Preserved Catalytic Activity in an Engineered Ribonucleotide Reductase R2 Protein with a Nonphysiological Radical Transfer Pathway

THE IMPORTANCE OF HYDROGEN BOND CONNECTIONS BETWEEN THE PARTICIPATING RESIDUES*

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A hydrogen-bonded catalytic radical transfer pathway in *Escherichia coli* ribonucleotide reductase (RNR) is evident from the three-dimensional structures of the R1 and R2 proteins, phylogenetic studies, and site-directed mutagenesis experiments. Current knowledge of electron transfer processes is difficult to apply to the very long radical transfer pathway in RNR. To explore the importance of the hydrogen bonds between the participating residues, we converted the protein R2 residue Asp^{237}, one of the conserved residues along the radical transfer route, to an asparagine and a glutamate residue in two separate mutant proteins. In this study, we show that the D237E mutant is catalytically active and has hydrogen bond connections similar to that of the wild type protein. This is the first reported mutant protein that affects the radical transfer pathway while catalytic activity is preserved. The D237N mutant is catalytically inactive, and its tyrosyl radical is unstable, although the mutant can form a diferric-oxo iron center and a R1-R2 complex. The data strongly support our hypothesis that an absolute requirement for radical transfer during catalysis in ribonucleotide reductase is an intact hydrogen-bonded pathway between the radical site in protein R2 and the substrate binding site in R1. Our data thus strongly favor the idea that the electron transfer mechanism in RNR is coupled with proton transfer, i.e. a radical transfer mechanism.

The enzyme ribonucleotide reductase (RNR) supplies all living organisms with the precursors for DNA synthesis by catalyzing the reduction of ribonucleotides to the corresponding deoxyribonucleotides. *Escherichia coli* class I RNR is composed of two components, proteins R1 and R2, both essential for catalytic activity (for a recent review, see Ref. 1). The R1 protein contains the active site, where a radical-based reaction takes place (2, 3), and a stable tyrosyl radical involved in the reaction is located at Tyr^{122} in the R2 protein (4).

The three-dimensional structures of both proteins have been resolved to high resolution (5–8) and used to model a structure of the R1-R2 complex. The model suggests that the active site in R1 and the stable tyrosyl radical in R2 are connected via an array of conserved residues, which constitutes the radical transfer pathway. These residues are Tyr^{122}, Asp^{237}, His^{118}, Asp^{237}, Trp^{356}, and Tyr^{274} in the R2 protein and Tyr^{730}, Tyr^{731}, and Cys^{439} in the R1 protein (see Fig. 1).

The importance of the radical transfer route has been demonstrated extensively by site-directed mutagenesis of the *E. coli* enzyme and also in experiments using selected residues in the mouse enzyme. Each residue of the radical transfer pathway has been replaced by another nonphysiological residue, and none of the mutant proteins could perform ribonucleotide reduction (4, 9–14). In all reported cases, the side chain of the particular amino acid residue was changed to a side chain with other hydrogen bonding properties than those of the original residue in the wild type protein.

The very long (35–40-Å) radical transfer pathway in RNR is hard to reconcile with current electron transfer theories (15–19), since the transfer of an electron over such a distance should, according to theory, take longer time than the actual enzymatic reduction of substrate. In the current study, our intention was to investigate further the function of the radical transfer pathway. By altering the properties of the side chain of one residue, we wanted to assess the importance of the hydrogen bonds. We converted Asp^{237} in the R2 protein into an asparagine and a glutamate residue in two separate experiments. Asp^{237} plays an essential role; it forms hydrogen bonds with His^{118}, Trp^{356} and Gla^{43} and thereby functions as a connecting point for three helices. It also participates both in the catalytic radical transfer (12) and in the generation of the tyrosyl radical (20, 51). Our data emphasize the requirement for hydrogen bonds between the conserved residues in the radical transfer route and strongly suggest that the catalytic radical transfer process in RNR is a coupled electron/proton transfer.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides used for mutagenesis and sequencing were synthesized and purified by Scandinavian Gene Synthesis AB. CzDP was obtained by cleavage of its CTP derivative by incubation with myosin and subsequent purification on Centricon-10. CzTP was purchased from Amersham Pharmacia Biotech. Bacterial Strains—*Escherichia coli* CJ336 (lacI q, ung-1, thi-1, relA/pCJ105 Cm r) and *E. coli* MV1190 (Δlac-proAB), thi, supE, Δ(srl-recA306::Tn10/F pruD36, pnuAB, lacP*ZAM15), obtained from Bio-Rad, were used for mutagenesis and cloning. *E. coli* MC1000 (ΔlacIP0ZAYAH74, galE, galK, strA, Δaro-leu7897, araD139, recA, srl::Tn10/pGP1–2, obtained from Amersham Pharmacia Biotech, was used for expression. Plasmids—pTZ18R, obtained from Amersham Pharmacia Biotech, contains a T7 promoter upstream of a cloning cassette (22). The plasmid...
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ptB2 is a derivative of ptZ18R, containing the E. coli nrdB gene, coding for the R2 protein (14). The plasmid pGP1–2, obtained from S. Tabor, codes for T7 polymerase under control of the λPr promoter and a heat-sensitive cl857 repressor under the control of the Lac promoter (23).

Oligonucleotide-directed Mutagenesis—Mutagenesis of ptB2 was performed using the Muta-Gen phagemid in vitro mutagenesis kit from Bio-Rad, based on a method described by Kunkel (24). Initial verifications of the mutations were done by polymerase chain reaction of the entire nrdB gene and cleavage with a restriction enzyme specific for the mutations, MnII for the D237E mutation, and BsrDI for the D237N mutation. The entire gene was then sequenced.

Expression of R2 Protein—The concentrated samples were loaded onto a Superdex 200 column and concentrated by Centricon 10/100 (Amicon Inc.) using a gradient of NaCl in 50 mM Tris buffer, pH 7.6. The further purification of the purified protein was then accomplished by a gel filtration step on a Superdex 200 column and concentrated by Centricon centrifugation.

Iron and Radical Analysis—The apo D237N mutant protein was reconstituted with 6 and 3 iron/R2 protein. The reconstitution was performed at 25 °C by mixing apoprotein in an oxygen-containing buffer with ferrous iron in an anaerobic solution. The uv-visible absorbance spectra were collected on a Perkin-Elmer Lambda 2 spectrophotometer. The contents of wild type and mutant proteins were determined from light absorption spectra using an extinction coefficient of 2,110 M cm⁻¹ for the tyrosyl radical (26). Iron contents were determined colorimetrically as described previously (27). The radical stability in the D237N mutant was determined spectrophotometrically by measuring the 410-nm absorbance at 25 °C every 10th minute for 3 h, with a scan rate of 240 nm/min. Recording of the first spectrum was started approximately 30 s after the addition of excess R1. The specific activity of R2 D237N was determined approximately 2 min after reconstitution of the protein.

Crystallization and Data Collection—The D237E mutant was crystallized in space group P2₁2₁2₁ with cell dimensions of 74.4, 85.6, and 115.7 Å. The crystallization of D237E was carried out in Petri dishes by the hanging drop method using the same conditions as for the wild type R2 protein (29). The reservoir contained 18 or 20% polyethylene glycol 4000 in 50 mM MES buffer, pH 6.0, with 0.2 M sodium chloride and 1.0 M ethylenetrihydroxy thiosalicylate as additives. The drops contained 5 μl of protein solution at a concentration of 20 mg/ml and 5 μl of reservoir solution. Diffraction data were collected to 1.95 Å on a Mar Research exposure using Cu Kα radiation at 100 K. The Rmerge of 8.5% and a completeness of 96%. Further data reduction was performed using programs of the CCP4 package (31) and the software package BIOMOL (Protein Crystallography Group, University of Groningen, The Netherlands).

RESULTS

Construction and Overproduction of Mutant R2 Proteins—The constructs R2 D237N and D237E contain only the desired GAC to AAT (D237N) and GAC to GAG (D237E) mutations as verified by dyeoxy sequencing. The D237N R2 protein was overexpressed in the presence of an iron chelator which could then be reactivated immediately prior to the experiments. No radical was present in D237N purified from material produced under normal growth conditions.

Three-dimensional Structure of the Asp → Glu Mutant Protein—The structure of the D237E mutant showed that a glutamic acid at position 237 is able to form the same hydrogen bonds as an aspartic acid in the wild type protein (Fig. 1). The lengths of the hydrogen bonds in the mutant protein differ slightly from those in the wild type protein. The closest distance between the two ligands of His118 and Asp237 or Glu237 were similar within experimental error, 2.7 Å in wild type R2 and 2.6 Å in the mutant R2. Also connected to Asp237 is Trp48, another residue in the proposed radical transfer pathway in wild type R2. Asp237 has only one possibility to form a hydrogen bond to Trp48, whereas Glu237 in theory can form two types of hydrogen bond connections to Trp48. The bond distances were...
3.3 and 2.9 Å in the D237E mutant and 2.9 Å in the wild type protein (Fig. 2, A and B). However, the shorter bond in the mutant has an angle that may be unfavorable for a hydrogen bond. Gln43, the third residue connected to Asp237, is positioned identically in the D237E mutant and in wild type R2, with a hydrogen bond distance of 3.3 Å in the mutant and 3.0 Å in the wild type.

Iron Binding, Tyrosyl Radical Formation, and Interaction with the R1 Protein of the Asp → Glu and Asp → Asn Mutant R2 Proteins—Light absorption spectra for wild type and mutant R2 proteins are shown in Fig. 1. Active wild type R2 shows a sharp peak at 410 nm, related to the tyrosyl radical, and absorbances at 325 and 370 nm related to the diiron-oxo center (Fig. 3A). Both mutant proteins could form a tyrosyl radical and also an oxo-bridged iron center (Fig. 3, B and C), although the spectrum of the D237N mutant protein was not as well resolved as were the wild type and the D237E spectra.

The iron and radical contents of wild type and mutant R2 proteins are presented in Table I. The data of wild type R2 and D237E are almost identical, whereas the iron content was higher, and the amount of radical formed was slightly lower in the D237N than in the wild type protein. However, these differences are small and not likely to be a major cause of loss of enzyme activity (see below).

The radical of the D237E mutant protein was as stable as the radical of the wild type protein. In contrast, the radical of the D237N mutant decayed with a rate constant of 0.37/h at 25 °C, which corresponds to a half-life of about 110 min (Table I).

We determined the R1-R2 interaction constants for the mutant R2 proteins. Table I shows that both mutant proteins have interaction constants similar to that of the wild type. From these data, we concluded that the mutant R2 proteins are not affected in their ability to bind to wild type R1.

The Asp → Glu Mutant Is Catalytically Active, in Contrast to the Asp → Asn Mutant—The specific activities of both mutant proteins were measured and are presented in Table II. The D237E was shown to have a significant enzyme activity, about 7% of the wild type activity. This is the first observation of a mutant affecting the radical transfer route in RNR that can still catalyze ribonucleotide reductase reduction. The low activity measured in the D237N mutant protein (0.3% of the wild type activity) corresponds approximately to the estimated amount of chromosomally encoded wild type protein in preparations from MC1009/pTB2/pGP1–2 constructs. It is therefore plausible that the D237N mutant protein per se is incapable of ribonucleotide reduction and that the low activity observed is due to a very small fraction of wild type contamination.

To differentiate between a low intrinsic enzyme activity in the mutant proteins and a low amount of contaminating wild type activity, we used the substrate analogue CzDP. CzDP is a suicidal substrate analogue that scavenges the tyrosyl radical of R2 in a single half-turnover reaction catalyzed by the holoenzyme (35–37). A protein with an intact radical transfer route will form a nitrogen-centered radical at the active site in R1 at the expense of the tyrosyl radical in R2, whereas a protein with a disrupted radical transfer route will not form the nitrogen-centered radical, and the tyrosyl radical will consequently not be lost. By CzDP inactivation experiments, we showed that the D237N mutant protein was unable to perform radical transfer. No nitrogen-centered radical was formed, and the tyrosyl radical decay had the same rate constant as in the isolated D237N protein. In the D237E mutant protein, the radical transfer was functional; we could observe a nitrogen-centered radical and a tyrosyl radical decay.

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Glu Mutant Protein—To determine an accurate formation rate for the nitrogen-centered radical in the D237E mutant protein and wild type R2 protein, we used stopped flow EPR spectroscopy at room temperature (Table II). The formation rate of the nitrogen-centered radical in the D237E protein was $0.084 \text{ s}^{-1}$, which is approximately 8 times slower than in the wild type. The corresponding tyrosyl radical decay was $0.044 \text{ s}^{-1}$, indicating that this mutant enzyme can perform radical transfer. The overall rate of the half turnover reaction in the mutant D237E is approximately 10% of the corresponding wild type rate (Table II). The only difference between the wild type and the mutant systems is the mutation in the R2 protein, since the same R1 wild type protein was used in both reactions.

CzDP Has the Same Binding Constant as CDP—The rate constant for formation of the nitrogen-centered radical in wild type protein was determined to be $0.7 \text{ s}^{-1}$ (Table II), which is faster than previously reported values of $0.23 \text{ min}^{-1}$ (36, 38), probably due to the efficient mixing technique used here. The decay rate of this radical (data not shown) agrees with the earlier published value (36). In comparison with $k_{\text{cat}}$ of the enzyme, which is about $7 \text{ s}^{-1}$, the CzDP-dependent reaction is a 10 times slower process. To exclude the possibility that the slower reaction rate with the CzDP substrate analogue was due to a nonsaturating CzDP concentration or a defective binding of the analogue to the active site, we determined the binding constant of CzDP to the wild type holoenzyme complex. The Lineweaver-Burke plot of Fig. 4 shows that the binding constant for CzDP is $0.27 \text{ mM}$. Using the method of Ormo et al. (39), we obtained a similar binding constant for the normal substrate CDP, a value that is in accordance with the previously reported CDP binding constant of $0.25 \text{ mM}$ obtained by equilibrium dialysis (40). From this, we concluded that the slower rate of the suicidal reaction with CzDP in wild type protein as compared with the multiple turnover of the CDP substrate is not due to decreased or defective binding of the analogue. Instead, it is most likely due to differences in the chemical reaction mechanism.

DISCUSSION

The existence of a dedicated radical transfer route in E. coli RR has been established by a combination of three-dimensional structure determinations, phylogenetic sequence comparisons, and site-directed mutagenesis experiments. As long ago as 1990 when the three-dimensional structure of the R2 protein was solved, Nordlund et al. (5) pointed out that an array of three strictly conserved residues connected the diiron site in R2 with the surface of the protein through hydrogen bonds. The subsequently solved three-dimensional structure of protein R1 showed that there was an array of three hydrogen-bonded residues that connected the active site of R1 with the surface of the protein (7). The modeling of an R1-R2 complex brought the two arrays of hydrogen-bonded residues together. A complete radical transfer route between the tyrosyl radical in R2 and the active site in R1 plausibly also comprises the R2 residue Tyr$^{256}$ (Fig. 1), which has been shown to be essential for binding of R2 to R1 (11) but which belongs to a disordered C-terminal part of the three-dimensional structure of the isolated R2 (5).

Extensive site-directed mutagenesis data support our hypothesis that this radical transfer pathway involves at least

![Fig. 3. Light absorption spectra of wild type and mutant R2 proteins.](Image)
nine conserved residues, six in R2 and three in R1 (cf. Fig. 1). Substitutions in four of these conserved residues in E. coli R2 (4, 11, 14) and all three in R1 (9, 10, 13) led to catalytically inactive enzymes. In related experiments in mouse R2, the two remaining conserved residues were substituted and shown to be part of the corresponding radical transfer pathway in this enzyme (12). However, all mutant proteins that have been constructed to date to affect the radical transfer pathway have been catalytically inert, since the active groups of the amino acid side chains have been removed by the engineering.

In this study, we have used site-directed mutagenesis to explore further the mechanism of catalytic radical transfer. We constructed two mutants, D237E and D237N, into one of the residues along the radical transfer pathway and showed that it can function with a mutant residue, provided that the hydrogen bonds and their character are preserved, as is the case in D237E. The glutamic acid in position 237 can form the same hydrogen bonds to Trp and His as in the wild type. The slightly longer distance of the bond to Trp (2.9 Å in wild type R2 and 3.3 Å in D237E) in the mutant suggests a weaker hydrogen bond, which could explain the reduced enzymatic activity of the D237E mutant.

The D237N mutant was inactive, although an asparagine residue is similar in size to aspartate and theoretically has the ability to form hydrogen bonds with other residues. To date, we have not been able to crystallize the D237N mutant. However, the preserved iron content, initial radical content, and R1-R2 interaction of the D237N mutant as compared with wild type R2 suggest that the loss of activity and the destabilization of the tyrosyl radical are due to a local structural change around the mutation. In yeast cytochrome c peroxidase (CCP), where a homologous His-Asp-Trp electron transfer pathway exists, it has been shown that the corresponding Asp → Asn mutant protein lacks the hydrogen bond to Trp because the indole side chain has flipped as a result of the mutation (41, 42).

A similar engineering experiment has also been performed in mouse protein R2, where it was shown that an Asp → Ala mutation at position 266 (corresponding to Asp237 in the E. coli enzyme) is catalytically inert (12). In contrast to the mouse D266A R2, which is a stable mutant, an alanine in the 237 position of E. coli R2 destabilizes the protein, which is extensively degraded during the purification procedure (43).

It has previously been shown that the hydrogen bonds between Cys496, Tyr730, and Tyr731 in protein R1 are essential for radical transfer in RNR (13). Engineering of either of the two tyrosines into phenylalanines in separate experiments led to catalytically inert mutants. The conclusion was that the preserved π-interaction between the aromatic rings in these mutant R1 proteins is not enough for radical transfer to occur (13), again emphasizing that an intact hydrogen-bonded network is important.

The radical transfer pathway in RNR is very long, about 35–40 Å, and therefore difficult to reconcile with current electron transfer theories (15–17, 19), which predict electron transfer rates that are orders of magnitude slower (<0.005 s⁻¹; Ref. 19) than the actual catalytic rate of the enzyme (1). Even the fact that hydrogen-bonded connections are more efficient than covalent bonds in electron transfer (18) is not sufficient to account for the catalytic rate of RNR. Recently a quantum chemical model study of hydrogen transfer between amino acids in the presence of radicals has been presented (44). These data suggest that many processes normally considered as electron transfer reactions in biological systems could be better explained as radical transfer processes, i.e. an electron concomitantly transferred with a proton. A radical transfer process would preserve neutrality and is energetically more favorable than a charge separation mechanism. Since charge separation is not required during RNR catalysis, radical transfer seems to be the most efficient and rational way to communicate the electron and fits very well with our experimental data on catalysis in RNR. A coupled electron/proton transfer process has also been suggested to occur in both E. coli and mouse protein R2 during the reconstitution reaction, where one of the four electrons needed to reduce molecular oxygen to water is suggested to use the Trp-Asp-His triad (20, 51).

CCP and its specific redox partner, cytochrome c, have been studied as a model system for intermolecular electron transfer process in proteins (45–49). There are striking similarities between the radical transfer routes in CCP and RNR. In both proteins, a hydrogen-bonded triad, consisting of His175-Asp235-Trp191 in CCP and His118-Asp237-Trp48 in R2 RNR, has its histidine residue coordinated to the metal site and its tryptophan residue at the protein surface. Asp235 in CCP (corresponding to Asp237 in RNR) has been converted into asparagine, alanine, and glutamate by site-directed mutagenesis experiments (42). Both D235N and D235A were unable to catalyze cytochrome c oxidation (<0.1% of the wild type CCP activity), but D235E showed 41% wild type activity. Structural data showed that the asparagine and alanine mutants completely destroyed the hydrogen bonding interaction in CCP, whereas the substitution to glutamate introduced only subtle changes in the hydrogen bonding interaction with Trp191 and His175 (41, 42).

The data presented in this study show that the radical transfer route in ribonucleotide reductase is not amino acid-specific but that the ability to form similar hydrogen bonds to the surrounding residues needs to be conserved in a functional enzyme. Also, the efficiency of radical transfer seems to depend strongly on the nature of the hydrogen bonds. The importance of the hydrogen bonds implies that the catalytic radical transfer process in RNR is a coupled electron/proton transfer process.

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