Identification of structural domains within the large subunit of herpes simplex virus ribonucleotide reductase

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The large subunit (R1) of herpes simplex virus (HSV) ribonucleotide reductase is a bifunctional protein consisting of a unique N-terminal protein kinase domain and a ribonucleotide reductase domain. Previous studies showed that the two functional domains are linked by a protease sensitive site. Here we provide evidence for two subdomains, of 30K and 53K, within the reductase domain. The two fragments, which were produced by limited proteolysis and were resistant to further degradation, remained tightly associated in a complex containing two molecules of each. They were capable of binding the R2 subunit of HSV ribonucleotide reductase with approximately the same affinity as the intact protein but the complex did not complement the small subunit (R2) to give an active enzyme. At low concentrations (0.4 μg/ml) of trypsin or V8 protease, cleavage between the subdomains was prevented by the presence of the N-terminal protein kinase domain. At higher protease concentrations (1 μg/ml) the N-terminal domain is extensively proteolysed and the 30K and 53K domains were generated. Identical results were obtained using purified R1 isolated from infected cell extracts or following expression in Escherichia coli. The origin of the two domains was investigated by N-terminal sequencing of the 53K fragment and by examining their reactivity with a panel of R1-specific monoclonal antibodies which we isolated and epitope mapped for that purpose. The trypsin cleavage site was found to lie between arginine 575 and asparagine 576, and proteolysis in this region was not prevented by the presence of R2 or the nonapeptide YAGAVVNDL. We propose that the ribonucleotide reductase region of HSV R1 exists in a two domain structure, and that the interdomain linking region is protected by the unique N terminus.

Introduction

Ribonucleotide reductase (EC 1.17.4.1) is an essential enzyme in the de novo synthesis of DNA and catalyses the conversion of all four ribonucleoside diphosphates to the corresponding deoxyribonucleotides (Reichard, 1988). The enzyme from Escherichia coli, which serves as the prototype for eukaryotic ribonucleotide reductase, consists of homodimeric R1 and R2 subunits in an α2β2 configuration. Each R1 subunit provides a substrate binding site and redox-active thiols, and the R2 subunits (the three-dimensional structure of which has been solved; Norlund et al., 1990) contribute a stable tyrosyl radical associated with two binuclear iron centres (Stubbe, 1990; Nordlund et al., 1990). A number of herpesviruses, including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus and Epstein–Barr virus, encode their own ribonucleotide reductase and the enzyme may be essential for viral pathogenesis (Cameron et al., 1988; Jacobson et al., 1989; reviewed by Conner et al., 1994). Recently the R1 subunit was identified in pseudorabies virus, and ribonucleotide reductase deficient mutants of this virus were avirulent in pigs (de Wind et al., 1993).

The nonapeptide YAGAVVNDL, which corresponds to the C terminus of the HSV R2 subunit, is an effective and specific inhibitor of herpesvirus ribonucleotide reductase (Dutia et al., 1986; Cohen et al., 1986) and acts by inhibiting the normal association between R1 and R2 (McClements et al., 1988; Cohen et al., 1988; Darling et al., 1990). A feature common to the class of ribonucleotide reductase enzymes having two non-identical subunits is the interaction of the R1 subunit with the C terminus of the R2 subunit (Cohen et al., 1987; Telford et al., 1990; Paradis et al., 1988; Consentino et al., 1991; Climent et al., 1991; Filatov et al., 1992). Rational drug design based on the herpesvirus inhibitory nonapeptide (Bio-Mega, 1990) may result in an effective antiviral agent.

A feature of HSV R1 is the presence of a unique N-terminal domain of approximately 310 amino acids (Nikas et al., 1986). An autophosphorylating (Conner et al., 1992a; Paradis et al., 1991) and a protein kinase activity (Chung et al., 1989; Luo & Aurelian, 1992) have been reported for HSV types 1 and 2 unique N-terminal domains, respectively, and these activities may be required during viral pathogenesis. The unique N terminus of HSV-1 R1 is susceptible to degradation both...
in vivo and in vitro (Ingemarson & Lankinen, 1987; Lankinen et al., 1989). Purified E. coli-expressed R1 was specifically cleaved by trypsin at amino acid 305 to generate a 33K N-terminal fragment which possessed autophosphorylating activity, and an 87K C-terminal polypeptide which complemented R2 to give a functional ribonucleotide reductase (Conner et al., 1992a, b). The two fragments remained associated and, at the concentration of protease used (0.4 µg/ml), were resistant to further degradation. At higher protease concentrations the unique N terminus was susceptible to further degradation.

We have recently described the properties of a series of random N-terminal deletions of HSV-1 R1 expressed in E. coli, which we used to identify regions essential for subunit interaction (Conner et al., 1993). These are located between amino acids 349 to 373 and 996 to 1137. However, the relative contribution of these two regions of R1 for R2 binding is unknown at present and we wish to examine by X-ray crystallography the structure of a truncated protein of R1 in complex with the inhibitory nonapeptide. We have previously shown that an N-terminal deletion of R1, dN247R1, bound YAGAVVNDL with the same affinity as the intact protein (Conner et al., 1993) but further studies are required to demonstrate that the truncated protein is folded in an identical manner to R1 produced in infected cells. Limited proteolysis is an excellent means of probing protein folding and has been used extensively to identify stable domains (Price & Johnson, 1989). We report here on stable fragments produced by limited proteolysis with trypsin and V8 protease from intact R1 expressed in infected cells or in E. coli and compare them with the products obtained from two truncated R1 proteins lacking most of the unique N terminus.

We identify a protease sensitive site within the reductase domains of R1 using truncated proteins lacking 243 and 245 amino acids from the N terminus. The fragments generated by the cleavage were resistant to further proteolysis, remained associated and, although they bound R2, did not form an active enzyme. Under the same conditions this cleavage did not occur in intact R1 and higher concentrations of protease were required before identical proteolysis occurred within the reductase domains. We suggest that the reductase region of R1 has a distinct two domain structure and this structure is not compromised by expression in E. coli. Additionally, in the intact protein, the region between these two domains is protected by the unique N terminus.

Methods

Proteolytic digestion. R1 and the N-terminally truncated proteins dN245R1 and dN247R1 were expressed in E. coli and purified as described previously (Furlong et al., 1991; Conner et al., 1993). The N-terminally truncated proteins retain the R1 initiator methionine and commence at R1 amino acids 245 and 247. R1 was isolated from infected cell extracts as described by Paradis et al. (1991) except that R2 immobilized to CNBr-activated Sepharose was used to bind R1 instead of peptide. Trypsin and V8 protease (Sigma) were incubated with R1 proteins at 25 °C for various time periods up to 2 h. In general, between 5 to 10 µg of purified proteins were incubated with 4 ng of protease, although in some cases intact R1 was treated with 10 ng of protease. In several experiments 5 µg of purified HSV-1 R2 (Lankinen et al., 1991) or 1 µg of the peptide YAGAVVNDL were included in the incubation mix.

Antibodies and epitope mapping. The polyclonal antiserum 106, raised against purified dN245R1, was used as described in Conner et al. (1993). Polyclonal antisera R1 and F3, raised against fusion proteins containing amino acids 3 to 181 and 282 to 436 of R1 respectively (Lankinen et al., 1993), were used as described in Conner et al. (1992b). Monoclonal antibodies (MAbs) were generated as described in Cross et al. (1987) except that the BALB/c mice were immunized with dN245R1. Three intraperitoneal injections of 1 µg protein/mouse (one with complete Freund’s adjuvant and two with incomplete) given at intervals of 1 week were followed 6 weeks later by boosting with 28 µg protein/mouse in PBS given intraperitoneally. Mice were bled and the mouse with the strongest antibody reaction was given a further boost of 50 µg of dN245R1 in PBS intraperitoneally 4 days before the fusion. Antibodies were detected by ELISA. Epitopes recognized by the MAbs were mapped using as antigens R1-β-galactosidase fusion proteins (Lankinen et al., 1993), 31 different N-terminally truncated R1 polypeptides and two defined C-terminally truncated R1 polypeptides (Conner et al., 1993).

R1/R2 interaction assays. The ELISA method for determining R1/R2 interactions was performed as described previously (Conner et al., 1993). Briefly, R1 or truncated proteins treated with or without trypsin were added to ELISA wells coated with 0.3 µg of purified R2. Protein concentrations were determined before and after proteolysis and equimolar amounts of protein were added to the wells. Dilutions of proteins prior to addition to the ELISA wells were such that no proteolytic degradation of R2 was observed (data not shown). Bound proteins were detected using MAb 11,453 (Conner et al., 1993) followed by sheep anti-mouse IgG-horseradish peroxidase conjugate and colour development was achieved with 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) at 0.5 mg/ml in 0.1 M citrate-phosphate buffer, pH 4.0, with 0.05% (v/v) H2O2. In competition experiments with the peptide YAGAVVNDL, proteins and increasing concentrations of peptide were added simultaneously. The IC50 value for the peptide was determined as the concentration required to reduce the A405 by 50% of control values obtained in the absence of peptide.

Purified HSV-1 R2 was immobilized on CNBr-activated Sepharose at a concentration of 1 mg/ml wet gel and this R2 matrix was used to bind truncated R1 proteins treated with proteases. Bound proteins were visualized by SDS-PAGE and staining of the gel with Coomassie brilliant blue.

N-terminal sequencing and protein purification. N-terminal sequencing was performed on an Applied Biosystems 477A protein sequencer (Conner et al., 1992b). Ribonucleotide reductase activity assays were performed as described by Darling et al. (1987) with the inclusion of 0.1 units/100 µl of α2-macroglobulin to inhibit proteases (Conner et al., 1992b).

Chromatography was performed on 1 ml Mono Q (Pharmacia) or 2 ml Cibacron blue 3GA-agarose (Sigma) columns on an FPLC system. The starting buffer for both was 25 mm-HEPES, pH 7.6, with 2 mM-dithiothreitol, and bound proteins were eluted with a 0 to 0.5 M or 0 to 2 M-NaCl gradient, developed over 15 ml, for the Mono Q or Cibacron blue 3GA-agarose columns respectively. Gel filtration was
Fig. 1. Proteolytic degradation products resulting from incubation of R1, dN245R1 and dN247R1 with trypsin or V8 protease. (a) Western blots developed with polyclonal antiserum 106 (lanes 1 to 5), fusion protein antiserum F1 (lanes 6 and 7) and MAb 11,427 (lanes 8 and 9). Lane 1, intact R1; lane 2, R1 incubated with 0.4 μg/ml trypsin for 1 h; lanes 3, 6 and 8, R1 incubated with 0.4 μg/ml V8 protease for 1 h; lane 4, R1 treated with 1 μg/ml trypsin for 1 h; lanes 5, 7 and 9, R1 treated with 1 μg/ml V8 protease for 1 h. (b) Coomassie stained SDS-polyacrylamide gels. Lane 1, dN245R1; lanes 2 and 3, dN245R1 incubated with 0.4 μg/ml trypsin for 1 h and 2 h, respectively; lane 4, dN247R1; lanes 5 and 6, dN247R1 incubated with 0.4 μg/ml V8 protease for 1 h and 2 h, respectively; lanes 7 and 8, dN247R1 incubated with 0.4 μg/ml trypsin for 1 h in the presence of YAGAVVNDL or R2, respectively. (c) Coomassie stained SDS-polyacrylamide gels (lanes 1 to 6) and Western blots (lanes 7 to 10) developed with antiserum 106 (lanes 7 and 8), MAb 11,543 (lane 9) and MAb 11,427 (lane 10). Lane 1, dN245R1; lanes 2 and 3, dN245R1 treated for 1 h with 0.4 μg/ml trypsin and V8 protease, respectively; lane 4, purified R1 from infected cell extracts; lanes 5 and 6, purified R1 incubated for 1 h with 1 μg/ml trypsin and V8 protease, respectively; lanes 7 and 8, purified R1 incubated for 1 h with 1 μg/ml trypsin and V8 protease, respectively; lanes 9 and 10, purified R1 treated with 1 μg/ml trypsin for 1 h.

Results

Proteolytic digestion of N-terminally truncated fragments of R1

The digestion products generated when intact R1 purified from E. coli extracts was treated with 0.4 μg/ml trypsin are shown by Western blotting in Fig. 1(a), lane 2. In agreement with previous results two fragments can be seen when R1 was incubated for 1 h with 0.4 μg/ml trypsin: the 87K fragment (dN305R1) contains the reductase domains of the protein and the 33K fragment is derived from the unique N terminus (Conner et al., 1992b). Similar products (though not identical) obtained when R1 was treated with 0.4 μg/ml V8 protease (Fig. 1a, lane 3) were probably the result of cleavage within the junction region between the distinct functional domains of the protein. The fragments derived from the unique N terminus stain poorly with Coomassie brilliant blue (Conner et al., 1992b) and are visualized in Fig. 1(a) by Western blotting. These fragments contrast with the products obtained when E. coli-expressed intact R1 was incubated with 1 μg/ml trypsin or V8 protease (Fig. 1(a), lanes 4 and 5). A polypeptide equivalent to dN305R1 was detected as well as novel 53K and 30K fragments. By means of antibodies we were able to show that this 30K fragment was distinct from the 33K fragment obtained with 0.4 μg/ml trypsin or V8 protease.

Antiserum F1 raised against R1 amino acids 3 to 181 detected a 33K N-terminal fragment only when R1 was
treated with 0.4 μg/ml V8 protease (lane 6). N-terminal fragments were not detected when R1 was treated with 1 μg/ml V8 protease (lane 7). MAb 11,427, which recognized an epitope between R1 amino acids 434 to 458 (see below), detected only dN305R1 when R1 was incubated at the low concentration of V8 protease (lane 8) but at 1 μg/ml of V8 protease a 30K fragment was detected as well as dN305R1 (lane 9).

The N-terminally truncated proteins dN245R1 and dN247R1 were treated with 0.4 μg/ml trypsin or V8 protease and the products analysed by SDS-PAGE and staining with Coomassie brilliant blue (Fig. 1 b). Incubation of dN245R1 (lane 1) with 0.4 μg/ml trypsin for 1 h (lane 2) or 2 h (lane 3) resulted in fragments of 53K and 30K. Incubation of dN247R1 (lane 4) with 0.4 μg/ml V8 protease for 1 h (lane 5) or 2 h (lane 6) also generated bands of 53K and 30K. A polypeptide equivalent to dN305R1 is visible (lanes 5 and 6) and some additional bands can be observed immediately above the 53K fragment. Inclusion of the inhibitory nonapeptide YAGAVVNDL (lane 7) or HSV-1 R2 (lane 8) did not affect the fragments produced when dN247R1 was incubated with 0.4 μg/ml trypsin.

Identical fragments of 53K and 30K were obtained when R1 isolated from infected cell extracts was treated with 1 μg/ml trypsin or V8 protease (Fig. 1 c). Incubation of dN245R1 (lane 1) with 0.4 μg/ml trypsin (lane 2) or V8 protease (lane 3) produced 53K and 30K fragments as described above. R1 isolated from infected cells by R2-affinity chromatography (lane 4) and treated with 1 μg/ml trypsin (lane 5) or V8 protease (lane 6) resulted in fragments of 53K and 30K. DN305R1 is also visible in these lanes. Incubation of R1 isolated from infected cells with 0.4 μg/ml trypsin yielded fragments of 87K (dN305R1) and 33K (data not shown). Western blotting, using polyclonal antiserum 106 (lanes 7 and 8) and MAb 11,453 (lane 9) and 11,427 (lane 10), which recognize epitopes between R1 amino acids 1072 to 1137 and 434 to 458 respectively (see below), of R1 isolated from infected cell extracts and incubated with 1 μg/ml trypsin or V8 protease, indicated that the 53K and 30K fragments were identical to those obtained under similar conditions with R1 expressed in E. coli (Fig. 1 a).

Mapping of the epitopes recognized by R1-specific monoclonal antibodies

The origin within R1 of the identified cleavage products was investigated using dN247R1 incubated with trypsin for 1 h and four MAbs. The data from which the epitopes of two of these MAbs were mapped is presented in Fig. 2. Panel (a) shows the mapping of MAb 12,488 and indicates that the antibody reacts with fusion fragment F5 (lane 5) which corresponds to R1 amino acids 566 to 737, but does not react with any other fusion fragment including F6 (lane 6) which contains R1 amino acids 677 to 789. The antibody reacts with N-terminally truncated proteins with deletions up to amino acid 598 (lanes 10 to 14) but not with the subsequent truncated protein which starts at amino acid 693 (lane 15). Numerous degradation products derived from the truncated proteins are also visible in lanes 10 to 14. We conclude that the epitope is located between R1 amino acids 598 to 677.

Numerous degradation products derived from the truncated proteins are also visible in lanes 10 to 14. We conclude that the epitope is located between R1 amino acids 598 to 677. As shown in panel (b), MAb 11,427 reacted with fusion protein F4 (R1 amino acids 411 to 566; lane 4) and with N-terminally truncated proteins up to dN421R1 (lanes 9 to 11) but not with the smaller truncated protein dN458R1 (lane 12) or any other fusion fragment. This antibody failed to react with the double deletion dN247/dC434R1 (lane 13) which contains R1 amino acids 247 to 434 and we conclude that the epitope for this MAb is located between R1 amino acids 434 to
Using similar methodology the epitopes for the two other MAbs, 11,453 and 12,964, were located between R1 and amino acids 1072 to 1137 and 305 to 324 respectively. (Fig. 3b).

**Origin of the 53K and 30K proteolytic fragments**

Fig. 3 shows Western blots demonstrating the reactivities of fragments obtained from treating dN247R1 with trypsin for 1 h, with the four MAbs and the polyclonal antiserum 106 and F3. The 30K fragment reacts with the polyclonal antiserum 106 and F3, and with MAbs 11,427 and 12,964 (lanes 1 to 4); the 53K fragment reacts with polyclonal antiserum 106, and with MAbs 11,453 and 12,488 (lanes 1, 5, 6). Similar results were obtained with V8 protease treated dN247R1 (data not shown). We conclude that the protease sensitive site is located between amino acids 434 and 677. The high molecular mass bands seen in Fig. 3 correspond to intact dN245R1 and dN305R1. Two faint bands are also visible above the 53K fragment with the polyclonal antiserum 106 and MAb 11,453, suggesting several cleavage sites within the region 434 to 677. These results are similar to those given in Fig. 1(a, c) and indicate that expression in *E. coli* or loss of most of the unique N terminus does not affect folding within the reductase domains of R1. N-terminal sequencing of the 53K band obtained from trypsin treatment of dN245R1 for five cycles gave a sequence of NYYXX, where X represents amino acids which were not positively identified. This sequence uniquely matches amino acids 576 to 578 of R1, which are preceded by arginine 575, and this result maps the principal cleavage
site to this residue. The 53K fragment from V8 treatment was not sequenced but since the fragments were of similar size the site must be close to residue 575. Potential cleavage sites are at aspartic acid 559 or glutamic acid 548.

**Properties of the 53K and 30K fragments**

To investigate the properties of the 30K and 53K fragments their behaviour during various chromatographic procedures was observed. Fig. 4a shows a Coomassie brilliant blue stained SDS–polyacrylamide gel of the elution of fragments generated by trypsin treatment of dN247R1 from a Mono Q column (lanes 1 to 5) or from a Cibacon blue column (lanes 6 and 7). Both fragments bound to the columns and eluted in a single fraction during the salt gradient. These observations suggest that the two fragments remain associated. Further evidence for this suggestion was obtained using an R2-affinity matrix and dN245R1 treated with V8 protease (Fig. 4b): both the 53K and 30K fragments (lane 1) bound to the matrix, and remained associated with it, even after washing with 1 m-NaCl (lane 2), 2 m-NaCl (lane 3), 5 m-LiCl (lane 4), 0.1 m-glycine–HCl, pH 2.5 (lane 5) or 50% (v/v) ethylene glycol (lane 6).

Gel filtration of dN247R1 gave a single peak with a molecular mass of 160K, equivalent to a homodimer of the protein (Fig. 5a). Following treatment of this protein with trypsin for 1 h, a single peak was obtained, also with a molecular mass 160K (Fig. 5b). Thus, after proteolysis the 30K and 53K domains of the protein remain tightly associated and exist as a dimer. Non-reducing SDS–PAGE resolved the 53K and 30K fragments, demonstrating that the polypeptides are not linked by disulphide bonds (data not shown).

The interaction of the 30K/53K complex with R2 was quantified using an R2 binding ELISA. Calibration curves were prepared (Fig. 6a) for intact R1, dN245R1 and dN245R1 treated with trypsin for 1 h, starting with approximately equimolar concentrations of protein (150 µg/ml R1, molecular mass 140K; 100 µg/ml dN245R1, molecular mass 96K; and 80 µg/ml 30K/53K complex, molecular mass 83K). Concentrations of the three proteins were chosen such that they gave an A405 in the ELISA of approximately 0.5 units after a 30 min incubation and IC50 values for the peptide YAGAVVNDL were calculated. Interpolation from the curves in Fig. 6(b) gave values of 30 µM, 37 µM and 40 µM for R1, dN245R1 and the 30K/53K complex, respectively, indicating that these proteins have the same affinity for R2. Although the complex generated by trypsin treatment of dN245R1 binds specifically to R2 the resulting enzyme had no detectable ribonucleotide reductase activity (data not shown).
Discussion

The results presented in this paper suggest that the R1 subunit of the HSV ribonucleotide reductase has a distinct, well-defined, three domain structure. In the intact protein two of these domains are within the ribonucleotide reductase region of the protein and the third consists of a unique N-terminal domain which can function in vitro as a protein kinase (Paradis et al., 1991; Conner et al., 1992a). The distinct N-terminal domain of the protein is fused to the reductase domains by a protease sensitive loop which is cleaved at low concentrations of protease to give 33K N-terminal and 87K (dN305R1) reductase polypeptides. The N-terminal and reductase domains of the protein remain associated and are functional in both protein kinase and ribonucleotide reductase assays, although the cleaved complex only complements R2 to 50% of the activity obtained with the intact protein (Conner et al., 1992a, b). At higher concentrations of protease the 33K N-terminal fragment is not detectable and a novel protease sensitive site is exposed within the reductase domains of the protein. Cleavage at this site generates stable 30K and 53K fragments. In the presence of the unique N-terminal domain the site is inaccessible to proteases. At higher concentrations of protease the unique N-terminal domain is degraded and the proteases have access to the site within the reductase domains of the protein.

Confirmation of this scheme and the precise location of the protease sensitive site within the reductase domain was obtained using dN245R1 and dN247R1, two N-terminally truncated proteins of R1 which lack most of the unique N terminus. When incubated with low concentrations of protease these proteins were readily cleaved at the site within the reductase domains. Cleavage with trypsin still occurred at amino acid 305 to give dN305R1 but the principal digestion products were 30K N-terminal and 53K C-terminal fragments, both of which were resistant to further proteolysis. We use these data to propose that these two fragments represent stable domains within R1 of HSV-1. These findings and conclusions are similar to results reported by Slabaugh et al. (1993) for vaccinia virus R1 expressed in E. coli, which has no unique N-terminal extension. However, these authors did not identify the regions of R1 from which the proteolytic fragments originated. A further observation from this study is that the tertiary structure of R1 is not compromised by expression in E. coli and, since here and in previous studies (Furlong et al., 1991; Conner et al., 1993) we have been unable to identify any differences in R1 isolated from infected cells or following expression in E. coli, we conclude that proteins from the latter source are suitable for R1 structural studies.

N-terminal sequencing of the 53K fragment generated by trypsin treatment identified the cleavage site at arginine 575 and we suggest that cleavage by V8 protease probably occurs at aspartic acid 559 or glutamic acid 548, giving rise to fragments of similar molecular mass. The 30K fragment reacted with MAb 12,946 (epitope within residues 305 to 324) and the 53K fragment reacted with MAb 11,453 (epitope within residues 1072 to 1137) suggesting that the N and C termini of the reductase domains remain largely intact after cleavage. The 30K fragment appears homogeneous, but additional minor bands are consistently observed with the 53K fragment, indicating that there are several alternative proteolytic sites within the domain linking region.

We have previously identified two regions of R1 required for the interaction with R2 using N- and C-terminally truncated proteins (Conner et al., 1993). They are located between amino acids 349 to 372 and 996 to 1137, within the 30K and 53K fragments respectively. We therefore attempted to separate the two domains by standard chromatography procedures in order to identify the relative contribution of each of these regions to the mechanism of interaction with R2. However, the domains remained tightly complexed and were only separable under protein denaturation conditions. This observation is indicative of a strong interaction between the two domains.

Proteases are extensively used to identify stable domains within polypeptides and are also useful probes for determining conformational changes in proteins following binding of subunits or substrata (Price & Johnson, 1989). We wished to determine whether the binding of R2 or YAGAVVNDL to R1 resulted in any domain rearrangements. Binding of R2 or peptide may result in a structural reorganization which masks the novel protease sensitive site described in this study or it may result in the exposure of a new proteolytic site. However, proteolysis of dN245R1 or dN247R1 was not affected by the presence of R2 or YAGAVVNDL (Fig. 1), suggesting that binding of R2 or peptide does not cause large structural rearrangements.

The 30K/53K complex contains two molecules of the 30K and 53K fragments and can bind R2 with the same affinity as intact R1. However, the resulting complex is inactive in ribonucleotide reductase assays. Proteolysis occurs close to cysteine 582, a highly conserved residue (equivalent to cysteine 225 in E. coli R1) which is proposed to be in the active site and involved in substrate reduction (Aberg et al., 1989; Mao et al., 1989; Stubbe, 1990). Cleavage at arginine 575 may affect the active site conformation, rendering the enzyme inactive, or essential residues may be lost during additional proteolysis in this region.

We have demonstrated that inactivation of R1 by proteolysis in vitro can be prevented by the presence of
the unique N terminus and it is possible that this observation may have some functional role in vivo. Western blotting of virus-infected cell extracts using the four MAbs described in this study did not identify degradation products equivalent to the 30K or 53K polypeptides (data not shown), and it is possible that the location of the N terminus in the R1 structure may protect this protease sensitive site.

References


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(Received 6 June 1994; Accepted 17 August 1994)