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Investigation of an Octapeptide Inhibitor of *Escherichia coli* Ribonucleotide Reductase by Transferred Nuclear Overhauser Effect Spectroscopy†

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ABSTRACT: Several peptides contained within the C-terminal sequence of the B2 subunit of *Escherichia coli* ribonucleotide reductase (RNR) were investigated for their ability to inhibit the enzyme, presumably by interfering with association of the B1 and B2 subunits. AcYLVGQIDSE, corresponding by sequence homology to a nonapeptide that inhibits herpes simplex RNR [Gaudreau et al. (1987) *J. Biol. Chem. 262*, 12413] shows no inhibition of the *E. coli* enzyme ($IC_{50} > 3$ mM), whereas AcDDLSNFQL, the C-terminal octapeptide of the *E. coli* B2 subunit, is a noncompetitive inhibitor (K_i = 160 μM). Neither bradykinin (RPPGFSPFR) nor the pentapeptide AcSNFQL inhibits the *E. coli* enzyme. Transferred nuclear Overhauser enhancement spectroscopy was used to probe the conformation of AcDDLSNFQL when it is bound to the B1 subunit. These experiments suggest that the peptide adopts a turn in the region of Asn_5 and Phe_6 and that a hydrophobic cluster of the phenylalanine and leucine side chains is involved in the interaction surface.

Ribonucleotide reductase (RNR, EC 1.17.4.1)[1] catalyzes the conversion of ribonucleotides to the corresponding deoxyribonucleotides. Since this enzyme is responsible for the first committed step in de novo DNA biosynthesis, considerable effort has been devoted to its mechanistic and structural elucidation (Reichard, 1988; Eriksson & Sjöberg, 1989; Stubbe, 1989, 1990a,b). The enzyme from *Escherichia coli* is composed of two readily dissociated nonequivalent subunits, designated B1 (171 kDa) and B2 (87 kDa) (Carlson et al., 1984), each of which is in turn homodimeric; the overall composition is therefore $\alpha_2\beta_2$. The substrate-binding sites are located on the B1 subunit; however, the B2 subunit is required for activity since it contains a tyrosine-based radical, stabilized by a binuclear μ-oxo-bridged iron center, that is thought to initiate the radical reaction (Thelander & Reichard, 1979). The electrons necessary for reduction of the ribosyl center are provided by redox-active cysteines on the B1 subunit, which are in turn reduced by thioredoxin or glutaredoxin to complete the catalytic cycle. The binding affinity of the B1 and B2 subunits is Mg^{2+} dependent and relatively weak (Thelander & Reichard, 1979).

A number of virally encoded RNRs have been identified, including those from the herpes simplex viruses (HSV) 1 and 2 (Cohen et al., 1974), equine herpes virus type 1 (Cohen et al., 1977), Epstein-Barr virus (Henry et al., 1978), pseudorabies virus (Lankinen et al., 1982), and vaccinia virus (Tengelsen et al., 1988). These enzymes are heterodimeric, like the RNR from *E. coli*, and significant sequence homology is observed between the *E. coli* enzyme and those from HSV, Epstein-Barr virus, and vaccinia virus (Sjöberg et al., 1985). The best characterized of the viral enzymes is that from HSV-1 (Preston et al., 1984). Two proteins of 136 and 36 kDa are associated with the activity, but, unlike the *E. coli* enzyme, these two subunits form a tight complex whose formation is not Mg^{2+} dependent (Ator et al., 1986).

It has been shown that the peptide YAGAVVNDL, corresponding to the C-terminus of the small subunit of HSV-1 RNR, inhibits the enzyme with a K_i value of 15 μM (Dutia et al., 1986; Cohen et al., 1986; Paradis et al., 1991). This peptide also shows high specificity for the viral over the mammalian RNR. The reversible, noncompetitive nature of the inhibition led Cohen et al. (1986) to suggest that the peptide inhibits association of the enzyme subunits. Affinity labeling (Paradis et al., 1988) and immunoprecipitation experiments (McClements et al., 1988) subsequently confirmed this interpretation.

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[1] Abbrevations: Ac, acetyl; RNR, ribonucleotide reductase; HSV, herpes simplex virus; Fmoc, (9-fluorenyl)methoxycarbonyl; HOBT, hydroxybenzotriazole; BOP, benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate. For identification of NOE interactions, each hydrogen involved is identified by the amino acid, residue number, and position on the carbon framework; e.g., Q7α/L8NH represents a cross-peak between the α-hydrogen on Gln_7 and the NH of Leu_8.

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HSV inhibitor YAGAVVNDL

HSV-2 TNFFECRSTSYAGAVVNDL

E. coli VAPQEVEVSSYLVGQIDSEVDTDDLSNFQL

AcYLVGQIDSE

AcDDLSNFQL

FIGURE 1: Sequence alignment of C-termini of RNR B2 subunits [boldface indicates structurally similar residues and underlined residues are identical; after Sjöberg et al. (1985)], along with HSV-inhibitory nonapeptide described by Gaudreau et al. (1987) and peptides described in this work.

The discovery of a system in which relatively small peptides inhibit an enzyme by interfering with subunit association presents an unusual opportunity for pursuing a new approach to inhibitor design, namely, the development of noncompetitive inhibitors that are not directed toward the substrate-binding site. Of critical importance to such an approach is information on the conformation of the peptide when it is associated with the target protein; such information would assist in the design of conformationally restricted smaller peptides or of nonpeptidic molecules as structural mimics of the lead peptide. Although no structural information on the viral RNRs is yet available, the X-ray crystal structure of the B2 subunit of the *E. coli* enzyme has been reported recently (Nordlund et al., 1990). Unfortunately from the point of view of inhibitor design, the C-terminal residues are not located in the electron density map, either due to disorder in the crystal or their prior loss through proteolysis.

Information on the conformation of a small molecule bound to a macromolecule can be obtained from transferred nuclear Overhauser effect (TRNOE) NMR experiments (Clore & Gronenborn, 1982, 1983). The foundation for this technique is the transfer of proton nuclear Overhauser (cross-relaxation) effects (NOE) in the bound state to the free state by means of chemical exchange. For the TRNOE technique to be effective, exchange of the ligand between bound and unbound states must be rapid on the NMR time scale. As a result, inhibitors with modest affinity and therefore relatively rapid dissociation rate constants are required. This technique has been applied successfully to the determination of the conformation of nucleotides in their protein-binding sites (Gronenborn et al., 1984) as well as the conformation of protein-bound peptides (Clore et al., 1986; Meyer et al., 1988; Ni et al., 1989a,b, 1990).

At the outset of our work, inhibition of *E. coli* RNR dimerization by peptides had not been demonstrated, although Sjöberg et al. (1987) had shown that loss of the C-terminal 30 amino acids of the B2 subunit abolishes its affinity for B1. Their results strongly suggested that, as for the HSV enzyme, the C-terminal residues play a critical role in the interaction of the two subunits. This portion of the sequence is shown in Figure 1, along with the homologous alignment of C-termini of the two viral sequences.

In an effort to map this interaction region in the B2 subunit of the *E. coli* RNR, we prepared AcYLVGQIDSE and AcDDLSNFQL (Figure 1) and variations thereof as possible dimerization inhibitors. The first was chosen for its homology to the peptide inhibitor demonstrated for the HSV enzyme, while the second was selected on the basis of analogy, that is, as the C-terminal octapeptide. The N-termini of these peptides were acetylated to provide better mimics of their internal location in the native protein, and because blocking the ionic nitrogen leads to an improvement in the HSV peptide inhibitors (Gaudreau et al., 1987; Paradis et al., 1991).

Materials and Methods

Peptide Synthesis, General. Fmoc-protected amino acids were obtained from Milligen/Biosearch; alkoxybenzyl resin loaded with the C-terminal amino acid was obtained from Bachem. *O-tert*-Butyl protection was employed for the side chains of Ser, Asp, Glu, and Tyr; the side chains of Gln and Asn were protected as their *N*-trimethoxybenzyl derivatives. HOBT active esters were prepared by treatment of the amino acid derivative with one equivalent each of HOBT and BOP and 1.5 equivalents of *N*-methylmorpholine in dimethylformamide for 10 min (Hudson, 1988). Amino acid analyses were performed by the Peptide Facility of the University of California, Davis.

Ac-Tyr-Leu-Val-Gly-Gln-Ile-Asp-Ser-Glu, AcYLVGQIDSE. Solid-phase peptide synthesis was performed on a scale of 0.5 mmol according to the protocol of Atherton et al. (1981) employing four equivalents of the active esters in the coupling steps. Completion of each coupling was verified by qualitative ninhydrin tests. The peptides were deprotected and cleaved from the resin by treatment with 70% trifluoroacetic acid, 25% dichloromethane, and 5% dimethyl sulfide for 1 h. The peptide was purified by preparative reverse-phase HPLC on a 22-mm × 50-cm Whatman Partisil 10 ODS-3 C-18 silica column, eluting with a linear gradient of 10–70% acetonitrile in water with 0.3% TFA. The total isolated peptide was 141 mg. Amino acid analysis was normalized to Glx = 2.0; Ser, 0.83; Asx, 0.99; Ile, 0.95; Gly, 0.98; Val, 0.95; Leu, 0.98; Tyr, 0.96. HRMS (FAB): calcd for $C_{47}H_{72}N_{10}O_{18}$+H, m/z 1065.5073; Found, m/z 1065.5089.

Ac-Asp-Asp-Leu-Ser-Asn-Phe-Gln-Leu, AcDDLSNFQL. Prepared by the same protocol as described above; the total isolated peptide was 150 mg. Amino acid analysis was normalized to Leu = 2.0; Glx, 1.03; Phe, 1.02; Asx, 3.02; Ser, 0.85. HRMS (FAB): calcd for $C_{43}H_{64}N_{10}O_{17}$+H, m/z 993.4479; found, m/z 993.4504.

Ac-Ser-Asn-Phe-Gln-Leu, AcSNFQL. *tert*-Butoxycarbonyl-protected amino acids were obtained from Bachem and employed for the synthesis of this peptide; the Ser side chain was protected as the benzyl ether. Coupling was performed with dicyclohexylcarbodiimide according to the protocol described by Stewart and Young (1984), except for Asn and Gln, which were coupled via their respective *p*-nitrophenyl esters in dimethylformamide. The peptide was deprotected and cleaved from the resin by catalytic transfer hydrogenation (Anwer et al., 1983). Purification was effected by preparative reverse-phase HPLC on the column described above, eluting with 28% acetonitrile in 25 mM aqueous ammonium acetate, pH 7. The free acid was converted to the sodium salt by ion exchange. The ^{1}H NMR spectrum was consistent with the expected sequence and showed no significant contaminants. HRMS (FAB): calcd for $C_{22}H_{31}N_4O_6Na$+H, m/z 471.2213; found, m/z 471.2219.

Enzyme Purification, General. RNR subunit B2 from *E. coli* was a generous gift from Professor J. Stubbe (MIT). RNR subunit B1, thioredoxin, and thioredoxin reductase were prepared from overproducing clones also provided by Professor Stubbe. Protein concentrations were determined by the method of Lowry et al. (1951).

RNR Subunit B1. RNR subunit B1 was purified from *E. coli* strain C600/pMB1 according to the procedure described by Salowe (1987; J. Stubbe, personal communication), had a specific activity of 420 Units/mg, and was stored frozen in 50 mM Tris (pH 7.6), 20% glycerol, 15 mM $Mg(OAc)_2$, and 10 mM DTT, at a concentration of 28 mg/mL.

Thioredoxin. Thioredoxin was purified from *E. coli* strain SK3981 according to a modification of the procedure of Lunn et al. (1984; J. Stubbe, personal communication), had a specific activity of 260 Units/mg, and was stored frozen in 50 mM Tris (pH 7.4) and 3 mM EDTA, at a concentration of 1.4 mg/mL.

Thioredoxin Reductase. Thioredoxin reductase was purified from *E. coli* strain K91/pPMR14 according to a modification of the procedure of Pigiet and Conley (1977; J. Stubbe, personal communication), had a specific activity of 640 Units/mg, and was stored frozen in 1 mM NADPH, 50 mM potassium phosphate (pH 7.6), and 3 mM EDTA, at a concentration of 5.0 mg/mL.

Enzyme Assay. All assays were performed on a Kontron Uvikon 860 UV spectrophotometer operating in the kinetics mode.

Ribonucleotide Reductase. A procedure modified from that of Thelander et al. (1978) was employed, following NADPH consumption in a coupled assay with RNR, thioredoxin, and thioredoxin reductase. In a final volume of 500 μL, the assay mixture contained buffer (50 mM HEPES pH 7.6, 15 mM $MgSO_4$, 1 mM EDTA), 1.6 mM ATP, 1.0 mM CDP (or appropriate concentration), 0.16 mM NADPH, and appropriate amounts of thioredoxin and thioredoxin reductase (determined empirically). The background rate was recorded for several minutes, the reaction was initiated by addition of RNR, and the decrease in absorbance at 340 nm was monitored. The linear portion of the initial rate, corresponding to <10% depletion of substrate and corrected for the background rate, was used for calculation of activity. One unit of activity is defined as the amount of B1 or B2 subunit that catalyzes the formation of 1 nmol/min of dCDP at 25 °C under these conditions.

Inhibition of Ribonucleotide Reductase. A working solution containing 1.3 mg/mL of thioredoxin and 0.33 mg/mL of thioredoxin reductase was prepared from the stock solutions. RNR subunits B1 and B2 were combined in a 1:1 stoichiometric ratio and diluted to a concentration of 40 μM with 50 mM Tris (pH 7.6), 20% glycerol, 15 mM $Mg(OAc)_2$, and 10 mM DTT. Both of these solutions were stored in an ice/salt bath during use. A 2-μL aliquot of the RNR subunit solution was employed in each assay (final volume 0.5 mL) to give a final concentration of 160 nM. For the determination of IC_{50} values, a single concentration of CDP = K_m (50 mM) was employed; for the extended kinetic analyses and the determination of K_m, CDP concentrations of 20, 25, 35, 50, and 100 μM were used. For IC_{50} determinations, the peptides were evaluated over the following concentration ranges: AcYLVGQIDSE, AcSNFQL, and bradykinin (1–3 mM); AcDDLSNFQL (150–900 μM). For extended analysis of the mode of inhibition by AcDDLSNFQL, it was assayed at final concentrations of 150, 300 and 600 μM and the data were analysed with Cleland's COMP program (Cleland, 1979).

NMR Experiments. Sample Preparation. All operations were carried out at 4 °C. One liter of 50 mM Tris-sulfate (pH 7.0, 15 °C), 0.1 mM EDTA, and 250 mL of 50 mM d_{11}-Tris-sulfate (pH 7.0, 15 °C), 0.1 mM EDTA in 10% D_2O were prepared, and each solution was eluted through a short column of Chelex (Bio-Rad). $MgSO_4$ (Aldrich Gold Label) was added to both buffers to a final concentration of 15 mM, and each was sparged overnight with a stream of argon. DTT was added immediately prior to use to a concentration of 10 mM for the protio-buffer (buffer H) and 0.5 mM for the deutero-buffer (buffer D). A 1.2-mL aliquot of RNR subunit B1 [26 mg/mL in 20% glycerol, 50 mM Tris-HCl (pH 7.6), 15 mM $Mg(OAc)_2$, 10 mM DTT] was diluted to 2 mL with buffer H, concentrated to 0.5 mL by ultrafiltration (Amicon Centricon-30), and dialyzed for 3 h against 1 L of buffer H and then for 3 h against 250 mL of buffer D. Argon was bubbled through the dialysis buffers at all times. The final concentration of the B1 subunit was determined by absorbance [ϵ_{280} = 1.08 mg^{-1} mL; Thelander et al. (1978)] to be 0.165 mM. A 5.2-mg sample of AcDDLSNFQL was dissolved in 600 μL of the B1 solution (final concentration of peptide = 8.25 mM), and the sample was centrifuged for 2 min at 2000*g*. A 550-μL aliquot was placed in a 5-mm NMR tube, purged with argon for 10 min, and the tube was sealed under a mild vacuum. An identical sample was prepared without the B1 subunit.

NMR Measurements. NMR spectra were acquired on a Bruker AMX-400 NMR spectrometer; spectral analysis and plotting were carried out on a Bruker X-32 workstation. Two-dimensional spectra were acquired in the phase-sensitive mode (Aue et al., 1976), employing time-proportional phase incrementation (Redfield & Kuntz, 1975) along the t_1 direction (Marion & Wüthrich, 1983; Bodenhausen et al., 1984). TOCSY spectra (Braunschweiler & Ernst, 1983; Bax & Davis, 1985b; Rance, 1987) were recorded with a mixing time of 46 ms for the peptide alone and 36 ms for the peptide in the presence of B1 subunit (MLEV 17 mixing, 60° final pulse, 2-ms trim pulses) with a jump–return (delay = 217 μs) sequence according to the procedure of Bax (1989). ROESY spectra (Bothner-By et al., 1984; Bax & Davis, 1985a; Kessler et al., 1987; Rance, 1987) with a mixing time of 250 ms were recorded with the pulse sequence of Otting and Wüthrich (1989) by employing a train of 45° pulses separated by delays 10 times longer than the width of the individual pulses for the spin-lock. The selective pulses applied to the water were Gaussian-shaped starting at 1% of their maximum value. The low-power transmitter output was reduced by 53 dB for generation of the 10-ms 90° pulses employed. The exact pulse length of the selective pulse was adjusted to give maximal water suppression in a two-pulse experiment in which a Gaussian pulse with phase x is immediately followed by a nonselective 90° pulse of phase $-x$. NOESY spectra (Jeener et al., 1979; Macura & Ernst, 1980) were recorded with the pulse sequence of Otting and Wüthrich (1989) by employing the same Gaussian pulses for selective excitation. Zero-quantum coherences were suppressed by random variation of the mixing time. The post-acquisition delay was set to 0.6 s for spectra acquired in the presence of the B1 subunit and to 0.9 s for spectra acquired in its absence due to the enhanced T_1 relaxation in the presence of the protein. A total of 800 FIDs of 4096 complex points were collected for each spectrum, except the ROESY spectra for which only 400 FIDs of 2048 complex points were collected. A total of 64 scans per FID for the ROESY and TOCSY spectra and 96 scans per FID for the NOESY spectra were recorded.

The FID matrices for the ROESY and TOCSY spectra were premultiplied by a 45°-shifted sine bell in both dimensions, and the NOESY data were premultiplied by a 60°-shifted squared sine bell in both dimensions. Appropriate zero filling was carried out to yield final two-dimensional matrices of 1K × 2K real points. The final matrices were baseline corrected separately on each side of the water signal in F_2 with a third-order polynomial.

Results

Inhibition of RNR by Oligopeptides. None of the peptides inhibits the reduction of thioredoxin by thioredoxin reductase, the coupling enzyme employed in the assay of RNR. Only

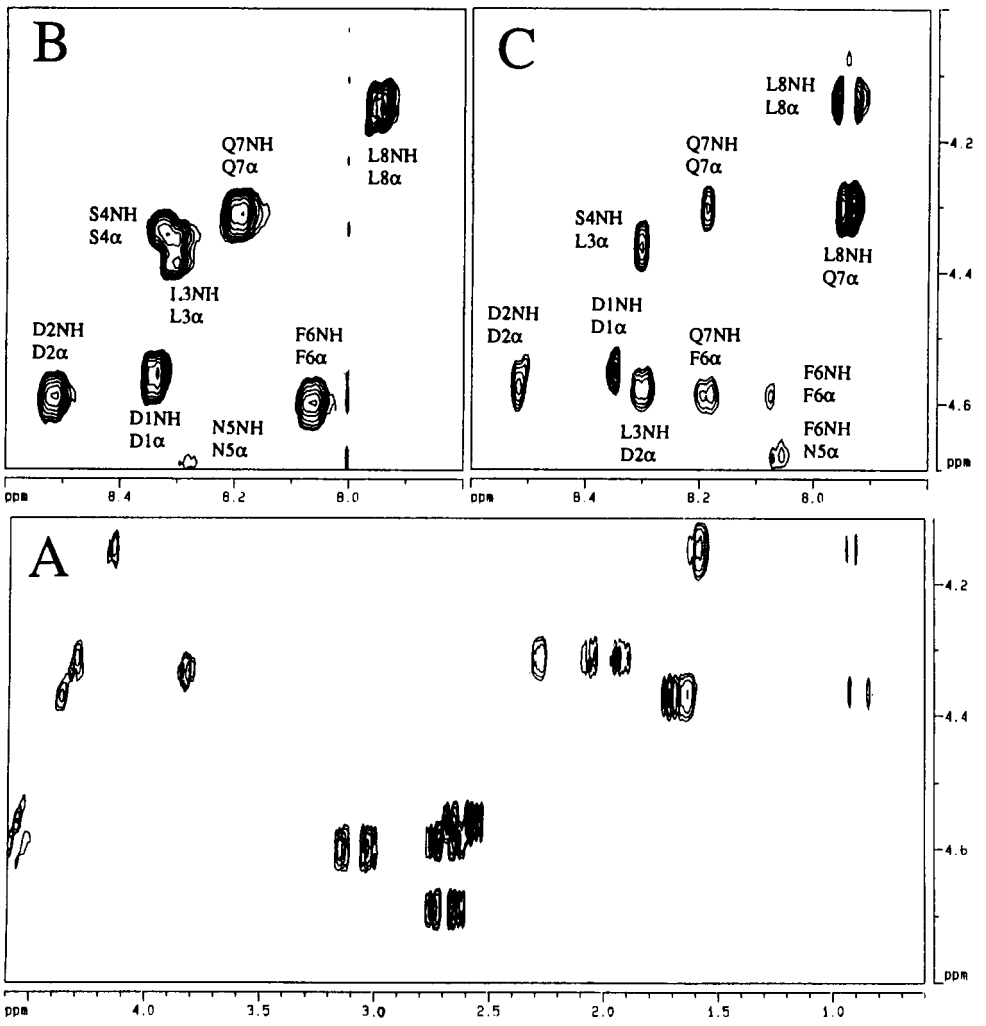

FIGURE 2: (A and B) Plot of segments of the 46-ms TOCSY spectrum of AcDDLSNFQL: (A) β,γ,δ(F2)/α(F1) region; (B) NH(F2)/α(F1) region. (C) Plot of the NH(F2)/α(F1) region of the 250-ms ROESY spectrum of AcDDLSNFQL.

AcDDLSNFQL showed any appreciable inhibition of RNR itself, with an IC_{50} value of 310 μM; the IC_{50} values of the other peptides were found to be in excess of 2 mM. The binding of AcDDLSNFQL is noncompetitive with CDP as substrate with an inhibition constant K_{IS} = 160 μM (Cleland, 1979).

Stability of B1 Subunit. The stability of the B1 subunit over the extended time periods necessary for acquisition of the NMR data was of concern, particularly since a large excess of DTT could not be included in the sample for reasons of signal interference. There are a large number of free thiols on this subunit, and their oxidation leads to inactivation and dissociation (Thelander et al., 1978). Preparation of the NMR samples therefore involved removal of heavy metals from the buffer and dialysis solutions via Chelex complexation and dialysis against argon-purged buffer to remove DTT under nonoxidizing conditions. In samples prepared under these conditions, the protein lost no activity over the course of 36 h at 15 °C.

Choice of NMR Sample Conditions. The selection of pH 7 for the NMR experiments reflected a compromise between considerations of protein stability (RNR loses activity at lower pH; J. Stubbe, personal communication) and suppression of NH exchange (which increases with pH). Nevertheless, at pH 7, presaturation of the water signal results in bleaching of all but one of the amide resonances (Leu_8); hence various forms of selective excitation had to be employed in order to record the 2-D spectra. TOCSY spectra were obtained using the pulse sequence of Bax (1989) with a jump–return detection pulse. NOESY and ROESY spectra were recorded with the method of Otting and Wüthrich (1989) with selective pulses on the water and a spin-lock purge pulse for water suppression.

NMR Assignment of AcDDLSNFQL. TOCSY and ROESY spectra were used to assign the spectrum of AcDDLSNFQL. ROESY was employed to obtain the sequential connectivities since peptides of this size are known to be near the $\omega_0/\tau_c \approx 1$ regime, so that NOE effects are small or null (Marion, 1985). The separate amino acid spin systems for the peptide were readily extracted from the TOCSY spectrum (Figure 2A,B). The long side-chain spin systems of glutamine and the two leucines were easily identified. Among the five ACMX spin systems, serine and phenylalanine were assigned from the chemical shift values. Connectivity information obtained from the ROESY spectrum was used to assign the remaining three spin systems of Asp-1, Asp-2, and Asn-5. The following ROEs were observed: acetyl/D_1NH, $D_2\alpha/L_3$NH, $L_3\alpha/S_4$NH, $N_5\alpha/F_6$NH, and $Q_7\alpha/L_8$NH (Figure 2C). The signal for N_5NH was weak in all spectra due to rapid exchange. Confirmation of the Phe_6 assignment was obtained from the ROEs observed between $F_6\beta$ and $F_6\delta$ (aromatic) protons. The Gln_7 and Asn_5 assignments were further confirmed by NOEs observed between the $N_5\beta$ and $Q_7\gamma$ protons and their respective side chain amide protons in the TRNOE spectra described below. In order to verify the chemical shifts in the presence of the B1 subunit, a TOCSY spectrum was recorded on the sample employed for the

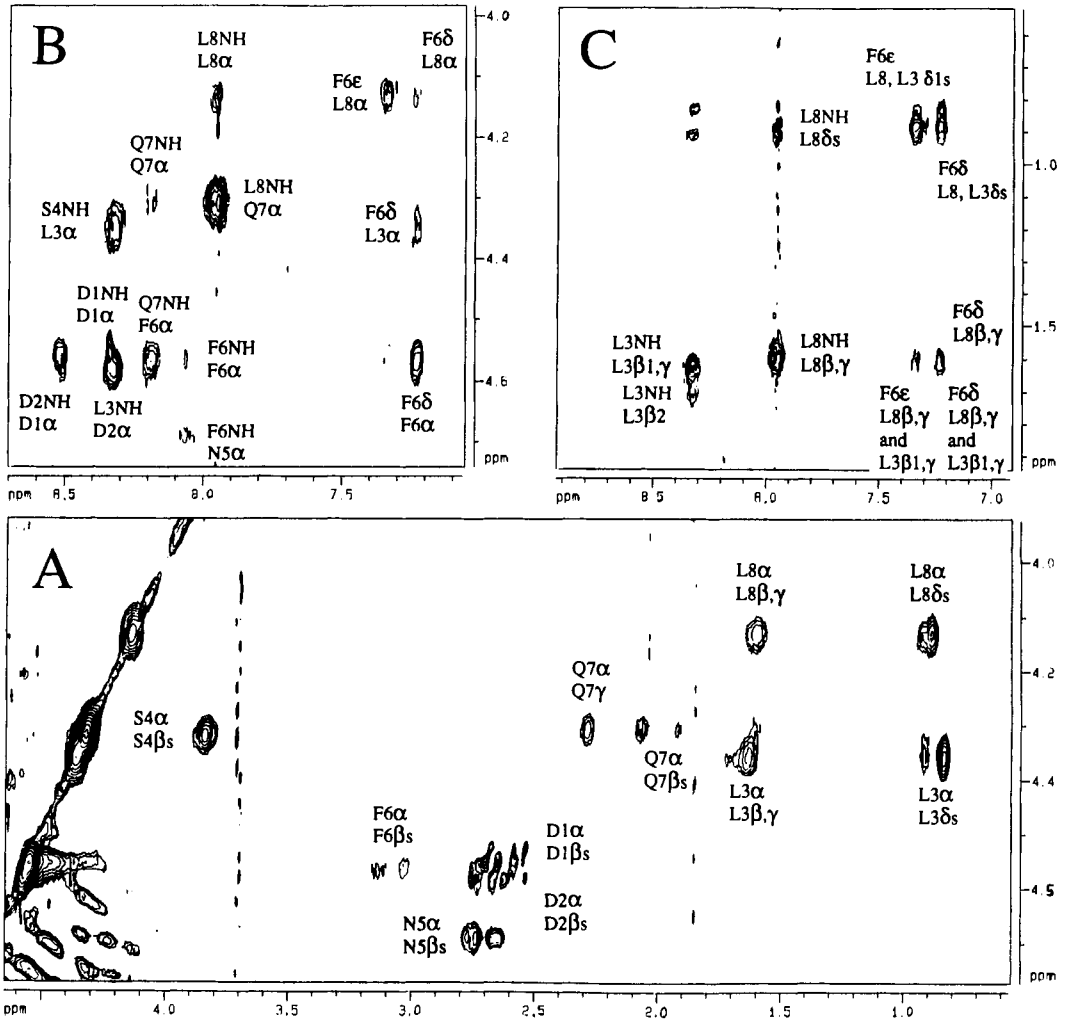

FIGURE 3: Plot of segments of the NOESY spectrum of AcDDLSNFQL and B1 subunit (positive contours only). (A) β,γ,δ(F2)/α(F1) region; (B) NH,aromatic(F2)/α(F1) region; (C) NH,aromatic(F2)/Leu-β,γ,δ(F1) region.

TRNOE experiment. The chemical shifts of all the protons of AcDDLSNFQL in the presence of the B1 subunit (i.e., under the conditions of the TRNOE experiment) are listed in Table I.

Assignment of the TRNOE Spectrum of AcDDLSNFQL in the Presence of the B1 Subunit. NOESY spectra were first recorded at a molar ratio of AcDDLSNFQL/subunit B1 of 30:1 (e.g., a ratio of peptide to binding site of 15:1). However, the broad line width under these conditions made it difficult to obtain a TOCSY spectrum in order to verify the peptide chemical shifts in the presence of the B1 subunit. These problems were surmounted at a ratio of peptide to subunit of 50:1. Spin diffusion effects were minimized by keeping the NOESY mixing time below 120 ms.

The negative NOEs identified from the NOESY spectrum (Figure 3) are tabulated in Figure 4. The high selectivity of the NOEs observed (for example, $L_3\alpha/L_3\delta_1,\delta_2$, $Q_7\alpha/Q_7\beta_1,\beta_2$, and $L_8\alpha/L_8\delta_1,\delta_2$; Figure 3A) indicates the absence of large spin-diffusion effects. A number of strong sequential NOEs from CO–NH to Cα hydrogens (NH–α) were observed (Figure 3B): $D_1\alpha/D_2$NH, $D_2\alpha/L_3$NH, $L_3\alpha/S_4$NH, $F_6\alpha/Q_7$NH, and $Q_7\alpha/L_8$NH. A very weak NOE is observed for $N_5\alpha/F_6$NH and none for $S_4\alpha/N_5$H.

Table I: ^{1}H NMR Chemical Shifts of AcDDLSNFQL in the Presence of the B1 Subunit[a]

residue	NH	CαH	CβH	other
acetyl				2.04
Asp_1	8.34	4.55	β_1 2.58	
			β_2 2.68	
Asp_2	8.51	4.58	β_1 2.65	
			β_2 2.75	
Leu_3	8.32	4.37	β_1 1.63	γ 1.63
			β_2 1.72	δ_1 0.84, δ_2 0.91
Ser_4	8.32	4.33	3.84	
Asn_5	8.28[b]	4.69	β_1 2.65	γ_1 6.88
			β_2 2.75	γ_2 7.61
Phe_6	8.06	4.57	β_1 3.02	δ 7.23
			β_2 3.14	ϵ 7.36, ω 7.31
Gln_7	8.19	4.31	β_1 1.90	γ 2.28
			β_2 2.04	δ_1 6.88, δ_2 7.65
Leu_8	7.96	4.12	β,γ 1.58	δ_1 0.88, δ_2 0.92

[a] pH 7.0, 15 °C. [b] Low intensity.

Discussion

Our observation that a short peptide from the C-terminal region of the B2 subunit of RNR inhibits the enzymatic activity noncompetitively is fully consistent with the results described previously for the HSV enzyme (Cohen et al., 1986; Gaudreau et al., 1987, 1990) and more recently for the *E. coli* enzyme (Climent et al., 1991), equine herpes virus (Telford et al., 1990), and mouse enzymes (Yang et al., 1990). However, the effectiveness of the octapeptide AcDDLSNFQL (K_{IS} = 160 μM), and the lack of activity of nonapeptide AcYLVGQIDSE (IC_{50} > 3 mM), indicate that the *E. coli* and HSV systems are not strictly analogous. Whereas AcYLVGQIDSE is the sequence counterpart to the HSV inhibitor, there is no region in the HSV B2 subunit that

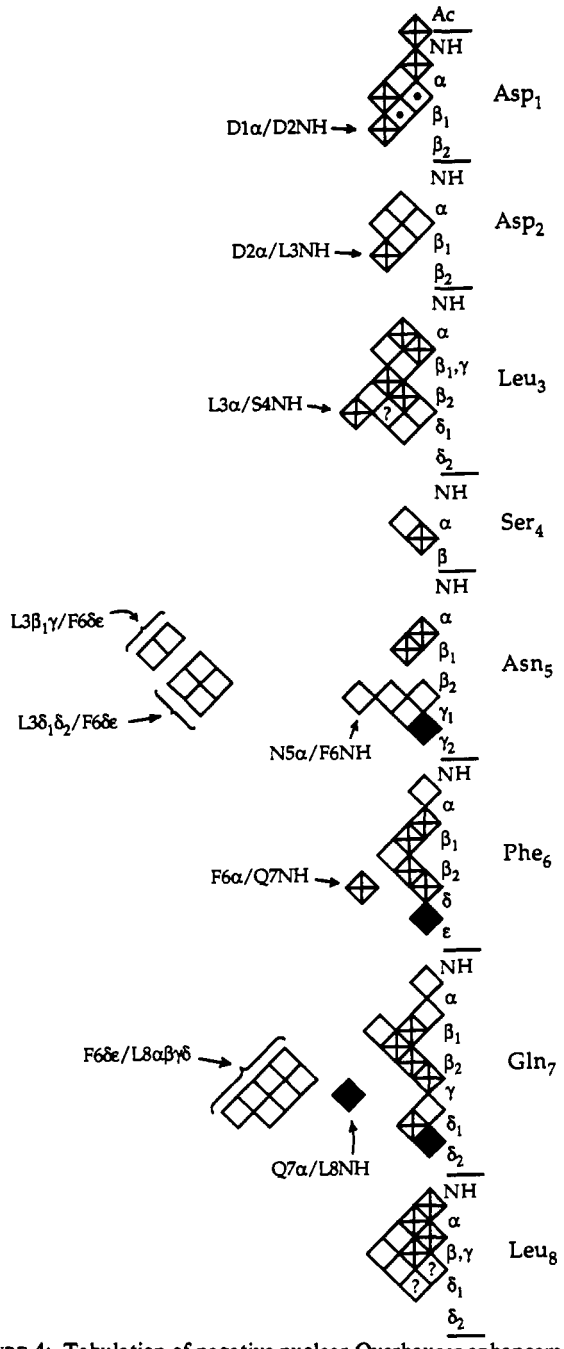

FIGURE 4: Tabulation of negative nuclear Overhauser enhancements observed in the NOESY spectrum of AcDDLSNFQL in the presence of the B1 subunit. The intensities of the cross-peaks were determined by counting contour levels and are categorized relative to the Phe_6-δ/Phe_6-ϵ cross-peak and adjusted for the number of protons: (solid square) strong; (crossed square) medium; (open square) weak; (?) overlap obscures accurate intensity determination; (square with dot) zero-quantum peak. Ambiguous cross-peaks were assigned by line-shape comparison with cross-peaks of known identity; a fuller analysis of these assignments is presented in the supplementary material.

corresponds to the C-terminal octapeptide of the *E. coli* protein from which AcDDLSNFQL is derived. The lack of inhibitory activity on the part of AcYLVGQIDSE, as well as by bradykinin, indicates that nonspecific effects by peptides of this size are negligible. In addition, the inactivity of the pentapeptide AcSNFQL defines the minimally active segment of the peptide as encompassing six to eight residues.

In concurrent work, Sjöberg and co-workers have localized the region of interaction within the C-terminal domain of the B2 subunit to the last 20 amino acids (Climent et al., 1991).

Table II: Oligopeptide Inhibition of *E. coli* RNR

peptide	K_i (μM)
LVSDNVQVAPQEVEVSSYLVGQIDSEVDTDDLSNFQL	18[a]
YLVGQIDSEVDTDDLSNFQL	20[a]
AcDDLSNFQL	160[b]
AcSNFQL	>2000
AcYLVGQIDSE	>2000

[a] From Climent et al. (1991). [b] Climent et al. (1991) report K_i = 370 μM.

They found, for example, that the C-terminal 37-mer and 30-mer have no greater affinity as inhibitors than the 20-mer (Table II). In comparison to the latter peptide, the octapeptide AcDDLSNFQL is reduced in affinity only 20–40-fold. That fact, as well as the finding that AcYLVGQIDSE shows no affinity, narrows this binding determinant even more and suggests that it may be restricted to a relatively small region. This interpretation further increases the attractiveness of RNR as a system for the investigation of structure-derived noncompetitive enzyme inhibitors and therefore the importance of information that can reveal details of the conformation of this peptide when it is bound to the B1 subunit.

The NOESY spectrum of AcDDLSNFQL in the presence of the B1 subunit reveals a large number of identifiable negative NOEs (displayed in Figure 4), confirming that this peptide does indeed bind to the B1 subunit. This observation provides further support for the suggestion that the peptide inhibits *E. coli* RNR by binding to the heterodimer interface in competition with the B2 subunit (but not in competition with substrate), as shown for the HSV system (Paradis et al., 1988). Observation of transferred NOEs also shows that the peptide is in fast exchange between the free and bound states, as would be expected on the basis of the modest K_i value and barring any slow steps in the association process. Although considerable information is contained in the transferred NOESY spectrum of AcDDLSNFQL, there are an insufficient number of distance constraints to provide a detailed structure or backbone conformation by distance-geometry methods (Braun & Go, 1985; Havel & Wüthrich, 1984). Nevertheless, some significant structural conclusions can be drawn from the data.

The strong sequential NH–α NOEs (Figure 4) along with the absence of any observable NH–NH sequential NOEs precludes a helical conformation for the bound peptide. The data would be consistent with an extended conformation for the peptide chain, except that there are detectable NOEs between the ortho protons of the aromatic ring of Phe_6 and the side-chain protons of Leu_3 (Figure 3B,C), which could not be observed in an extended form. These NOEs indicate that the peptide binds to the B1 subunit in a reverse-turn conformation that brings the side-chains in close proximity. This turn appears to involve residues Phe_6 and Asn_5, because of the presence of the strong sequential NH–α NOEs along the chain except at these positions. However, positive evidence for this assignment is missing because the low intensity of the Asn_5–NH signal prevents observation of any NH–NH NOE that could localize the turn to a specific sequence. Moreover, none of the long-range NH–α NOEs associated with classical tight turns are observed (Wüthrich, 1986), which suggests that a nonclassical turn or β-bulge is involved.

The Phe_6 aromatic ring also displays NOEs to the side-chain of Leu_8 (Figure 3C), reflecting their proximity in the bound conformation. Finally, the aromatic resonances for Phe_6 are extremely broadened in the presence of the B1 subunit, indicating that the side chain of this residue interacts with the protein. Taken together, this information suggests that a

hydrophobic cluster of the Leu_3, Phe_6, and Leu_8 side chains forms upon binding of the peptide to the B1 subunit, constituting a major site of interaction. Protein–protein interfaces are frequently populated with hydrophobic amino acids, like the interior of a protein, so such a cluster seems quite plausible. Confirmation of this interpretation and refinement of a model for the conformation of C-terminal peptides in their interaction with the B1 subunit will await further binding and NMR investigations with related peptides.

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Supplementary Material Available

A detailed analysis of assignments involving overlapping resonances (4 pages). Ordering information is given on any current masthead page.

Registry No. RNR, 9047-64-7; AcDDLSNFQL, 133101-38-9.

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Inactivation Mechanism of Tetrameric β-Galactosidase by γ-Rays Involves both Fragmentation and Temperature-Dependent Denaturation of Protomers†

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ABSTRACT: The radiation inactivation method is widely used to estimate the molecular size of membrane-bound enzymes, receptors, and transport systems in situ. The method is based on the principle that exposure of frozen solutions or lyophilized protein preparations to increasing doses of ionizing radiations results in a first-order decay of biological activity proportional to radiation inactivation size of the protein. This parameter is believed to reflect the "functional unit" of the protein defined as the minimal assembly of structure (protomers) required for expression of a given biological activity. We tested the functional unit as a concept to interpret radiation inactivation data of proteins with *Escherichia coli* β-galactosidase, where the protomers are active only when associated in a tetramer. γ-Irradiation of β-galactosidase at both −78 and 38 °C followed by quantitation of the residual unfragmented protomer band by SDS–polyacrylamide gel electrophoresis yielded the protomer size, indicating that only one protomer is fragmented by each radiation hit. By following the enzyme activity as a function of dose it was found that only the protomer that has been directly hit and fragmented at −78 °C was effectively inactivated. In contrast, at 38 °C, it was the whole tetramer that was inactivated. β-Galactosidase cannot have two different functional units depending on temperature. The inactivation of the whole β-galactosidase tetramer at 38 °C is in fact related to protomer fragmentation but also to the production of stable denatured protomers (detected by gel-filtration HPLC and differential UV spectroscopy) due to energy transfer from fragmented protomers toward unhit protomers. We conclude that β-galactosidase inactivation is the result of a two-step mechanism involving (1) fragmentation of the protomer directly hit by an ionizing radiation and (2) temperature-dependent radiation-induced denaturation of associated unfragmented protomers. Therefore, the radiation inactivation size reflects the size of the fragmented protomer and, when irradiation is carried out at higher temperatures, the transfer of energy from fragmented protomers toward other protomers inside the oligomer.

The radiation inactivation and fragmentation method is used extensively to estimate the molecular size of enzymes, receptors, and transport systems [reviewed by Kempner and Schlegel (1979); Beauregard et al., 1987a; Kempner & Fleisher, 1989]. The method is especially useful with membrane-associated proteins since it yields the molecular size of a protein as it exists in situ without prior purification or solubilization by detergent. The target theory is the basic framework used to analyze radiation inactivation data (Lea, 1955). It postulates that direct hit by an ionizing radiation on a protein will cause complete and irreversible inactivation whereas unhit molecules will retain full activity. The analysis of biological activity decay curves as a function of radiation dose gives the radiation inactivation size (RIS)[1] (Beauregard et al., 1987a) of the protein based on an empirical calibration curve established with proteins of known molecular weight (Kepner & Macey, 1968).

Most oligomeric proteins studied by radiation inactivation yield the protomer size, but RIS values corresponding to the whole oligomer have also been obtained (Kempner & Schlegel, 1979). In the latter case, it has been proposed that the integrity of each protomer in the oligomer is required for expression of biological activity and that the RIS reflects the minimal functional unit of the oligomer (Kempner & Schlegel, 1979; Steers et al., 1981). Alternatively, the energy absorbed by a hit on one subunit may be transferred to the other protomers in the oligomer, causing the loss of biological activity of the whole oligomer (Saccomani et al., 1981). The functional unit concept has been widely accepted to interpret radiation inactivation experiments, but its validity has not been thoroughly tested with well-characterized oligomeric proteins. To do so, we selected the homotetrameric enzyme β-galactosidase

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[1] Abbreviations: ONPG, *o*-nitrophenyl β-D-galactopyranoside; MUG, 4-methylumbelliferyl β-D-galactopyranoside; SDS, sodium dodecyl sulfate; GuHCl, guanidine hydrochloride; RIS, radiation inactivation size; TS, target size.

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