Evidence by Site-Directed Mutagenesis Supports Long-Range Electron Transfer in Mouse Ribonucleotide Reductase†

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ABSTRACT: Mammalian ribonucleotide reductase consists of two nonidentical subunits, proteins R1 and R2, each inactive alone. The R1 protein binds the ribonucleotide substrates while the R2 protein contains a binuclear iron center and a tyrosyl free radical, essential for activity. The crystal structures of the corresponding *Escherichia coli* proteins suggest that the distance from the active site in R1 to the tyrosyl radical buried in R2 is about 35 Å. Therefore, an electron pathway was suggested between the active site and the tyrosyl radical. Such a pathway could include a conserved tryptophan on the suggested R1 interaction surface of R2 and a conserved aspartic acid hydrogen bonded both to the tryptophan and to a histidine iron ligand. To find experimental support for such an electron pathway, we have replaced the conserved tryptophan in mouse R2 with phenylalanine or tyrosine and the aspartic acid with alanine. All the mutated R2 proteins were shown to bind metal with the same affinity as native R2 and to form the binuclear iron center. In addition, the W103Y and D266A proteins formed a normal tyrosyl free radical while only low amounts of radical were observed in the W103F protein. Neither the kinetic rate constants nor the equilibrium dissociation constant of the R1/R2 complex was affected by the mutations as shown by BIAcore biosensor technique. However, all mutant R2 proteins were completely inactive in the enzymatic assay, supporting the hypothesis that the tryptophan and aspartic acid residues are important links in an amino acid residue specific long-range electron transfer.

The enzyme ribonucleotide reductase (EC 1.17.4.1) plays a crucial role in DNA synthesis by catalyzing the direct reduction of all four ribonucleotides to the corresponding deoxyribonucleotides (Thelander & Reichard, 1979; Reichard, 1988). Mammalian ribonucleotide reductase belongs to the class I ribonucleotide reductases (Reichard, 1993; Thelander & Gräslund, 1994). In this class the active enzyme is a heterotetramer between two nonidentical homodimers called the R1 and R2 proteins, each inactive alone. The large subunit, protein R1, binds the substrates and allosteric effectors and contains redox active disulfides participating as electron donors during catalysis as shown for the *Escherichia coli* R1 protein (Thelander, 1974; Åberg et al., 1989; Mao et al., 1992). Each polypeptide of the small subunit, protein R2, contains a binuclear non-heme ferric iron center, which, during its formation, generates a stable tyrosyl free radical essential for activity (Ehrenberg & Reichard, 1972).

In the formation of deoxyribonucleotides, the hydroxyl group at the 2′-position of the ribonucleotide is reduced. The central theme in the mechanism is a transient transfer of radical properties from the enzyme to the substrate (Stubbe, 1990). The tyrosyl radical of protein R2 could be considered as storage of an oxidation equivalent which mediates the abstraction of a hydrogen atom from the 3′-position of the ribonucleotide bound to protein R1. This initial step yielding a free radical at the 3′-position facilitates the leaving of the protonated 2′-OH group. The cation radical intermediate is then reduced by the redox active dithiols of protein R1. Finally, the hydrogen atom abstracted by the tyrosyl radical in the first step is returned to the 3′-position and the tyrosyl radical is regenerated.

By site-directed mutagenesis the free radical of the *E. coli* R2 protein was located to Tyr122 (Larsson & Sjöberg, 1986). The 3-D structure of the R2 protein showed Tyr122 to be buried 10 Å inside the protein (Nordlund et al., 1990; Nordlund & Eklund, 1993). Although the crystal structure of the holoenzyme has not yet been solved, recent X-ray studies of the *E. coli* R1 protein suggest that the distance from the most likely R2−R1 interaction area to the active site of the R1 protein is approximately 25 Å (Uhlin & Eklund, 1994).

The involvement of the tyrosyl radical in the reduction of the substrate can therefore only be realized by either of two alternatives: a direct interaction through considerable conformational changes in the proteins R1/R2 during complex formation, or via long-range electron transfer from the protein R1-bound substrate to Tyr122 in protein R2. Structural changes are unlikely due to the very rigid α-helical structure of protein R2 (Nordlund et al., 1990). Furthermore, it has previously been shown that the tyrosyl radical EPR spectrum is the same for isolated R2 from *E. coli* and the R1/R2 complex (Sahlin et al., 1987). This argues in favor of the presence of long-range electron transfer from protein R1 to protein R2.

A connecting link of such a pathway may be found at the suggested R1−R2 interface. Amino acid residues with this function may have been conserved through evolution. A comparison between the amino acid sequences of the small

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subunits of ribonucleotide reductase of nine different species, ranging from bacteria to mammals and mammalian viruses, shows a low homology (Eriksson & Sjöberg, 1989). Only 16 residues are conserved in protein R2. Most of them are iron ligands, residues close to the iron center and to tyrosyl residue 122. However, one of the conserved residues, Trp48 in *E. coli* protein R2 (Trp103 in mouse R2), has been proposed as a connecting link for an electron transfer pathway from the R1 protein to the R2 protein (Nordlund et al., 1990; Nordlund & Eklund, 1993). Trp48 is found half buried at the proposed R1 interaction area of protein R2 and participates in a hydrogen-bonded network which connects it to the iron center. This network also includes the side chains of Asp237 (Asp266 in mouse R2) and His118, which is one of the iron ligands. Therefore, the proposed electron transfer pathway is composed of the amino acid triad Trp-Asp-His, which links the surface of protein R2 to the iron site through hydrogen bonds. Several lines of evidence suggest that Trp48 is involved in redox reactions at the iron site: Trp48 is postulated to form a transient radical during reconstitution of the iron site in the reaction between apoprotein R2, ferrous iron, and molecular oxygen (Bollinger et al., 1994). It is also suggested to be the site of a transient free radical in the corresponding reconstitution reaction of the point mutant R2 Y122F (Bollinger et al., 1994; Sahlin, personal communication).

Electron transfer is a fundamental process for proteins involved in the respiratory and photosynthetic chains. It has now been accepted that transport of electrons occurs between well separated redox centers within proteins and protein complexes (Winkler & Gray, 1992). A controversy in the literature concerns models of the nature of long-range electron transfer reactions in proteins: whether (1) there exists a uniform electronic barrier, leading to an exponential distance dependence of the transfer rate, independent of the nature of the detailed protein structure (Moser et al., 1992) or whether (2) the transfer rate is dependent on distance as well as local barrier heights (Beratan et al., 1992), which in turn depend on the detailed protein structure. In the latter model there is a role for redox active components and amino acid residues as bridges between donor and acceptor. Several lines of evidence favor model 2 in a general situation (Franzen et al., 1993). The results discussed in the present study should be regarded in the framework of model 2, including a preferred protein "pathway" for specific long-range electron transfer, and are in fact evidence in strong support of such a model in the present context.

Yeast cytochrome *c* peroxidase and its redox partner, cytochrome *c*, have been used as a model system for the intermolecular electron transfer process in proteins (Poulos & Kraut, 1980a,b; Waldemeyer et al., 1982; Ho et al., 1985; Cheung et al., 1986; Hazzard et al., 1987). There is a striking similarity between the radical site of cytochrome *c* peroxidase (Edwards et al., 1988) and the *E. coli* R2 protein. The indole nitrogen of a tryptophan (Trp191) is in hydrogen bond contact with an aspartic acid (Asp235) and a proximal histidine (His175) of the heme group. The free radical in cytochrome *c* peroxidase is localized on Trp191 (Sivaraja et al., 1989). Its EPR spectrum is unusual for a free radical and arises because the Trp radical is weakly coupled to a high valent state of the heme iron (Houseman et al., 1993). As suggested in the case of Trp48 in protein R2, the Trp191 is also located close to the interaction surface with its redox partner (Pelletier & Kraut, 1992). Site-directed mutagenesis of Trp191 → Phe showed a dramatic decrease in the catalysis of peroxide-dependent ferrocytochrome *c* oxidation (Mauro et al., 1988). The conclusion was that Trp191 may be an important link in an electron transfer pathway which was disrupted by the mutation.

In this paper we characterize three different mutants of the mouse R2 protein, in which Trp103 has been replaced by a phenylalanine or a tyrosine and Asp266 by an alanine. The effects of the substitutions on the properties of the tyrosyl radical−iron center, subunit interaction, and enzyme activity were studied.

MATERIALS AND METHODS

Plasmids. Full-length cDNA encoding the mouse ribonucleotide reductase protein R2 was previously cloned and the coding region ligated into the T7 RNA polymerase vector pET3a (Mann et al., 1991). This construct, called pETR2, was used as a source of DNA for site-directed mutagenesis. To simplify the construction of the Asp266 → Ala mutant protein (D266A), the pETR2 plasmid was first digested completely with *Hin*dIII and partially with *Eco*R1 to remove an *Eco*R1 and a *Cla*1 site in the vector. The resulting plasmid, lacking a 24 bp fragment, was then religated creating the plasmid pETR2-Cla/R1 with one unique *Cla*1 and *Eco*R1 restriction site in the R2 sequence.

Oligonucleotide-Directed Mutagenesis. Mutagenesis of Trp103 to phenylalanine or tyrosine (W103F/W103Y), and Asp266 to alanine, was made according to the Kunkel method as described in Sambrook et al. (1989). Oligonucleotides used for mutagenesis (underlining denotes mismatched nucleotides): W103F, (5′-CTCCTCGGCAGTGAAAAAG-GAGGCCT-3′); W103Y, (5′-CTCCTCGGCAGTGTAAAAG-GAGGCCT-3′) were purchased from Symbicon, Umeå, Sweden. The nucleotide sequence for D266A (5′-TAAAC-CCTCGGCTCTGCTAAT-3′) was synthesized on an Applied Biosystem 392 DNA/RNA synthesizer. The mutations in the R2 gene were verified by dideoxyribonucleotide sequencing in M13 (Tabor & Richardson, 1987). In the case of W103Y and W103F the correctly mutated fragment was recovered from M13 RF II DNA by digestion with *Pst*1 and *Nco*1, resulting in a fragment of 450 bp. This fragment was then cloned in pETR2 partially digested with *Pst*1 and completely with *Nco*1. The mutated D266A fragment from M13 RF II DNA was recovered by *Cla*1 and *Eco*R1 digestion and cloned in pETR2-Cla/R1 digested with the same enzymes. The constructs were transfected into the *E. coli* strain MC1061. After plasmid preparations, the constructs were again verified by dideoxyribonucleotide sequencing. The constructs were finally transfected into *E. coli* strain BL21(DE3)pLysS (Studier et al., 1990), that contains an IPTG inducible chromosomal copy of the T7 RNA polymerase gene and a plasmid with the T7 lysozyme gene.

Expression and Purification of Mutant R2 Proteins. The previously described expression system was used with minor exceptions to produce mutant R2 proteins (Mann et al., 1991).

Overnight cultures of BL21(DE3) pLysS containing the pETR2 plasmids were grown in LB medium containing carbenicillin (100 μg/mL) and chloramphenicol (25 μg/mL) for 12 h at 37 °C. The overnight cultures were diluted 1:100 in 1 L of TB medium (terrific broth) containing the same

antibiotic concentrations and shaken vigorously at 30 °C (400 rpm in a New Brunswick G-25KC incubator). At OD_{595} = 1 the incubator temperature was set at 15 °C, and when the culture had reached OD_{595} = 2, R2 expression was induced with IPTG (0.2 mM). After a subsequent 12 h of incubation, the culture was quickly chilled and centrifuged at 2500*g* for 15 min at 2 °C. The pellet was resuspended in 50 mM Tris, pH 7.6, and frozen in liquid nitrogen.

Frozen bacteria were gently thawed in a 25 °C water bath and centrifuged at 44000*g* for 40 min at 4 °C. Purification procedures for the mutated R2 proteins were performed as previously described for R2 native protein (Mann et al., 1991). Purification included nucleic acid precipitation with streptomycin sulfate at a final concentration of 2.5% (w/v), ammonium sulfate precipitation of the R2 protein at 40% saturation, desalting of the dissolved pellet on a Sephadex G-25 column, and chromatography on DEAE-cellulose.

Protein purity was analyzed by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis. The concentration of pure proteins was calculated using the extinction coefficient $\epsilon_{280-310}$ = 62 000 $M^{-1}cm^{-1}$ (Mann et al., 1991).

Light Absorption Spectra. Iron center formation of the mutated proteins was studied by light absorption spectroscopy in the wavelength range 280−600 nm. The spectra were recorded at 10 °C on a Varian Cary 4 spectrophotometer. Pure protein, about 50 μM, in a final volume of 400 μL of 50 mM Tris-HCl, pH 7.0, and 0.1 M KCl, was incubated with increasing amounts of a 16 mM ferrous ammonium sulfate solution freshly prepared under anaerobic conditions in the same buffer as above. The samples were well mixed before the spectra were recorded.

Immediately after the last optical recording, aliquots of the mixtures were transferred to EPR tubes and frozen in liquid nitrogen for later EPR measurement.

Electron Paramagnetic Resonance (EPR) Spectroscopy. EPR spectra (77 K) were obtained on a Bruker ESP 300 spectrometer equipped with a cold finger Dewar for liquid nitrogen. The tyrosyl free radical concentration was determined at 9.35 GHz, 20 mW by comparison with a standard sample of mouse protein R2 with a radical concentration of 49 μM (Mann et al., 1991).

Manganese Binding Assay. The affinity for manganese of the R2 proteins was determined using a filter binding assay modified after Söderman and Reichard (1986). All experiments were preformed at room temperature (25 °C). ^{54}Mn-labeled $MnCl_2$ (Du Pont, specific activity >1.48 TBq/g) was diluted with 100 μM nonlabeled $MnCl_2$ in 50 mM Tris-HCl, pH 7.6, and 0.1 M KCl, to give a specific activity of approximately 10 000 cpm/nmol. A constant amount (35−45 μg) of proteins R2 (native, W103Y, W103F, or D266A) was incubated for 10 min in a final volume of 1 mL of 50 mM Tris-HCl, pH 7.6, and 0.1 M KCl with $^{54}MnCl_2$ at increasing concentrations (0.5−10 μM). The incubation mixture was aspirated into a 2 mL plastic syringe and filtrated through a nitrocellulose filter attached to a filter holder (cf. Nyholm et al., 1993). ^{54}Mn bound to the R2 protein was determined by liquid scintillation counting of the filter. To minimize quenching, the metal ions were released by 10 min incubation in 0.5 mL of 1 M HCl. Unspecific binding of ^{54}Mn to the filter was determined at each concentration of $^{54}MnCl_2$ and then subtracted. The specific radioactivity of $^{54}MnCl_2$ was determined by adding 10 μL of 100 μM $^{54}MnCl_2$ directly to a filter followed by incubating 10 min with 0.5 mL of 1 M HCl.

Biosensor Analysis. Subunit interaction between the R1 protein and the mutated R2 proteins was studied using the Pharmacia Biosensor (BIAcore) method (Fägerstam, 1991; Jönsson et al., 1991). All experiments were performed at 22 °C. The mutated R2 proteins and the native R2 (30−80 μg) dissolved in 50 mM potassium phosphate buffer, pH 7.0, were immobilized on a Sensor Chip CM5 (Pharmacia Biosensor AB, Uppsala, Sweden) using an amine coupling method as previously described (Johnsson et al., 1991). Surfactant P-20 (20% Tween 20) and an Amine Coupling Kit containing EDC (*N*-ethyl-*N*-(diaminopropyl)carbodiimide), NHS (*N*-hydroxysuccinimide), and 1 M ethanolamine hydrochloride, pH 8.5, were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). The carboxylic acid groups of the dextran matrix were activated with 30 μL of a mixture of 0.2 M EDC and 0.05 M NHS. The R2 protein solution, 35 μL, was then injected over the surface, followed by the injection of 30 μL of 1 M ethanolamine hydrochloride, pH 8.5, to transform remaining active esters into amides. A flow of HBS buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 0.15 M NaCl, 0.05% Surfactant P-20, 10 mM $MgCl_2$, pH 7.4) was kept constant at 5 μL/min throughout the immobilization procedure. This procedure allows protein R2 to be covalently coupled via exposed amino groups to the (carboxymethyl)dextran-modified gold surface. Approximately 1200−1900 resonance units (RU), where a response of 1000 RU corresponds to a surface concentration of 1 ng of reactant/mm^2 of the 100 nm thick dextran layer, of the R2 proteins were immobilized.

Kinetics for the R1−R2 interaction were determined by allowing the immobilized R2 proteins to interact with increasing concentrations of pure recombinant R1 protein (see below) in a solution of HBS buffer also containing 2 mM dithiothreitol (DTT) and 0.125 mM dTTP. A constant flow, 5 μL/min, of the same HBS buffer used in the immobilization procedure was used as eluent during the subunit interaction. Therefore, no dTTP is present during the dissociation phase. The surface was regenerated between each protein R1 injection with 10 μL of 0.5 M KCl in HBS buffer.

When the immobilized ligand is allowed to interact with a constant concentration of free ligand through a continuous flow of buffer, complex formation is detected as a change in response over time. The rate of formation of surface complexes could then be described as:

$$dR/dt = k_{assoc}CR_{max} - (k_{assoc}C + k_{dissoc})R$$

where R corresponds to the response of the surface plasmon resonance detector, C is the concentration of the free ligand, $R_{max} - R$ is the remaining free binding sites at time t, and k_{assoc} and k_{dissoc} are the association and dissociation kinetic rate constants respectively (cf. O'Shannessy, 1994).

A plot of dR/dt vs R in the association phase for the different concentrations of the analyte, in this case the R1 protein, gives straight lines where the slope of the individual lines, k_s, is equal to $-(k_{assoc}C_0 + k_{dissoc})$. The association rate constant (k_{assoc}) can then be determined from a plot of k_s vs the concentration of protein R1, where the slope of the line gives the value of k_{assoc}.

After approximately 330 s when no R1 protein passes over the surface, bound R1 protein starts to dissociate from the immobilized R2 protein. This region can then be used to determine k_{dissoc} by making a $\ln(R_1/R_n)$ vs time $(t_n\text{-}t_1)$ plot. R_1 corresponds to the relative response at the start of the dissociation phase. The dissociation time at this point is set to zero. R_n and t_n represent subsequent response and time values during the dissociation phase. From this kind of plot, k_{dissoc} can be calculated as the slope of the line. At low protein concentrations, rebinding of the R1 protein affects the value of the rate constant k_{dissoc}. Therefore, the value of k_{dissoc} is chosen from the slope of the line that corresponds to the highest concentration of protein R1. The equilibrium dissociation constant (K_D) can be determined directly from k_{dissoc}/k_{assoc}.

Reactivation of Iron Center and Radical. Iron center and tyrosyl radical of protein R2 was regenerated according to the anaerobic procedure described earlier (Mann et al., 1991).

Enzyme Activity. The enzymatic activity of the different R2 proteins was measured in the presence of an excess of pure recombinant mouse R1 protein using the [^{3}H]CDP reduction assay as described earlier (Engström et al., 1979).

Mouse Protein R1. Recombinant mouse R1 protein was expressed and purified to homogeneity as described (Davis et al., 1994). The protein concentration was determined by the A_{280} absorbance using an $E^{1\%}_{1\ cm}$ of 12 (Thelander et al., 1980).

RESULTS

Construction of Mouse Protein R2 Expression Plasmids Having the Conserved Trp103 Replaced by Tyr or Phe, or the Conserved Asp266 Replaced by Ala. Recombinant mouse R2 protein expressed in *E. coli* using the T7 RNA polymerase pET expression system has been described earlier (Mann et al., 1991). Site-directed mutagenesis was now performed using the Kunkel method as described in Sambrook et al. (1989). All constructs were verified twice by dideoxynucleotide sequencing.

Protein Expression and Purification. To avoid possible formation of inclusion bodies, we choose to grow our mutant R2 expressing cells at 15 °C (cf. Davis et al., 1994). Instead of LB we used TB (terrific broth) medium which allows a much higher yield of bacteria per milliliter of medium. With these modifications, we obtained 30−50 mg of mutant protein/L of culture. The purification procedure was made according to a method previously described (Mann et al., 1991).

SDS−polyacrylamide gel electrophoresis showed that the purified mutant proteins had the same purity as the native R2 protein (Figure 1). All preparations contained about the same amounts of N-terminal truncated R2 protein which in the case of native R2 was shown earlier to be active (Mann et al., 1991).

The iron content of the purified mutated apo-R2 proteins was the same as for the native apo-R2 protein, i.e., less then 15%, where 100% represents two irons per polypeptide chain (Mann et al., 1991).

Immunoblotting using the monoclonal antibody YL 1/2 (Engström & Rozell, 1988) confirmed that no C-terminal proteolysis occurred during the expression and purification of the mutated proteins (data not shown).

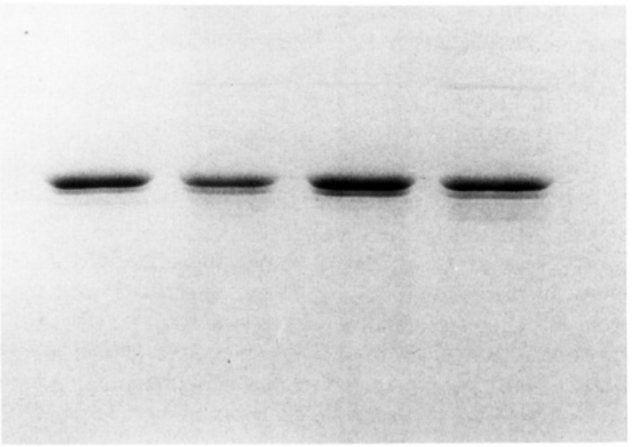

FIGURE 1: SDS−polyacrylamide gel electrophoresis of purified native protein R2, W103F protein R2, W103Y protein R2, and D266A protein R2. All samples (3 μg) were analyzed on a 12% polyacrylamide gel. Lane 1 (going from left to right), native protein R2. Lane 2, W103F protein R2. Lane 3, W103Y protein R2. Lane 4, D266A protein R2.

Light Absorption Spectra. Light absorption spectra for aerated native and mutated R2 apoprotein solutions after mixing with increasing amounts of an anaerobic ferrous ammonium sulfate solution are shown in Figure 2. The diferric iron center has prominent high absorption bands between 300 and 400 nm and the tyrosyl radical around 390−410 nm with a characteristic sharp feature around 410 nm (Petersson et al., 1980; Mann et al., 1991). The diferric iron center light absorption band is clearly visible at 329 nm, and the light absorption of the tyrosyl radical is apparent at 417 nm. The data suggest that all the mutated proteins formed an iron center, although the spectra were not as well resolved as the wild type spectrum. In addition, the W103Y and D266A R2 proteins after reconstitution with ferrous iron and oxygen exhibit the light absorption bands characteristic of the tyrosyl radical, while no significant radical absorbance could be detected in the W103F R2 protein.

Tyrosyl Free-Radical Content. More accurate quantification of the tyrosyl free radical generated in the different R2 proteins was made by EPR spectroscopy. Purified native and mutated R2 proteins reactivated with ferrous ammonium sulfate solution showed that W103Y and D266A could regenerate a tyrosyl radical with the same characteristics as for the native R2 protein (Figure 3) and in amounts comparable to native R2, where radical yields typically amount to 0.5−0.8 radicals/R2. In contrast, only a very weak radical was detected in the W103F protein. However, by studying the EPR microwave saturation at low temperature (Gräslund et al., 1994), we could show that this radical EPR signal was indeed mouse specific (data not shown). CD spectroscopy of this mutant showed a similar α-helical content as the native R2 protein (data not shown), indicating that the low radical content is not due to major conformational changes.

Manganese Binding Assay. Even if the spectroscopic data indicated that all the mutated R2 proteins could form an iron center, we wanted to measure if the mutations affected the affinity for iron. Since protein R2-bound Fe^{2+} is very easily oxidized to Fe^{3+} (Mann et al., 1991), we used the more stable Mn^{2+} as a probe for Fe^{2+} to study the affinity for iron of the native and mutated R2 proteins.

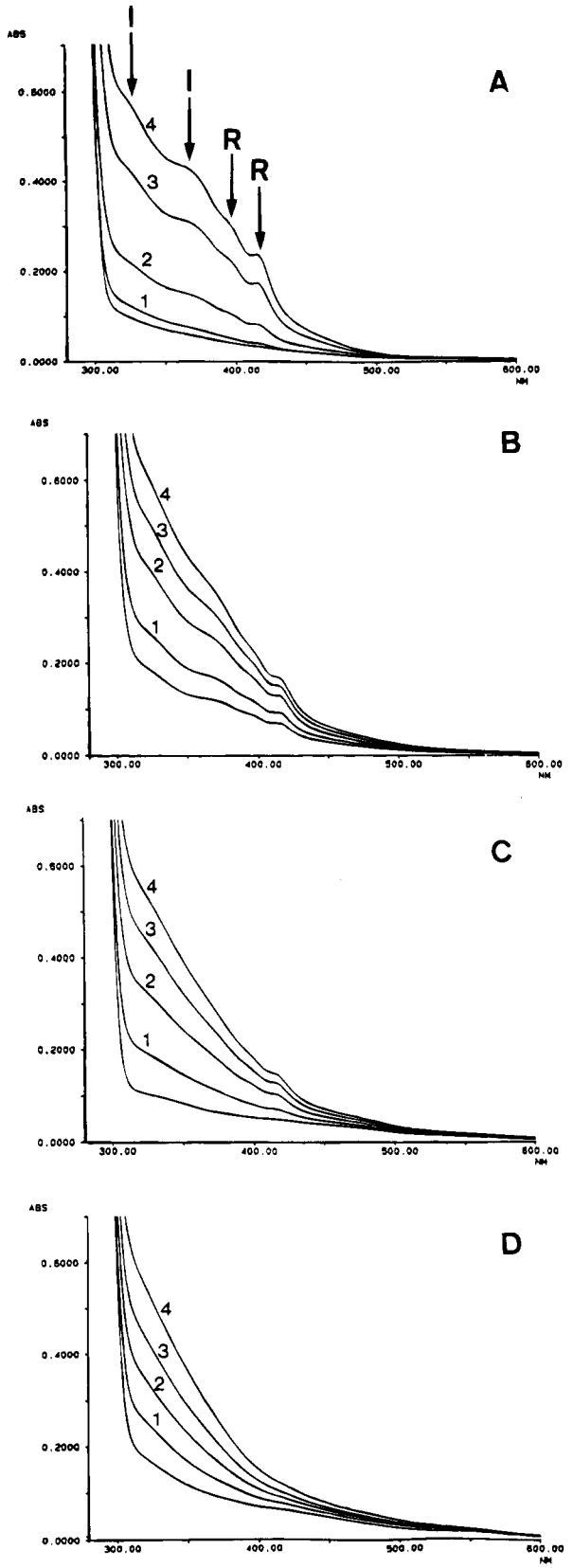

FIGURE 2: Light absorption spectra of native and mutated R2 proteins dissolved in 50 mM Tris-HCl, pH 7.0, and 0.1 M KCl. Pure apoprotein was incubated with increasing amounts of an anaerobic ferrous ammonium sulfate solution. (A) 52.6 μM native protein R2. (B) 49.7 μM W103Y protein R2. (C) 53.1 μM D266A protein R2. (D) 48.3 μM W103F protein R2. The numbers above each curve denote the molar ratio Fe/R2 added to the sample where the molar amounts of R2 are calculated using the polypeptide molecular weight of 45 000. The arrows denote the iron center (I) and tyrosyl radical (R) light absorption bands.

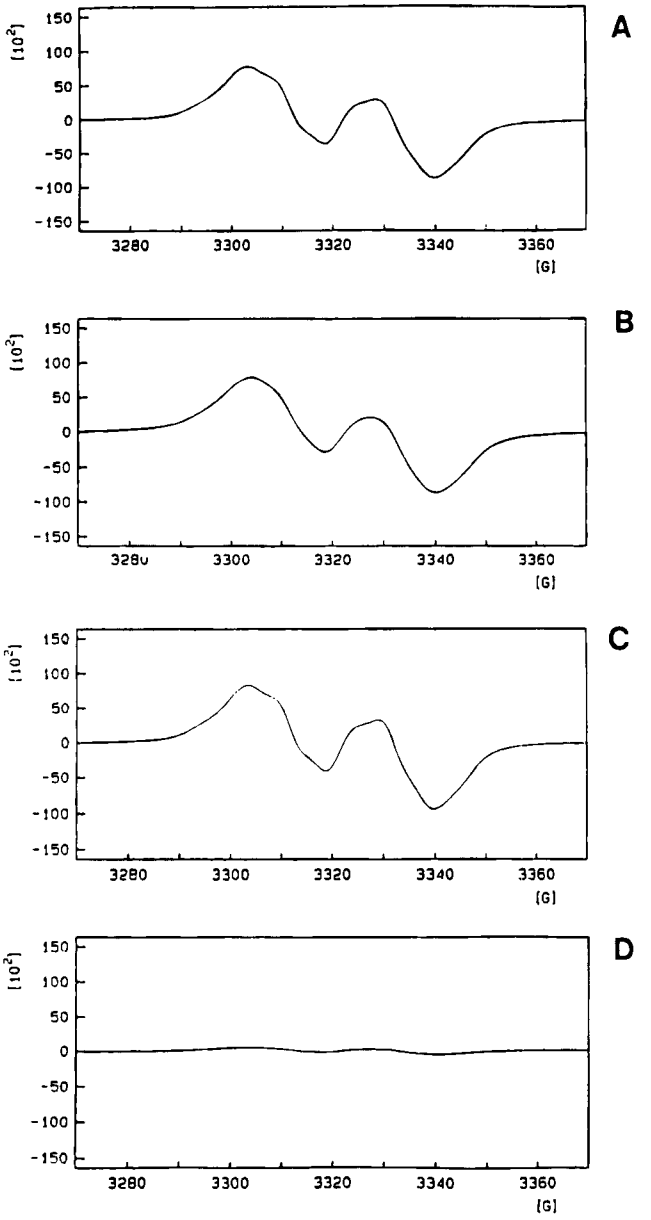

FIGURE 3: EPR spectra of R2 protein tyrosyl radicals recorded at 77 K. (A) Native protein R2. (B) W103Y protein R2. (C) D266A protein R2. (D) W103F protein R2. The solutions were prepared in a similar way as the ones used in Figure 2 (see Materials and Methods) but are not identical. The tyrosyl radical contents per polypeptide are as follows: native protein R2, 0.6; W103Y protein R2, 0.6; D266A protein R2, 0.6; and W103F protein R2, 0.03 mol/mol.

It has been reported that the *E. coli* R2 subunit has a very strong affinity for Mn^{2+} ions (Atta et al., 1992). X-ray crystallography showed that the manganese ions occupy the normal iron-binding sites, and therefore, Mn-R2 is suggested as a good model for the native diferrous form of protein R2.

Manganese binding to mouse protein R2 was studied using a nitrocellulose filter binding assay and ^{54}Mn (Materials and Methods). Using an excess of $^{54}MnCl_2$ and increasing amounts of protein R2, the binding increased in a linear way up to 60 μg of protein when the filter started to be saturated. Therefore, we chose to use 35−45 μg of protein for each point. Figure 4 shows a typical curve for the binding of $^{54}Mn^{2+}$ to native R2 protein. The unspecific binding of $^{54}Mn^{2+}$ to the filter increased in a linear way when the

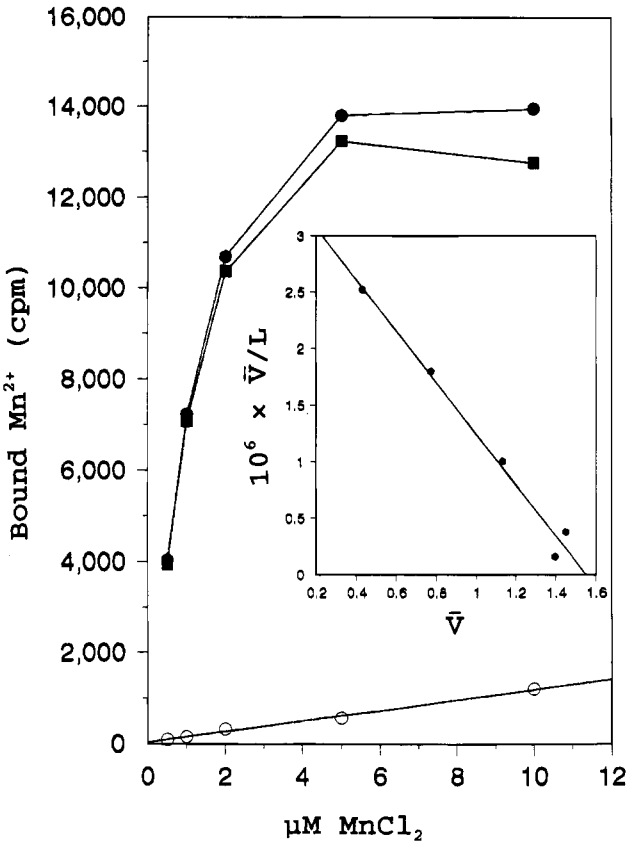

FIGURE 4: Binding of $^{54}Mn^{2+}$ to protein R2. Increasing amounts of $^{54}MnCl_2$ were incubated with a constant amount of R2 protein (42 μg) in 1 mL of 50 mM Tris-HCl, pH 7.6, and 0.1 M KCl as described in Materials and Methods. The *open circles* represent the unspecific binding without protein R2, the *closed circles* represent binding with protein R2, and the *squares* represent the specific binding, i.e., the difference between values obtained in the presence and absence of R2. The insert shows a Scatchard plot for the binding of Mn^{2+} to the native R2 protein. L represents the concentration of free manganese in μM, and V the amount of bound manganese per mole of R2 protein using a polypeptide molecular weight of 45 000.

Table 1: Equilibrium Dissociation Constant for Manganese Binding to Native and Mutated R2 Proteins

protein R2	K_D (μM)[a]
native	0.44
W103Y	0.52
W103F	0.68
D266A	1.2

[a] In the calculations, a polypeptide molecular weight of protein R2 of 45 000 was used.

concentration of $MnCl_2$ was increased. The specific binding was corrected for this background by subtracting unspecific filter binding from total binding at each concentration. The specific binding showed a similar saturation behavior for all the tested R2 proteins.

The dissociation constant, K_D, for manganese binding to protein R2 could be determined from Scatchard plots (Table 1), which also showed that around 1.5 mol of manganese bound per mole of R2 polypeptide chain (Figure 4, insert). No significant difference in binding stoichiometry or affinity for Mn could be detected for the mutated proteins when compared to the native R2 protein. Manganese most probably has the same affinity and binding sites as iron also for mouse R2 and therefore can be used to probe iron binding. We conclude that the mutations had not affected the iron binding.

Table 2: Rate Constants and Equilibrium Constants for the Binding of R1 Protein to Immobilized R2 Proteins

protein R2	k_{assoc}[a] (M^{-1} s^{-1})	k_{dissoc}[a] (s^{-1})	K_D[b] (μM)
native	3.3×10^5	5.5×10^{-2}	0.17
W103Y	3.8×10^5	4.7×10^{-2}	0.13
W103F	3.0×10^5	4.5×10^{-2}	0.15
D266A	3.0×10^5	4.8×10^{-2}	0.16

[a] The rate constants, k_{dissoc} and k_{assoc}, were determined using BIAlogue Kinetics Evaluation software using a dimeric molecular weight for protein R1 of 180 000. [b] The equlibrium constant, K_D, was calculated directly from the kinetic parameters k_{dissoc}/k_{assoc}.

Table 3: Ribonucleotide Reductase Activity of Native R2 Protein and the Different Mutated R2 Proteins

R2 protein	total protein (μg)	formation of dCDP/30 min at 37 °C (nmol)[a]
native	0.6	6.07
W103F	0.46	0.042
W103F	0.92	0.046
W103F	1.38	0.035
W103Y	0.82	0.046
W103Y	1.64	0.044
W103Y	2.46	0.054
D266A	2.0	0.027
D266A	4.0	0.110
D266A	8.0	0.095

[a] The activity of the protein was measured in the presence of 25 μg of pure recombinant mouse R1 protein (see Materials and Methods). In order to avoid iron depletion, which can inhibit the assay when high amounts of apo-R2 are used (cf. Filatov et al., 1992), we chose to use reactivated D266A R2 protein. The column background is approximately 0.04 nmol and has not been substracted from the values.

Subunit Interaction. Changes in surface plasmon resonance upon binding of protein R1 were used to study the kinetics of the R1/R2 complex formation. The immobilized R2 proteins were allowed to interact with increasing concentrations of pure recombinant mouse R1 protein in a buffer solution containing DTT and the allosteric effector dTTP. This effector was shown earlier to increase subunit interaction (Thelander et al., 1980). Figure 5A shows typical response curves when native R2 protein was allowed to interact with six different concentrations of the R1 protein. The association rate constant, k_{assoc}, and the dissociation rate constant, k_{dissoc}, were determined from Figure 5, panels B and C, respectively. All the binding parameters for the R1−R2 interaction are put together in Table 2. No significant difference in the kinetics of R1 interaction was found for native R2 protein and our mutated R2 proteins. Therefore, the point mutations had no effect on the R1−R2 binding.

Enzyme Activity. The specific activities of native R2 protein and the mutated R2 proteins were measured in the presence of an excess of pure recombinant mouse R1 protein. No significant activity could be detected for the mutated proteins (Table 3). At very high concentrations, the D266A mutant showed values above background. However, the values are not proportional to the amount of protein and therefore most likely are not significant. The same results were obtained with reactivated R2 proteins, as with apo-R2 proteins making it less likely that the mutations had affected the ability to regenerate the iron-radical center during assay conditions (cf. Mann et al., 1991). Furthermore, EPR spectroscopy showed that the tyrosyl radical of reactivated

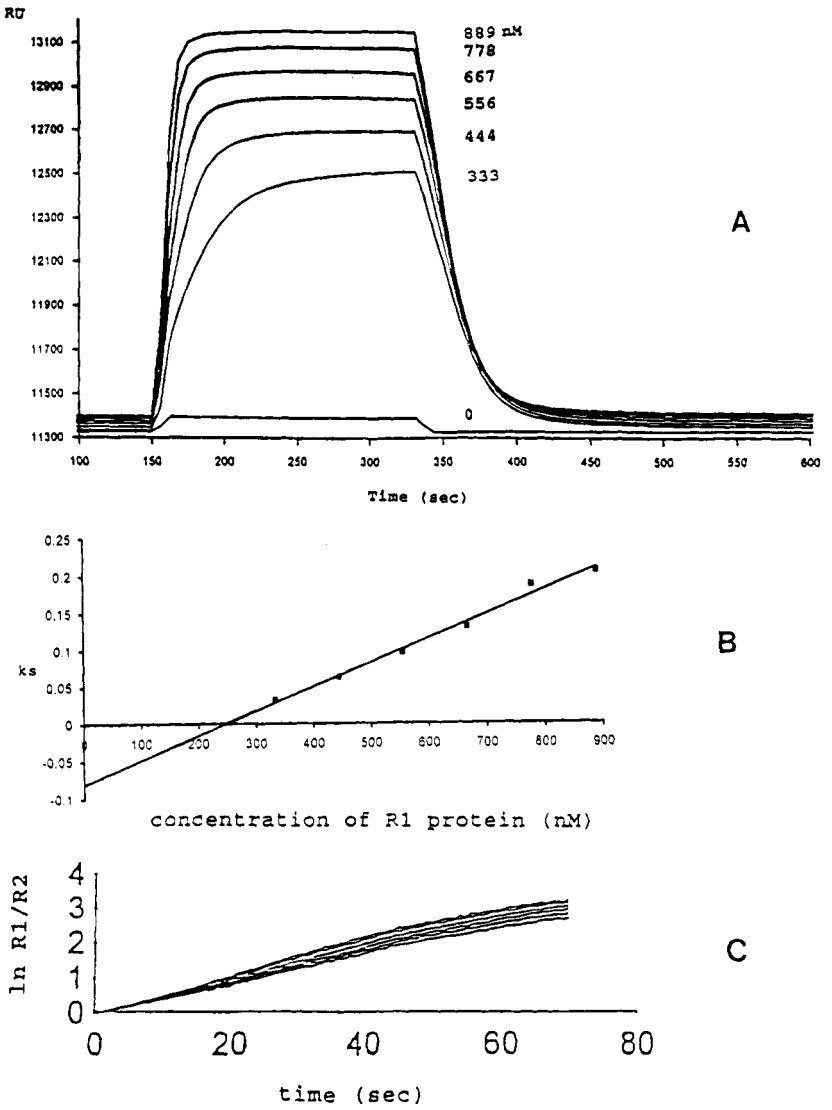

FIGURE 5: (A) Sensorgram showing interaction between R1 and native R2 protein at increasing concentrations of protein R1 (concentration given beside each curve). For details see Materials and Methods. The amount of R2 protein immobilized on the surface of the sensor chip was 1281 RU. The association phase starts after approximately 150 s, and the dissociation phase at around 330 s. In a control experiment were no protein was immobilized, injection of a 2100 nM R1 protein solution gave no significant response in RU. (B) The k_{assoc} was determined as the slope of the line from a plot of k_s vs the concentration of the R1 protein using a dimeric molecular weight of 180 000. (C) A $\ln(R_1/R_n)$ vs time plot at the same concentrations of protein R1 as in panel A. The slope of the line of the highest concentrations of protein R1 gives the value of k_{dissoc}.

W103Y protein, like the radical of reactivated native R2, was not decreased after incubation for 10 min at 25 °C in 10 mM DTT, indicating a similar stability (data not shown).

DISCUSSION

Nordlund et al. (1990) were the first to suggest that catalysis by the enzyme ribonucleotide reductase should be dependent on long-range electron transfer, since the 3-D structure of the *E. coli* R2 protein revealed that there is no direct contact between the tyrosyl radical of the R2 protein and the substrate at the active site of the R1 protein. A specific electron transfer should involve highly conserved residues of the R1 and R2 proteins to link the substrate binding site of protein R1 with the radical site on protein R2. For the R2 subunit, likely candidates for amino acids in such a pathway are the highly conserved residues Trp48 (*E. coli* R2 numbering) and Asp237. Both residues are coordinated to the binuclear iron center via a hydrogen-bonded network. The Trp48 is located half buried at the proposed interaction area of the R1 protein and the ϵ-hydrogen atom is hydrogen bonded to one δ-oxygen atom of Asp237, which also makes a hydrogen bond to the ϵ-nitrogen atom of the histidine iron ligand.

In the postulated model for the complex between proteins R1 and R2 (Uhlin & Eklund, 1994) the upper part of the R2 protein is positioned in an open cavity of the R1 protein with a 25 Å distance between Trp48 and the active site. Remodeling of the complex did not bring the Trp48 any closer to the active site. Long-range electron transfer from the conserved Cys439 at the active site of protein R1 to the tyrosyl radical in protein R2 is supposed to generate a thiyl radical. This Cys439 thiyl radical is then suggested to be responsible for the initial abstraction of the 3′-hydrogen of the substrate (Mao et al., 1992). From the 3-D structure of the *E. coli* R1 protein an electron transfer pathway between the active site of protein R1 and the tyrosyl radical of the R2 protein has been suggested to involve the conserved residues Tyr730 and Tyr731 (Uhlin & Eklund, 1994). It is

still unclear which residue in the R2 protein could link this chain to Trp48. According to site-directed mutagenesis of the R2 protein, a conserved tyrosine (Tyr356) is very important for enzyme activity (Climent et al., 1992; Filatov et al., 1992). It is possible that this tyrosine is the linking residue, but due to the very flexible C-terminal region of *E. coli* R2, this residue could not be localized in the 3-D structure (Nordlund et al., 1990).

Curious about the postulated long-range electron transfer, we wanted to study if we, by point mutating the suggested candidates, Trp103 and Asp266 in the mouse R2 protein, could find any evidence for the existence of this pathway. We chose to replace the tryptophan with a tyrosine or a phenylalanine and the aspartic acid with an alanine. Homology modeling of the 3-D structures with those replacements did not suggest any major disruption of the structure (Eklund, personal communication). The W103F R2 protein would create a mutant which lacks the possibility to form a hydrogen bond between position 103 and the δ-oxygen atom of Asp266. The W103Y mutant with its hydroxyl group should still be able to form this bond. The D266A mutant could no longer form a hydrogen bond to the ϵ-hydrogen atom of His173.

The most profound observation of this study is the complete loss of enzyme activity for the mutated R2 proteins. Even if the amounts of the mutated proteins added to the assay were 3−10 times more then the native protein, no significant activity could be detected.

The mutations did not affect the formation of the R1/R2 complex, as shown by binding studies using the very sensitive BIAcore technique. We chose to immobilize the R2 protein on the sensor chip due to difficulties in finding a suitable coupling method for the R1 protein. The dissociation constant for the *E. coli* R1/R2 complex has been determined to around 0.1 μM in the presence of 1.5 mM ATP (Climent et al., 1991). The K_D for the mouse R1/R2 proteins in the presence of 0.125 mM dTTP is 0.15 μM, which is very similar to the value for the bacterial enzyme. It is clear that the mutant mouse R2 proteins are able to bind to the R1 protein and therefore lack of enzyme activity is not due to an impaired R1−R2 binding. Binding of all three mutated R2 proteins to the R1 protein was also evident by their similar ability to compete with native R2 protein in the enzyme assay (data not shown).

All mutated R2 proteins had the same affinity for manganese binding and could regenerate a diferric iron center similar to that of the native R2 protein. Since this center is necessary for the formation of the tyrosyl radical, one might expect that all three mutants would be able to generate a tyrosyl radical. This is also the case for the W103Y and D266A R2 proteins. Interestingly, EPR spectroscopy of reactivated W103F R2 protein showed only a very low radical content. We have not yet been able to determine if this is due to inability to form the tyrosyl radical or if the radical when formed rapidly decays.

In conclusion, our data support the postulated amino acid residue specific long-range electron transfer between the active site of protein R1 and the tyrosyl free radical of protein R2. The R1/R2 system of ribonucleotide reductase may be an example of extremely long-range biological electron transfer using a very precise route along amino acids involving aromatic side chains as well as hydrogen bonds between residues.

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