Two Conserved Tyrosine Residues in Protein R1 Participate in an Intermolecular Electron Transfer in Ribonucleotide Reductase*

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The enzyme ribonucleotide reductase consists of two nonidentical proteins, R1 and R2, which are each inactive alone. R1 contains the active site and R2 contains a stable tyrosyl radical essential for catalysis. The reduction of ribonucleotides is radical-based, and a long range electron transfer chain between the active site in R1 and the radical in R2 has been suggested. To find evidence for such an electron transfer chain in Escherichia coli ribonucleotide reductase, we converted two conserved tyrosines in R1 into phenylalanines by site-directed mutagenesis. The mutant proteins were shown to be enzymatically inactive. In addition, the mechanism-based inhibitor 2'-azido-2'-deoxy-CDP was incapable of scavenging the R2 radical, and no azido-CDP-derived radical intermediate was formed. We also show that the loss of enzymatic activity was not due to impaired R1-R2 complex formation or substrate binding. Based on these results, we predict that the two tyrosines, Tyr-730 and Tyr-731, are part of a hydrogen-bonded network that constitutes an electron transfer pathway in ribonucleotide reductase. It is demonstrated that there is no electron delocalization over these tyrosines in the resting wild-type complex.

The enzyme ribonucleotide reductase is essential for all living organisms. By catalyzing the reduction of ribonucleotides to the corresponding deoxyribo nucleotides, the enzyme furnishes cells with precursors for DNA synthesis. To maintain a stable and balanced supply of nucleotides during cell proliferation, cells with precursors for DNA synthesis. To maintain a stable tyrosyl radical essential for catalysis. The substrate binding site in R1 includes a cysteine triad that is involved in catalysis (3-5). The smaller protein R2 contains essential tyrosyl radical that is generated and stabilized by an oxo-bridged diiron center (6-9). The oxidized tyrosyl radical of R2 probably participates in the reaction mechanism as a transient electron sink.

The reaction catalyzed by ribonucleotide reductase is the reduction of the 2'-hydroxyl group of a ribonucleoside diphosphate. The mechanism involves the initial generation of a transient protein radical in R1 close to the bound substrate. The protein radical abstracts a hydrogen atom from the 3' position of the substrate thereby generating an oxidized substrate radical that enables leaving the protonated 2'-hydroxyl group. The resulting substrate radical cation intermediate is reduced by a redox-active cysteine pair, which in turn is oxidized to a disulfide (10). The 3'-hydrogen atom is reintroduced by the same amino acid that initially abstracted it and that again forms the transient protein radical (11).

The crystal structure of the R2 protein shows that the R2 radical is buried inside the protein structure about 10 Å from the closest surface (9). Two possibilities have been suggested by which the tyrosyl radical in R2 and the active site in R1 could possibly interact: a direct interaction through considerable conformational changes in the proteins or an indirect interaction via a long range electron transfer. Since the spectroscopic characteristics of the tyrosyl radical are unchanged in the holoenzyme complex as compared with the R2 protein alone, no major conformational changes are likely to occur at the site of the R2 radical during R1-R2 complex formation (12). This argues in favor of the presence of a long range electron transfer chain within the ribonucleotide reductase enzyme.

The three-dimensional structures for both protein R1 and protein R2 have been solved to a high resolution (5, 9), but the structure of the holoenzyme complex is not yet known. A plausible model of the R1-R2 complex suggests that the distance between the radical in R2 and the active site in R1 is about 35 Å. In this part of the modeled complex, there is an array of conserved residues that has been proposed to constitute the electron transfer chain. The residues are Tyr-122, Asp-84, His-118, Asp-237, Trp-48, and Tyr-356 in the R2 protein and Tyr-730, Tyr-731, and Cys-439 in the R1 protein (cf. Fig. 1). These residues form a hydrogen-bonded network running through the holoenzyme complex. For five of the R2 residues and the cysteine in R1, site-directed mutagenesis showed that substitutions of any of the residues resulted in a catalytically inactive mutant protein (3, 8, 13, 14). Furthermore, site-directed mutagenesis of Trp-103 and Asp-266 in the mouse enzyme (corresponding to Trp-48 and Asp-237 in the E. coli enzyme) suggests that they participate in a corresponding electron transfer chain in mouse ribonucleotide reductase (15). When considered together, this implies that the electron is transferred via a specific route comprised of at least nine amino acids.

The two tyrosines in the R1 protein, Tyr-730 and Tyr-731, are located close to the active site. Tyr-730 is hydrogen-bonded to Tyr-731 and Cys-439, the latter of which has been proposed to be the residue that abstracts the 3'-hydrogen atom during catalysis. Tyr-731 is located close to the R1-R2 interaction area in the model-built holoenzyme complex. In this paper, we altered each of the two conserved R1 tyrosines to phenylalanines by site-directed mutagenesis and characterized the mutant proteins. We showed that both mutant proteins were inactive in the presence of normal substrate as well as the substrate...
analogue 2'-azido-2'-deoxy-CDP (CzDP),

1 even though both mutant proteins could form an R1-R2 complex and bind substrate. These data strongly suggest that the two tyrosine residues, Tyr-730 and Tyr-731, participate in an electron transfer chain between the R2 radical and the R1 active site.

**EXPERIMENTAL PROCEDURES**

Materials—The oligonucleotides used for mutagenesis (underlining denotes mismatched nucleotide), Y730F (5'-AGTGGCCG-3') and Y731F (5'-AGATCTGGATACCATCG-3') were synthesized and purified by Scandinavian Gene Synthesis AB. Single-stranded sequencing primers, me6 d(5'-CCG-3') analogue 2', (d(5'-AGTGGCCG-3') and me19 (d(5'-TAACTGGATACGTTCCG-3')) were synthesized and purified by Scandinavian Gene Synthesis AB, and Universe-40 (d(5'-GTTTCCC-A/GTTTCCGAC-3')) was purchased from U.S. Biochemical Corp. The 2'-azido-2'-deoxy-CDP was obtained by deavage of its CTP derivative by incubation with myosin and then purified on a DEAE-cellulose column. The 2'-azido-2'-deoxy-CTP was purchased from Amersham Corp.

Bacterial Strains—E. coli C36 (dut-1, ung-1, thi-1, relA/pR105 Cmy) and E. coli MV1190 (Δlac-proAB), thi, supE, Δ(serC-recD:306::Tn10), thd-MC1009/pGP1–2 (Δ(sufU-srl), sup30, thi-1, rel-1, ung-1, thi-1, rec-1, traD36, proAB, lacI::Tn10), obtained from Bio-Rad, were used for mutagenesis and cloning. E. coli MC1009/pGP1-2 (Δ(lacPOZYAX7, galA, galK, strA, Δ(ara-leu)-7697, adaI39, recA, srl::Tn10), obtained from Pharmacia Biotech Inc., was used for expression.

Plasmids—pT218R, obtained from Pharmacia, contains a T7 promoter upstream of a cloning cassette (16). The plasmid pT218 is a derivative of pT22, which contains the λ gene coding for the R1 protein (3). The plasmid pGP1-2, obtained from S. Tabor, codes for T7 polymerase under the control of the T7 promoter and a heat-sensitive lac repressor under the control of the Lac promoter (17).

Oligonucleotide-directed Mutagenesis—Mutagenesis of pT218 was performed using the Muta-Gene Phagemid in vitro mutagenesis kit from Bio-Rad based on a method described by Kunkel (18). Initial verifications of the mutations were done by the Sanger chain termination DNA sequencing method using the Sequenase version 2.0 kit from U.S. Biochemical Corp. A 750-base pair long SplI/SfuI fragment of the mutants was sequenced using the Taq Dye Deoxy Terminator cycle sequencing kit and cloned into a wild-type pT218 plasmid. The sequencing reactions were performed with polymerase chain reaction and analyzed with an automated laser fluorescent DNA sequencer from Applied Biosystems.

Expression of Mutant R1 Protein—MC1009/pGP1-2, containing either pT218 Y730F or Y731F was grown overnight at 30 °C in 50 ml of LB broth supplemented with kanamycin (50 μg/ml) and carbenicillin (50 μg/ml). The next day, 4 liters of the same medium were inoculated with 2 ml of overnight culture and grown overnight (300 rpm) at 30 °C. At A600 = 0.5, the temperature was raised to 42 °C to induce the T7 promoter by heat inactivation of the CI857 repressor. Growth was continued until late log phase (A = 1.7). After incubation (about 4 h), the culture was quickly chilled and centrifuged at 4000 × g. The pellets were frozen and stored at −80 °C for further purification.

**RESULTS**

The strength of the R1-R2 interactions was determined in a series of inhibition experiments where the mutant R1 proteins were used as inhibitors of wild-type ribonucleotide reductase activity. Enzyme activity was measured according to the [3H]CDP assay (see below) with a constant R2 concentration of 0.03 μM, and the R1 concentrations were varied between 0.04 and 0.7 μM. The concentration of R1 Y730F and Y731F were 0.0, 0.07, 0.26, and 1.05 μM for R1 Y730F and 0.0, 0.07, 0.26, and 0.53 μM for R1 Y731F. The inhibition constants (K) for the mutant proteins were determined graphically by plotting K versus inhibitor concentration.

**Nucleotide Binding Assay**—Substrate binding of GDP to the mutant proteins was performed according to the method of direct partition through ultralfiltration (21). The experiments were carried out at 25 °C, using 7.4 mM R1, 6–50 μM tritium-labeled GDP as substrate, and 40 μM dTTP as effector.

**Assay of Enzyme Activity**—Assays were performed and analyzed as described for the [3H]CDP assay (22) in a final volume of 50 μl with 1.5 mM ATP as effector and 0.5 μM [3H]CDP (63,000 cpm/mmol) as substrate and in the presence of the thioredoxin-reducing system. One enzyme unit is defined as the amount of R1 reducing 1 nmol of product/min at room temperature in the presence of an excess of R2.

**CdZP Inactivation**—Experiments were done essentially as described previously (13, 23) except that 40 μM R1, 20 μM R2, 0.2 mM dTTP, 4.2 μM CdZP, and 3.5 mM CDPP were used. The samples were incubated at 25 °C for different times and subsequently frozen in liquid nitrogen.

**Crystallization and Data Collection**—The Y730F and Y731F proteins were crystallized in the space group R32 with hexagonal cell axes a = b = 226 Å and c = 341 Å as in the wild type (20). The crystals were obtained by hanging drops of 10 μl (5 μl of protein mixture + 5 μl of reservoir solution). The protein mixture contained 15 mM MgCl2, 1 mM dithiothreitol, and 20% glycerol, which contained 17% lithium sulfate and 10 mM magnesium chloride in 25 mM citrate buffer, pH 6.0. Diffraction data for both mutants were collected to 2.8 Å resolution at the synchrotron in Hamburg, Germany (station X31, EMBL outstation). Data were indexed and integrated with Denzo, scaled and reduced with Scalepack (24, 25), and truncated with programs from the CCP4 suite (26). The resulting data for the Y730F mutant are comprised of 78,000 unique reflections with an averaged redundancy of 3.2, an Rmerge of 8.7%, and a completeness of 94.8%. The corresponding values for Y731F were 82,000, 2.4, 9.7, and 98.4%, respectively.

**Structural Refinement**—The coordinates of wild-type E. coli R1 ribonucleotide reductase (5) were used as a starting model for refinement with TnT (27). The refined models were evaluated and corrected using O (28) and then further refined with TnT. Y730F and Y731F proteins have R-factors of 18 and 19%, respectively, with good geometry and root mean square differences in bond lengths of 0.023 Å and 0.020 Å and root mean square differences in angles of 2.3° and 2.5°.

**EPR Measurements**—EPR spectra measured at 77 K were recorded on an ER 200D Bruker spectrometer using a cold finger Dewar flask for liquid nitrogen temperatures. Quantitation of the EPR signals was performed by a comparison with a standard R2 sample of 1.5 mM tyrosyl radical, previously quantitated from the comparison of double integrals of the protein sample with a Cu-EDTA standard (1–10 mM). Spectra at 9 K were recorded on an ESP 300 Bruker spectrometer equipped with an Oxford instrument cryostat. Subtractions at 9 K and double integrals were performed using the ESP 300 software.

**RESULTS**

Construction, Expression, Purification and Structure of Tyr → Phe Substitutions in Protein R1—The constructs R1 Y730F and Y731F contained only the desired TAT → TTT mutations as verified by dideoxy sequencing. Expression and purification of mutant proteins yielded about 50 mg of protein of 60% purity from 20 g of heat-induced cells. The mutant proteins behaved like R1 wild-type protein during expression and purification except for a tendency to precipitate early in the purification procedure that could be overcome by inclusion of 20% glycerol in all purification steps. The three-dimensional structures of the two mutant R1 proteins were solved by using the wild-type structure as the initial model (5). The refined structures confirm that the mutants contain phenylalanines at positions 730 or 731, respectively. The phenylalanines occupy the same positions as the wild-type tyrosines, maintaining the p-p interaction between them (Fig. 1). The structural differences are small in the vicinities of the phenylalanines and negligible within experimental error, indicating that the phenotypic differences compared with the wild-type R1 are due only to the
loss of either of the phenolic hydroxyl groups. The Y731F mutant shows no other major differences compared with the wild-type R1 protein. There is one major difference compared with the wild-type R1 in the Y730F crystal structure. Whereas wild-type R1 crystallized in the absence of reductants was obtained in the oxidized form, the redox-active cysteine pair (Cys-225 and Cys-462) was reduced in the Y730F crystals when crystallised under identical conditions. These two cysteines are, however, far from the substituted tyrosine, and there is no obvious interaction between the reduced cysteinyl residues and phenylalanine 730. This interesting feature will be further explored in a separate study.

The Mutant R1 Proteins Can Bind R2 and Substrate—The inhibition of ribonucleotide reductase activity by mutated R1 proteins was used to study the kinetics of R1-R2 complex formation. The inhibition constants ($K_i$) for Y730F and Y731F were determined from Lineweaver-Burk plots. All parameters for the R1-R2 interactions are summarized in Table I. Both mutant proteins are able to bind R2, and only a small difference in $K_i$ values is observed between the mutant proteins and the wild type. The apparent R1-R2 binding constant for the wild type is approximately 0.11 $\mu M$ (13), and the differences in the mutant proteins could reflect either the position of mutated residues per se or a perturbed holoenzyme conformation induced by the mutation. The weaker binding between Y731F and R2 may reflect that exchange of this residue which is located close to the R1-R2 interaction area, affects the interaction in a negative way. The stronger binding between R1 Y730F and R2 could be due to a small structural change caused by R1-R2 complex formation or by the loss of the hydroxyl group in the 730 position. However, the differences in binding strength are small and are not, per se, likely to cause any significant changes in the enzymatic activity.

We also tested the mutant proteins for ability to bind substrate. The GDP binding data presented in Table II show that both mutant proteins have $K_d$ values similar to that of the wild-type protein (21). From this, we conclude that the substrate binding is not affected by the mutations.

The Mutant R1 Proteins Are Catalytically Inert and Incapable of Electron Transfer—The specific activities of mutant and wild-type R1 proteins are summarized in Table III. The low but significant enzyme activity measured in the mutant preparations (1.6% of the wild-type activity) corresponds approximately to the estimated contaminating, chromosomally encoded, wild-type protein in preparations from MC1009/pTB1/pGP1–2 constructs (3). Thus the mutant proteins were almost certainly catalytically inactive.

CzDP inactivation of ribonucleotide reductase was used to substantiate the lack of activity in the mutant proteins and to...
determine if the lack of activity is due to a disturbed electron transfer chain. CzDP is a suicidal substrate analogue that destroys the tyrosyl radical in a single half-reaction catalyzed by the holoenzyme (23, 29, 30). A protein with an intact electron transfer chain will thus be captured in the substrate radical state, whereas a protein with a disturbed electron transfer chain will never reach this state because the tyrosyl radical is never reduced and the 3'-hydrogen atom of the substrate never abstracted. As can be seen from Fig. 2, incubation of isolated R2 protein with CzDP showed no measurable change in the radical signal during 6 h of incubation. The R2 radical of the R1-R2 wild-type complex is rapidly inactivated during the first 20 min of incubation with CzDP, and azidosubstrate radical is already observed after 18 s. In contrast, the mutant R1 proteins in a complex with R2 wild-type protein showed no formation of an azidosubstrate radical during the incubation and no loss of tyrosyl radical during the first 20 min of incubation (Fig. 2). Even after 6 h of incubation with either CDP or CzDP, approximately 40% of the radical remained. In a parallel incubation of wild-type holoenzyme with CzDP, approximately 15% remained after 6 h of incubation (Fig. 2). We estimate that this nonspecific inactivation of the radical in the presence of CDP, and CzDP in the case of the mutant protein complexes, is 0.05% compared with wild-type R1. With this knowledge, the binding of substrate and R2 to the mutant proteins was established that the three-dimensional structures of the proteins contain normal protonated tyrosines in the presence of CzDP (•), R1Y730F/R2 in the presence of CzDP (■), R1 Y731F/R2 in the presence of CzDP (○), R1-R2 wild type in the presence of CDP ( ), R1 Y730F/R2 in the presence of CDP ( ), and R1Y731F/R2 in the presence of CDP ( ▲).
tional change. In summary, this means that all residues suggested to be involved in the electron transfer reaction in ribonucleotide reductase have been shown to be essential for catalysis.

The distance between the radical in R2 and the active site in R1 is 30–40 Å (Fig. 1), which is the longest naturally occurring electron transfer described to date. According to current understanding of electron transfer theory (34), this implies an extremely slow transfer process. For distances longer than e.g. 25 Å, the rate of transfer would be considerably slower than 1 s\(^{-1}\) since the exponential coefficient of decay of electronic coupling (\(\beta\)) is 1.4 Å\(^{-1}\) in several experimentally observed systems. The \(k_{\text{cat}}\) of wild-type ribonucleotide reductase is, however, on the order of 4–11 s\(^{-1}\) (30, 36); and in studies with isotopically labeled substrates, Stubbe (37) showed that the initial step of catalysis is not rate-limiting. Therefore it is not straightforward to describe the electron transfer in ribonucleotide reductase according to the theoretical model.

The original theory describing electron transfer in proteins is based on a mechanical tunneling mechanism (34). A tunneling pathway is defined as a combination of interacting bonds, i.e. bonds sharing a common atom, hydrogen bonds, or atoms in van der Waals forces, that link donor with acceptor. It is characteristic of an electron tunneling process that the rate of electron transfer rapidly decreases with distance. The model suggests that the coupling decay across a hydrogen bond is approximately equivalent to that across two covalent bonds, and the decay across a typical space-jump is equivalent to that across 10 covalent bonds (38). Current understanding of a long range electron transfer mechanism is, however, still incomplete, and in a recent experimental study, no difference in the rate of transfer was observed between a covalent bond and a hydrogen bond in a model compound (39).

The site-directed mutagenesis studies on ribonucleotide reductase (8, 13–15) suggest an amino acid-specific electron transfer pathway where the participating residues (aromatics, cysteine, histidine, and carboxylates) are part of a hydrogen-bonded network with additional \(\pi-\pi\) interaction in R1. The structural data on the two Tyr \(\rightarrow\) Phe mutants show that there are no major changes in the three-dimensional structure and that the mutations do not affect the aromatic interaction. From this, we can conclude that conservation of the \(\pi-\pi\) interaction is not enough for electron transfer to occur.

An explanation for the fast electron transfer rate in wild-type ribonucleotide reductase, in relation to the considerable distance between the tyrosyl radical in R2 and the active site in R1, would be that the radical was delocalized over the entire hydrogen-bonded pathway. In experiments with deuterium-labeled R2 protein and unlabeled R1 protein, we found, however, no indication of a delocalized electron in the resting R1-R2 wild-type complex. One feature of the Tyr \(\rightarrow\) Phe mutations is an interrupted hydrogen-bonded network. This characteristic is in fact true for all mutant constructs affecting the electron transfer chain in ribonucleotide reductase. Perhaps a coupled proton/electron transfer process is essential and determines the rate of transfer in the catalytic process.

Acknowledgments—We are grateful to M. Ormø for valuable discussions all through the work and to I. Climent for help with the R1-R2 interaction studies.

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