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# QUATERNARY STRUCTURE OF VACCINIA VIRUS THYMIDINE KINASE

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Thymidine kinase enzymes isolated from a variety of sources are generally considered to have a native molecular weight of 80-90 kDa composed of two 40-45 kDa subunits. Although these parameters may accurately describe the atypical deoxypyrimidine kinases expressed by members of the *Herpesviridae*, the nucleotide sequences of thymidine kinase genes isolated from human, mouse, chicken and a variety of poxviruses (vaccinia virus, monkeypox virus, variola virus, fowlpox virus and capripoxvirus) predict molecular weights on the order of 20-25 kDa for the derived primary translation products. To resolve this apparent dilemma, velocity sedimentation centrifugation, gel filtration chromatography and protein cross-linking procedures were employed to provide experimental evidence that enzymatically-active vaccinia virus thymidine kinase is a homotetrameric complex of 20 kDa monomers with a native $M_r$ of 80 kDa. 

Thymidine kinase (TK, EC 2.7.1.21) is a central enzyme in the nucleotide salvage pathway. The importance of this enzyme is underscored by its conservation throughout a divergent group of organisms including human (1), mouse (2), chicken (3), herpesviruses (4), bacteriophage T4 (5) and a large number of poxviruses [vaccinia virus (6), variola, monkeypox (7), fowlpox virus (8) and capripoxvirus (9)]. Although TK enzymes from a variety of sources have been subjected to detailed biochemical analyses, major discrepancies exist in both the native molecular weights and the subunit compositions which have been reported for these enzymes. Most of the information available in the literature suggests that functional TK enzymes have a native molecular weight of 80-90 kDa composed of dimers of identical 40-45 kDa subunits (10). In contrast, based on the size of the predicted open reading frame of the vaccinia virus TK gene (20 kDa), Hruby and Ball (11) suggested that the viral-encoded enzyme functions as a tetrameric complex. In order to resolve this issue, the experiments reported in this paper have used velocity sedimentation, protein cross-linking and molecular sieving

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Abbreviations: VV, Vaccinia Virus; TK, thymidine kinase; HSV, Herpes Simplex Virus; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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procedures to provide evidence that VV TK (and by analogy most other TK enzymes) functions as a complex of four 20 kDa subunits.

## EXPERIMENTAL PROCEDURES

*Expression and analysis of VV thymidine kinase in vitro.* Biologically-active VV TK transcripts were produced by transcribing linearized pT7:TKII plasmid DNA with T7 RNA polymerase using conditions previously described by Wilson et al. (12). The capped TK transcripts were translated into protein, in the presence or absence of L-[$^{35}$S]methionine using an mRNA dependent rabbit reticulocyte lysate (13). The synthesis of VV TK was monitored either by the incorporation of L-[$^{35}$S]methionine into trichloroacetic acid precipitable material or, in the case of unlabeled TK, by measuring the ability of the extract to convert [$^{3}$H]thymidine to dTMP using a filter binding assay (13).

*Glycerol gradient sedimentation.* Radiolabeled or unlabeled pT7:TKII RNA translation products were subjected to velocity sedimentation in 10 to 30% glycerol gradients prepared in TMDT buffer [20 mM Tris-Cl (pH 7.6), 2 mM magnesium acetate, 1 mM dithiothreitol and 50 μM thymidine] in a SW50.1 rotor at 37,000 rpm for 20 hours at 4°C.

*Determination of molecular weight by gel filtration.* A standard curve of protein molecular weights was generated by passing a mixture of protein gel filtration molecular weight markers [1 mg/ml each of ß-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa), (MW-GF-200 kit from Sigma, St. Louis, MO.)] through a 1 x 34 cm Sephacryl-300 (Pharmacia LKB Biotechnology, Uppsala, Sweden) gel filtration column equilibrated with TENDT buffer [50 mM Tris (pH 7.6), 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, and 0.015% Triton X-100]. Fractions (250 μl) of the eluate were collected (100 fractions, 25 ml total volume) and alternate samples from number 30 to 100 were subjected to electrophoresis in 12% polyacrylamide gels containing SDS (14). The gels were then stained with Coomassie Brilliant Blue R-250 to visualize the individual proteins. VV TK synthesized *in vitro* in the presence or absence of L-[$^{35}$S]methionine was chromatographed under identical conditions. The fractions containing VV TK were identified either on the basis of radioactivity or enzyme activity.

*Chemical cross-linking of glycerol gradient-purified TK.* VV TK which had been synthesized *in vitro* in the presence of L-[$^{35}$S]methionine was partially purified by glycerol gradient centrifugation. Aliquots of the peak fraction containing radiolabeled TK were incubated in the presence of the various concentrations of glutaraldehyde for one hour (Sigma, St. Louis, MO.) at room temperature. Gel loading buffer was added, the samples heat denatured for 3 min at 100°C and then subjected to electrophoresis in 12% polyacrylamide gels containing SDS (14) followed by autoradiography.

## RESULTS AND DISCUSSION

The transcription vector, pT7:TKII (12), was used to produce VV TK mRNA which was translated in a reticulocyte lysate in the presence or absence of L-[$^{35}$S]methionine to produce enzymatically-active VV TK (13). Radiolabeled and unlabeled VV TK enzymes produced *in vitro* were subjected to velocity sedimentation through glycerol gradients in a buffer designed to maintain enzyme activity. From the data shown in Figure 1 it is evident that the peaks of radioactivity and enzyme activity are coincident. Furthermore, analysis of the peak fractions of radiolabeled and unlabeled TK enzyme by polyacrylamide gel electrophoresis and autoradiography (top

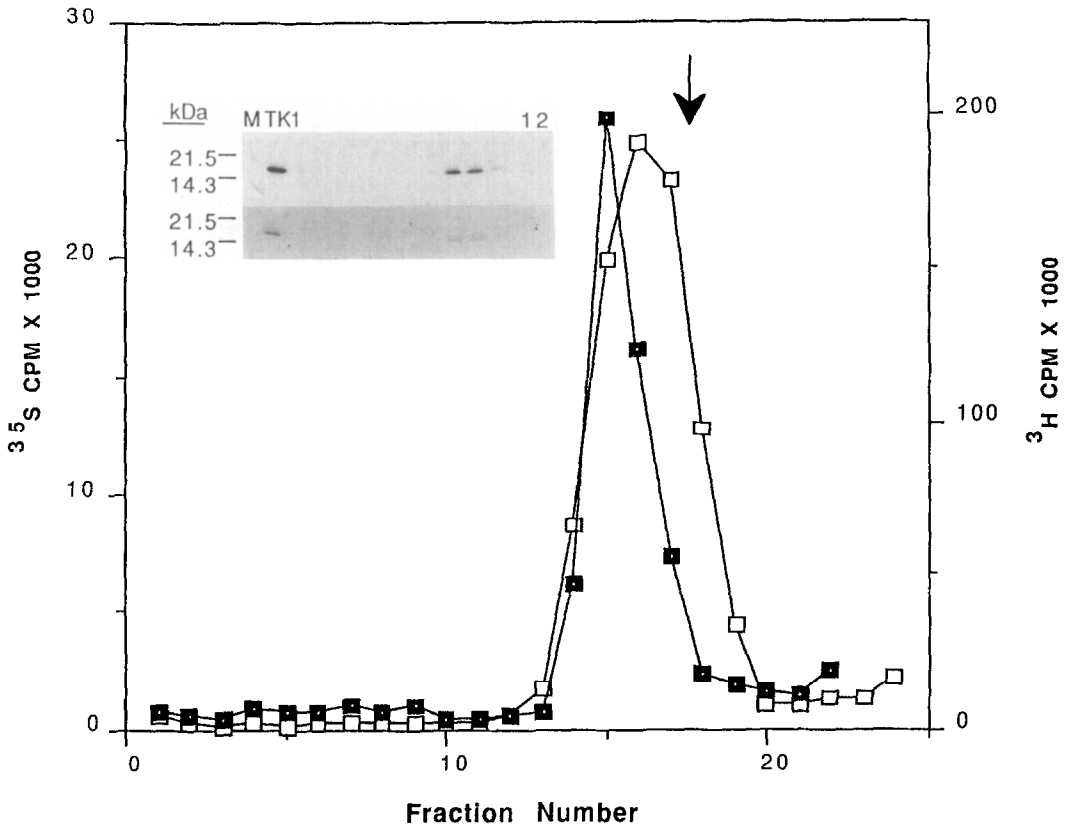


**Figure 1. Glycerol gradient separation of VV TK enzyme activity and radiolabeled protein.** pT7:TKII-derived capped VV TK RNA was translated in the presence or absence of L-[$^{35}$S]methionine. The translation products were then subjected to velocity sedimentation in 10 to 30% glycerol gradients as described in "Experimental Procedures". Fractions were collected (200µl) from the bottom and assayed for hot TCA precipitable radioactivity (white squares) or for enzyme activity (black squares). The sedimentation position of hemoglobin (64.5 kDa) is indicated by the arrow. Radiolabeled cell-free translation products (lane 1) as well as every other fraction (lanes 2-12) from the glycerol gradient were subjected to discontinuous gel electrophoresis in a 12% polyacrylamide gel (14) and autoradiography (top inset panel). A duplicate gel was subjected to immunoblot analysis using polyclonal antiserum to a *trp*E-TK fusion protein (Black et al., submitted for publication, bottom inset panel).

inset of Figure 1) or immunoblot analysis (bottom inset of Figure 1), respectively, provided evidence that a 20 kDa protein which was immunoreactive with anti-TK serum co-sediments with enzyme activity. Relative to the internal 64.5 kDa hemoglobin marker in the gradient, the VV TK enzyme complex sediments faster with an apparent molecular weight of approximately 80 kDa, suggesting the complex has a subunit stochiometry of four.

As an alternative means to estimate the native molecular weight of the VV TK enzyme, VV TK synthesized *in vitro* was subjected to gel filtration chromatography through a Sephacryl-300 matrix (Figure 2). Fractions containing VV TK were identified

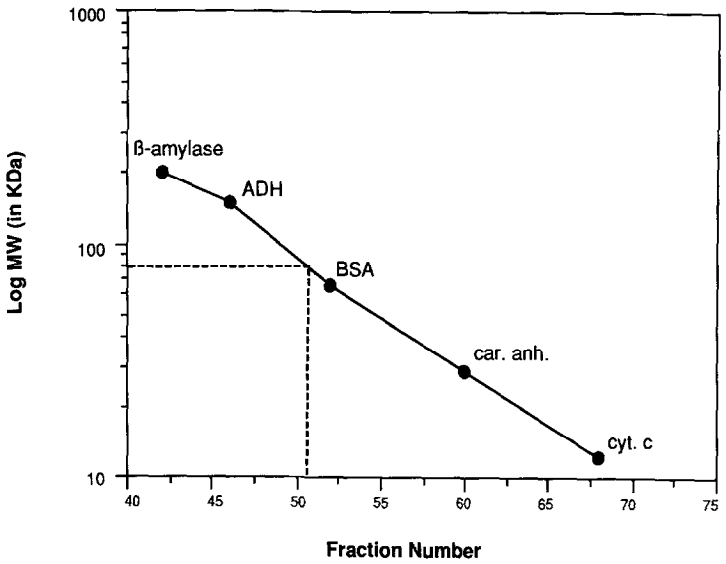


**Figure 2. Sephacryl-300 gel filtration of TK for native molecular weight determination.** A standard curve of protein molecular weights was generated by passing a mixture of protein gel filtration molecular weight markers [ß-amylase (200 kDa), alcohol dehydrogenase (ADH, 150 kDa), bovine serum albumin (BSA, 66 kDa), carbonic anhydrase (car. anh., 29 kDa) and cytochrome c (cyt. c, 12.4 kDa)] through a 1 x 34 cm Sephacryl-300 gel filtration column as described in the "Experimental Procedures". VV TK synthesized *in vitro* in the presence or absence of L-[$^{35}$S]methionine was chromatographed under identical conditions. The fractions containing VV TK were identified either on the basis of radioactivity or enzyme activity. The dashed line corresponds to the chromatographic position of the VV TK complex and its estimated molecular weight.

on the basis of enzyme activity or radioactivity. In both cases, the VV TK enzyme complex chromatographed, eluted in a peak centered around fraction 51. By reference to a standard curve generated using a mixture of protein gel filtration molecular weight markers, an apparent molecular weight of 80 kDa for the functional VV TK enzyme was obtained. This is in close agreement with the results of the velocity sedimentation analysis shown in Figure 1.

There are two potential subunit structures which would explain the apparent 80 kDa molecular weight of the VV TK enzyme. First, and most likely, is that the enzyme is a tetramer of four identical virus-encoded 20 kDa monomers. Alternatively, one or more 20 kDa proteins could associate with a cellular protein present in both the infected cell and the reticulocyte lysate to form the active enzyme complex. As an approach to distinguish between these two possibilities, protein cross-linking procedures were employed. Radiolabeled VV TK was synthesized *in vitro* and partially purified by velocity sedimentation in a glycerol gradient as described in Figure 1. Portions of the peak fraction were incubated at room temperature in the presence of increasing concentrations of the protein cross-linking agent, glutaraldehyde. After cross-linking, the reaction products were analyzed by polyacrylamide gel electrophoresis (Figure 3).

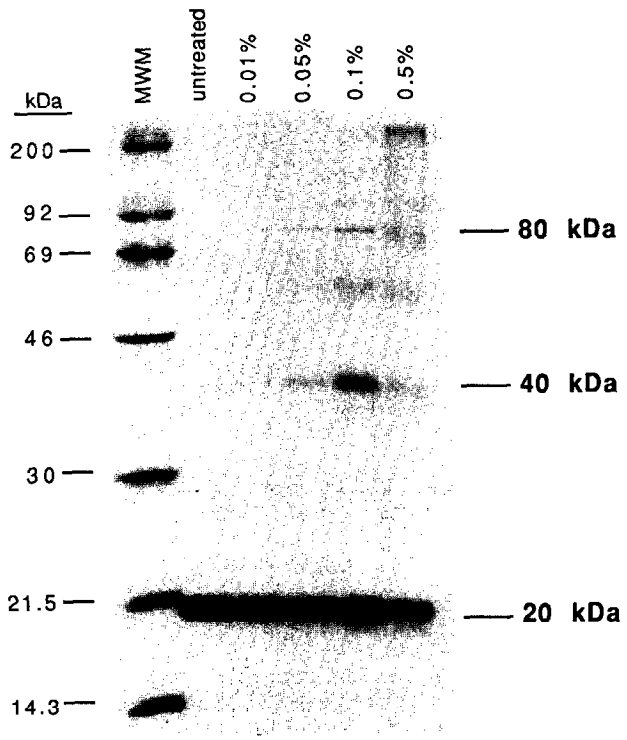


**Figure 3. Glutaraldehyde cross-linking of glycerol gradient-purified TK.** L-[$^{35}$S]methionine-labeled TK was partially purified by glycerol gradient centrifugation. Aliquots of the peak fraction containing radiolabeled TK were incubated in the presence of the indicated concentrations of glutaraldehyde (0.01%, 0.05%, 0.1% or 0.5%) for one hour at room temperature. Gel loading buffer was added, the samples heat denatured for 3 min at 100°C and then analyzed by electrophoresis in 12% polyacrylamide gels containing SDS (14), followed by autoradiography. The positions and sizes of protein molecular weight standards (MWM) included on the gel are indicated on the left.

In the absence of glutaraldehyde, the only protein species evident is the 20 kDa monomer. With increasing concentrations of glutaraldehyde the relative intensity of the 20 kDa protein band decreases and new major (40 kDa and 80 kDa) and minor (60 kDa and 100 kDa) cross-linked products become evident. An immunoblot of a duplicate gel indicated that each of the new bands contain proteins which are immunoreactive with anti-TK serum (data not shown). Since the cross-linked species increase by discrete 20 kDa increments, it is most likely that the 80 kDa VV TK enzyme is a homotetramer and not associated with any other specific ancillary factors.

Based solely on amino acid sequence comparisons and substrate specificities it appears that two classes of TK enzymes exist. The first group (type I) contains the Herpes Simplex Virus-like TK enzymes [Herpes Simplex Virus (15), Epstein-Barr Virus (16), Feline Herpes Virus (4), Marmoset Herpes Virus, (17) and Varicella-Zoster Virus (18)]. The second group (type II), which is quite distinct from HSV-like enzymes, is comprised of thymidine kinases similar to that of VV (6), namely those expressed by

monkeypox (7), variola (7), fowlpox (8), capripox (9), mouse (2) chicken (3), human (1), bacteriophage T4 (5) and *Escherichia coli* (Black & Hruby, submitted for publication). Type I TK genes encode an open reading frame with a predicted molecular weight of approximately 40 kDa and the encoded enzyme behaves as a dimer with a molecular weight of 80 kDa (15) Initial studies of the type II prototype TK enzyme encoded by VV suggested that it was also a dimer of 40 kDa subunits (19). However, when the VV TK gene was mapped and sequenced (6) the nucleotide sequence predicted a protein with a molecular weight of 20 kDa. Since, like the type I enzymes, the functional VV TK enzyme had an apparent native molecular weight of 80 kDa (20), this suggested that type II enzymes must function as a tetrameric complex. This hypothesis has been directly tested in the experiments reported in this paper. The results obtained by three independent lines of investigation are all consistent with the notion that VV TK is active as a homotetrameric complex. Given the high degree of amino acid sequence conservation between the different members of the type II TK enzymes (though less so for bacteriophage T4 and *E. coli* TK enzymes) it is likely that all of these enzymes will function as tetramers and not dimers. This theory is supported by biochemical studies on another type II TK enzyme, namely the human TK (21), purified from tissue culture cells.

As more detailed structural information (e.g., X-ray crystallographic data) becomes available on different TK enzymes it will be of great interest to determine whether the dimeric type I and tetrameric type II enzymes assemble into similar or dissimilar three dimensional structures for binding their substrates and co-factors in order to carry out their catalytic and regulatory functions.

## ACKNOWLEDGMENT

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