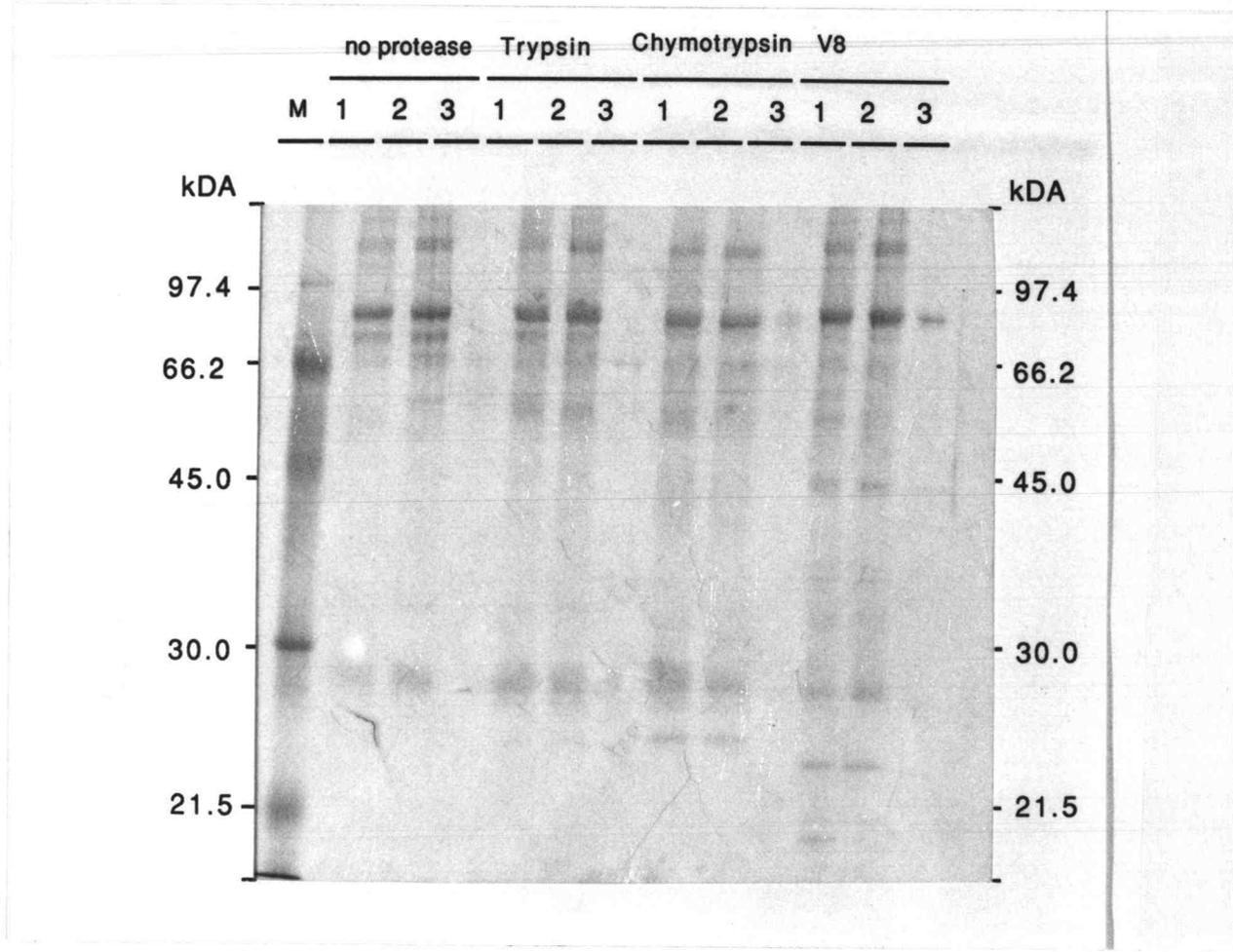


Fig.III.2

**Fig.III.3** Proteolytic digest of [ $^{32}\text{P}$ ]dTTP-labeled R1-His

- (A) Colloidal Coomassie stained 12% SDS PAGE gel of 20 min. digested [ $^{32}\text{P}$ ]dTTP labeled R1-His
- (B) Autograph of the same gel as in (A)
- (C) Colloidal Coomassie stained 12% SDS PAGE gel of 90 min. digested [ $^{32}\text{P}$ ]dTTP labeled R1-His
- (D) Autograph of the same gel as in (C)

Fig. III.3



Panel A

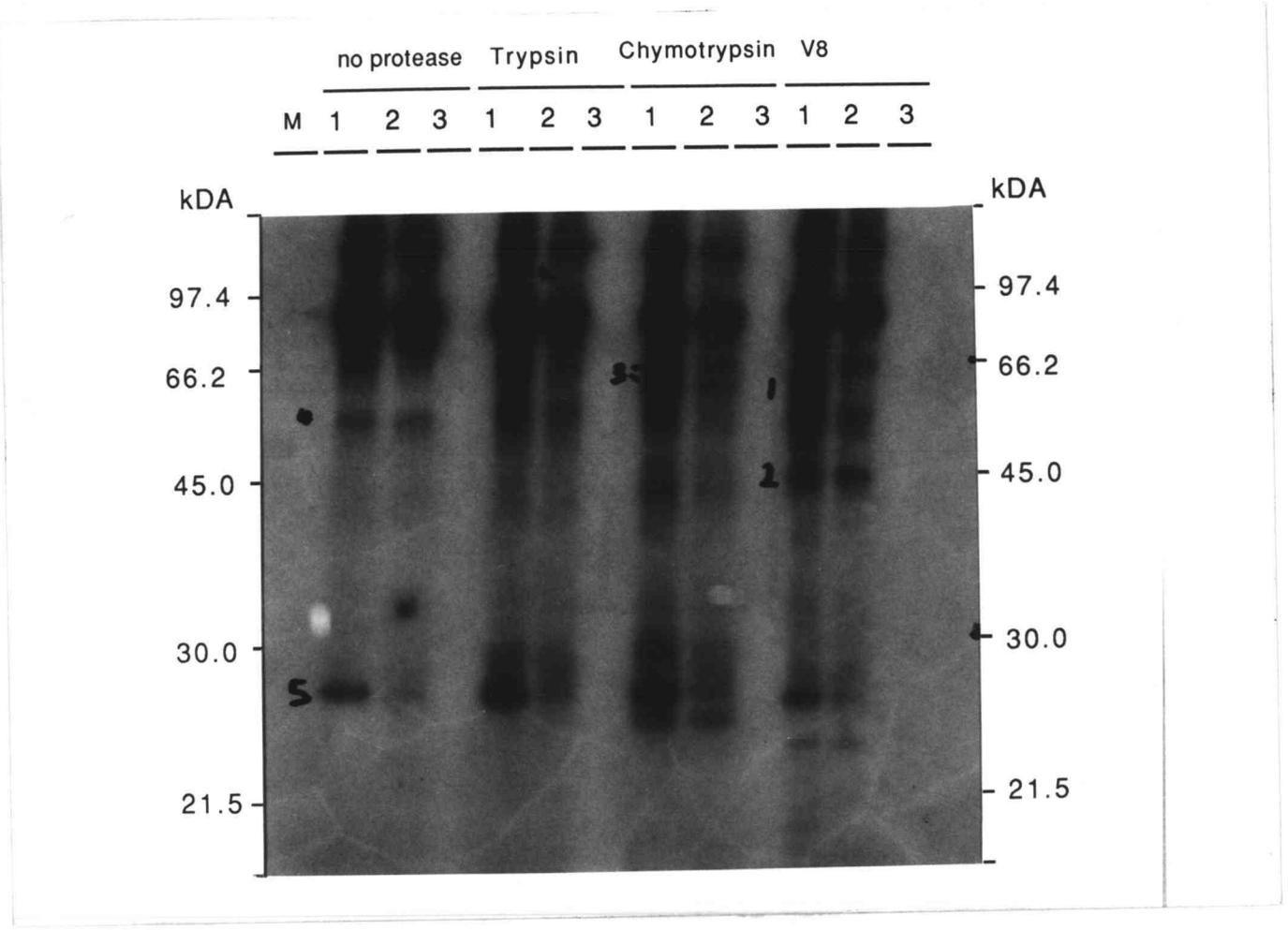


Fig. III.3, Continued

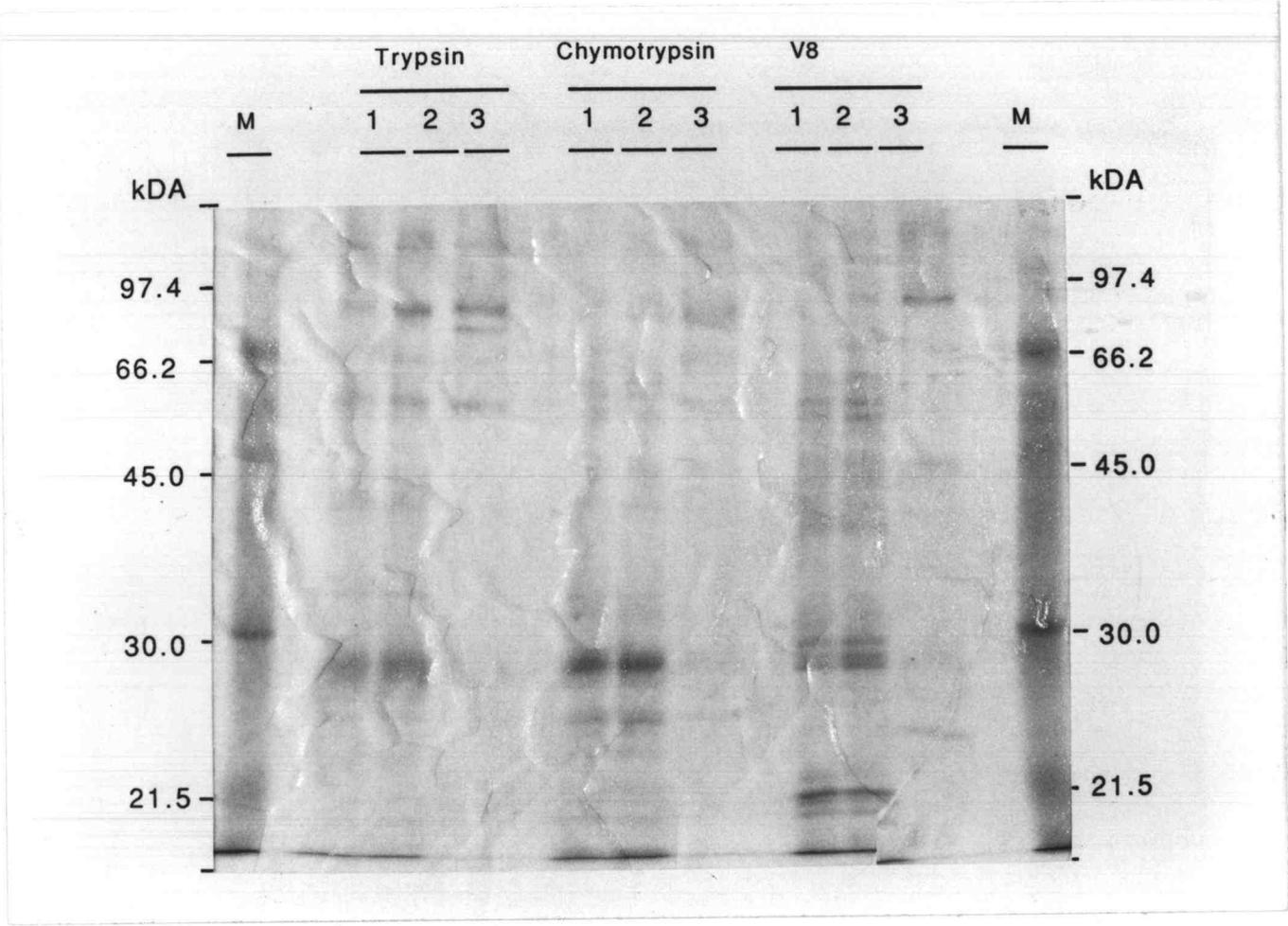
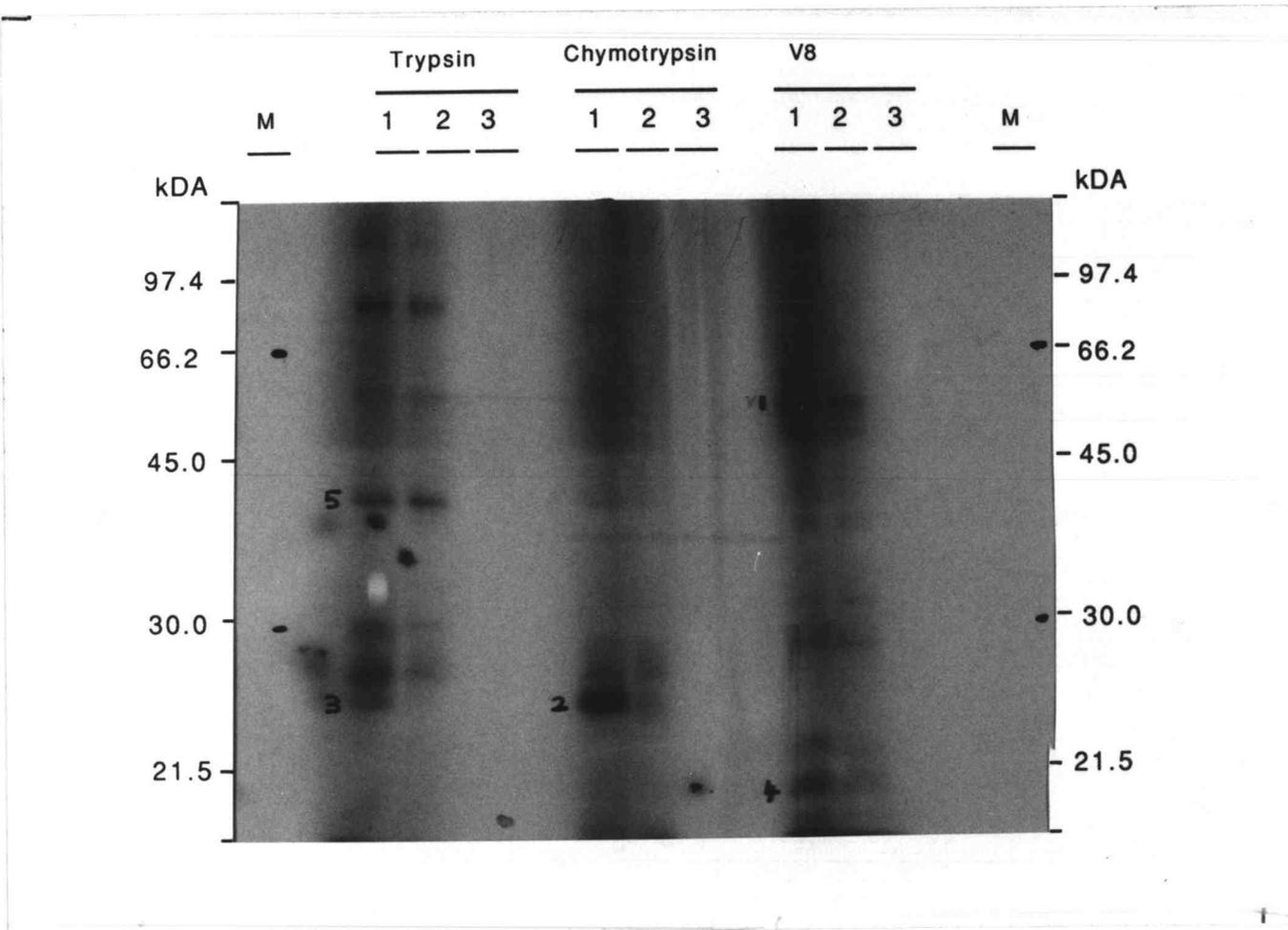


Fig.III.3, Continued

Fig.III.3, Continued

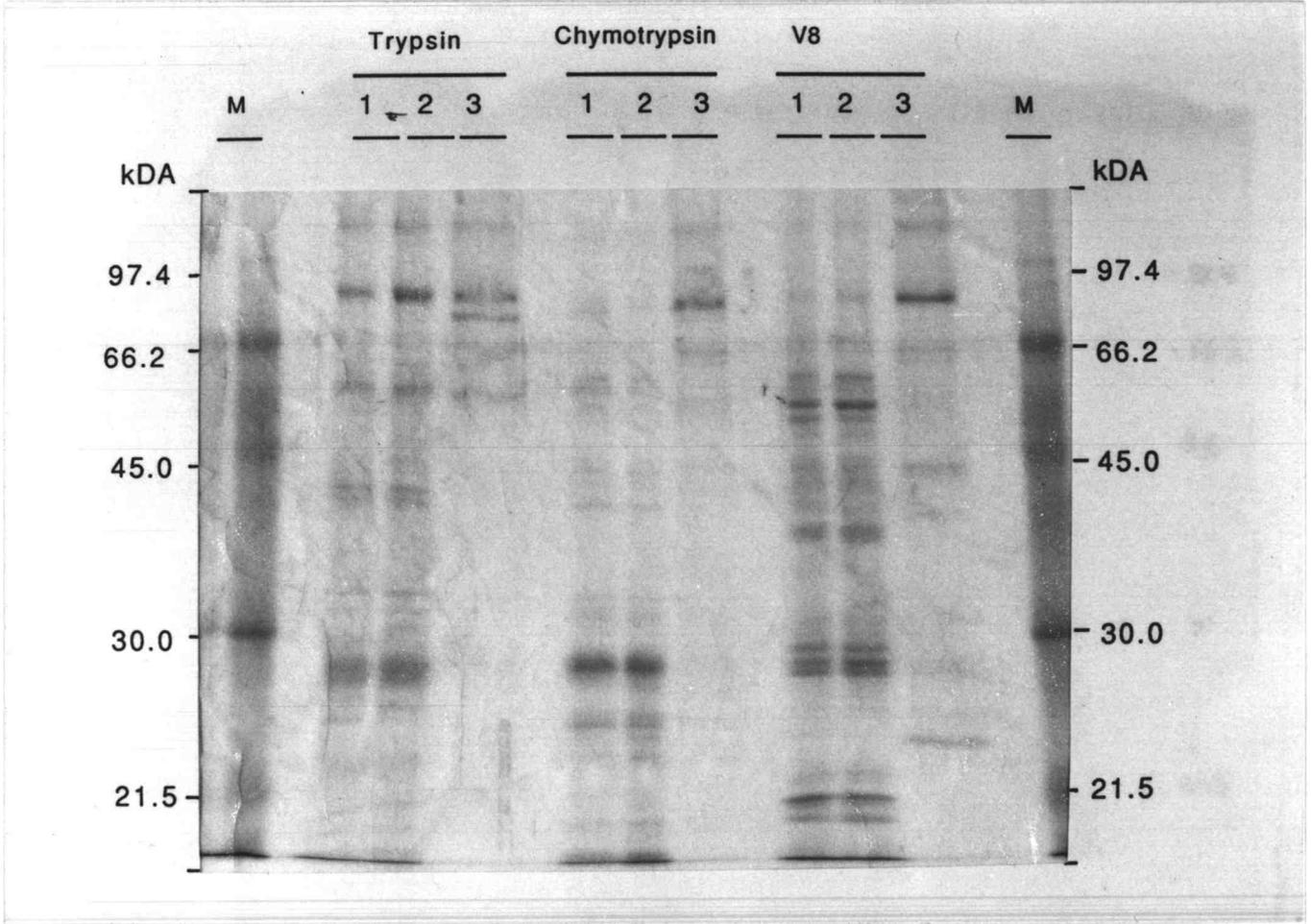


Panel D

**Fig.III.4** Proteolytic digest of [ $^{32}\text{P}$ ]dATP-labeled R1-His

- (A) Colloidal Coomassie stained 12% SDS PAGE gel of 90 min. digested [ $^{32}\text{P}$ ]dATP labeled R1-His
- (B) Autograph of the same gel as in (C)

Fig. III.4



Panel A

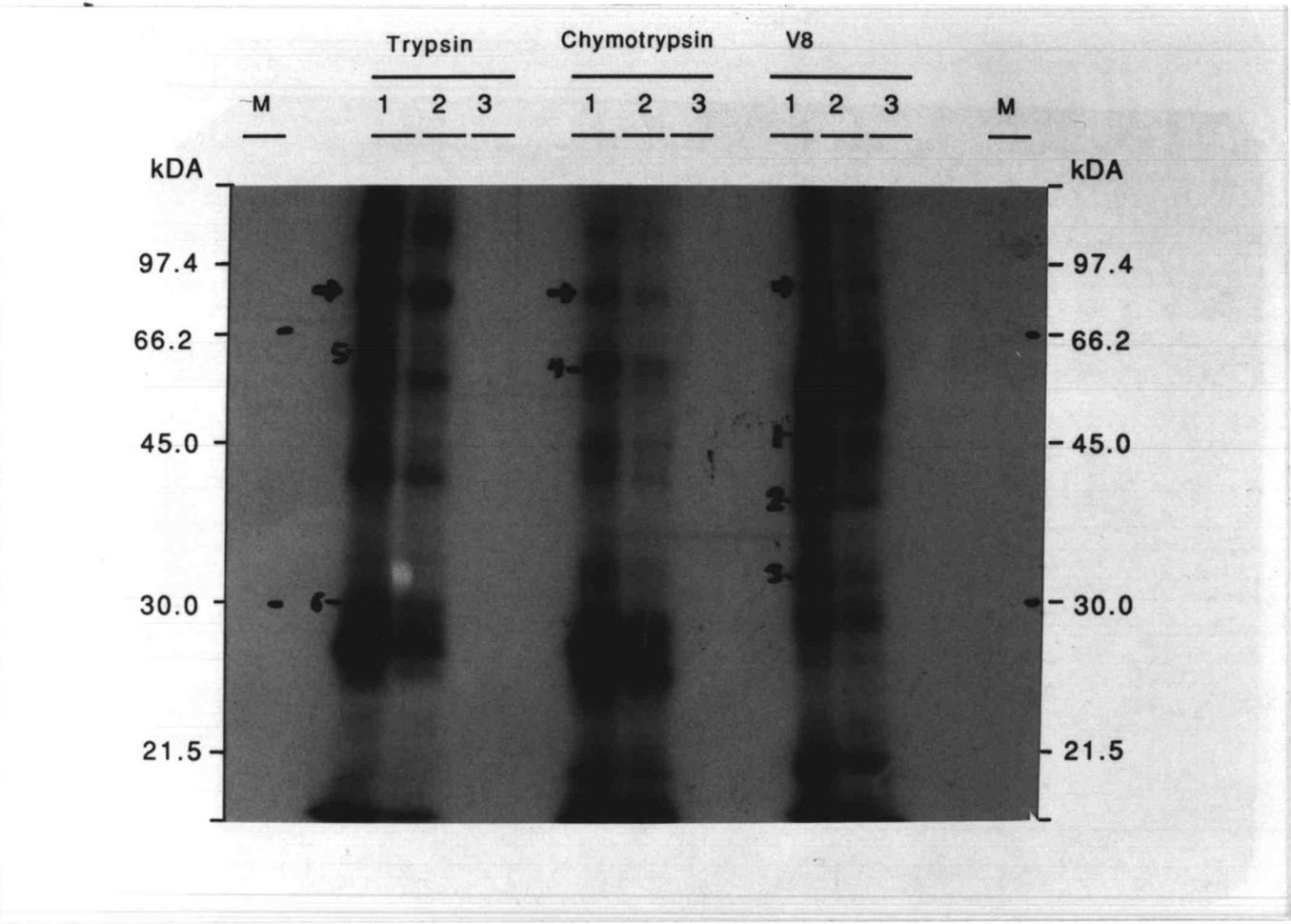


Fig. III.4, Continued

**Fig.III.5** Mass spectrum from dATP labeled 90 min. digest with V8.

(A) full spectrum

(B) selected region of the spectrum in (A)

(continued)

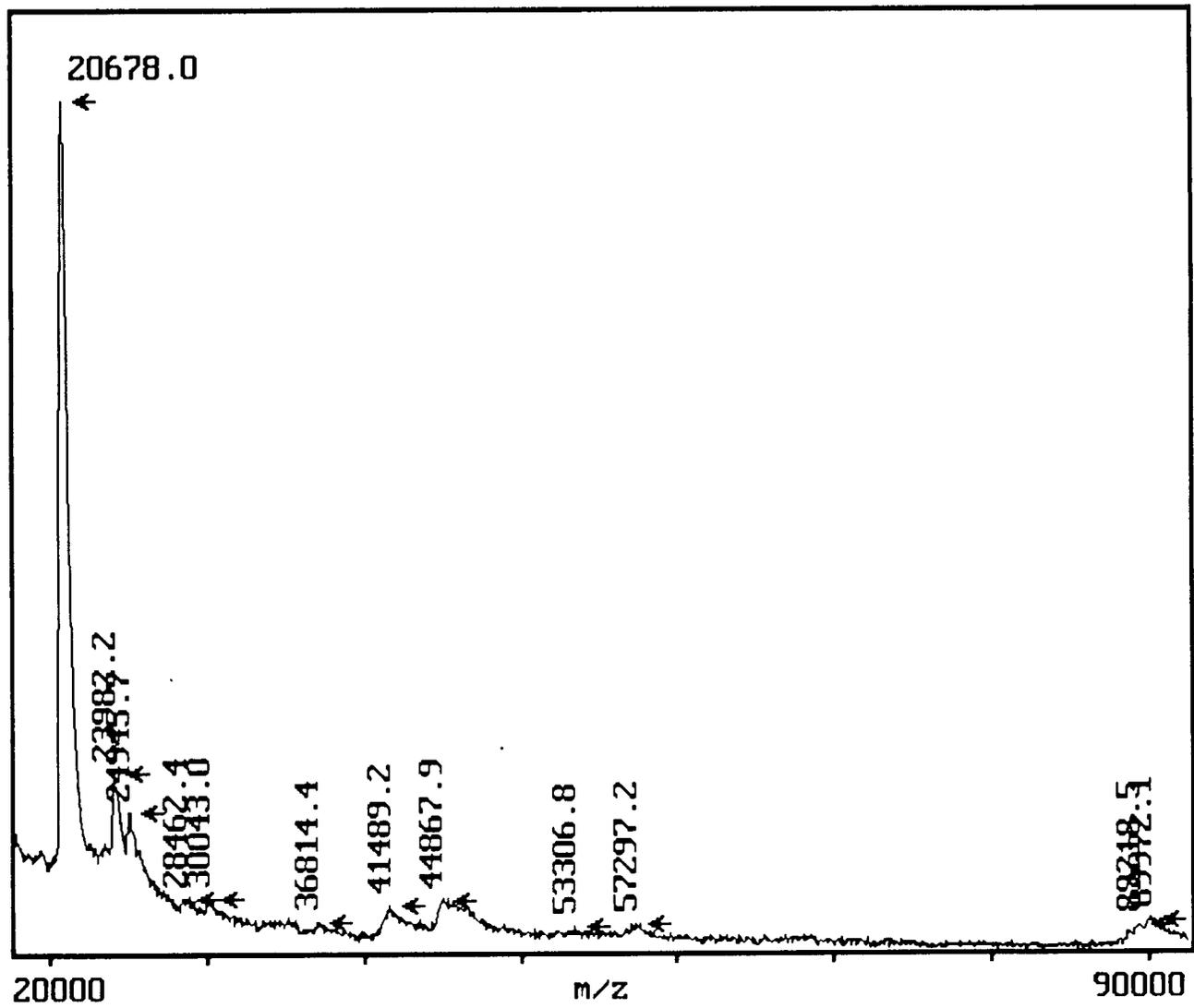


Fig.III.5 Panel A

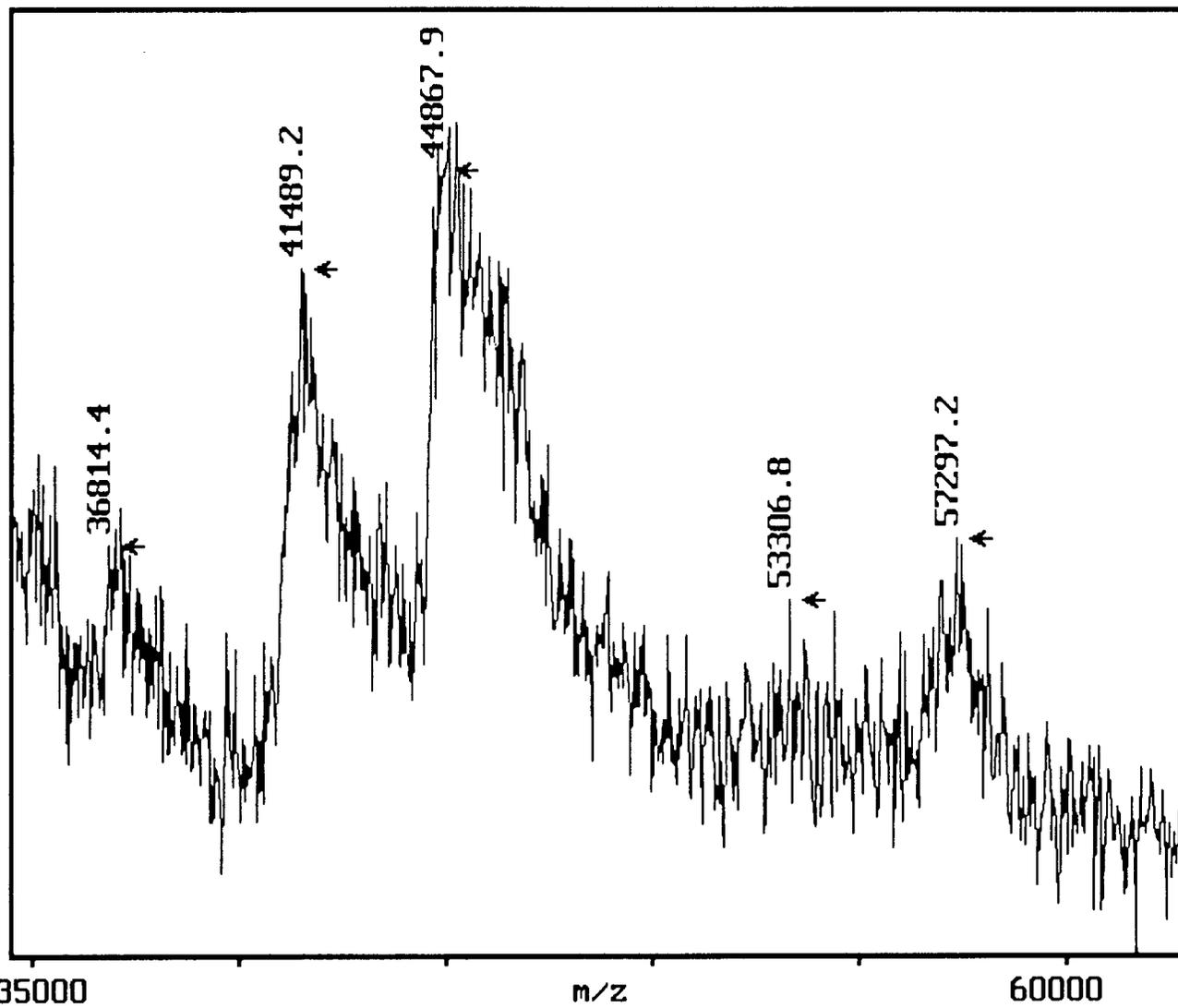


Fig.III.5, Continued Panel B

### III.4. Discussion

#### *Photoaffinity labeling of R1-His and BSA with [<sup>32</sup>P]dTTP and [<sup>32</sup>P]dATP*

It was possible to label R1-His with [<sup>32</sup>P]dTTP and [<sup>32</sup>P]dATP in a photoaffinity labeling experiment similar to the observations of Eriksson *et al.* (1982) and Caras *et al.* (1982). It is an interesting finding that the competition experiment with the dTTP-labeled protein yielded bands of reduced intensity for the full-length but not for the C-terminal deleted one. It is possible to speculate that the full-length protein is necessary to facilitate specific binding to dTTP, and that the loss of the C-terminal end increases non-specific nucleotide binding .

The labeling experiment with dATP is interesting in respect to the labeling of the full-length but not of R1-His-70kD. This indicates that the C-terminal end is required for either the activity site or the specificity site to bind to dATP

The specificity of the labeling experiment must be carefully examined. As shown with BSA as the control it is possible to label proteins nonspecifically with dTTP and dATP. Even the competition experiments with non-radioactive nucleotides yielded similar results compared to R1-His. However, BSA might not be a good choice as negative control. Titrating the amount of nucleotides used in the photoaffinity labeling experiment should help optimizing the condition in favor of specific binding. The concentration of MgCl<sub>2</sub> might also affect specificity of binding and should be addressed in a titration experiment.

*Proteolytic digestion of dTTP-labeled R1-His and dATP-labeled R1-His*

To really understand the specificity of photoaffinity labeling a digestion was performed. Nonspecific binding should yield a wide range of peptides carrying label, whereby specific binding should yield few peptides. The control experiment with non-radioactive nucleotides should further show reduced intensity of the bands compared with the labeled one. Examining the digests performed with the dATP-labeled protein showed only 4 bands. dTTP, on the other hand, yielded a wide variety of bands from which about 6 looked like specific labeling based on comparison with the control lane.

*Mass spectrometric analysis of proteolytically cleaved R1-His*

To identify the sequence of labeled fragments, mass spectrometry was used. The samples were analyzed without further treatment. The resulting spectra were difficult to interpret since the peaks were very broad and the spectra were mostly limited to peaks under 40 mass units. In addition it appeared impossible to assign the bands from the gel to the peaks especially where several bands were clustered together in the gel.

Gels with a better resolution in the molecular weight range of the bands of interest need to be run. The best solution would be to run a 2-dimensional gel from which the spots carrying the label would be cut out of the gel to elute the peptides. To identify these spots a film would be exposed from the gel. These peptides could then be subjected to mass spectrometric analysis to determine their exact molecular weights. If this procedure would be done with three different proteases, different fragments would be obtained. In the best case they would overlap in a

narrow sequence range, to which the possible labeling site could be assigned.

Another way to identify the labeled fragment would be through reverse-phase HPLC after a complete proteolytic digestion. The separated fragments could then be subjected to microsequencing to obtain sequence information.

### **III.5. Conclusion**

Photoaffinity labeling of proteins is a commonly used technique to probe for nucleotide binding sites. The main problem in these studies is to verify the specificity of the labeling reaction. In the study presented here the control experiment with BSA yielded a rather surprising result in that it featured almost the same labeling pattern as R1-His. This needs to be kept in mind when examining the results obtained with the dTTP and dATP labeling of R1-His. The proteolytic digestion performed after labeling did indeed show fragments which seem to carry the label specifically based on comparison with the control experiment. To identify these fragments, mass spectrometric analysis was attempted but was not possible due to poor spectra and problems assigning the peaks to bands observed on the gel. This does not mean it is not possible to locate the label on the R1-His sequence. A different approach in which the dots from a 2D gel would be cut out and analyzed should yield clean enough samples to be suitable for mass spectrometric analysis.

It is also of importance to realize that the R1-His still needs to be examined for its similarity to R1. The His-tag might alter the structure of

the protein in such a way that it also affects the binding site. An enzyme assay needs to be done to address this question. If the enzyme assay does not show any activity, a filter binding assay comparing non-tagged R1 with His-tagged R1-His should be done to see if at least the monomer shows the same nucleotide binding properties.

Since the R1-His featured the same labeling characteristics as the mouse ribonucleotide reductase, this work provides further evidence that the vaccinia virus ribonucleotide reductase is a close relative to the mammalian enzyme. In addition the results presented here indicate that the mapping of the nucleotide binding sites of R1-His is possible, if specificity of binding can be further confirmed and the subsequent peptide mapping can be improved. The availability of large amounts of R1-His through the use of 6xHis/Ni-NTA-chromatography should also open the door for other studies. Little is known about the assembly of the holoenzyme in the presence of different nucleotides. The formation of the large subunit dimer in the presence of different ATP and dTTP concentration could be followed in an analytical ultracentrifugation experiment. It is also of interest what conformational changes take place upon binding of nucleotide effectors, which could be followed by a circular dichroism experiment.

## **Appendix A**

### *A.1 Isopropanol precipitation*

To the DNA-containing solution 0.6 volume of isopropanol was added and mixed well. The mix was stored for 10 minutes at room temperature (25°C). After this time the nucleic acid was recovered by centrifugation for 10 minutes at 5000 rpm in a Sorvall GS3 rotor.

### *A.2 Removal of Ethidium Bromide from DNAs Purified by Equilibrium Centrifugation in CsCl-Ethidium Bromide Gradients*

To the solution of DNA in a glass tube an equal volume of isoamyl alcohol was added and then vortexed. The mixture was subsequently centrifuged at 1500 rpm for 3 minutes at room temperature in a bench centrifuge. Using a Pasteur pipette the lower, aqueous phase was transferred to a clean glass tube. The extraction was repeated six times until all the pink color disappeared.

### *A.3 Restriction digest of pET-11c-R1 with SalI, PstI, BamHI, KpnI, and NcoI*

Buffers:

*SalI* buffer: 150 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (pH 7.9 at 25°C)

*PstI* buffer: 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM dithiothreitol (pH 7.9)

*BamHI* buffer: 150 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (pH 7.9)

*KpnI* buffer: 10 mM Bis Tris Propane-HCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (pH 7.0)

*NcoI* buffer: 20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol (pH 7.9)

10  $\mu\text{g}$  of plasmid DNA was digested in 50  $\mu\text{l}$  reaction volume with 10 units of restriction enzyme for 2 hours at 37°C.

#### *A.4 RNA synthesis*

Buffer: 10X Transcription Buffer  
 400 mM Tris HCl (pH 7.5 at 37°C)  
 60 mM MgCl<sub>2</sub>  
 20 mM spermidine HCl  
 50 mM NaCl

After ethanol precipitation the DNA was redissolved in RNAase-free T.E. (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) at a concentration of 1  $\mu\text{g}/\mu\text{l}$ . The following components were mixed at room temperature in the order shown:

Template DNA (1 $\mu\text{g}/\mu\text{l}$ )	5 $\mu\text{l}$
RNAase-free H <sub>2</sub> O	11 $\mu\text{l}$
100 mM dithiothreitol	5 $\mu\text{l}$
Bovine Serum Albumin (2.5 mg/ml)	2.5 $\mu\text{l}$
5 mM rATP, rUTP, rCTP	2.5 $\mu\text{l}$ each
500 $\mu\text{M}$ rGTP	2.5 $\mu\text{l}$
Cap Analog (GpppG at 5 mM)	5.0 $\mu\text{l}$
10 X Transcription Buffer	5.0 $\mu\text{l}$
Placental RNAase Inhibitor (10 units/ml)	2.5 $\mu\text{l}$
T7 RNA Polymerase (15 units/ $\mu\text{l}$ )	3.0 $\mu\text{l}$

The reagents were mixed by gently tapping the outside of the tube and incubating for 1 hour at 37°C.

#### *A.5 Liposome mediated transfection*

5  $\mu\text{g}$  DNA/well of cells

The medium in each well was replaced with 1 ml Opti-MEM and incubated for 20 min at 37°C. During this time a liposome suspension

was prepared by placing 1 ml of Opti-MEM I in separate 12x75-mm polystyrene tubes (one for each well to be tested). To each of these tubes 15 ul of liposomes were added and vortexed briefly. Then 5  $\mu$ g of plasmid DNA was added and mixed gently. This suspension was added to the cells after aspirating off the 1 ml Opti-MEM at the the end of the 20 minute incubation.

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