Site-Directed Mutagenesis and Deletion of the Carboxyl Terminus of *Escherichia coli* Ribonucleotide Reductase Protein R2. Effects on Catalytic Activity and Subunit Interaction†

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ABSTRACT: Ribonucleotide reductase from *Escherichia coli* consists of two dissociable, nonidentical homodimeric proteins called R1 and R2. The role of the C-terminal region of R2 in forming the R1R2 active complex has been studied. A heterodimeric R2 form with a full-length polypeptide chain and a truncated one missing the last 30 carboxyl-terminal residues was engineered by site-directed mutagenesis. Kinetic analysis of the binding of this protein to R1, compared with full-length or truncated homodimer, revealed that the C-terminal end of R2 accounts for all of its interactions with R1. The intrinsic dissociation constant of the heterodimeric R2 form, with only one contact to R1, 13 μM, is of the same magnitude as that obtained previously [Climent, I., Sjöberg, B.-M., & Huang, C. Y. (1991) *Biochemistry 30*, 5164–5171] for synthetic C-terminal peptides, 15–18 μM. We have also mutagenized the only two invariant residues localized at the C-terminal region of R2, glutamic acid-350 and tyrosine-356, to alanine. The binding of these mutant proteins to R1 remains tight, but their catalytic activity is severely affected. While E350A protein exhibits a low (240 times less active than the wild-type) but definitive activity, Y356A is completely inactive. A catalytic rather than structural role for these residues is discussed.

Ribonucleotide reductases catalyze the reduction of ribonucleotides to the corresponding deoxyribonucleotides in all organisms and thereby supply cells with the four precursors for DNA synthesis. The enzyme from *Escherichia coli* has been extensively studied and serves as prototype for eukaryotic ribonucleotide reductases. It consists of two nonidentical dimeric proteins (α_2 and β_2), denoted R1 and R2 (previously called B1 and B2 in *E. coli* enzyme), respectively [for recent reviews, cf. Eriksson and Sjöberg (1989), Stubbe (1990), and Fontecave et al. (1992)]. The R1 protein binds substrates and allosteric effectors and contains the redox-active cysteines (Reichard, 1988 Åberg et al., 1989; Lin et al., 1987; Stubbe, 1990). Protein R2 carries the cofactor required for nucleotide reduction, a stable tyrosyl radical next to a dinuclear iron center (Larsson & Sjöberg, 1986; Lynch et al., 1989; Nordlund et al., 1990).

Ribonucleotide reductases from *E. coli*, viral, and most eukaryotic sources form the active enzyme upon association of the two proteins into an $\alpha_2\beta_2$ complex. We and others have suggested that the carboxyl-terminal region of protein R2 is involved in the binding to protein R1. The binding to R1 of a heterodimeric R2, which has 1 full-length chain and 1 truncated chain missing the last 24 C-terminal residues, is substantially impaired, whereas the doubly truncated R2 is incapable of binding (Sjöberg et al., 1987). Synthetic peptides corresponding to the C-terminal end of R2 protein from *E. coli*, herpes simplex virus, and mouse ribonucleotide reductases specifically inhibit the enzyme activity (Cohen et al., 1986; Dutia et al., 1986; Yang et al., 1990; Climent et al., 1991).

We have recently established that the C-terminal peptides are competitive inhibitors of the bacterial enzyme, thus providing evidence that these peptides and R2 bind to the same region on R1 (Climent et al., 1991). We also concluded that the C-terminal region of R2 is the major determinant in subunit interaction. An amino acid sequence comparison of R2 proteins from nine different species [cf. Nordlund et al. (1990)] shows a low but significant degree of homology among them. Crystallographic studies with the *E. coli* R2 have identified most of the invariant residues either to be ligands of the iron center or to be vicinal to the Tyr-122 free radical involved in catalysis (Nordlund et al., 1990). The structure of R2 further reveals that the invariant tyrosyl radical is buried inside the protein. Its participation in the reduction reaction, therefore, implicates a possible route for electron transfer through the C-terminal contacts with R1. Two invariant residues, glutamic acid-350 and tyrosine-356, are located in the C-terminal region of R2 protein.

To gain insight into the interaction between R1 and R2 in the active complex of the bacterial enzyme and to elucidate the structural and functional roles of the invariant C-terminal residues, we have carried out a kinetic analysis of the binding of two R2 proteins mutationally altered at these conserved residues. In addition, several forms of an inactive R2 in which Tyr-122 has been mutated to Phe, with either an intact or a truncated C-terminus, have also been studied to serve as a basis for comparison with the wild-type R2 protein.

Experimental Procedures

Materials. Oligonucleotides used for mutagenesis (underlining denotes mismatched nucleotide) [E350A-BS60, d(5′-CTTCCACAGCCTGCGG-3′); E350A-BS61, d(5′-CCGCAGGCTGTGGAAG-3′); Y356A-BS58, d(5′-GACCAGAGCAGAACTG-3′); and Y356A-BS59, d(5′-CAGTTCTGCTCTGGTC-3′)] were synthesized and purified at the Department of Immunology, University of Uppsala,

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Uppsala, Sweden. M13 reverse sequencing primer and M13 ss primer were purchased from Pharmacia, Sweden. Polymerase chain reactions were performed using the GeneAmp Kit (Perking Elmer Cetus). *E. coli* thioredoxin and thioredoxin reductase (NADPH) (EC 1.6.4.5) were kindly supplied by Dr.A. Holmgren, Department of Physiological Chemistry, Karolinska Institute, Stockholm, Sweden. 2'-Azido-2'-deoxy-CDP (CzDP)[1] was a gift of Dr. F. Eckstein, Department of Chemistry, Max-Planck-Institut, Göttingen, Germany. The 30-residue C-terminal peptide of R2 protein was synthesized as previously described (Climent et al., 1991).

Bacterial Strains. *E. coli* JM105 [Δ(*lac-pro*), *thi*, *strA*, *endA*, *sbc*B15, *hsd*R4, F′ [*tra*D36, *pro*AB, *lac*I^{q}ZΔM15]], obtained from Pharmacia, was used for cloning and mutagenesis. *E. coli* MC1009 [Δ(*lac*IPOZYA)X74, *gal*E, *gal*K, *str*A, Δ(*ara-leu*)7697, *ara*D139, *rec*A, *srl*::*Tn*10], obtained from Pharmacia, and JM109(DE3) [*end*A1, *rec*A1, *syr*A96, *thi*, *hsd*R17 (r_{K^-}, m_{K^+}), *rel*A1, *sup*E44, Δ(*lac-pro*AB), F′ [*tra*D36, *pro*AB, *lac*I^{q}ZΔM15]], obtained from Dr. E. Haggård-Ljungquist, Department of Microbial Genetics, Karolinska Institute, were used for expression.

Plasmids. pGP1-2, obtained from Dr. S. Tabor at the Department of Biological Chemistry, Harvard Medical School, Boston, MA, codes for T7 RNA polymerase under the control of the λP_L promoter and a heat-sensitive cI857 repressor under the control of the lac promoter (Tabor & Richardson, 1985). pTZ18R, obtained from Pharmacia, contains a T7 promoter upstream of a cloning cassette (Mead et al., 1986). pMK5 and pMK5Δ30C are recombinant derivatives of pTZ18R, containing a mutant *nrdB* gene in which the codon for residue Y122 has been mutated to a phenylalanine codon. The last one has in addition a 90-nucleotide deletion, resulting in an R2 protein lacking the 30 C-terminal residues (M. Karlsson and V. Johnsson, unpublished results). pTB2 is a pTZ18R derivative containing the *nrdB* gene coding for protein R2. pTB2 was constructed from pMK5, by substituting an *Eco*RI/*Asp*718 fragment of 1 kb containing the Y122F mutation by a *Stu*I/*Asp*718 fragment from pAL7 containing the wild-type Y122 codon (Larsson & Sjöberg, 1986), using an *Eco*RI linker.

Oligonucleotide-Directed Mutagenesis. Mutagenesis of Y356 or E350 to alanine was done by overlap extension, using PCR (Ho et al., 1989). Briefly, a set of two complementary primers, containing the mismatched nucleotides, and two external primers (M13 universal and reverse) annealing upstream and downstream of the *nrdB* gene in pTB2 were used. The template DNA was linearized by digestion with *Sca*I, and two independent PCR reactions were carried out, one for each pair of primers (mismatch and external). Amplification of DNA fragments was done in 50-μL final volume as described (Ho et al., 1989), except that 0.25–0.5 μM primers were used. Low-melting agarose (1% Sea plaque or 3% Nusieve, FMC-BioProducts) was used to purify the amplified fragments. Bands were cut out from the gel and mixed in a fusion PCR reaction. This reaction was done as described before, except that M13 universal and reverse primers were not added until after the first 10 cycles. The amplified fragments were purified by DEAE paper and cloned in pTB2 using *Eco*RI/*Bam*HI restriction sites. Mutant plasmids were screened, and the entire coding region was identified by DNA sequencing using the dideoxynucleotide chain termination method. With this approach, three out of three and one out of two sequenced clones had the desired Y356A and E350A mutations, respectively. One and two additional mutations within the *nrdB* gene were also present in the isolated Y356A and E350A clones, respectively. They were eliminated by subcloning a *Bam*H1/*Asp*718 fragment of approximately 1 kb into pTB2 digested with the same restriction enzymes.

Expression of Mutant R2 Proteins. MC1009/pGP1-2 containing pTB2Y356A was grown overnight at 30 °C in 1 L of SLBH medium (Green et al., 1974) supplemented with 0.4% glucose, 0.1 M potassium phosphate, pH 7.3, kanamycin (40 μg/mL), and carbenicillin (50 μg/mL). The next day, ca. 8 L of the same medium without antibiotics was inoculated with 0.5 L of the overnight culture and grown at 30 °C in a Microferm fermentor (New Brunswick Scientific). At a density of approximately 4×10^9 cells/mL, the temperature was raised to 42 °C and growth continued until late log phase (about 3 h). Cells were harvested by continuous-flow centrifugation at 13000*g*. The pellets were frozen and stored at −85 °C for further purification. For expression of E350A mutant protein, JM109(DE3) containing pTB2E350A was grown overnight in LB medium (Luria & Burrows, 1957) containing 50 μg/mL carbenicillin and inoculated into 1-L batches of 2xYT medium (Messing, 1983) supplemented with 0.2% glucose. Cultures were grown at 37 °C, and at a density of approximately 1×10^9 cells/mL, IPTG was added to 0.4 mM. Growth was continued for ca. 4 h. Cells were harvested by centrifugation as described above, and pellets were stored at −85 °C.

Protein Purification. Wild-type *E. coli* ribonucleotide reductase R1 and R2 proteins were purified from the overproducing strains C600/pLSH1 and C600/pBS1, respectively, as previously described (Larsson et al., 1988), using molecular sieving instead of ion-exchange FPLC in the last purification step. R2Y122F and R2Y122FΔ30C proteins were purified from overproducing strains K38/pGP1-2/pMK5 and K38/pGP1-2/pMK5Δ30C, respectively. All mutant R2 proteins were purified as described for wild-type R2 protein, except that 0.1 mM PMSF instead of 10 μM was used during the purification of R2E350A. Mutant proteins behaved as wild-type R2 protein during the different purification steps, except for the E350A protein which, as expected from the substitution of a negatively charged residue for a neutral one, eluted at a slightly lower NaCl concentration than the wild-type R2 in DEAE chromatography. In SDS electrophoresis, E350A protein has a slightly increased mobility compared with wild-type R2.

Assay of Enzyme Activity. Assays were performed and analyzed as described for the [^{3}H]CDP assay in the presence of the thioredoxin–thioredoxin reductase redox system (Climent et al., 1991), except that 1 mg/mL bovine serum albumin was present.

Protein Determination. The concentrations of R1 protein and the different R2 proteins were determined from their absorbance at 280 nm minus that at 310 nm. Molar extinction coefficients of 180 000 and 120 000 M^{-1} cm^{-1}, respectively, were used. No corrections of molar extinction coefficients were introduced for the truncated or mutant R2 proteins, since theoretical estimates indicated a decrease of less than 5%.

CzDP Inactivation. Experiments were done essentially as previously described (Sjöberg et al., 1987), except that 93 μM R1 protein, 35 μM wild-type, E350A, or Y356A R2 proteins, 0.2 mM dTTP, and 2.5 mM CzDP were used.

[1] Abbreviations: CzDP, 2'-azido-2'-deoxy-CDP; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; DEAE, diethylaminoethyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Table I: Inhibition Constants for Different R2 Proteins

R2 form	K_i (μM)
wild-type	0.18[a]
Y122F	0.18 ● 0.04
heterodimer of $\beta^{F'}\beta^{F}$	6.50 ± 1.00
Y122FΔ30C	NI[b]
E350A	0.48 ± 0.07
Y356A	0.44 ± 0.08

[a] K_m value obtained by extrapolation to a saturating level of thioredoxin [taken from Climent et al. (1991)]. [b] No inhibition.

Sucrose Gradient Centrifugation. Linear sucrose gradients of 5–20% with a volume of 3.6 mL were prepared as described (Hames, 1987). Gradients were buffered with 50 mM Tris-HCl, pH 7.6, and contained 10 mM DTT, 0.1 M KCl, 15 mM $MgCl_2$, and 0.1 mM dTTP. Each tube contained a 0.1-mL cushion of 60% sucrose in the same buffer solution. Protein samples were prepared in 0.11 mL of the buffer solution given above, except that KCl was omitted. Before centrifugation, samples were incubated at 25 °C for 20 min. After addition of catalase (5 μg, $s_{20,w}$ = 11.4 S), 0.1 mL of the protein mixture was loaded on the gradient and centrifuged at 77000*g* (average) for 14 h at 20 °C in an LKB-RPS56T rotor. Fractions of approximately 0.09 mL were collected manually. At the end of the sedimentation experiment, the specific activities of R1 and R2 were 75% of those before centrifugation. Protein was quantified by a modification of the Lowry method (Peterson, 1977), and the amount of R2 was determined from SDS–PAGE of fraction aliquots, followed by Coomassie Blue R-250 staining and tracing in a Molecular Dynamics computing densitometer.

Kinetic Data Analysis. When the mutated R2 proteins served as competitive inhibitors to the wild-type R2 protein, inhibition constants, K_i were determined graphically from a K_{app} vs I_t (total mutant protein concentration) plot according to the equation:

$$K_{app} = K_1(1 + I_t/K_i) \quad (1)$$

where K_{app} and K_1 are the Michaelis constants for protein R2 in the presence and absence of I, respectively. K_{app} was generally obtained from a double-reciprocal plot of velocity vs [R2] (β_t). The total R2 concentration was the sum of the concentrations of R2 added in the assay plus that of the contaminating wild-type R2 present in the mutants, qI_t, at a given I_t; q is the fraction of wild-type R2 present in the mutant preparations. When necessary, e.g., at low I concentration, K_{app} was calculated with appropriate corrections according to an equation similarly derived as previously described (Climent et al., 1991):

$$K_{app} = K_1[1 + (1 - q)I_t/K_i] = (\beta_t + qI_t)[(V_m/v) - 1] - \alpha_t[1 - (v/V_m)] \approx K_1(1 + I_t/K_i) \text{ if } q \ll 1 \quad (2)$$

where α_t is the total R1 concentration. q was determined from velocity measurements using no added wild-type R2, according to the equation:

$$q = \frac{v_1v_2[\alpha_t(I_2v_1 - I_1v_2) - V_m(K_1 + \alpha_t)(I_2 - I_1)]}{V_m^2I_1I_2(v_1 - v_2)} \quad (3)$$

where v_1 and v_2 are velocities observed at two I_t concentrations, I_1 and I_2, respectively. Equation 3 can be readily obtained from eq 2 by setting $\beta_t = 0$.

When the 30-mer C-terminal peptide served as competitive inhibitor, K_i was determined from the equation:

$$K_{app} = K_1(1 + I/K_i)^2 \quad (4)$$

In the complementation experiment where both the 30-mer peptide (I) and the partially truncated R2 (I′) were present, the expression of K_{app} would vary according to one of the following mechanisms:

(a) I and I′ mutually exclusive

$$K_{app} = K_1[(1 + I/K_i)^2 + I'/K_i'] \quad (5)$$

(b) Simultaneous binding of I and I′ to different sites

$$K_{app} = K_1(1 + I/K_i)(1 + I/K_i + I'/K_i') \quad (6)$$

(c) Complementation occurs upon simultaneous binding of I and I′

$$K_{app} = K_1[(1 + I/K_i)^2 + (I'/K_i')(1 + I/K_i'')] \quad (7)$$

where $K_i'' = K_d$ for binding of I to the R1–I′ complex.

Results

Inhibition of E. coli Ribonucleotide Reductase Activity by Inactive R2 Proteins. Inhibition of ribonucleotide reductase by full-length $(\beta^F{}_2)^2$ and fully $(\beta^{F'}{}_2)$ or partially $(\beta^{F'}\beta^F)$ truncated R2 proteins was performed in order to determine the affinity of varying forms of R2 for protein R1. Due to the mutation Y122F, these proteins are inactive for lack of tyrosyl radical (Larsson & Sjöberg, 1986) and thereby can be used as inhibitors of ribonucleotide reductase activity. Structurally, the mutant $\beta^F{}_2$ protein has the intact C-termini, which should permit it to serve as a control for the wild-type R2 in terms of subunit interaction. Double-reciprocal plots of velocity vs free wild-type R2 concentrations at three levels of $\beta^F{}_2$, 0.2, 1, and 2 μM, yielded a set of lines intersecting on the ordinate (not shown). This pattern is consistent with the expected competitive inhibition of Y122F R2 against the wild-type R2 protein. The same inhibition pattern was obtained for the partially truncated $\beta^{F'}\beta^F$ R2 protein form at three concentrations, 3, 15, and 31 μM. The fully truncated $\beta^{F'}{}_2$ R2 protein did not inhibit the enzyme activity at all (data not shown). Inhibition constants (K_i) for the different R2 proteins were calculated according to the method described under Experimental Procedures and are summarized in Table I. The full-length homodimer $\beta^F{}_2$ binds to R1 with a K_i of 0.18 ± 0.04 μM. This value is in excellent agreement with the K_m of the wild-type R2 protein extrapolated to a saturating level of thioredoxin (Climent et al., 1991), suggesting that mutation at the invariant tyrosyl radical does not affect the binding to R1. However, a 36-fold decrease in affinity (K_i = 6.5 ± 1.0 μM) was observed for the heterodimer $\beta^{F'}\beta^F$ as compared to the full-length Y122F or wild-type R2. These results confirm that the C-terminal region of R2 is the key contact for binding to R1 and that dual contacts significantly improved the affinity. As will be discussed later, they also show that the binding energy for a single contact between R2 and R1 proteins can be accounted for from data obtained with C-terminal peptides.

Inhibitor-Complementation Experiments with Heterodimeric ($\beta^{F'}\beta^F$) R2 Protein and Synthetic 30-mer Peptide. From experiments presented in the foregoing section, one may wonder whether the partially truncated $\beta^{F'}\beta^F$ R2 protein can be complemented by the missing 30-residue C-terminal peptide, like S-protein and S-peptide of ribonuclease (Richards, 1958), to form a complex with a K_i approaching that of the

[2] Nomenclature of mutant R2 proteins is as follows: $\beta^F{}_2$, R2Y122F homodimer; $\beta^{F'}{}_2$, R2Y122FΔ30C homodimer; $\beta^{F'}\beta^F$, R2 heterodimer consisting of one β^F and one $\beta^{F'}$ polypeptide chain.

Table II: Kinetic Constants for the Inhibition of Ribonucleotide Reductase by Partially Truncated R2 Protein and C-Terminal Peptide[a]

inhibitor	concn (μM)	K_{app}[b] (μM)	K_i[b] (μM)
none		0.11	
30-mer	20	0.55	16
heterodimer of $\beta^{F'}\beta^{F}$	22	0.55	5.5
30-mer + heterodimer	20 + 22	1.0	

[a] Experiments were done with 0.036 μM R1 by varying the wild-type R2 concentration from 0.041 to 0.654 μM in the absence and presence of the indicated inhibitor concentrations. [b] Calculated as described under Experimental Procedures. In the absence of inhibitor, $K_{app} \approx K_I$.

Table III: Properties of Protein R2 Carboxyl-Terminal Mutants

protein B2	radical content[a] (mol/mol)	iron content[b] (mol/mol)	act.[c] (units/mg)
wild-type	1.0	2.4	3300
E350A	1.1	2.8	17
Y356A	1.0	2.4	15

[a] Determined by the absorptivity at 412 nm. [b] Iron analysis (Sahlin et al., 1990). [c] [^{3}H]CDP assay with 0.1 μM R2 proteins and 0.84 μM R1 protein.

full-length β^{F}_2 R2 protein. Thus, inhibition experiments against wild-type R2 protein in the presence of 30-mer peptide and heterodimer R2 protein $\beta^{F'}\beta^{F}$, individually or jointly, were performed. Table II summarizes the K_i values observed in this particular experiment. Although K_i values of 16 and 5.5 μM were obtained at a single inhibitor concentration of the 30-mer and the heterodimeric R2, respectively, they are comparable to those calculated from several inhibitor concentrations, 15–20 and 6.5 ± 1.0 μM [see Climent et al. (1991) and Table I]. When the peptide and the heterodimer R2 protein were present together, the observed K_{app} value, 1.0 μM (Table II), was very close to the predicted K_{app} for a mechanism in which each of them binds to R1 alone (1.03 μM), suggesting that these two inhibitors not only do not complement each other but also are mutually exclusive. If the two inhibitors could bind simultaneously to R1 at different sites, the predicted K_{app} would be significantly higher (1.54 μM). However, such a value is, nonetheless, within experimental error, and the mechanism cannot be excluded. If complementation occurs and gives rise to a tight inhibitory complex, a much larger K_{app} value is expected. For example, when the K_d for the 30-mer peptide binding to the R1–$\beta^{F'}\beta^{F}$ becomes 1 μM (i.e., $K_i'' = 1$ μM in eq 7), the expected K_{app} is 9.8 μM.

Characterization of R2 Proteins with C-Terminal Point Mutations. The results summarized in Table III show that R2 proteins mutated to alanine at the conserved residues Y356 or E350 are identical to wild-type R2 protein regarding the tyrosyl radical and iron content. However, the major difference was the enzymatic activity. Although a low residual activity could always be detected, basically both mutants were unable to support CDP reduction when assayed in the presence of an excess of R1 protein. To examine the possibility that the absence of a normal level of activity was caused by proteolytic cleavage at the C-terminal end during purification, carboxypeptidase C-terminal sequence analysis was done. Both mutants were found to be full-length proteins (data not shown), indicating that the low activity was not due to the lack of a major binding domain. Examination of other components present in the assay system also ruled out the R1 protein and thioredoxin/thioredoxin reductase preparations as sources of traces of contaminant R2 protein. Consequently, the low activity observed either is intrinsic to the mutants or is due to wild-type contamination of the mutant preparation or both.

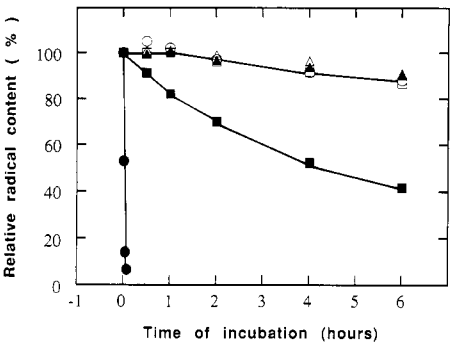

FIGURE 1: Time-dependent inactivation of wild-type and C-terminal mutated R2 proteins in the presence of the suicidal substrate analogue CzDP at 25 °C. Wild-type R2 in the absence (○) and in the presence (●) of R1; E350A in the absence (□) and in the presence (■) of R1; Y356A in the absence (△) and in the presence (▲) of R1. The amplitude of the R2-specific EPR signal, recorded at 77 K at indicated intervals, was used as a measure of the radical content and is expressed as percentage of the content at zero time, before addition of CzDP. Reaction conditions are as described under Experimental Procedures.

Inactivation of R2 Proteins with C-Terminal Point Mutations by the Suicidal Substrate CzDP. In order to distinguish a low intrinsic catalytic activity from a wild-type R2 contamination in the E350A and Y356A R2 proteins, we used CzDP, a suicidal substrate analogue which has been shown to destroy the tyrosyl radical of the R2 protein in a single turnover catalyzed by the holoenzyme complex (Thelander et al., 1976; Sjöberg et al., 1983). Thus, a tyrosyl radical-containing R2 protein must be catalytically competent to cause its own demise. If the low activity observed with a given mutant R2 is due solely to contaminating wild-type, only ≈0.5% of the radical should be destroyed, and no measurable change of the EPR signal could be seen. If the mutant R2 is capable of catalysis, regardless of the reaction rate, total loss of the free radical signal should be detected in the presence of R1 protein.

As can be seen from Figure 1 in the wild-type R2 control, the tyrosyl radical was found to be almost completely inactivated after 5–10 min ($t_{1/2}$ = 1.25 min) of incubation with CzDP and saturating R1 protein. Surprisingly, the tyrosyl radical of E350A R2 protein was also susceptible to inactivation, albeit with drastically different rates. From Figure 1, we estimate that 50% of the radical was lost after 5 h of incubation. The loss of radical was not due to instability since, in the absence of R1, less than 15% of the tyrosyl radical was lost after 6 h of incubation. The 240-fold decrease in the rates of CzDP inactivation for E350A mutant compared to the wild-type is consistent with the observed low activity when the [^{3}H]CDP assay was used (Table III). In contrast, Y356A R2 protein was unaffected by CzDP. Less than 10% of the radical was lost after 6 h of incubation, both in the presence and in the absence of R1 protein. It is thus plausible that the low activity of Y356A (0.45%, calculated according to eq 3) is due to chromosomally encoded wild-type R2 contamination. In fact, mutant Y122F, incapable of catalysis for lack of tyrosyl radical, was found to exhibit 0.5% activity relative to that of wild-type. It is likely that the E350A mutant also contains some chromosomally encoded wild-type R2 and that its intrinsic activity is less than the 0.5% determined in the CDP assay. The results with CzDP inactivation show that, while mutation of Y356 or E350 to alanine in R2 severely reduces the enzymatic activity, some catalytic exchange between R1 and R2 does occur in the E350A holoenzyme complex but not in the Y356A complex.

Inhibition of Ribonucleotide Reductase Activity by R2 Proteins with C-Terminal Point Mutations. To examine

whether the low level or lack of activity in E350A and Y356A R2 mutants, respectively, was due to an impaired binding of these proteins to R1, inhibition experiments using the mutant R2 proteins as inhibitors were performed. For this particular experiment, the total residual activity of R2 mutant proteins was considered wild-type R2 contamination and corrected for as described under Experimental Procedures. Reciprocal plots of velocity vs free wild-type R2 concentration at different concentrations of mutant proteins yielded typical competitive inhibition plots. The inhibition constants (K_i) for E350A and Y356A proteins were obtained by replots as described under Experimental Procedures. As shown in Table I, both C-terminally altered R2 proteins have similar K_i values in the range of 0.4–0.5 μM, indicating strong affinities for R1. Although these K_i values are 2–3-fold higher than the K_m for wild-type R2, or the K_i for Y122F, the binding remains tight. The free energy of binding merely changes from 9.2 to 8.6 kcal/mol. These results indicate that substitution of either of the invariant residues at the C-terminal end of R2 does not significantly affect the binding of these proteins to R1.

Sucrose Gradient Centrifugation. The binding of the two C-terminal mutants to R1 was also analyzed by sucrose gradient centrifugation. These experiments were done in the presence of equimolar concentrations of R1 protein and wild-type, E350A, or Y356A R2 proteins at ≈20 μM. Figure 2 shows the sedimentation profiles after 14-h centrifugation. Under the conditions used in these experiments, all three R2 proteins formed a complex with R1 protein that sedimented with an s value of 9.1 S. However, as expected from the differences in K_i values (see Table I), the C-terminal R2 mutants formed less R1R2 complex than did wild-type R2, and part of the mutant R2 proteins sedimented as free forms with an s value of 5.8 S. The ratio between free and bound R2 was slightly different for R2E350A and R2Y356A proteins, even though their K_i values were very similar (Table I). The long period of incubation during centrifugation, the presence of sucrose, the lack of the thioredoxin system, etc. probably account for the observed discrepancy. It should be mentioned that ≈75% activity was recovered after centrifugation. In one experiment, a mixture of R1 and R2Y356A was sedimented in a sucrose gradient containing CDP and ATP (instead of dTTP) in the buffer solution. These conditions are more similar to those of the inhibition experiments. However, no difference was found as compared to the result of Figure 2 (data not shown), suggesting that the presence of substrate has little effect on complex formation.

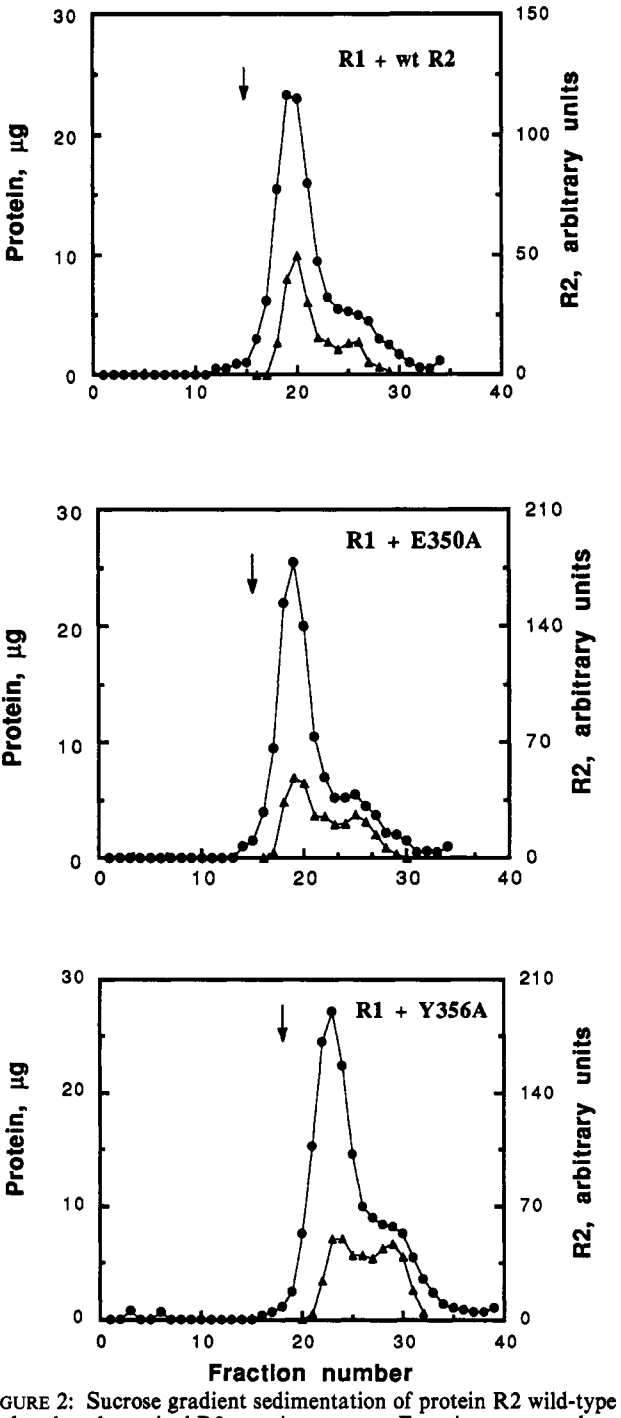

FIGURE 2: Sucrose gradient sedimentation of protein R2 wild-type and carboxyl-terminal R2 protein mutants. Experiments were done in the presence of 20 μM R1 protein and 22 μM wild-type and C-terminal mutants. (●) Total protein and (▲) R2 protein trace in arbitrary units. The arrows denote the 11.4S position of catalase.

Discussion

We have shown previously (Climent et al., 1991) that the carboxyl-terminal region of *E. coli* ribonucleotide reductase protein R2 plays a major role in the formation of the R1R2 holoenzyme. To study the role of the C-terminus in greater details, we employed two R2 mutants which have been mutated at the conserved residues in this region. As a control, we constructed homo- and heterodimeric forms of *E. coli* protein R2 using the Y122F mutant, which is inactive due to the lack of the tyrosyl radical but structurally nearly identical to the wild-type, and the Y122FΔ30C mutant, which lacks the last 30 residues at the C-terminal end. The truncated homodimer did not inhibit the enzyme activity, indicating that it does not bind to R1, as was previously shown by sedimentation analysis for the truncated wild-type R2 (Sjöberg et al., 1987). The heterodimer $\beta^F\beta^F$ containing a single C-terminus, on the other hand, is inhibitory with a K_i of 6.5 μM. It can be shown that this value translates into an intrinsic K_i that is essentially the same as the intrinsic K_i of the C-terminal peptides of 20 residues or longer. The latter K_i was calculated on the basis of equivalent binding of two molecules of peptide per dimeric R1 protein, α_2 (Climent et al., 1991):

$$\alpha_2 + I \underset{k_{-i}}{\overset{2k_i}{\rightleftharpoons}} \alpha_2 I + I \underset{2k_{-i}}{\overset{k_i}{\rightleftharpoons}} \alpha_2 I_2 \qquad (8)$$

The statistical factor "2" indicates the number of sites available for peptide association and dissociation. The K_i is determined by the relationship given in eq 4, and is therefore the intrinsic dissociation constant reflecting the ratio of off- and on-rate constants:

$$K_i = k_{-i}/k_i \qquad (9)$$

Only one molecule of heterodimeric R2, however, can bind

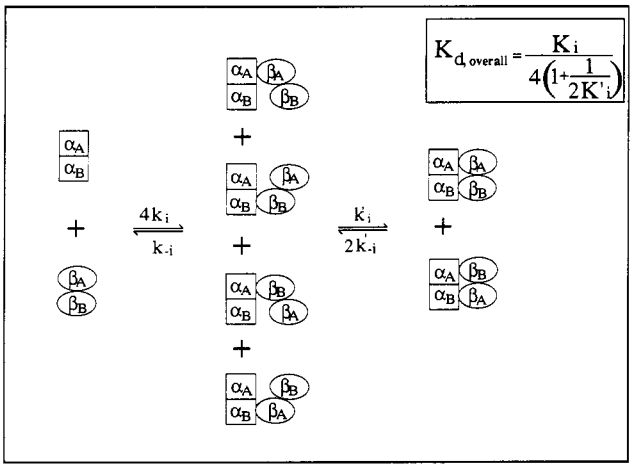

FIGURE 3: Illustration of the binding between *E. coli* R2 and R1 ribonucleotide reductase proteins. The scheme shows all possible subunit association–dissociation contacts. The symbols represent the following: α_A and α_B squares, each of the two equivalent polypeptide chains of protein R1; β_A and β_B ovals, each of the two equivalent polypeptide chains of full-length protein R2. k_i, k'_i, k_{-i}, and k'_{-i} are rate constants; $K_i = k_{-i}/k_i$, $K'_i = k'_{-i}/k'_i$. The equation for calculation of the overall dissociation constant, K_d is given in the inset.

to R1, and its binding can be described by the first part of eq 8:

$$\alpha_2 + \beta^{F'}\beta^{F} \underset{k_{-i}}{\overset{2k_i}{\rightleftharpoons}} \alpha_2\beta^{F'}\beta^{F} \qquad (10)$$

Thus, for $\beta^{F'}\beta^{F}$, the inhibition constant has the expression

$$K_i = k_{-i}/2k_i \qquad (11)$$

From the K_i of 6.5 ± 1.0 μM for $\beta^{F'}\beta^{F}$, one can calculate that the intrinsic dissociation constant (k_{-i}/k_i) is 13 ± 2.0 μM, which is very close to the values of 15–18 μM obtained for the C-terminal peptides. This strongly suggests that the C-terminal region is solely responsible for binding to R1 and that the presence of the bulk of R2 protein in the heterodimer contributes little to this binding. In other words, the C-terminal part, whether attached to R2 or not, interacts with R1 protein with a similar binding energy of 6.4–6.7 kcal/mol. It is believed that the C-terminus is a rather flexible region, since it could not be resolved by X-ray crystallography (Nordlund et al., 1990). The lack of complementation between $\beta^{F'}\beta^{F}$ and the 30-mer peptide also suggests that the C-terminal structure is quite independent of the rest of R2.

The full-length homodimer $\beta^{F}{}_2$, as expected, is a strong competitive inhibitor of R2 with a K_i of 0.18 μM. This value is in excellent agreement with the K_m for wild-type R2 at saturating thioredoxin (Climent et al., 1991). It can be shown from steady-state treatment that such a K_m under several conditions represents the true dissociation constant. The close agreement between these two values strengthens this notion. Knowing K_d for a single contact and the overall K_d for dual contacts between the two subunits of ribonucleotide reductase, we can estimate the contribution of an additional contact in a manner analogous to the approach of Jencks (1981).

The complex formation between R1 and the full-length homodimer R2 protein is illustrated in Figure 3. The binding could be envisaged as a two-step process. An initial bimolecular reaction leads to the formation of four formally identical intermediates, each with one contact between the proteins, with an intrinsic K_d of 13 μM ($=k_{-i}/k_i$). The second step involves a unimolecular rearrangement that leads to the formation of the holoenzyme via two pairwise contacts between the R1 and R2 subunits. The dissociation constant (K'_i) for the second step can be calculated from the expression of the overall K_d of 0.18 μM according to the equation given in Figure 3. The K'_i so computed has a value of 0.029. From the ratio of the rate constants in the second step ($k'_i/2k'_{-i}$ = 1/0.058 = 17/1), we can see that, when the initial contact has been established, the formation of the second one is favored by a factor of 17 to 1. Taking statistical factors into consideration, we calculate the contribution to the free energy of binding to be 7.5 and 1.7 kcal/mol for the first and second steps, respectively.

The fact that the C-terminus of R2 is mainly responsible for binding to R1 leads to several interesting questions: Is its role purely structural or at once catalytic? What are the functions of amino acid residues in this region? In this study, we have highlighted the importance of the two invariant C-terminal residues E350 and Y356 by site-directed mutagenesis. As is often observed with the alteration of conserved residues, both R2E350A and R2Y356A had very poor or no activity at all. The lack of activity was due neither to an impaired capacity of these mutant R2 proteins to generate the iron center/tyrosyl free radical nor to a significantly diminished binding to R1. The K_i values for the mutant proteins, 0.4–0.5 μM, are comparable to that of R2 with intact C-termini, 0.18 μM. Since the R1R2 complex formation among ribonucleotide reductases from *E. coli*, animal viruses, and most eukaryotic sources is highly species-specific, conceivably some of the nonconserved residues at the C-terminal end of each R2 protein are responsible for the specificity and are therefore the key determinants for binding of subunits. Thus, since mutation of E350 or Y356 has a strong effect on the enzymatic activity, these conserved residues are likely to be catalytically important.

From the X-ray crystal structure of R2 protein (Nordlund et al., 1990), it is clear that catalysis requires long-range electron transfer between the radical in R2 and the substrate bound to R1, since Tyr-122 is at least 10 Å away from the nearest R2 surface and seemingly remains so in the R1R2 complex (Sahlin et al., 1987). The current concept of long-range electron flow proposes a specific pathway connecting the donor and the acceptor, which is determined by the structural motif of the protein (Beratan et al., 1991). It is plausible that the role of E350 and perhaps also Y356 remains structural; alteration of these residues may simply result in defective orientation or arrangement of the pathway. On the other hand, direct participation in catalysis of these conserved residues, particularly Y356, remains an intriguing possibility. By using the suicidal substrate CzDP (cf. Results in Figure 1), we were able to demonstrate that the E350A mutant is capable of definitive, though rather inefficient, catalysis. Since the Y356 residue is unaltered in this mutant, its presence may permit electron transfer to proceed by serving as a relay between the substrate and the tyrosyl radical. The involvement of a tyrosyl group as an intermediary in electron transfer has been implied in other protein systems: photosystem II (Metz et al., 1989) and the blue copper proteins stellacyanin (Farver & Pecht, 1989) and plastocyanin (He et al., 1991). Recently, Y356 has been suggested to give rise to the transient radical observed in the reconstitution of Y122F R2 protein with limiting Fe^{2+} and hence to play a possible role in catalysis (Bollinger et al., 1991). The latter suggestion is certainly in line with the results presented in this study.

In summary, the present study using mutant R2 proteins has shown that the C-terminal of R2 protein is the predominant determinant in the subunit interaction of *E. coli* ribonucleotide reductase. In addition, our results indicate that the invariant glutamic acid-350 and tyrosine-356 at the C-terminal

region of R2 protein are not the key elements in the binding between subunits but are more likely involved in or affecting the electron-transfer pathways between R1 and R2.

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