^{1}H NMR Studies of Mouse Ribonucleotide Reductase: The R2 Protein Carboxyl-Terminal Tail, Essential for Subunit Interaction, Is Highly Flexible but Becomes Rigid in the Presence of Protein R1†

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ABSTRACT: Mouse ribonucleotide reductase consists of two nonidentical subunits, proteins R1 and R2, each inactive alone. It has earlier been shown that the carboxyl-terminal part of the R2 protein is essential for subunit association to form the active enzyme complex. We now demonstrate that protein R2 gives rise to a number of sharp ^{1}H NMR resonances, significantly narrower than the major part of the resonances. This line narrowing of certain resonances indicates segmental mobility in the molecule. In two-dimensional ^{1}H TOCSY spectra of protein R2, cross-peak patterns from about 25 amino acid residues are visible. Most of these were assigned to the carboxyl-terminal part of the protein by comparisons with cross-peak patterns of oligopeptides corresponding to the carboxyl terminus of mouse R2 and to the patterns of a seven amino acid residue carboxyl-terminal truncated form of protein R2. These results and the magnitude of the chemical shifts of the assigned residues demonstrate that the carboxyl-terminal part of mouse R2 protein is highly mobile compared to the rest of the protein and essentially unstructured. When protein R1 is added to a solution of protein R2, the sharp resonances are broadened, suggesting that the mobility of the carboxyl-terminal tail of protein R2 is reduced. The possibility of making direct observations of subunit interaction in native and mutagenized R1/R2 proteins should allow discrimination between effects of amino acid replacements on the catalytic mechanism and effects on subunit interaction.

Mouse ribonucleotide reductase (EC 1.17.4.1) is composed of two nonidentical subunits, proteins R1 and R2, each of which is a homodimer with a molecular mass of about 180 and 90 kDa, respectively. Protein R1 contains redox-active thiol groups and binds the substrate and allosteric effectors. Protein R2 contains two binuclear ferric iron sites and stable free radicals localized to tyrosyl residues close to the iron sites (Thelander & Gräslund, 1993).

The interaction between the subunits is essential for enzyme activity since each subunit is inactive alone. For the herpes simplex virus type I enzyme it was shown that a nonapeptide corresponding to the C-terminal of the small subunit could effectively inhibit enzyme activity (Dutia et al., 1986; Cohen et al., 1986). The reason was that the nonapeptide inhibited subunit interaction involving specifically the C-terminal of protein R2 (McClements et al., 1988). Similar inhibitory effects were also demonstrated for the mammalian and for the *Escherichia coli* enzymes by oligopeptides with sequences corresponding to the respective C-termini (Yang et al., 1990; Consentino et al., 1991; Climent et al., 1991). Subsequently, it was reported that a truncated mutant HSV1[1] protein R2, lacking the last seven C-terminal residues, could not bind protein R1 and had no enzymatic activity, thus showing that the necessary subunit interaction is crucially dependent on the C-terminal residues of protein R2 (Filatov et al., 1992).

The three-dimensional crystal structure of *E. coli* protein R2 has been determined (Nordlund et al., 1990) and shows that the iron/tyrosyl sites are well inside the protein, about 10 Å from the nearest surface. In the electron density maps of *E. coli* protein R2, 32 C-terminal residues were missing. The suggested reason was dynamic disorder of this part of the protein.

Preliminary reports (LaPlante, personal communication; LaPlante et al., 1993) indicated that NMR could be used to study the mobility and conformation of the C-terminus of the herpes simplex virus ribonucleotide reductase R2 protein. In the present study we report results for mouse ribonucleotide reductase showing that the C-terminal end of the R2 protein is indeed highly dynamic and unstructured in solution. The high degree of mobility makes it possible to observe well-resolved ^{1}H NMR resonances from this flexible tail, well distinguishable from the rest of the protein R2 resonances which are broad due to the large molecular weight of the protein. When protein R1 is bound to protein R2, the high degree of mobility of the R2 C-terminal tail is lost and the previously well-resolved resonances are broadened beyond individual detection.

MATERIALS AND METHODS

Mouse R2 protein was produced in *E. coli* by expression from its cDNA using the T7 RNA polymerase system (Studier et al., 1990) and purified to homogeneity as described elsewhere (Mann et al., 1991). A new plasmid was constructed, expressing a truncated R2 lacking seven amino acid residues in the carboxyl terminus. Complementary oligonucleotides 5′-AAT TCG ACA GAG AAC TCT TAA G-′3 and 5′-GAT CCT TAA GAG TTC TCT GTC G-′3 were synthesized. After annealing, the resulting double-stranded oligonucleotide

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[1] Abbreviations: BSA, bovine serum albumin; DANTE, delays alternating with nutations for tailored excitation; dATP, deoxyadenosine triphosphate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; HSV, herpes simplex virus; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect, NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; SDS, sodium dodecyl sulfate; TOCSY, total correlation spectroscopy; Tris, 2-amino-2-(hydroxymethyl)-1,3-propandiol.

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^{358} GKTNFFEKRVGEYQRMGVMSNSTENSFTLDADF^{390} mouse R2

VISNSTENSFTLDADF Oligopeptide I

FTLDADF Oligopeptide II

FIGURE 1: Amino acid sequences of the carboxyl-terminal tail of mouse ribonucleotide reductase protein R2 and of the two corresponding oligopeptides used for NMR studies.

was introduced between a unique *Eco*R1 restriction site in the coding region of mouse R2 cDNA and a unique *Bam*H1 restriction site in the pET3a expression vector. The oligonucleotide contained a new stop codon TAA replacing a phenylalanine codon TTT. The truncated R2 was expressed and purified in the same way as the native protein (Mann et al., 1991). Recombinant mouse R1 protein was produced from its cDNA, using the same expression system as for the R2 proteins, and purified to homogeneity by affinity chromatography on dATP-Sepharose (Davis, Thelander, and Thelander, to be published).

The proteins were transferred, by gel filtration on a Sephadex G-25 column, to a buffer containing 20 mM Tris-HCl, 150 mM KCl, and 2 mM $MgCl_2$, pD 7.6, in 99.8% D_2O. The Tris buffer was prepared from 98% deuterated 1 M Tris base solution (supplied by Glaser AG, Basel, Switzerland). $MgCl_2 \cdot 6H_2O$ was dissolved in 99.8% D_2O and lyophilized prior to buffer preparation. Also a solution of bovine serum albumin (supplied by Sigma) was prepared in the same buffer. The following solutions were prepared for NMR spectroscopy: 0.09 mM R2, 0.09 mM R1, 0.09 mM R2 + 0.09 mM R1, 0.09 mM R2 + 0.14 mM bovine serum albumin, and 0.14 mM bovine serum albumin. 1 mM R2 and 0.4 mM truncated R2 were prepared in 10 mM potassium phosphate and 150 mM KCl, pH 6.8, in D_2O or 90% H_2O/10% D_2O. The protein concentrations were determined from the UV absorbance difference at 280 and 310 nm. The molar extinction coefficient used was 62 000 M^{-1} cm^{-1} for protein R2 and truncated R2 (Mann et al., 1991) and 110 000 M^{-1} cm^{-1} for R1 (Thelander et al., 1980) expressing concentrations as mol/L of 45- and 90-kDa polypeptide, respectively.

Synthetic oligopeptides with the sequences VISNSTENSFTLDADF (here denoted oligopeptide I) and FTLDADF (here denoted oligopeptide II) were provided by Bio Méga/Boehringer Ingelheim Research Inc., Laval, Québec, Canada. The peptides were characterized by HPLC, amino acid analysis, and FAB-MS. The amino acid compositions were compatible with the sequences, and the molecular masses were determined to be within one mass unit from the expected. These peptide sequences correspond to the carboxyl-terminal part of mouse R2 except that the methionine corresponding to Met376 is exchanged for an isoleucine to simplify the synthesis of oligopeptide I (Figure 1). The synthetic oligopeptides were dissolved to a concentration of approximately 1 mM in 90% H_2O/10% D_2O, and the pH was adjusted to close to neutral by adding small quantities of NaOH solution.

The NMR spectra were recorded on a Bruker AM500 spectrometer. Two-dimensional TOCSY spectra (Braunschweiler & Ernst, 1983) were recorded from all protein and oligopeptide solutions using the phase-sensitive mode via time-proportional phase incrementation (Marion & Wüthrich, 1983). NOESY spectra (Jeener et al., 1979; Macura & Ernst, 1980) were recorded from protein R2 and oligopeptide I. A ROESY spectrum (Bothner-By et al., 1984; Bax & Davis, 1985) was recorded from oligopeptide II. In the TOCSY experiments a MLEV-17 spin-lock sequence (Bax et al., 1987) was used with a mixing time of 75 ms. Relaxation delays of 1.7–2 s were used with presaturation of the water resonance

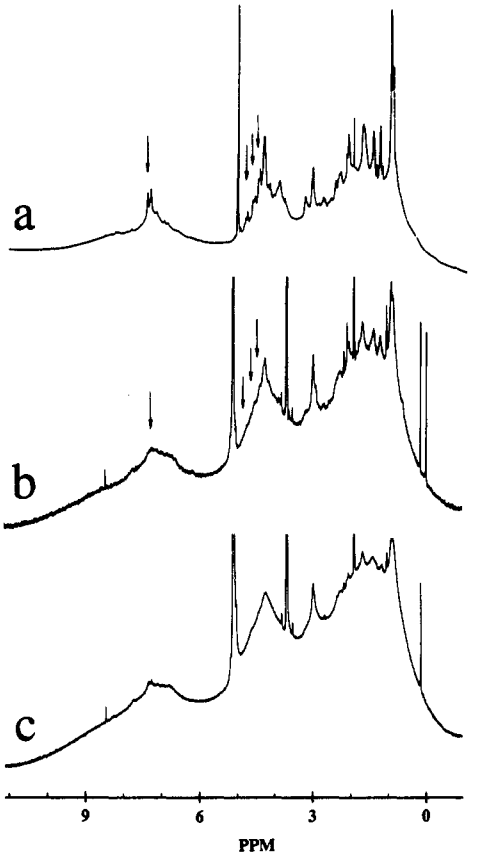

FIGURE 2: 500-MHz proton NMR spectra of (a) 1 mM mouse ribonucleotide reductase protein R2 in 10 mM potassium phosphate and 150 mM KCl, pH 6.8, in 99.8 % D_2O at 15 °C, (b) an equimolar mixture (0.09 mM) of ribonucleotide reductase protein R1 and protein R2 in 20 mM Tris-HCl, 150 mM KCl, and 2 mM $MgCl_2$, pH 7.6, in 99.8% D_2O at 7 °C, and (c) 0.09 mM of ribonucleotide reductase protein R1 in 20 mM Tris-HCl, 150 mM KCl, and 2 mM $MgCl_2$, pH 7.6, in 99.8% D_2O at 7 °C. Some of the comparably narrow peaks in free R2 and the corresponding positions in the R1–R2 complex have been indicated by arrows.

using a DANTE type of pulse sequence (Morris & Freeman, 1978). The solvent suppression pulse sequence was composed of 5-μs pulses at intermediate power followed by 15-μs delays. Typically the spectra were measured with a spectral width of 12 ppm with the carrier placed at the water resonance frequency. A total of 16–112 transients with two dummy scans were recorded per t_1 increment, and 1024 real data points were collected in the t_2 domain and 256–512 increments in the t_1 domain.

RESULTS

Figure 2a shows the one-dimensional ^{1}H NMR spectrum of mouse ribonucleotide reductase protein R2. This protein, with a molecular mass of 2 × 45 kDa, is not expected to give rise to well-resolved resonances. However, the figure shows a number of relatively sharp resonances (approximately 10-Hz line width) on top of a broad envelope spectrum. When a two-dimensional TOCSY spectrum is recorded, cross-peaks from the broad protein resonances are suppressed and only the cross-peak patterns from 25–30 amino acid residues are observed (Figure 3a). The sharp lines may be explained by the presence of a segment in the molecule with a much larger mobility compared to the rest of the protein. It has also recently been reported that protein R2 from herpes simplex virus type I shows a number of resolved resonances assigned to the carboxyl terminus (LaPlante et al., 1993).

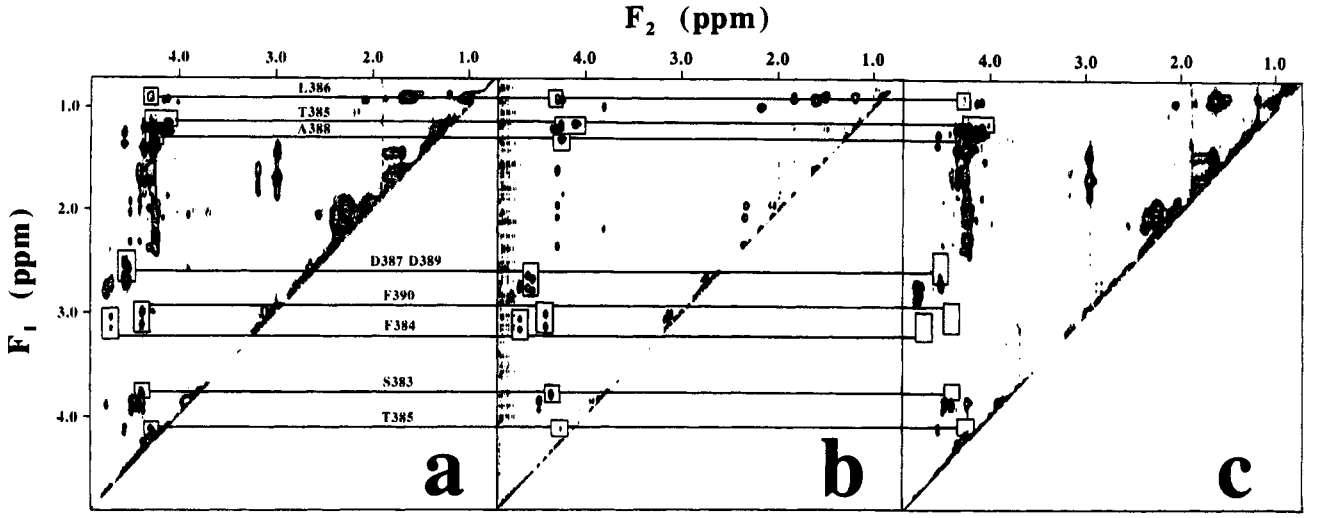

FIGURE 3: Comparison of 500-MHz TOCSY spectra from mouse R2, oligopeptide I, and truncated R2 lacking seven residues at the carboxyl terminus. The spin-lock time was 75 ms using a MLEV-17 pulse sequence with a DANTE type of solvent saturation. (a) Upper half of the aliphatic region in a TOCSY spectrum from 1 mM mouse R2 in 10 mM potassium phosphate and 150 mM KCl, pH 6.8, in 99.8% D_2O at 15 °C. (b) Upper half of the aliphatic region in a TOCSY spectrum from 1 mM oligopeptide I (VISNSTENSFTLDADF) in 90% H_2O/10% D_2O, at 7 °C. (c) Upper half of the aliphatic region in a TOCSY spectrum from 0.4 mM truncated R2, lacking seven amino acid residues at the C-terminus, in 10 mM potassium phosphate and 150 mM KCl, pH 6.8, in 99.8% D_2O at 7 °C. The cross-peaks assigned to the eight amino acid residues at the carboxyl terminus of R2 are indicated by boxes and connected to corresponding areas in the oligopeptide I and truncated R2 spectra.

Another more trivial explanation for observing narrow NMR resonances could be the progressive liberation of small peptides due to proteolytic cleavage of protein R2 during the NMR experiments. To exclude this possibility, one-dimensional spectra of a freshly prepared R2 protein sample were compared with spectra after two-dimensional NMR experiments. A spectrum after a 15-h incubation at 15 °C was identical with a spectrum of the fresh sample. SDS–polyacrylamide gel electrophoresis of the samples before and after NMR measurements also demonstrated that there were no major proteolytic degradation during the time course of the experiments (data not shown).

Assignment of ^1H NMR Resonances in Protein R2. Usually the sequential proton resonance assignment of small proteins can be made from NMR spectra of the protein alone (Wüthrich, 1986). However, in the present case this approach is obstructed by the very small chemical shift dispersion of the observed amide- and α-proton resonances. Also the fact that different parts of the protein may contribute to the NMR spectrum prevents unambiguous assignments from spectra of the intact R2 alone. Therefore the assignments are based on comparisons between spectra of intact R2, truncated R2 lacking seven C-terminal residues and synthetic peptides corresponding to the C-terminal sequence of R2 (Figure 3a–c).

The TOCSY spectrum of intact R2 was compared with TOCSY spectra of oligopeptide I corresponding to 16 C-terminal residues, and of truncated R2, lacking seven C-terminal residues (Figure 3). Comparisons were also made with spectra of oligopeptide II corresponding to the seven C-terminal residues (Figure 4). Comparing the TOCSY spectra of protein R2 with oligopeptides I and II, we observed almost perfect overlap between the R2 cross-peaks and oligopeptide cross-peaks for 14 amino acid residue cross-peak patterns in the 16-residue peptide (Figure 3a,b) and six patterns in the seven-residue peptide (data not shown). The patterns in the peptides that did not coincide with patterns in intact R2 were assigned to the N-terminal Phe in oligopeptide II and to the N-terminal Val and the Ile in oligopeptide I. The Ile in oligopeptide I does not have a corresponding counterpart

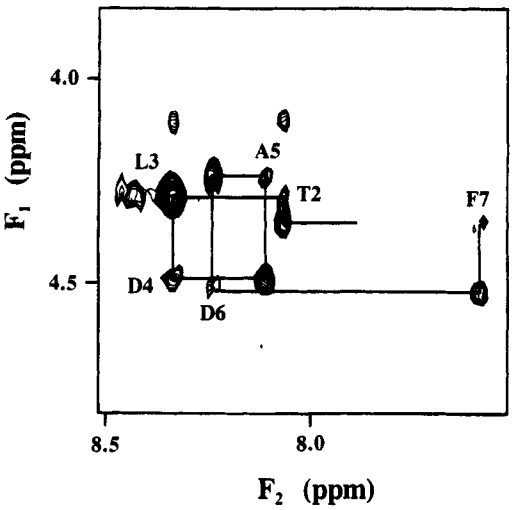

FIGURE 4: "Fingerprint" region ($C^\alpha H$–NH) of a 500-MHz ROESY spectrum from 1 mM oligopeptide II (FTLDADF) in 90% H_2O/10% D_2O at 27 °C. The mixing time used was 220 ms. The assignments of intraresidual $C^\alpha H_i$–NH_i cross-peaks are indicated. A "sequential walk" is shown by connecting intraresidual $C^\alpha H_i$–NH_i cross-peaks and sequential $C^\alpha H_i$–NH_{i+1} cross-peaks with a solid line.

in R2 but is used instead of a Met to facilitate chemical synthesis. Significantly altered chemical shift values are frequently observed for residues at the termini of a peptide, as noticed for the Val residue in oligopeptide I and one Phe residue in oligopeptide II.

Both oligopeptides I and II contain two Phe residues with cross-peaks that coincide with the aromatic resonances observed from intact R2, which makes it plausible that they correspond to Phe384 and Phe390. The observation of sequential ROESY $C^\alpha H$–NH cross-peaks, in oligopeptide II, between a Phe and a Thr residue and between an Asp and a Phe residue settled the sequential assignment of the aromatic residues (Figure 4). Similarly, sequential cross-peaks between an Asp and the Ala, between the Ala and an Asp, and an Asp to a Phe secured the assignments of the two Asp residues in oligopeptide II. The other three residues are unique, which completed the sequential assignment of oligopeptide II. The

Table 1: Proton NMR Resonance Assignments of the C-Terminal Part of Mouse Ribonucleotide Reductase R2 Protein As Inferred by the Assignments of Oligopeptides I and II[a]

residue	NH	C$^\alpha$H	C$^\beta$H	C$^\beta$H	C$^\gamma$H	C$^\gamma$H	others
Met376							
Ser[b]	8.40	4.48	3.83	3.93			
Asn[b]	8.61	4.66	2.69	2.72			
Ser[b]	8.62	4.48	3.83	3.83			
Thr380	8.29	4.36	4.28		1.18		
Glu381	8.32	4.28	2.33	2.33	2.04	1.94	
Asn[b]	8.46	4.74	2.73	2.80			
Ser383	8.23	4.36	3.77	3.77			
Phe384	8.22	4.70	3.14	3.04			7.30, 7.40[c]
Thr385	8.05	4.28	4.10		1.14		
Leu386	8.28	4.29	1.58	1.58	1.58		0.87, 0.92
Asp387	8.39	4.52	2.68	2.56			
Ala388	8.20	4.26	1.30				
Asp389	8.23	4.56	2.64	2.51			
Phe390	7.58	4.38	3.11	2.99			7.30, 7.40[c]

[a] The chemical shift values (in ppm) are relative to DSS. The measurements were performed at 7 °C. [b] The serine and asparagine residues could not be sequentially assinged due to spectral overlap. [c] The aromatic protons could not be sequentially assigned due to overlapping resonances.

corresponding resonances of seven C-terminal residues in protein R2 were assigned by analogy (Table 1). The resonances corresponding to oligopeptide II were absent in the spectra of the truncated R2 lacking seven C-terminal residues (Figure 3c), which unambiguously demonstrates the validity of the assignments also in the R2 spectrum. However, the absence of peaks corresponding to Leu386 in the spectrum of truncated protein R2 is not obvious due to overlapping peaks. Similar comparisons with oligopeptide I containing 16 residues (Figure 3b) led to putative assignments of resonances from 14 amino residues in the R2 spectrum (Table 1). Of the 14 assigned cross-peak patterns in oligopeptide I, nine were sequentially assigned. Assignment of Ser383 is inferred by a significant shift of a Ser/Thr/Gly C$^\alpha$H–C$^\beta$H cross-peak in the TOCSY spectrum of truncated protein R2 compared to intact R2 and oligopeptide I, probably due to this residue becoming C-terminal. Due to spectral overlap and missing sequential cross-peaks in the NOESY spectra of oligopeptide I, the Ser and Asn residues could not be sequentially assigned. These data demonstrate that most of the mobile amino acids in mouse R2 can be attributed to the carboxyl-terminal segment of the protein. A number of resolved resonances, corresponding to 10–15 residues, still remain to be assigned, which indicates that the flexible carboxy-terminal segment in mouse R2 may be longer than 14 residues. Sensitivity to proteolytic degradation at the N-terminus, which does not affect activity or subunit interaction (Mann et al., 1991), suggests that the amino terminus may also be exposed and mobile and contribute to the resolved NMR resonances.

Interactions between Proteins R1 and R2. When the NMR spectrum of an equimolar mixture of proteins R2 and R1 was recorded, most of the well-resolved resonances were significantly broadened, and the cross-peaks in the TOCSY spectrum were lost as demonstrated in Figures 2b and 5b. This demonstrates that the mobility of the C-terminal segment is significantly reduced upon binding to R1. The sharp resonances of R2 still remains unchanged when R2 is mixed with bovine serum albumin, to which R2 is not expected to bind (Figure 5a). Mouse R1 alone, on the other hand, does not possess any highly mobile segment giving rise to well-resolved NMR resonances. The one-dimensional spectrum is broad and featureless (Figure 2c). The TOCSY spectrum of

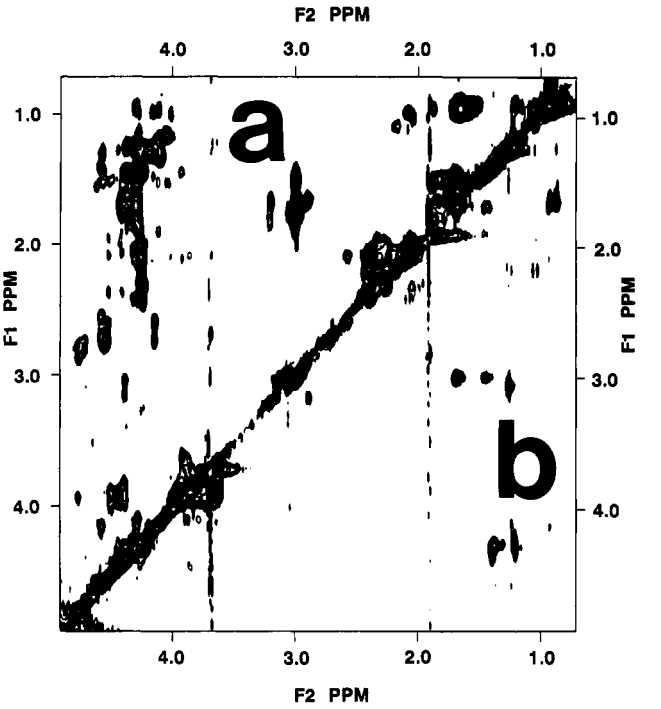

FIGURE 5: Comparison between 500-MHz TOCSY spectra of mouse ribonucleotide reductase protein R2 mixed with BSA or with ribonucleotide reductase protein R1. (a) Above the diagonal: Part of a TOCSY spectrum, showing the aliphatic region, of a mixture of 0.09 mM R2 + 0.14 mM BSA in 20 mM Tris, 150 mM KCl, and 2 mM $MgCl_2$ in 99.8% D_2O at 7 °C. (b) Below the diagonal: Corresponding part of a TOCSY spectrum of a mixture of 0.09 mM protein R2 + 0.09 mM protein R1 in 20 mM Tris, 150 mM KCl, and 2 mM $MgCl_2$ in 99.8% D_2O at 7 °C. The spin-lock time was 75 ms using a MLEV-17 pulse sequence with a DANTE type of solvent saturation.

protein R1 only shows four cross-peaks which are compatible with Ala/Thr/Lys/Arg sidechains (data not shown). Appearance of similar cross-peaks are frequently observed in TOCSY spectra of large proteins (Lycksell, unpublished observations).

Line Width Estimations and Rotational Correlation Times. The crystal structure of *E. coli* protein R2 shows a heart-shaped globular protein (Nordlund et al., 1990). A simple estimation of rotational correlation times based on molecular weight, a partial specific hydrated volume for a protein of 1 cm^3/g, viscosity, and temperature (Cantor & Schimmel, 1980) gives τ_c = 55 ns for protein R2 in water at 7 °C. Using the Solomon–Bloembergen equation relating NMR relaxation times to rotational correlation times and assuming a dipolar relaxation mechanism, one can estimate T_2 to be of the order of 2 ms in a 500-MHz spectrometer (Wüthrich, 1986, particularly p 27). This gives an expected line width of about 150 Hz for resonances arising from protons in rigid parts of protein R2. As anticipated, this line width is too broad for individual resonance resolution and efficient TOCSY coherence transfer which prevents assignments for the major part of protein R2 resonances. The corresponding estimation for oligopeptide I, assuming it has a rigid and globular shape, would yield τ_c = 1 ns, T_2 = 0.1 s, and an expected line width of 3 Hz. In reality, from the one-dimensional ^{1}H NMR spectra of protein R2 one can estimate the line widths of some resolved proton resonances from residues in the R2 C-terminal tail to be of the order of 10 Hz. For oligopeptide I the measured line width is about 4 Hz. A line width of 10 Hz corresponds to a rotational correlation time of 3 ns estimated as above. The significant difference between resolved resonances and the

envelope of most protein resonances indicates that the major contribution to rotational motion of the carboxyl-terminal tail is from local mobility. On the other hand, the line width of the oligopeptide I is about 4 Hz, considerably less than the protein R2 C-terminal resonance line widths, which indicates that the tail certainly is less mobile than a free oligopeptide in solution.

DISCUSSION

The direct observation of subunit interactions in ribonucleotide reductase has up to now proven difficult. Attempts to monitor interactions by induced conformational changes using, e.g., circular dichroism spectroscopy or intrinsic fluorescence spectroscopy have proven unsuccessful (A. Gräslund, unpublished results). The present study is the most direct demonstration of the site and also the extent of the subunit interaction. The observations demonstrate a particular type of protein–protein interaction operating in ribonucleotide reductase: The flexible tails of one subunit recognize particular surfaces on the other subunit, and presumably the tails become the rigid "glue" between the two proteins. This type of interaction may fit into a model of macromolecular associations where unstructured, polymeric domains give rise to increased rates of specific associations (Pontius, 1993). In the present case a flexible structure in protein R2 recognizes a particular surface in protein R1, whereupon the interaction causes the flexible structure to become rigid and structured. The structuring of the specific inhibitory peptide interacting with *E. coli* protein R1 was also previously shown in studies of transferred NOE effects in the presence of small amounts of protein R1 (Bushweller & Bartlett, 1991).

The methodology outlined here for studies of subunit interactions in ribonucleotide reductase should be useful for studies of site specific mutants in the C-terminal tail of protein R2. The C-terminal tail, or rather the area of interaction between the two subunits, is believed to be important also for the long-range electron transfer suggested to take place between the active site in protein R1 and the iron/radical center inside protein R2 during the enzymatic reaction (Filatov et al., 1992; Mao et al., 1992; Climent & Sjöberg, 1992). For understanding the enzymatic mechanism it will be most important to be able to separate effects of mutated amino acids on subunit interactions or electron transport. Whereas the carboxyl-terminal tail of the R2 subunit is clearly identified as one interacting partner, the corresponding structure of the R1 subunit is still not identified. Here NMR studies using mutagenized R1 protein interacting with R2 protein might give valuable information.

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SUPPLEMENTARY MATERIAL AVAILABLE

Three figures showing the amide proton regions of the TOCSY spectra, including the NH–C$^{\alpha}$H/aliphatic region of protein R2 and the corresponding parts of the TOCSY spectra from oligopeptide I and oligopeptide II, respectively (3 pages). Ordering information is given on any current masthead page.

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