Demonstration of segmental mobility in the functionally essential carboxyl terminal part of ribonucleotide reductase protein R2 from *Escherichia coli*

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Abstract The C-terminus of protein R2 is important for the formation of the enzymatically active complex between proteins R1 and R2 of ribonucleotide reductase from *Escherichia coli*. Some residues in this part of R2 may also be involved in intramolecular electron transfer. We now demonstrate that 26 amino acid residues at C-terminus of protein R2 are mobile in the free protein, and can be studied by ^{1}H NMR. Spectral assignment of narrow resonances was made by comparison of TOCSY and NOESY spectra from wild-type R2 with corresponding spectra of a mutant protein R2, lacking 30 residues at the carboxyl terminus.

Key words: Ribonucleotide reductase; *Escherichia coli*, Deoxyribonucleotide synthesis; ^{1}H NMR; Protein–protein interaction

1. Introduction

The enzyme ribonucleotide reductase catalyzes the reduction of ribonucleotides to their corresponding deoxyribonucleotides, an essential step in the chain of reactions leading to DNA synthesis. The *Escherichia coli* enzyme is composed of two non-identical homodimeric proteins, R1 and R2, respectively. The molecular masses are 2×87 kDa for protein R1 and 2×43 kDa for protein R2. The active enzyme is a 1:1 complex of the two subunits. Protein R1 binds substrates and allosteric effectors and contains redox-active thiol groups. Active protein R2 contains two binuclear ferric ion centers and stable free radicals on tyrosyl (Tyr 122) residues [1].

The formation of the complex between the R1 and R2 subunits is essential for enzymatic activity. It has been shown that the carboxyl terminal part of protein R2 is important for the binding of protein R2 to protein R1 both in the *E. coli* enzyme [2–4] and in the mammalian and Herpes simplex type I enzymes [5–9]. Truncations of C-terminal residues of protein R2 impair binding of protein R1 [2], and point mutations of conserved residues in the C-terminus reduces or abolishes catalytic activity [3–4]. This demonstrates the importance of the R2 C-terminus for anchorage of the two proteins and as well for catalytic function. The C-terminal part of the protein is therefore an interesting target for antiviral and antiproliferative drugs. Successful attempts are made to develop antiviral drugs that mimic the C-terminal part of viral protein R2 [10]

The spatial structure of protein R2 from *E. coli* has been determined by X-ray diffraction [11]. It was, however, not possible to find any localized electron density from the 32 last amino acid residues at the carboxyl terminus. The suggested reason was that this part of the protein was disordered in the crystals. Subsequently it was reported that sharp ^{1}H-NMR resonances could be observed from the corresponding parts of protein R2 from HSV1 [12] and mouse [13] due to flexibility at the C-terminus. Six mobile residues were assigned to the C-terminus of protein R2 from HSV1. In mouse R2 a larger number of flexible residues were observed. Eight of these were assigned to the carboxyl terminal part, but it was also suggested that the mouse R2 protein contains other flexible parts beside the C-terminal segment.

In this paper we present data showing that 26 residues in the carboxyl terminal segment of protein R2 from *E. coli* are flexible, and that the protein does not contain any other highly mobile domains, which is in contrast to the mammalian protein.

2. Materials and methods

Overproduction of mutant protein R2Δ30C, lacking 30 amino acid residues in the C-terminus compared to wild type protein, was obtained from *E. coli* strain K38 containing plasmids pGP1–2 and pTB2Δ30C [4]. Wild-type protein R2 was purified as described earlier [14]. R2Δ30C was purified as wild-type except that the gradient for elution from the DEAE was performed with a gradient of 0.1–0.25 M KP_i pH 7.0. Molar absorbance indices ($\varepsilon_{280-310}$) used were for protein R2 120,000 $M^{-1}cm^{-1}$, protein R2Δ30C 107,000 $M^{-1}cm^{-1}$ and protein R1 180,000 $M^{-1}cm^{-1}$. The proteins were transferred to 25 mM phosphate buffer pH 7.5 in D_2O or 90% ^1H_2O/10% ^2H_2O by gel filtration on NAP10 (Pharmacia, Uppsala, Sweden) columns and then concentrated on Centricon30 (Amicon, Beverly MA, USA) filters to a final protein concentration of 0.8–2 mM. NMR samples were checked for proteolytic degradation running SDS denaturing PAGE gels, homogenous 7.5%, before and after NMR runs. The condition of the samples were also checked by light absorption spectroscopy.

The NMR spectra were recorded at a ^{1}H resonance frequency of 500 MHz on a Bruker AMX2–500 spectrometer or on a Varian Unity 500 spectrometer. Two-dimensional TOCSY [15] and NOESY [16,17] spectra were recorded in the phase-sensitive mode using time proportional phase incrementation (TPPI) [18]. In TOCSY spectra a MLEV-17 spin-lock sequence [19] was used with delays before and after 180° pulses to suppress ROE peaks [20]. The delays were $2.6 \times 90°$ pulse length. The TOCSY mixing times were 18–75 ms. Water suppression was achieved by presaturation during the relaxation delays, which were 1.4–2 s. A spectral width of 12 ppm was used with the carrier frequency placed on the water resonance. Typically 16–64 transients were recorded per t_1 increment with two dummy scans at the beginning of the experiment. 1024 real data points were acquired in the t_2 domain and 256–512 increments in the t_1 domain. Spectra were recorded at 7°C, 15°C, 22°C

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Abbreviations: NMR, nuclear magnetic resonance; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; ROE, rotating frame Overhauser effect; HSV1, herpes simplex virus type I; KP_i, potassium phosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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and 35°C. Data processing was performed on Silicon Graphics Indigo R4000 work stations using Bruker UXNMR software. Data were typically zerofilled to 1024 data points in the t_1 dimension and a sine-bell apodization function, shifted 36–60°, was applied prior to fourier transformation. Chemical shift values are relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

3. Results and discussion

In general, a protein with the molecular weight of 86 kDa is not expected to give rise to a well resolved ^{1}H-NMR spectrum. However the one-dimensional spectrum of protein R2 from *E. coli* shows a number of relatively sharp lines on top of a broad envelope spectrum (Fig. 1). In TOCSY spectra crosspeaks are only expected between relatively narrow lines. Coherence transfer should be effective only between sharp resonances. Therefore a limited number of crosspeaks are observed in the TOCSY spectrum of protein R2 (Fig. 2), and these can be attributed to the narrow lines. The presence of narrow ^{1}H-NMR resonances can be explained by a reduced rotational correlation time for a few residues due to segmental mobility in some parts of the protein. To exclude the possibility of these resonances originating from proteolytic fragments released during the experiments, one-dimensional spectra were recorded before and after two-dimensional experiments. These spectra were compared and found to be identical (data not presented). Samples were also run on SDS-PAGE before and after the NMR experiments, showing that the protein was not degraded by proteolysis.

The sequence-specific assignment was carried out in a sequential manner from TOCSY, and NOESY spectra [21]. The spin systems of 27 amino acid residues were delineated. However, the resonances from three glutamate residues overlap extensively. This overlap could not be completely resolved by measurements at different temperatures. The small chemical shift dispersion of the $C^{\alpha}H$ protons of the valine residues next to the glutamates also causes ambiguities in their assignments. However, small chemical shift differences at different temperatures make one solution of the sequential assignment more likely than the other. A 'sequential walk' connecting intraresidual NOESY $C^{\alpha}H$-NH peaks with corresponding sequential peaks in the 'fingerprint region' is presented in Fig. 3, upper panel. Most of the sequential steps could also be confirmed by sequential NH-NH NOESY crosspeaks (Fig. 3, lower panel). The assignments are presented in Table 1. The chemical shift

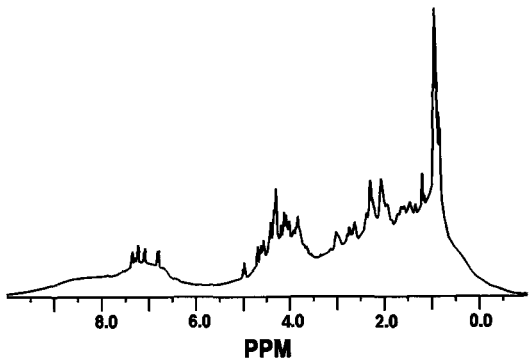

Fig. 1. 500 MHz proton NMR spectrum of 0.8 mM *E. coli* ribonucleotide reductase protein R2 in 25 mM phosphate, pH 7.5 in 99.8% D_2O at 6°C.

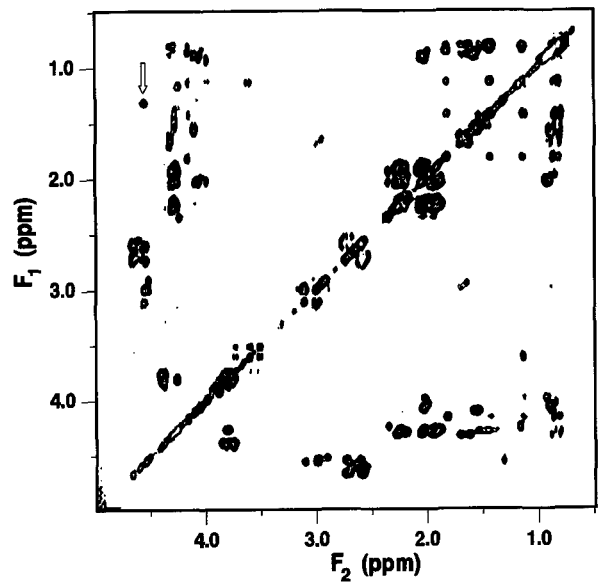

Fig. 2. Aliphatic region of a TOCSY spectrum (500 MHz) of protein R2 in 25 mM phosphate, pH 7.5 in D_2O at 6 °C. The mixing time was 75 ms using a MLEV17 spin lock sequence. The unassigned crosspeak, which is missing in the spectrum of truncated R2, is indicated with an arrow.

values are close to those for random-coil peptides. This, together with the absence of long range NOEs, strong $C^{\alpha}H_i$-NH_{i+1} NOEs and weak intraresidual $C^{\alpha}H$-NH NOEs, are indications of an extended structure with a high degree of flexibility.

The spectra of full length R2 were compared with spectra of a truncation mutant protein lacking 30 amino acid residues in the C-terminus. Spectra were also compared with spectra from a synthetic peptide corresponding to the 20 C-terminal residues. The TOCSY spectrum of the 20 amino acid residues peptide showed almost perfect overlap with peaks in the full length R2 protein spectrum except for the N-terminal tyrosine residue in the peptide (data not shown). The similarity between the synthetic peptide, corresponding to the R2 C-terminus, implies that the resolved resonances are due to the C-terminal part of protein R2. This is then further proved by the absence of resolved NMR lines in the truncated R2 protein, lacking 30 amino acid in the carboxyl terminus. Most of the resonances found in the full length protein were lacking in the TOCSY spectrum of the truncated protein (Fig. 4). Only very few crosspeaks were visible, compatible with side chains from methyl containing residues and arginines and lysines. Such crosspeaks are frequently observed in TOCSY spectra of large proteins (Lycksell, unpublished observations). Also one cross peak pattern compatible with a glutamate or glutamine residue was present in the spectrum of the truncated protein. This pattern may be due to Gln-345 becoming C-terminal in the truncated R2. Beside the crosspeaks assigned to the C-terminal segment, there is one additional crosspeak missing in the spectrum of the truncated R2 (from 4.60 ppm to 1.34 ppm). This crosspeak is compatible to a $C^{\alpha}H$-$C^{\beta}H_3$ crosspeak of an alanine residue. It is therefore likely that the methyl group of Ala-347 is sufficiently mobile to give rise to this TOCSY crosspeak.

The carboxyl terminal part of R2 is essential for enzyme

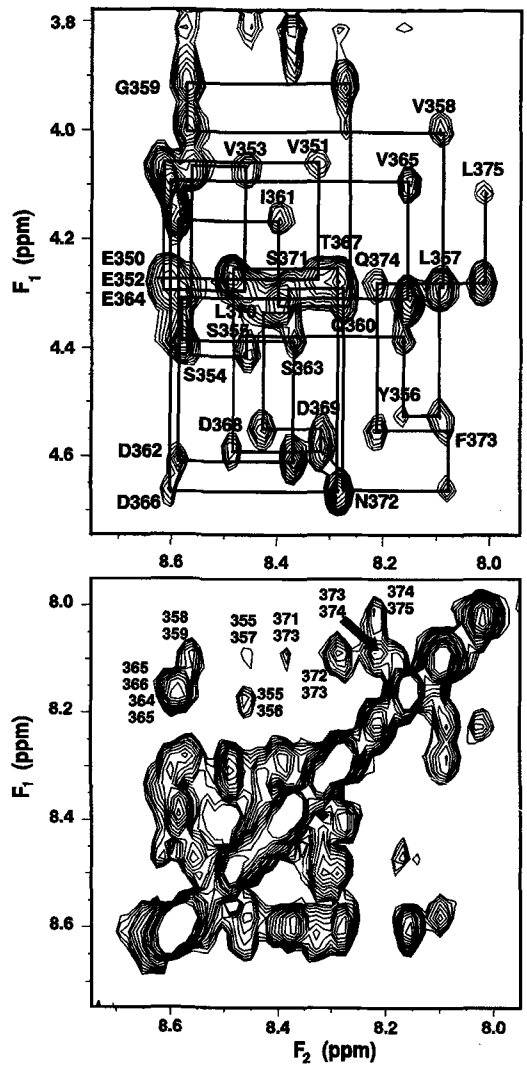

Fig. 3. Upper panel: the NH-C$^{\alpha}$H region of a NOESY spectrum of protein R2. The intraresidual crosspeaks are labelled with their residue number and connected with lines through their corresponding sequential peaks. Lower panel: NH-NH region of the same NOESY spectrum. The mixing time was 250 ms. Some isolated crosspeaks are labelled with their residue numbers.

activity. This part of R2 has an important role in the association with the R1 protein [2–9]. The NMR observable segment also contains the conserved residues, Tyr-356 and Glu-350, that are invariable between species [22]. Since indirect evidence implicate a free radical mechanism for the reduction of ribonucleotides [23,24], electron transfer processes have been proposed to connect the tyrosyl radical to the enzymatic function as soon as the structure of *E. coli* protein R2 was solved [11]. This since the structure revealed that both tyrosyl radical and binuclear iron center are buried deep inside the protein far from any surface. A hydrogen binding network connecting the radical carrying residue 122 with a conserved tryptophan on the surface suggested to interact with protein R1, was put forward as a possible route for electron transfer [11, 25]. Recently the structure of protein R1 was solved [26] revealing that also the substrate binding site is buried in the protein, far from any surface. A 20-mer peptide, corresponding to the C-terminus of protein R2, was required for crystals to form. However, in the structure only eight of the twenty amino acid residues were resolved. Model building has been used to dock the protein R2 to protein R1. The complex thus formed is an entity with the R1 dimer on top of the R2 dimer [26].

The proposed extended long range electron transfer pathway, between tyrosyl radical 122 and the substrate binding site, was summarized by Sjöberg in [27]. The distance for the electron to cover is 35–40 Å and the pathway is proposed to involve residues Tyr-122, Asp-84, Fe-1, His-118, Asp-237, Trp-48, Tyr-356 and possibly Glu-350 in protein R2 and Tyr-731, Tyr-730 and Cys-439 in protein R1. The extension of the electron transfer route hence includes the two invariant residues of the R2 C-terminus, Tyr-356 and Glu-350, studied in this paper. That Tyr-356 is involved in electron transfer is implicated from studies on binding and enzyme activity [4]. Furthermore, a recent study, where possible participants in electron transfer pathways were monitored during reconstitution of the binuclear iron center of the Y122F mutant, identified one intermediate as Tyr-356 [28].

The flexibility of the carboxyl terminus of free protein R2 may facilitate the recognition of the corresponding interaction surface on protein R1. Upon formation of the R1:R2 complex we observe the disappearance of flexible residues (data not shown). This is in accordance both with the model built structure and previous studies of holocomplexes of the mammalian enzyme [13,26] showing that the C-terminus becomes rigid when the active enzyme is formed. However there seem to be differences between species. Protein R2 from *E. coli* contains one flexible segment of 26 amino acid residues. The corre-

Table 1
^{1}H chemical shifts of ribonucleotide reductase protein R2 from *Escherichia coli* at 6°C and pH 7.5
The values are in ppm relative to DSS.

Residue	NH	C$^{\alpha}$H	C$^{\beta}$H	Others
Glu-350	8.57	4.27	2.02, 1.89	γ-CH$_2$ 2.21†
Val-351	8.31	4.05	2.01	γ-CH$_3$ 0.88, 0.84
Glu-352	8.57	4.27	2.02, 1.89	γ-CH$_2$ 2.21†
Val-353	8.45	4.06	2.03	γ-CH$_3$ 0.90†
Ser-354	8.55	4.41	3.82, 3.76	
Ser-355	8.44	4.38	3.78, 3.76	
Tyr-356	8.14	4.51	2.99, 2.90	C2,6H 7.06, C3,5H 6.77
Leu-357	8.08	4.28	1.52, 1.44	γ-CH 1.36, δ-CH$_3$ 0.83, 0.78
Val-358	8.08	4.00	2.02	γ-CH$_3$ 0.92†
Gly-359	8.56	3.90†		
Gln-360	8.25	4.25	2.03, 1.92	γ-CH$_2$ 2.25†, δ-NH$_2$ 7.69, 6.91
Ile-361	8.39	4.15	1.80	γ-CH$_2$ 1.41, 1.11, γ-CH$_3$ 0.85, δ-CH$_3$ 0.80
Asp-362	8.57	4.60	2.68, 2.56	
Ser-363	8.35	4.38	3.85, 3.79	
Glu-364	8.56	4.29	2.02, 1.89	γ-CH$_2$ 2.21†
Val-365	8.14	4.07	2.03	γ-CH$_3$ 0.89†
Asp-366	8.59	4.68	2.70, 2.58	
Thr-367	8.26	4.25	4.25	γ-CH$_3$ 1.16
Asp-368	8.47	4.60	2.67, 2.59	
Asp-369	8.30	4.54	2.72, 2.59	
Leu-370	8.41	4.32	1.69, 1.60	γ-CH 1.60, δ-CH$_3$ 0.86, 0.78
Ser-371	8.36	4.26	3.85, 3.77	
Asn-372	8.26	4.68	2.71, 2.58	γ-NH$_2$ 7.63, 6.91
Phe-373	8.06	4.56	3.11, 2.99	C3,5H 7.32, C4H 7.27, C2,6H 7.21
Gln-374	8.19	4.26	2.02, 1.86	γ-CH$_2$ 2.23, δ-NH$_2$ 7.68, 6.91
Leu-375	7.99	4.10	1.55	γ-CH 1.55, δ-CH$_3$ 0.87†

†Degenerate or overlapping peaks.

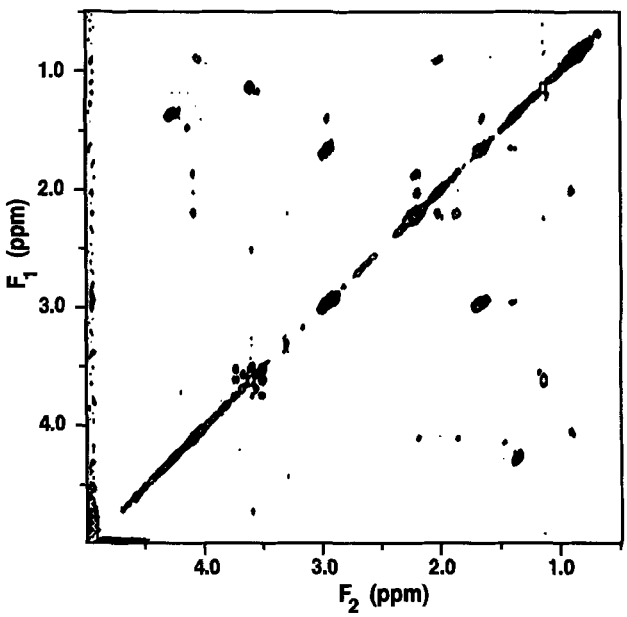

Fig. 4. Aliphatic region of a TOCSY spectrum (500 MHz) of truncated ribonucleotide reductase protein R2, lacking 30 residues in the C-terminus, in D_2O at 6°C. The mixing time was 75 ms using a MLEV17 spin lock sequence.

sponding part of the herpes simplex protein is significantly shorter, about 15 residues, of which only six are unambiguously assigned to the C-terminus [12]. A significant number, approximately 30 residues, are flexible and observed by NMR in the mouse protein R2. However some of these seem to originate also from other parts beside the carboxyl terminal segment. These data indicate differences in the interaction patterns between the R1 and R2 proteins of different species. Such knowledge may prove useful in the development of antiproliferative or antiviral drugs based on inhibition of ribonucleotide reductase by blocking R1-R2 interaction.

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