

# Vaccinia Virus Ribonucleotide Reductase Expression and Isolation of the Recombinant Large Subunit\*

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The vaccinia virus gene encoding the 87-kDa protein that comprises the large subunit of ribonucleotide reductase (vvR1) was cloned into a bacterial expression vector under the control of an inducible promoter. Culture of *Escherichia coli* cells harboring the recombinant plasmid under standard induction conditions (0.4 mM isopropyl β-D-thiogalactopyranoside, 37 °C) resulted in synthesis of a completely insoluble product. Production of soluble vvR1 was achieved by growing bacteria at low temperature (15 °C) during the induction period, initiating induction at low cell density, and using a low concentration (0.05 mM) of the inducer isopropyl β-D-thiogalactopyranoside. Hydroxyurea, an inhibitor of ribonucleotide reductase, increased production of soluble vvR1 in a dose-dependent manner. Recombinant vvR1 was purified from a high salt extract of the *E. coli* lysate in four steps, the last utilizing an affinity column consisting of the carboxyl-terminal seven amino acids of the vvR2 protein linked to an insoluble resin. Using purified recombinant vvR2 to reconstitute active enzyme, we determined that maximizing the rate of CDP reduction required pH 8.0–8.8, 50 mM dithiothreitol, and 2 mM ATP. Specific activity of purified vvR1 was 122 nmol/min/mg. Limited proteolysis of the vvR1 protein revealed protease-resistant fragments approximately 30 and 58 kDa in size. To our knowledge, this study represents the first expression, solubilization, and isolation of a recombinant "eukaryotic" form of ribonucleotide reductase large subunit.

Pox viruses, like other large DNA viruses such as the herpes viruses and bacteriophage T4, have acquired genes that encode the enzyme ribonucleotide reductase (EC 1.17.4.1). This activity is the only *de novo* route to deoxyribonucleotides and is thus a necessary enzyme in DNA synthesis (reviewed in Stubbe (1990)). Whether the virus-encoded enzymes are essential for viral replication has been tested in herpes simplex and vaccinia viruses by engineering null mutants (Goldstein

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and Weller, 1988; Child *et al.*, 1990). In actively growing host cells, viral reproduction was not impaired; however, the mutant viruses were markedly attenuated when infecting quiescent or nonreplicating cells. Since several studies have shown that ribonucleotide reductase is present only in cycling cells, consistent with its specialized role in DNA synthesis (Björklund *et al.*, 1990; Engström and Rozell, 1988), these results suggest that the presence or absence of host cell ribonucleotide reductase determines whether or not the viral enzyme is essential. We have recently determined that even in cycling tissue culture cells, vaccinia virus synthesizes sufficient ribonucleotide reductase to provide precursors for its own replication (Howell *et al.*, 1993).

Although the sequencing of cloned genes has demonstrated that the viral ribonucleotide reductases are homologous to their host cell counterparts, some of the viral reductases have acquired characteristics that may specifically favor the opportunistic lifestyle. For example, both the T4 and herpes virus ribonucleotide reductases are relatively insensitive to allosteric control by deoxyribonucleoside triphosphates *in vitro* (Berglund, 1972; Averett *et al.*, 1983). Additionally, the herpes enzyme is unique in exhibiting full activity in the absence of any positive nucleotide effector (Averett *et al.*, 1983). In contrast to the situation with T4 and the herpes virus family, the ribonucleotide reductase encoded by vaccinia virus displays the full complement of complex regulatory responses characteristic of this enzyme: overall activation by ATP, inhibition by dATP, and differential activation toward its four substrates by specific dNTP effectors (Slabaugh *et al.*, 1984, 1991). Alignment of deduced amino acid sequences revealed approximately 80% identity between vaccinia and mouse reductase proteins throughout both the small, or R2,<sup>1</sup> and the large, or R1, subunits (Slabaugh *et al.*, 1988; Tengelsen *et al.*, 1988). Therefore, the vaccinia enzyme can likely serve as a useful model for eukaryotic ribonucleotide reductase.

*Escherichia coli* ribonucleotide reductase was first purified to homogeneity more than 20 years ago (Brown *et al.*, 1969), and the catalytic and allosteric properties of the enzyme subunits have been well studied (reviewed in Eriksson and Sjöberg (1989)). A comparable understanding of the eukaryotic ribonucleotide reductase has lagged, however, due to difficulties in purifying the enzyme, which is present in cells at low levels. A 3400-fold purification of the holoenzyme from calf thymus (Engström *et al.*, 1979), and subsequent dissociation of the enzyme into subunits (Thelander *et al.*, 1980), allowed the first conclusive studies of the allosteric regulation

<sup>1</sup> The abbreviations used are: R1 and R2, large and small subunits of ribonucleotide reductase, respectively (Fontecave *et al.*, 1992); vv, vaccinia virus; bp, base pair(s); DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; AMP-PNP, adenylyl-5'-yl imidodiphosphate; Ap<sub>4</sub>A, P<sup>1</sup>,P<sup>4</sup>-di(adenosine 5')-tetraphosphate.

of the eukaryotic enzyme. These studies confirmed the regulatory similarities between the *E. coli* and eukaryotic enzymes. However, sedimentation velocity centrifugation of the purified calf thymus R1 protein indicated that the solution contained monomeric R1 in the absence of nucleotides (an inactive state), R1 dimers in the presence of dTTP (an activator for GDP reduction), and a mixture of monomers and dimers in the presence of ATP (an activator). These observations had no precedent in physical studies of the *E. coli* enzyme, where the protein is an  $\alpha_2\beta_2$  dimer (Brown and Reichard, 1969a), and indicated that further study of the eukaryotic ribonucleotide reductase might reveal unusual mechanisms of enzyme regulation by effects on associative properties. Other studies that will depend on availability of purified enzyme subunits include elucidation of the enzyme mechanism, study of the docking and electron transfer interactions between R1 and its proteinaceous, radical-generating cofactor, R2, and the interactions of R1 with thioredoxin and/or glutaredoxin, proteins that mediate the flow of reducing equivalents to ribonucleotide reductase *in vivo*.

The genes for both the R2 and R1 proteins of vaccinia virus ribonucleotide reductase have been characterized by ourselves and others (Slabaugh *et al.*, 1988; Tengelsen *et al.*, 1988; Schmitt and Stunnenberg, 1988). Overexpression of the vvR2 protein in *E. coli* allowed us to establish that the small subunit of the vaccinia enzyme carries a protein-based radical stabilized by a dinuclear iron center, similar to the well-characterized *E. coli* and mouse R2 subunits (Howell *et al.*, 1992).

In this paper, we report the cloning, overexpression, and purification of the vvR1 subunit protein. The purification of vvR1 represents a significant contribution to efforts in understanding this unique enzyme. We describe methodology for expressing the protein in soluble form in *E. coli* cells, presenting strategies that may be generally useful for overproduction of eukaryotic proteins in bacteria. We define assay conditions for ribonucleotide reductase that yielded a specific activity 9–15-fold higher than those previously reported for the purified calf thymus R1 (Thelander *et al.*, 1980) and recombinant HSV-1 R1 overexpressed in *E. coli* (Furlong *et al.*, 1991). Finally, we present preliminary results from limited proteolysis of the vvR1 protein.

#### MATERIALS AND METHODS

**Buffers**—Buffers used in this work were as follows. Buffer A was 0.05 M Tris-Cl (pH 8.1 at 25 °C), 0.15 M NaCl, 10 mM EDTA, 1 mM  $\beta$ -mercaptoethanol, 0.1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride. Buffer B was 0.05 M Tris-Cl (pH 8.1 at 25 °C), 0.1 M KCl, 2.5 mM EDTA, 1 mM  $\beta$ -mercaptoethanol, 0.1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol. Buffer C was 0.02 M Tris-Cl (pH 8.1 at 25 °C), 0.1 M KCl, 1 mM EDTA, 0.5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 20% glycerol. Solutions used for SDS-gel electrophoresis were as described by Laemmli (1970).

**Bacterial Strains and Cloning Vectors**—*E. coli* strain JM83 was used to initially establish stable recombinants containing vvR1 sequences because this host does not contain a T7 RNA polymerase gene; plasmids were transferred to *E. coli* strain BL21(DE3) ( $F^+$  *ompT* *rmb* [DE3]) to express recombinant vaccinia virus R1 sequences. BL21(DE3) cells, which contain a chromosomally encoded T7 RNA polymerase gene under the control of the inducible lacUV5 promoter, and the expression vector pET11c were generously provided by Dr. William Studier (Studier *et al.*, 1990).

**Cloning Procedures**—The gene for the 87-kDa vvR1 protein was previously localized to the left end of the HindIII I fragment of the vaccinia virus genome (Tengelsen *et al.*, 1988; Schmitt and Stunnenberg, 1988). A 3.3-kilobase *Xba*I fragment encompassing the 2315-bp coding sequence was cloned, using standard techniques, into the *Xba*I site in the polylinker of the phagemid vector pIB130 (International Biotechnologies, Inc., New Haven, CT). A *Nde*I site was created at the ATG encoding the first amino acid of the protein by mutagenizing

with the oligo 5'-GTTTAATGACAAACATATGTGTTGGTGTAA TA-3', using the methods described by Kunkel *et al.* (1987). The resulting plasmid was designated pM102. This manipulation, which changed two nucleotides just upstream of the start codon (underlined in the oligo sequence), did not alter the coding sequence of the vvR1 gene. Since the vvR1 gene contains an internal *Nde*I site, pM102 was first digested to completion with *Sall*, linearizing the plasmid at a site 430 bp beyond the 3' end of the vvR1 open reading frame. Subsequently, the entire R1 coding sequence was obtained by gel purification of a 2741-bp *Nde*I-*Sall* fragment after a partial *Nde*I digest. Expression vector pET11c was linearized with *Bam*HI, the overhanging ends filled in by using Klenow fragment, and *Sall* linkers (New England Biolabs) were ligated to the blunt ends. The vector was then digested to completion with *Sall* and *Nde*I, gel-purified, and ligated to the 2741-bp vvR1-containing DNA fragment. The resulting plasmid, pET11c-R1, was used to transform *E. coli* strain JM83 to ampicillin resistance.

Plasmids from which both vvR1 and vvR2 could be expressed were constructed by inserting a *Xba*I fragment 1200 bp in length containing the vvR2 coding sequence, obtained from the expression vector pET11d-M2 (Howell *et al.*, 1992), into a *Xba*I site upstream or downstream of the vvR1 gene in pET11c-R1. The dual vectors contained a single T7 promoter upstream of both genes and a single T7 termination sequence downstream of both genes. Gel analysis of soluble and insoluble proteins produced after IPTG-mediated induction revealed that very little vvR1 protein was produced from the construct in which vvR1 sequences preceded vvR2 sequences (pET11c-R1/R2). Expression of vvR2, on the other hand, was not affected by position. Therefore, further experiments utilized the construct pET11C-R2/R1.

**Analysis of vvR1 Solubility**—Samples of 1.5 ml from *E. coli* cultures were centrifuged and cell pellets were resuspended in 0.05 M Tris-HCl (pH 8.0 at 25 °C), 0.1 M NaCl, 1 mM EDTA, and 1 mg/ml lysozyme. After incubation at 25 °C for 15 min, Triton X-100 was added to 0.5% (v/v), and the suspension was cooled on ice. Cells were sonicated to ensure complete lysis, and insoluble material was separated from the soluble fraction by centrifugation. For gel analysis, 1/4 volume of a 5× SDS sample buffer solution was added to an aliquot of the soluble fraction, and the insoluble fraction was resonicated in 1× SDS sample buffer. Antibodies to vvR1 were prepared and utilized as previously described (Howell *et al.*, 1993).

**Induction of Gene Expression**—For large-scale production of recombinant vvR1 protein, LB medium (per liter: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 ml of 1 N NaOH) supplemented with ampicillin (100  $\mu$ g/ml) was inoculated from a frozen stock of *E. coli* BL21(DE3) cells harboring plasmid pET11c-R1. The culture was incubated overnight at 30 °C with vigorous shaking; a portion of this culture was diluted 100-fold into fresh LB-ampicillin, and bacterial growth continued at 30 °C until the  $A_{600}$  reached 0.2–0.3. The culture was transferred to a refrigerated shaking water bath held at 15 °C. After 30 min, IPTG and hydroxyurea were added to 0.05 mM and 5 mM, respectively (final concentrations). Growth of the culture was continued at 15 °C for 18–21 h with vigorous shaking. Induction of cultures at  $A_{600}$  greater than 0.3–0.4 resulted in lower recovery of soluble vvR1.

**Purification of Recombinant vvR1 Protein**—Cells were harvested by centrifugation at 7,600 × g for 10 min at 4 °C; the cell pellet was resuspended in 5 volumes of Buffer A and lysed in a French Press. All subsequent purification procedures were done at 4 °C. The lysate was brought to 0.5 M NaCl, stirred for 15 min, and transferred to 30-ml Corex tubes. After centrifugation at 17,000 × g for 30 min, solid streptomycin sulfate was added to the supernatant fraction to 0.8% (w/v). After stirring for 30 min, the insoluble material was removed by centrifugation. In order to concentrate the supernatant solution, solid ammonium sulfate was added to 55% saturation (351 mg/ml). The precipitate was recovered by centrifugation, dissolved in Buffer B at a protein concentration of 15–20 mg/ml, transferred to high molecular weight cutoff dialysis bags (Spectrapor No. 7, 25,000 MWCO), and dialyzed against Buffer B for 2 h with one change of dialysis buffer. After clarification by brief centrifugation, the protein solution was passed over a column of Blue Sepharose (Pharmacia LKB Biotechnology Inc.) pre-equilibrated in Buffer B. The column was washed with Buffer B until the  $A_{280}$  of the eluate dropped to a stable baseline and bound proteins were eluted with a linear gradient of increasing KCl concentration (0.1–1.2 M in Buffer B). Fractions containing vvR1 protein were identified by SDS-gel electrophoresis.

These were pooled, concentrated by ultrafiltration, and rapidly dialyzed into Buffer C. The protein solution was passed over a column of Affi-Gel 15 (Bio-Rad Laboratories) to which had been attached a synthetic heptapeptide (-Phe-Ser-Leu-Asp-Val-Asp-Phe-COOH) corresponding to the carboxyl-terminal seven amino acids of the vvR2 protein. (Prior to coupling, the crude peptide was purified by high pressure liquid chromatography. Coupling was carried out according to instructions accompanying the Affi-Gel 15 activated resin.) The peptide affinity column was washed with 6 M guanidinium hydrochloride and equilibrated with Buffer C just before each use.) The Affi-Gel 15 affinity column was washed with Buffer C until the  $A_{280}$  of the eluate had just begun to approach baseline; bound vvR1 protein was eluted with a linear gradient of increasing KCl concentration (0.1–1 M) in Buffer C. The eluted protein peak was concentrated by ultrafiltration, and the purified protein was flash frozen in small volumes and stored at –80 °C. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

**Enzyme Assays**—Ribonucleotide reductase activity was detected by measuring reduction of [ $^3\text{H}$ ]CDP to [ $^3\text{H}$ ]dCDP, using procedures described for assay of the vaccinia virus enzyme in crude extracts from virus-infected cells (Slabaugh *et al.*, 1984). For the purified, recombinant enzyme subunits, the following assay conditions were found to be optimal: 0.05 mM HEPES-KOH (pH 8.2 at 25 °C), 2 mM magnesium acetate, 2 mM ATP, 50 mM DTT, 20  $\mu\text{M}$  ferrous ammonium sulfate, and 0.1 mM [ $^3\text{H}$ ]CDP (Du Pont-New England Nuclear) at a specific activity of 20–40 cpm/pmol. Identical assay mixtures buffered with HEPES-NaOH exhibited 25% less activity. Prior to assay, purified enzyme subunits were diluted using a buffered solution containing 0.2 mg/ml bovine serum albumin and 0.5 mM DTT. Preliminary experiments revealed that maximal specific activity of vvR1 was achieved only in the presence of a molar excess of vvR2; expressed as monomer concentrations, vvR2 was routinely present at 0.75  $\mu\text{M}$  and vvR1 at 0.25  $\mu\text{M}$  in 25- $\mu\text{l}$  reaction mixtures. Generation of [ $^3\text{H}$ ]dCDP was linear for 10 min when assay mixtures were incubated at 30 °C. Assays were performed in duplicate or triplicate, and the results were averaged. One unit of ribonucleotide reductase activity was defined as 1 nmol of CDP reduced per min.

**Limited Proteolysis of vvR1**—Purified preparations of trypsin, chymotrypsin, and V8 protease were obtained from commercial suppliers. Each enzyme was diluted to concentrations of 0.1, 0.01, and 0.001 mg/ml in 0.05 M HEPES-NaOH (pH 8.0 at 25 °C), 1 mM DTT, 5% glycerol. Purified recombinant vvR1, diluted to a concentration of 1 mg/ml, was combined with an equal volume of each protease preparation and incubated at 25 °C. Aliquots were removed from each reaction mixture after 10, 20, and 30 min to tubes containing 5 × SDS sample buffer and heated immediately to 100 °C for 2 min. Proteolytic degradation products were analyzed by subjecting volumes containing 1.3  $\mu\text{g}$  of vvR1 protein to SDS-gel electrophoresis.

## RESULTS

**Low Temperature and Low Levels of Inducer-favored Production of Soluble vvR1 in *E. coli* Cells**—The vaccinia virus gene encoding the large subunit of ribonucleotide reductase was cloned into a prokaryotic expression vector, pETllc, which provided inducible transcription machinery and a bacterial ribosome binding site. The cloning strategy was designed such that translation of the cloned gene produced a protein identical in amino acid sequence with the natural protein. Nevertheless, induction of the construct with 0.4 mM IPTG at 37 °C resulted in accumulation of an 87-kDa protein entirely in insoluble inclusion bodies. The identity of the induced protein as vvR1 was confirmed by positive reaction with anti-vvR1 polyclonal antiserum that showed no cross-reactivity with the bacterial R1 protein. Induction at 30 °C and 25 °C demonstrated that lower induction temperature correlated with a larger fraction of the total vvR1 in soluble form. However, the positive effect of lower temperature was offset by higher levels of inducer.

Hypothesizing that lower temperatures might be slowing the rate of protein synthesis, and thereby allowing more time for productive folding of the enzyme, we induced vvR1 with

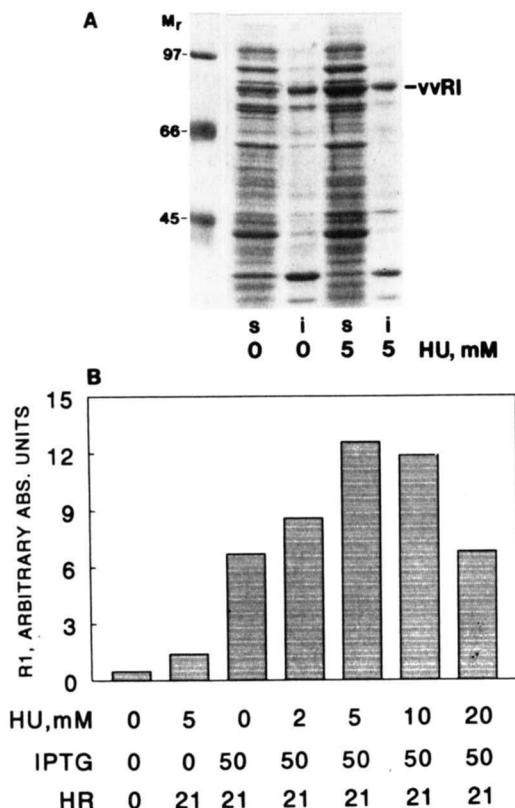
various concentrations of IPTG while shaking bacterial cultures at 15 °C for 20–24 h. Induced at this low temperature in the presence of 50  $\mu\text{M}$  IPTG, 50–70% of the vvR1 produced remained soluble, and the yield of soluble vvR1 was several-fold greater than previously achieved at 25 °C. Higher concentrations of IPTG, however, yielded increasingly insoluble vvR1.

**Hydroxyurea Increased Soluble vvR1**—Studies of both *E. coli* and mammalian ribonucleotide reductases have shown that the presence of the negative effector dATP promotes self-aggregation of the R1 subunit of the enzyme (Brown and Reichard, 1969a; Thelander *et al.*, 1980). In order to see if the solubility of vvR1 might be improved if the levels of intracellular dATP were lowered, we used the drug hydroxyurea. This agent is itself a specific inhibitor of ribonucleotide reductase because it reduces the organic radical present in the R2 subunit of the enzyme (Ehrenberg and Reichard, 1972), leading to inhibition of DNA synthesis due to lack of dNTP precursors. Pertinent to our intent here, studies of intracellular dNTP pools before and after hydroxyurea treatment have shown that in both bacterial and eukaryotic cells, the dATP pool is particularly sensitive to the drug (Neuhard, 1967; Bianchi *et al.*, 1986; Walters *et al.*, 1976; Slabaugh *et al.*, 1991). Indeed, as shown in Fig. 1A, hydroxyurea enhanced production of soluble vvR1 as a fraction of total soluble protein, while having little apparent effect on the amount of vvR1 present in insoluble inclusion bodies. Further experiments confirmed these results and revealed that the increase in soluble vvR1 in relation to total soluble protein was dose-dependent and maximal between 5 and 10 mM hydroxyurea (Fig. 1B). As quantitated by laser densitometry of Coomassie-stained vvR1 bands, 5 mM hydroxyurea approximately doubled the concentration of soluble vvR1 in *E. coli* extracts. The effect of the drug on bacterial growth during the induction period was also dose-dependent; rather than exhibiting an optimum, however, the drug was progressively more inhibitory as the concentration was increased.

To investigate the possibility that these observations represented a nonspecific effect of hydroxyurea on plasmid-encoded proteins, we tested its effects on synthesis of two other constructs in which vaccinia virus genes were cloned in pET expression vectors downstream of a T7 promoter: pET11d-R2, carrying the gene encoding the small subunit of vaccinia ribonucleotide reductase (Howell *et al.*, 1992), and pET11c-I3, a plasmid expressing a vaccinia virus DNA binding protein (Davis and Mathews, 1993). Expression from each gene was induced at 15 °C with a low level of IPTG in the presence or absence of hydroxyurea. As determined by gel analysis of total soluble protein in bacterial extracts and by Western blotting, the expression of neither vvR2 nor vvi3 was affected by hydroxyurea, but the drug increased soluble vvR1 2–3-fold (not shown). Although the underlying mechanism for the effect of hydroxyurea on recombinant vvR1 remained conjectural, the drug was incorporated into our standard induction protocol to maximize recovery of soluble vvR1.

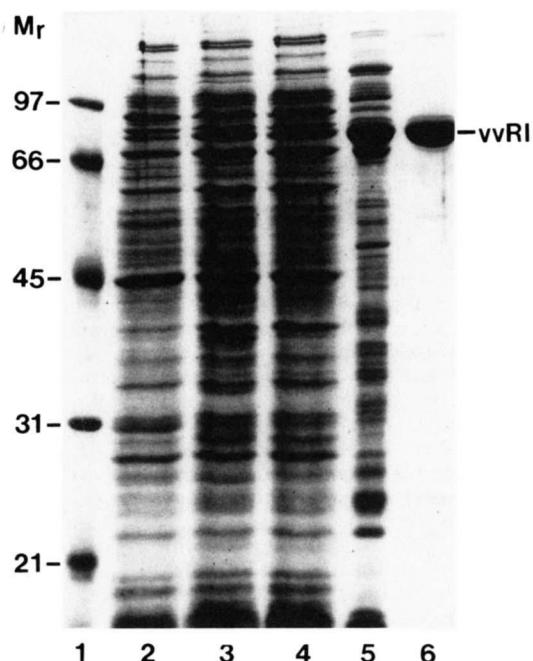
**Purification of Vaccinia Virus R1 from Overproducing *E. coli* Cells**—For purification of vvR1 protein, 1- to 4-liter cultures of BL21(DE3)/pETllc-R1 cells were induced by 50  $\mu\text{M}$  IPTG for 18–24 h at 15 °C in the presence of 5 mM hydroxyurea. Based on gel analysis of a number of independent preparations, we estimated that under these conditions, between 50 and 70% of the total vvR1 produced was present as soluble enzyme, and that vvR1 represented 1–2% of the soluble protein in the bacterial extract.

## Purification of Vaccinia Virus Ribonucleotide Reductase

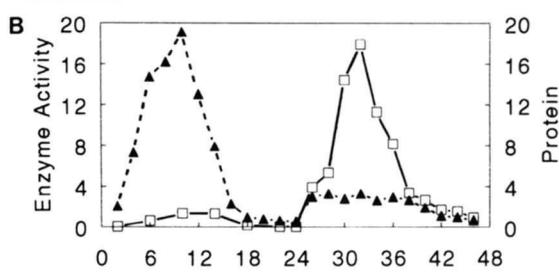
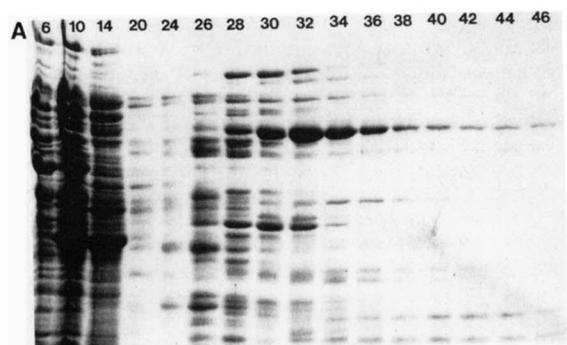


**FIG. 1. Effect of hydroxyurea on bacterial production of soluble vvR1.** Panel A, BL21(DE3)/pETllc-R1 cells were grown at 30 °C to an  $A_{600}$  of 0.2, then induced at 15 °C with 50  $\mu$ M IPTG in the presence or absence of 5 mM hydroxyurea. Soluble (s) and insoluble (i) protein fractions were prepared after 21 h. 10  $\mu$ g of soluble protein and an equivalent fraction of the insoluble material from each sample were subjected to electrophoresis. The relative molecular mass of standards shown is  $\times 10^{-3}$  in this and subsequent figures. Panel B, BL21(DE3)/pET11c-R1 cells were grown at 30 °C to an  $A_{600}$  of 0.3, then induced at 15 °C using the indicated concentrations of IPTG and hydroxyurea. After 20 h, extracts were prepared and 7.5  $\mu$ g of soluble protein from each extract was electrophoresed on an SDS gel. The fraction of total Coomassie stain present in the vvR1 band was quantitated by laser densitometry and is expressed as arbitrary absorbance units.

The vvR1 protein was purified using two precipitation steps and two columns. The protein content of the preparation at various stages is shown in Fig. 2. Following disruption of the bacterial cells, the ionic strength of the lysate was raised to 0.5 M by adding NaCl. The solution was clarified by centrifugation, and nucleic acids were precipitated from the supernatant fraction with streptomycin sulfate. Attempts to carry out streptomycin sulfate precipitation at lower ionic strength resulted in loss of all vvR1 with the polycation-nucleic acid pellet. The proteins were collected by precipitation with ammonium sulfate and desaltsed by rapid dialysis into a buffer containing 0.1 M KCl using high molecular weight cutoff membranes. Lengthy dialysis and/or dialysis to lower ionic strength resulted in loss of vvR1 protein by precipitation. Recombinant vvR1 bound tightly to Blue Sepharose resin at pH 8.3 and could be eluted with either ATP (10 mM, not shown) or 0.7 M KCl (Fig. 3). Enzyme activity was detected by complementing vvR1 with purified recombinant vvR2 and corresponded with a prominent 87-kDa protein identified as vvR1 by immunological criteria. The vvR1-containing fractions were pooled, concentrated using an Amicon device, and desaltsed by rapid dialysis. Typically, 70–80% of the applied



**FIG. 2. Purification of vvR1.** Lane 1, molecular weight markers; lane 2, 12  $\mu$ g of crude extract from control cells, *E. coli*/pETllc, 21 h postinduction. Lanes 3–6 contain proteins from vvR1-expressing cells, *E. coli*/pET11c-R1. Lane 3, 12  $\mu$ g of crude extract, 21 h postinduction; lane 4, 12  $\mu$ g of ammonium sulfate pellet; lane 5, 6  $\mu$ g of pooled Blue Sepharose fractions; lane 6, 2  $\mu$ g of pooled Affi-Gel 15 eluate.

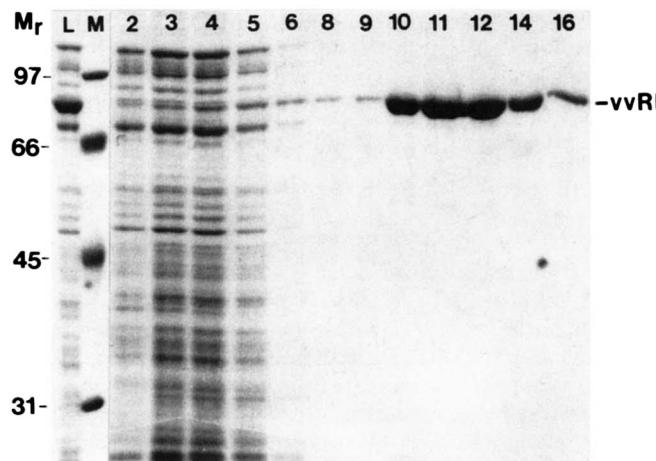


**FIG. 3. Blue Sepharose chromatography of vvR1.** Panel A, 215 mg of protein in 40 ml of Buffer B was applied to a 20-ml bed volume column of dye resin, and the resin was washed with Buffer B. Bound proteins were eluted with a 260-ml linear salt gradient (0.1 to 1.2 M KCl in Buffer B, initiated at fraction 22. Fractions of 300 drops were collected and analyzed by electrophoresis of 12.5  $\mu$ l on a 10% SDS gel. Panel B, enzyme activity was detected after combining 5  $\mu$ l of selected fractions with an excess of purified recombinant vvR2. Solid line, enzyme activity expressed as units/fraction; dashed line, mg of protein/fraction.

vvR1 activity was recovered in the pooled fractions.

The pooled Blue Sepharose fractions, in 0.1 M KCl, were immediately applied to an affinity resin composed of a heptapeptide identical with the carboxyl-terminal seven amino acids of the vvR2 protein, linked to an insoluble matrix. In utilizing this approach, we exploited accumulating evidence from other ribonucleotide reductase enzymes that the carboxyl-terminal part of the small subunit protein is involved in interactions with the large subunit protein (Dutia *et al.*, 1986; Cohen *et al.*, 1986; Yang *et al.*, 1990; Climent *et al.*, 1991). Indeed, the matrix selectively retained vvR1, while remaining contaminants were not bound (Fig. 4). A linear gradient of increasing salt concentration eluted vvR1 at 0.3 M KCl.

In Table I, a typical purification of recombinant vvR1 from a 1-liter culture is detailed. From a crude extract containing 365 mg of soluble protein, 1.2 mg of vvR1 was purified to >95% homogeneity. Based on measurements of enzyme activity after complementation with excess vvR2, we estimated an overall recovery of 20% of vvR1 activity and a 64-fold increase in specific activity. However, note that values given for enzyme activity in fractions prior to the final purification step are estimates due to the complications of assaying this activity



**FIG. 4. Chromatography of vvR1 on Affi-Gel 15-heptapeptide affinity column.** vvR1-containing fractions from Blue Sepharose chromatography (fractions 30–42, lane L) were dialyzed and concentrated before application to the affinity column. 6.2 mg of protein in 9.6 ml of Buffer C was applied to the column (6 ml bed volume) at a flow rate of 15 ml/h, followed by an 8-ml wash with Buffer C. At fraction 7, a 20-ml linear salt gradient (0.1–1.0 M KCl) in Buffer C was applied. vvR1 eluted at 0.3 M KCl.

(see "Discussion"). The total activity estimated in the crude extract may be lower than the estimated total activity after ammonium sulfate precipitation because of inhibitory substances present in the crude extract. A specific activity of 122 units/mg was determined for purified vvR1 by using optimized reaction conditions (3:1 molar ratio of vvR2 to vvR1 proteins, 50 mM DTT, 2 mM ATP). The rather substantial loss of activity that occurred during the final step in the purification can be attributed partly to the comparatively weak binding of vvR1 to the heptapeptide affinity column, resulting in leakage of vvR1 during loading and washing steps (see Fig. 4). A comparison of protein retention on this matrix at 25 °C and at 4 °C revealed that binding of vvR1 was stronger at the lower temperature, suggesting that hydrophobic interactions of vvR1 with the peptide ligand were not the major determinants of binding. This was somewhat surprising since the heptapeptide contains four hydrophobic amino acids. Additional losses were incurred during the purification by irreversible precipitation of vvR1 during dialysis and concentration steps. Once purified, vvR1 was quite susceptible to denaturation, but the protein was stable for several months at –80 °C.

**Optimization of Ribonucleotide Reduction Using Recombinant Enzyme Subunits—**Ribonucleotide reductase carries out an incompletely understood reaction in which the hydroxyl group present at the 2' position of the ribonucleotide ring is reduced to a hydrogen. *In vivo*, reducing equivalents for this reaction are transferred from NADPH to the active site via an electron transport chain that utilizes the redox proteins glutaredoxin or thioredoxin and two pairs of essential Cys residues in the R1 protein (reviewed in Stubbe (1990)). *In vitro*, the reducing agent DTT can substitute for NADPH and a redox-active protein. This small molecule apparently donates electrons directly to the active site Cys residues, since mutant *E. coli* R1, in which the Cys residues predicted to interact with glutaredoxin or thioredoxin have been changed to Ser or Ala, is inactive with NADPH and the redox proteins, but active with dithiothreitol (Åberg *et al.*, 1989; Mao *et al.*, 1989; Mao *et al.*, 1992). We investigated the effect of increasing DTT concentration on the rate of CDP reduction and found that activity was maximal when the agent was present at 50–75 mM, as shown in Fig. 5.

**Effects of ATP and ATP Analogs—**Ribonucleotide reductase accepts four ribonucleotide substrates (CDP, UDP, ADP, and GDP). Activity toward these substrates is influenced by nucleoside triphosphate effectors (ATP, dGTP, and dTTP) that bind to the large subunit of the enzyme (Brown and Reichard, 1969b; Eriksson *et al.*, 1979). Except in the case of

**TABLE I**  
**Purification of vvR1**

Purification step	Volume	Protein	Enzyme activity, ATP	Enzyme activity, AMP-PNP	Total activity <sup>a</sup>	Specific activity	Yield
	ml	mg	units	units	units	units/mg	%
Crude extract <sup>b</sup>	83	365	62.6	68.0	680	1.9	95
Ammonium sulfate <sup>c</sup>	41	262	67.1	71.8	718	2.7	100
Blue Sepharose <sup>d</sup>	15.7	10.2	217	39.2	392	38	55
Affinity resin <sup>e</sup>	4.9	1.2	146	14.6	146	122	20

<sup>a</sup> Total enzyme activity was estimated by applying a multiplication factor of 10 to the enzyme activity measured using AMP-PNP as activator.

<sup>b</sup> Crude extract denotes the supernatant fraction obtained after removal of cell debris.

<sup>c</sup> Ammonium sulfate denotes the 55% pellet fraction after resuspension and dialysis.

<sup>d</sup> Blue Sepharose denotes the pooled vvR1-containing fractions after concentration and dialysis.

<sup>e</sup> Affinity resin denotes the pooled vvR1-containing fractions after concentration and centrifugation to remove precipitated protein.

## Purification of Vaccinia Virus Ribonucleotide Reductase

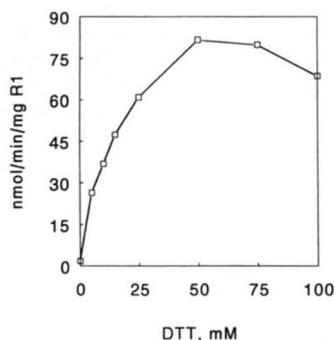


FIG. 5. Effect of DTT on enzyme activity. Enzyme assays were performed as described under "Materials and Methods" except that the concentration of DTT was varied.

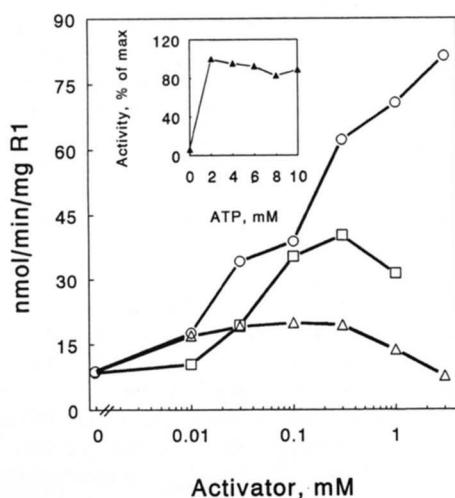


FIG. 6. Effect of adenine-containing nucleotides on CDP reduction. ATP (○—○), AMP-PNP (Δ—Δ), and Ap4A (□—□) were included in assays at 0–3 mM. Inset, effect of ATP (0–10 mM) on CDP reduction.

the herpes virus ribonucleotide reductases, ATP is required for activation toward pyrimidine-containing substrates. Fig. 6 shows the response of recombinant vaccinia virus ribonucleotide reductase to increasing concentrations of three adenine-containing nucleotides in a CDP reduction assay: ATP, the ATP analogue AMP-PNP, and Ap4A. Diadenosine tetraphosphate has been shown to have activating and inhibitory effects on several enzymes of nucleoside and nucleotide metabolism and has been suggested as a possible *in vivo* regulator of ribonucleoside reduction (Wasternack *et al.*, 1991). ATP was the most effective activator and was 10-fold more effective than its analogue AMP-PNP at 2 mM. Both AMP-PNP and Ap4A displayed stimulatory effects at low concentrations but were inhibitory at concentrations above 0.3 mM, suggesting complex interactions with vvR1. ATP, in contrast, was maximally stimulatory over the range 3–10 mM (Fig. 6, inset).

**Salt and pH Effects**—Maximal ribonucleotide reductase activity required the presence of magnesium ions. The optimal magnesium concentration varied with the concentration of nucleotide effector, with maximal activity at equimolar concentrations of magnesium and ATP. Similar to the results reported for the purified calf thymus enzyme (Engström *et al.*, 1979), and to our results using crude extracts (Slabaugh and Mathews, 1984), addition of magnesium to the assay approximately doubled activity. Enzyme activity was little affected by changes in the concentration of KCl over the range 0.01–0.2 M, but purified solutions of vvR1 tended to

precipitate when the ionic strength was taken below 0.1 M. The dependence of enzyme activity on pH was examined over the range of pH 6.8 to 8.8 and was maximal at pH 8.0 and above. Activity at pH 7.2 was 60% of that observed at pH 8.0–8.8.

**Partial Proteolytic Digestion of vvR1**—Substantial evidence exists that the R1 protein contains three types of nucleotide binding sites: the substrate binding site and two types of effector sites. An attractive mechanism for translation of information to the substrate binding site would be adjustments in domain geometry upon effector binding. However, little is currently known about the secondary or tertiary structure of R1. We sought preliminary evidence for the presence of discrete domains in the vvR1 protein by probing for protease-sensitive sites. Purified vvR1 was incubated with trypsin, chymotrypsin, and V8 protease for 10, 20, or 30 min at protease to vvR1 ratios of 0.001, 0.01, and 0.1 (w/w). Aliquots containing 1.3 µg of vvR1 were subjected to electrophoresis in the presence of SDS. The results from 10-min digestions are shown in Fig. 7.

Initial products of tryptic digestion were a 29-kDa fragment and a family of fragments of 54.5–59 kDa (Fig. 7, lane 2). The full-length protein (87 kDa) was simultaneously trimmed to a family of 76- to 86-kDa fragments not apparent on the stained gel shown in Fig. 7, but revealed by Western analysis. Therefore, we concluded that there were 2 or 3 trypsin-sensitive sites close to one end of the protein, and another readily-cleaved site approximately 260 amino acids from the other end. Treatment with the highest ratio of trypsin/vvR1 tested (0.1 g/g of vvR1) resulted in generation of additional fragments, but the 29-kDa fragment appeared to resist further degradation (lane 3). The lowest level of chymotrypsin tested (0.001 g/g vvR1) generated predominant products of 62 and 26 kDa (lane 4). At a 100-fold higher level of the protease, a 25-kDa fragment survived (lane 6).

V8 protease at 0.01 g/g of vvR1 generated 57-, 54.5-, 33.5-, and 31-kDa fragments (lane 8). The simplest interpretation of this pattern is that V8 protease initially cleaved at one of two accessible sites to generate 57- and 31-kDa and 54.5- and 33.5-kDa fragments. The two larger peptides were relatively stable, although with 10-fold higher levels of V8 protease, the

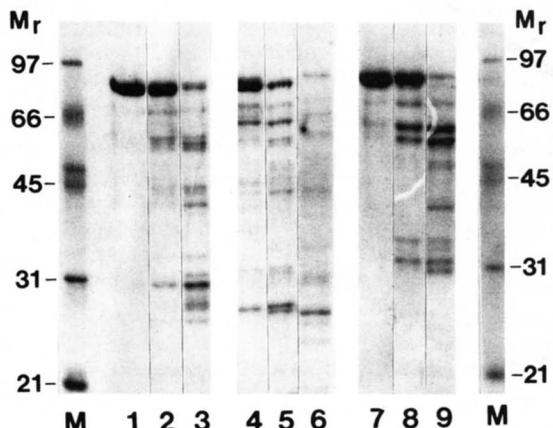


FIG. 7. Limited proteolysis of vvR1. vvR1 (0.5 mg/ml) was incubated at 25 °C for 10 min with trypsin (lanes 1–3), chymotrypsin (lanes 4–6), or V8 protease (lanes 7–9). Proteases were present at 0.001 g/g of vvR1 (lanes 1, 4, and 7), 0.01 g/g of vvR1 (lanes 2, 5, and 8), and 0.1 g/g of vvR1 (lanes 3, 6, and 9). A portion of each reaction (1.3 µg of vvR1 at the beginning of the reaction) was electrophoresed on a 12% SDS gel. Peptides in lanes 1–6 were sized based on migration of marker proteins shown on the left, and peptides in lanes 7–9 were sized based on the markers shown on the right.

54.5-kDa fragment increased in abundance (*lane 9*), probably reflecting cleavage of the 57-kDa fragment at the second site. Overall, this experiment suggests that vvR1 is folded into a larger domain of 57–62 kDa and a smaller domain of 25–30 kDa. Although mapping the various peptide fragments will require further experimentation, these results indicate that domains of the R1 protein might be isolated by limited protease digestion followed by peptide purification.

## DISCUSSION

Although cDNA clones encoding eukaryotic R1 protein from mouse were first isolated a number of years ago (Caras *et al.*, 1985; Thelander and Berg, 1986), no reports have yet appeared describing purification of recombinant mouse R1. The purification to homogeneity of herpes simplex type I R1 protein using a host/vector system similar to that in the present study was recently published, but the yield of R1 reported was approximately 10% of what we achieved using low temperature induction. The major obstacle to recovery of cloned eukaryotic R1 synthesized in bacteria has been the tendency of the protein to aggregate in inclusion bodies. Since we were unable to achieve renaturation of active protein after solubilizing vvR1-containing inclusion bodies in urea or guanidinium hydrochloride, we pursued methods to avoid formation of insoluble aggregates during the period of protein synthesis.

Reasoning that proper folding of vvR1 might be favored by the presence of the other subunit of the enzyme, vvR2, we constructed a dual-expression plasmid in which a single transcriptional unit contained the vaccinia virus genes arranged in the order, T7 promoter-vvR2-vvR1-T7 terminator. Co-expression of vvR2 and vvR1 in bacteria yielded some active viral ribonucleotide reductase but did not increase solubility of vvR1.

A more successful strategy was lowering the growth temperature of the bacterial culture to 15 °C at the time induction of the recombinant protein was initiated. Although bacterial growth was slowed markedly at this temperature, a large fraction of the vvR1 produced remained soluble. If low temperature promoted vvR1 solubility by favoring productive folding of the nascent protein, this positive effect could be overcome, even at 15 °C, by levels of inducer higher than 50–100 μM. Therefore, aggregation of vvR1 appeared to be concentration-dependent as well as temperature-dependent.

The finding that hydroxyurea favored recovery of soluble R1 is without precedent, and the mechanism remains conjectural. Our results are consistent with the hypothesis that reducing the intracellular dATP pool would decrease R1 self-aggregation. Sequestering of recombinant proteins in bacterial inclusion bodies, however, is generally attributed to nonspecific aggregation of improperly folded proteins; although the physical basis for the effect of dATP on R1 associate properties has not yet been elucidated, nonspecific aggregation seems unlikely. However, R1 proteins display a high affinity for dATP (Brown and Reichard, 1969a; Thelander *et al.*, 1980), and binding of this effector to a partially folded nascent protein might unfavorably affect the thermodynamics or kinetics of folding.

Purified vvR1 protein exhibited a specific activity that was 9-fold higher than the highest previously reported specific activity for a eukaryotic ribonucleotide reductase large subunit, purified calf thymus R1 (13.6 units/mg, Thelander *et al.* (1980)), and 15-fold higher than that reported for purified recombinant herpes simplex virus type I R1 (8.2 units/mg, Furlong *et al.* (1991)). This can be attributed not only to the

rapid purification possible from a bacterial expression system, but also to the optimization of reaction parameters.

A surprising result of the optimization studies was the difference in effectiveness between ATP and AMP-PNP as activating effector for CDP reduction. We and others have utilized this noncleavable analogue in studies of ribonucleotide reductase in crude extracts because it avoids the problem of ATP-promoted diversion of substrate to the triphosphate level due to phosphorylation of CDP by contaminating enzymes (Kucera and Paulus, 1982; Slabaugh and Mathews, 1984; Cunha and Costa, 1992). This difficulty is illustrated in the purification table in that the activity detected in impure fractions by using 2 mM ATP as activator was only a fraction of the total activity recovered at the end of the purification. Therefore, we assayed all fractions using both 2 mM AMP-PNP and 2 mM ATP and applied a correction factor derived from the experiment shown in Fig. 6 to estimate total activity throughout the purification. The activation pattern we observed using either AMP-PNP or AP<sub>4</sub>A is reminiscent of the effect of dATP on the *E. coli* enzyme (Brown and Reichard, 1969a). One interpretation is that these agents, like dATP, can interact with the R1 subunit at a stimulatory site as well as at an inhibitory site.

Ribonucleotide reduction requires a continuous supply of reducing equivalents, because active site thiols are oxidized with each turnover. The optimal DTT concentration for maximal reductase activity using recombinant vvR1 and vvR2 (at a molar ratio of 1:3) was 50–70 mM. Sequencing of the entire vaccinia virus genome recently revealed that the virus does encode a glutaredoxin homologue (Johnson *et al.*, 1991) that exhibits thiol transferase activity (Ahn and Moss, 1992). Experiments are underway to test the premise that this protein might interact with vvR1 and serve as hydrogen donor during enzymatic turnover.

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