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Chemical reduction of the diferric/radical center in protein R2 from mouse ribonucleotide reductase is independent of the proposed radical transfer pathway

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Dedicated to Professor A.G. Sykes

Abstract

The rates of reduction of the diferric/radical center in mouse ribonucleotide reductase protein R2 were studied by light absorption and EPR in the native protein and in three point mutants of conserved residues involved in the proposed radical transfer pathway (D266A, W103Y) or in the unstructured C terminal domain (Y370W). The pseudo-first order rate constants for chemical reduction of the tyrosyl radical and diferric center by hydroxyurea, sodium dithionite or the dihydro form of flavin adenine dinucleotide, were comparable with or higher (particularly D266A, by dithionite) than in native R2. Molecular modeling of the D266A mutant showed that the iron/radical site should be more accessible for external reductants in the mutant than in native R2. The results indicate that no specific pathway is required for the reduction. The dihydro form of flavin adenine dinucleotide was found to be a very efficient reductant in the studied proteins compared to dithionite alone. The EPR spectra of the mixed-valent Fe(II)Fe(III) sites formed by chemical reduction in the D266A and W103Y mutants were clearly different from the spectrum observed in the native protein, indicating that the structure of the diferric site was affected by the mutations, as also suggested by the modeling study. No difference was observed between the mixed-valent EPR spectra generated by chemical reduction in Y370W mutant and native mouse R2 protein.

Keywords: Ribonucleotide reductase; Tyrosyl radical reduction; Diiron–oxygen protein

1. Introduction

Ribonucleotide reductase is an essential enzyme in all living organisms as it catalyses the reduction of ribonucleotides to corresponding deoxyribonucleotides required for DNA synthesis [1–4]. Three classes of RNR have been described [1]. Mammalian and aerobically grown *Escherichia coli* ribonucleotide reductases belong to the class I enzymes. These enzymes consist of a 1:1 complex of two nonidentical homodimer proteins, named R1 and R2. Protein R1, the larger component, provides binding sites for the substrates and contains redox active dithiol groups participating in the reduction of the substrate.

Protein R2 contains a dinuclear μ-oxo-bridged Fe(III) center and a neighboring stable tyrosyl radical at residue 122 (*E. coli* numbering) [4]. This radical participates in the enzymatic process [5,6] whereas the dinuclear iron center is required for its generation and stabilisation. In active *E. coli* R2 protein, the radical can be reduced by the treatment with HU, HA [7–10]

Abbreviations: RNR, ribonucleotide reductase; RTP, radical transfer pathway; HU, hydroxyurea; HA, hydroxylamin; DA, dezaflavin; MV, methyl viologen; FAD, flavin adenine dinucleotide; DT, sodium dithionite; FMN, flavin mononucleotide.

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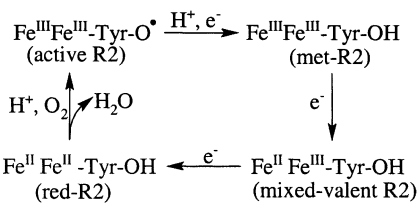
or dihydroflavins [11], leading to an inactive diferric protein without radical, called metR2 protein (Scheme 1).

More potent reductants can also transfer electrons to the diferric center resulting in a formation of reduced R2 protein with a diferrous site [12] (Scheme 1). A subsequent reaction of reduced R2 protein with molecular oxygen (called radical regeneration) leads to formation of active enzyme (Scheme 1).

The reduction reaction has been well studied in wild type *E. coli* R2 [8,9,11–14] but not in many mutants. Nevertheless, it has been demonstrated that the accessibility of the tyrosyl radical in *E. coli* R2 protein can be affected significantly by the mutations of its hydrophobic close environment [15].

For mouse R2, however, there is less quantitative data available concerning the reduction of the diferric/radical site. Early studies showed that mouse R2 is more susceptible to HU and analogues than *E. coli* R2 [16,17]. More recent reports concern reduction of mouse R2 by HU, DT or HA [18–21]. In the present study we have used three different reductants (HU, DT and $FADH_2$) to characterise the redox properties and investigate the mechanism of diferric/radical site reduction in native and mutant mouse R2 proteins.

X-ray crystallography and modeling studies on *E. coli* R1 protein suggest that the distance from the most likely R2-R1 interaction area to the active site in the R1 protein is approximately 25 Å [22]. Therefore, the involvement of the tyrosyl radical in the reduction of the substrate has been proposed to occur via a long range radical transfer pathway (hereafter called RTP) of conserved hydrogen-bonded residues from the bound substrate in protein R1 to the tyrosyl radical in protein R2 [23–29]. Theoretical [26] and experimental [29,30] studies suggest that the transfer is a coupled electron/proton radical transfer along the hydrogen bonds of the pathway. Three conserved amino acids of mouse R2 protein were originally suggested to participate in the RTP: H173, D266 and W103 [23]. The conserved Y370 was not seen in the crystal structure because it is a part of the flexible carboxy terminal tail of the protein. However, studies on catalytic activity have shown that it should be considered as part of the radical transfer chain operating in the enzyme reaction [31].



Scheme 1.

Previous studies have proposed [25,29] that the RTP is involved in the generation of the tyrosyl radical in mouse R2 by channeling a single reducing equivalent from external Fe^{2+} to the iron site in the protein. Mutation of any amino acid on the proposed pathway in mouse R2 results in a complete loss or significant reduction of enzyme activity although the D266A and W103Y mutants can form between 0.4 and 0.6 radicals/R2 [29] and Y370W up to 1.2 radicals/R2 [31]. Experimentally a maximum of about 1.6 radicals/R2 has been reported for native mouse R2 protein [32]. It was however demonstrated that the rate of tyrosyl radical formation in D266A and W103Y is approximately 20 times slower than in the native protein [29].

In the present study we wanted to probe if the same RTP is also involved in chemical reduction of the diferric/radical site. A combination of experimental and theoretical modeling studies on native and mutant mouse R2 proteins has provided some new insights about the structure of the iron site and the mechanism of its chemical reduction.

2. Experimental

Recombinant native mouse R2 protein and the mutants W103Y, D266A were prepared according to Refs. [33,25], respectively, and reconstituted by addition of 6 Fe(II) per R2 and molecular oxygen as described in Ref. [32]. The Y370W mutant was a generous gift of Dr. Ulrika Rova (Department of Medical Biosciences, Umeå University).

Mouse protein R2 concentration is based on the homodimer molarity ($\varepsilon_{280} = 124{,}000\ M^{-1}\ cm^{-1}$) [32]. The proteins were prepared and studied in 50 mM Tris–HCl, 100 mM KCl pH 7.6 buffer, containing 5% glycerol. The chemicals used are as follows: hydroxyurea (HU), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) (Sigma), sodium dithionite (DT), glycerol, ammonium ferrous sulfate (Mohr's salt) (Merck). The DT concentration was quantified by titration with potassium ferric cyanide ($\varepsilon_{420} = 1.03\ mM^{-1}\ cm^{-1}$).

Anaerobic samples for EPR and light absorption measurements were prepared by repeated cycles of evacuation and flushing with O_2-free argon gas. The mixed-valent EPR signals in mutant and native mouse R2 proteins were produced by illumination of the anaerobic sample, containing 150 μl protein/flavin (FMN) solution in the buffer, by projector light. After a certain time of reaction, the samples were frozen in liquid nitrogen and EPR spectra were recorded. EPR samples to monitor the reduction of tyrosyl radical by HU, DT and $FADH_2$ in native and W103Y mouse R2

proteins were produced in the following way: EPR tubes containing the reconstituted protein made anaerobic and a septum sealed bulb with the reductant (in solution) were thermostated at the reaction temperature (15 °C) in a water bath. A gas-tight Hamilton syringe, equipped with a long needle, was washed several times with the anaerobic solution to remove oxygen. Then the anaerobic reductant was mixed with the anaerobic protein in the EPR tube. The tubes were kept in the water bath, and after a certain time the reaction was stopped by immersing the EPR tubes into cold *n*-pentane (− 120 °C). For each series one sample was kept as a reference (not mixed with reductant).

2.1. EPR measurements

EPR signals from the mixed valent state were recorded in the temperature range 4–77 K on a Bruker ESP300 spectrometer equipped with an Oxford Instrument ESR 900 liquid helium flow system. Spectra to monitor the tyrosyl radical reduction were recorded on a Bruker Elexsys E500 system, using a rectangular mode Bruker EPR cavity (ER 4116DM) at 77 K. Spectra were evaluated using Xepr 2.0 software from Bruker.

2.2. Spectrophotometric measurements

Light absorption spectra were recorded on a Varian Cary 4 spectrophotometer in rectangular anaerobic cuvettes with a path length of 1 cm and a volume of 600 μl at 15 °C. The reaction was started by addition of microliter volumes of DT (or HU) to the anaerobic sample containing protein solution in the buffer. The kinetic curves were fitted using the program GRAFIT 3.09b.

2.3. Computer modeling of native and D266A mouse R2 proteins

The coordinates of native mouse R2 protein were downloaded from the Brookhaven Protein Data Bank (with entry code 1XSM) and used as starting point for model building of native and D266A and W103Y R2 structures. Since Fe_A was not seen in the crystal structure[2] of native mouse R2 [34], we first introduced a Fe^{3+} ion into the expected iron binding site, added a μ-oxo-bridge, and performed energy minimization. Then the single amino acid substitution was introduced and the structure was energy minimized.

Insight II was used to introduce the amino acid substitutions of the native structure on a Silicon Graphics workstation. Geometry optimization of the model molecules was performed by CHARMm, which is built into Insight II. Molecular visualization and measurements of distance and dihedral angles were performed by Chem3D Pro program from Cambridgesoft on a PC.

[2] The mouse R2 native protein crystal structure contains only one Fe ion (Fe_B) because the other one (Fe_A) was lost during the crystallisation procedure at low pH.

3. Results and discussion

One major question in the present study concerned the participation of the proposed hydrogen bonded RTP (H173, D266, W103) in chemical reduction. We have therefore compared the properties of native R2 protein with three point mutants located in the pathway (D266A, W103Y) or in the flexible tail (Y370W). We first look at a modeled iron cluster and observe the mixed-valent EPR spectra, then study the rates of reduction using HU, DT and $FADH_2$ as reductants.

To investigate how the mutations D266A and W103Y on the proposed RTP affect the structure of the diferric/radical site we have built models of these mutants based on the coordinates of native mouse R2 [34] available from the Protein Databank. As described in the Material and Methods section, first a diferric iron site was modeled into the native protein. Comparing the modeled native iron site with the crystal structure we observed only minor changes of the iron ligands (0.1–0.5 Å shifts of selected inter-ligand distances). The ligands in the mutants were somewhat more affected, particularly the positions of the two histidines (H173 and H270) and the aspartic acid (D139) ligand. Distances measured between these ligands and the Y177 phenyl oxygen were found to vary up to 0.8 Å as compared to the native protein crystal structure. The iron sites of the modeled native and mutant proteins are shown in Fig. 1(A–C).

Since the residue Y370 is not observed in a crystal structure, it was not possible to obtain any structural model for the Y370W mutant. However, since Y370 is located in the flexible C-terminus domain relatively far from the diferric/radical site, we assume that its mutation should not directly affect the ordered structure.

3.1. Structural modeling of D266A and W103Y mutants

3.1.1. Dinuclear iron site is gated by residue 266

Residue D266 in mouse R2 is located in the second ligand sphere of the dinuclear iron site (Fig. 1(A)). It is hydrogen bonded to the iron ion ligand H173 and the surface residue W103, thus fulfilling an important role in the proposed long distance coupled electron/proton transfer pathway. The structural model has shown (Fig. 1(B)) that substitution of aspartic acid 266 to alanine not only cuts off the proposed electron/proton transfer

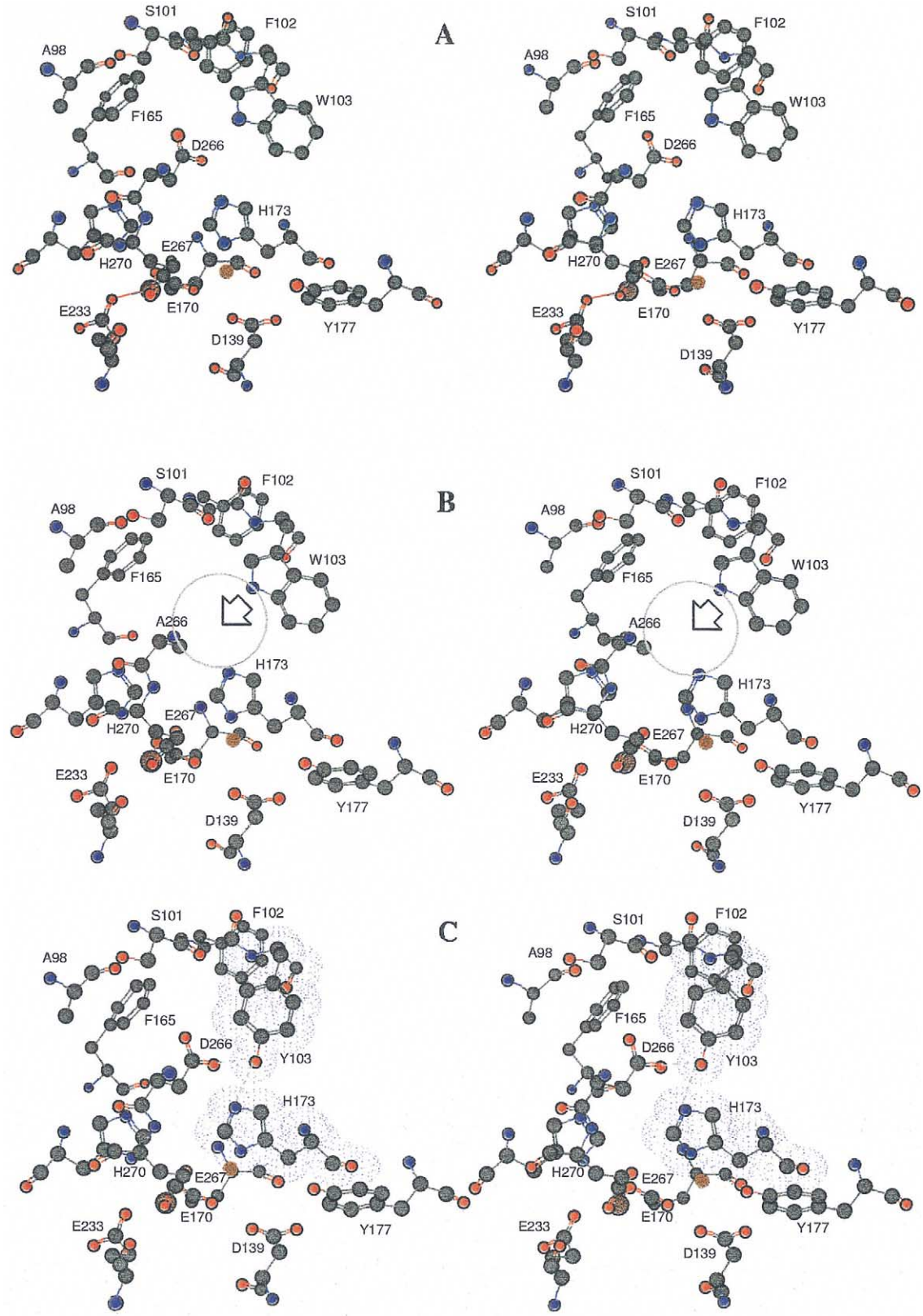


Fig. 1. (A) A model of native mouse R2 structure in a stereo view with offset of 10°. For clearer view only the radical site and radical transfer pathway residues are presented (the iron ions are shown in dark red colour); (B) a model of D266A adopted from the native protein coordinates by single amino acid substitution and geometry optimisation. The circle indicates a new hydrophobic cavity formed whereas the arrow indicates the direction from the protein surface towards the redox site; (C) a model of the W103Y mutant shows that tyrosine 103 is now in van der Waals contact with the iron ligand H173 (both are rendered with electronic sphere).

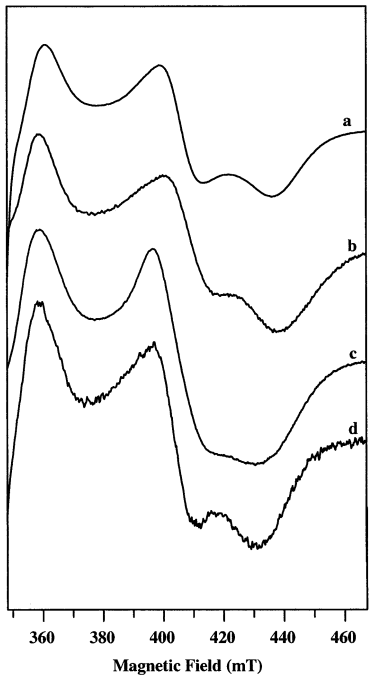


Fig. 2. EPR spectra of (a) 0.2 mM native mouse R2; (b) 0.2 mM W103Y mouse R2 mutant; (c) 0.2 mM D266A mouse R2 mutant; (d) 0.1 mM Y370W mouse R2 mutant after 3 min of illumination with 100 μM FMN in 50 mM Tris–HCl, 100 mM KCl, pH 7.6 buffer at 10 °C under anaerobic conditions. The measurement conditions are the following: microwave frequency 9.62 GHz, modulation frequency 100 kHz, modulation amplitude 10 G, microwave power 10 mW, temperature 4 K.

chain, but also makes the iron-radical environment less negatively charged as compared with the native protein. A striking difference from the native protein R2 is that external molecules have more direct access to the iron center, especially to the ligands E267, H173 and H270. The ligand residue H270 is already known to be accessible to the solvent from the surface and this has been proposed to be one major reason for the difference in reduction properties of mouse R2 and *E. coli* R2 [34]. There is no hydrogen bond to H173 from residue 266 in the mutant. An obviously hydrophobic cavity of approximately 8×10 Å composed of A98, S101, F102, F165 and A266 is an ideal location for the interaction with small molecules as indicated by the circle and arrow in Fig. 1(B).

3.1.2. Mutation W103Y introduces a hydrogen bond to the iron ligand H173

According to the structure model (Fig. 1(C)) the W103 residue is in van der Waals contact with the side chain of H173, an iron ligand. A hydrogen bond to H173 from W103, which is absent in the native protein, produces some direct effects on the ligands of the dinuclear iron center.

3.2. EPR characterisation of chemically generated mixed-valent diiron centres in D266A, W103Y and Y370W

It has been reported that unlike in *E. coli* R2 a stable mixed-valent Fe(II)Fe(III) state can be produced in active mouse R2 protein by a two electron chemical reduction [18,35]. The Fe(II)Fe(III) state gives rise to an anisotropic EPR signal [18,33], the shape of which depends on interactions between the Fe(II)Fe(III) site and surrounding ligands. We expected the EPR to be sensitive to the changes of ligand geometry that were suggested from the modeling results.

Fig. 2(a–c) shows that mixed-valent EPR signals generated by chemical reduction in native and the two mutant (D266A and W103Y) mouse R2 proteins have significant differences in the high field g_3 region. This observation provides evidence that the structural changes caused by point mutations have affected the interaction between the diferric site and its closest ligands, in agreement with the modeling results. The mixed-valent EPR signal observed in Y370W mutant is virtually identical to the mixed-valent signal generated by chemical reduction in native mouse R2 protein (Fig. 2(a,d)) suggesting that there is no significant effect of this mutation on the structure of the diferric/radical center in mouse R2 protein.

3.3. Reduction of D266A, W103Y and Y370W by HU, DT and $FADH_2$

3.3.1. Reduction by hydroxyurea

For the reduction studies we chose to compare native mouse protein R2 with two mutants on the proposed RTP (D266A, W103Y) and one in the flexible C terminal tail (Y370W). The reduction of the iron/tyrosyl radical sites in native, D266A and Y370W mutant mouse R2 proteins by 2 mM HU was monitored by light absorbance at 15 °C. The spectra were collected with 3 min interval after the start of the reaction. A typical kinetic curve is shown in Fig. 3. The data points at 414 nm were used to evaluate the rate and order of the reaction (Fig. 3, (insert)). Due to precipitation of the protein upon reconstitution, the reduction of the iron/tyrosyl radical site in W103Y mutant mouse R2 protein could not be monitored by light absorbance (because of light scattering from the precipitated protein). By EPR, however, the precipitation does not significantly affect the amount of detected radical. To make sure that the two methods were compatible we produced EPR samples of native mouse R2 protein reduced in the same manner as the samples produced for light absorption measurements. We found that the rate constants determined by the two different methods and repeated measurements on the native protein could vary by about a factor of 2. We therefore consider our results to show significant differences between rate con-

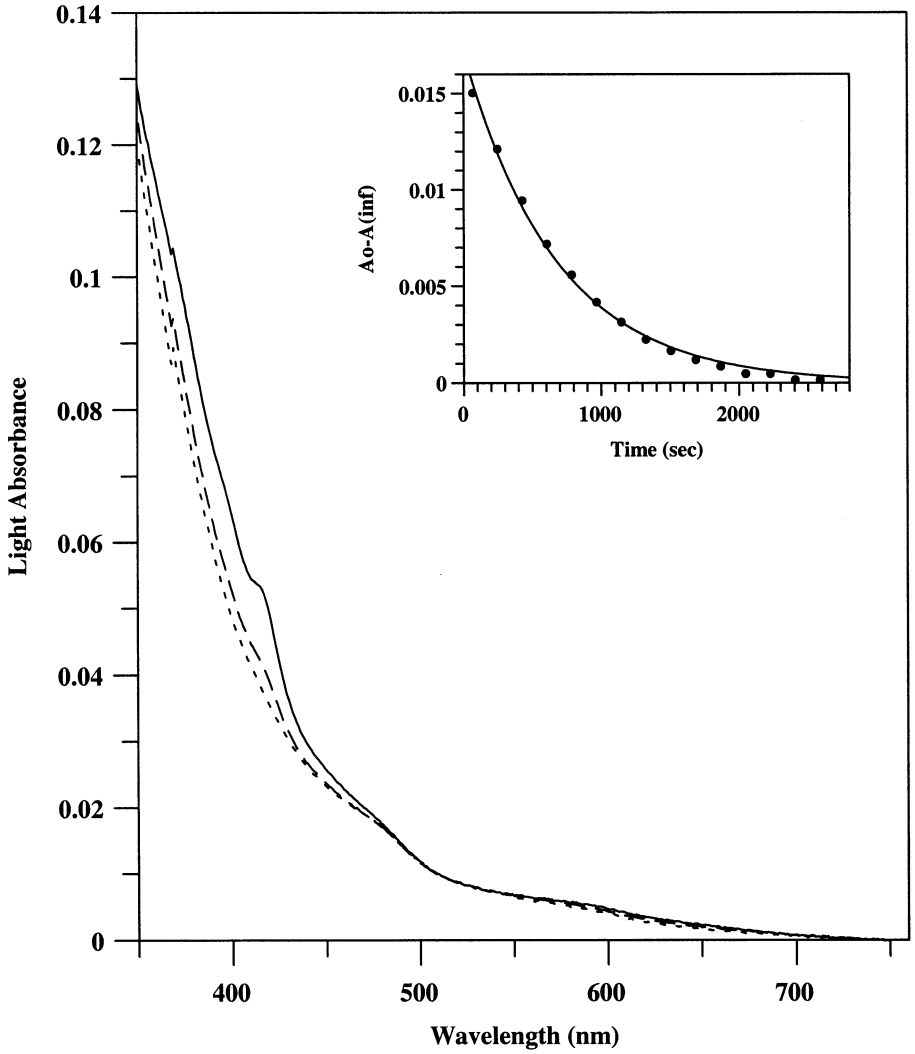


Fig. 3. Light absorption spectra recorded at the beginning (—) and after 970 s (– – –) and 7100 s (---) of anaerobic reaction of 13 μM D266A mouse R2 mutant with 2 mM HU at 15 °C. Insert: time dependence of the tyrosyl radical absorbance measured at 414 nm in the reaction of 10 μM D266A mouse R2 mutant with 2 mM HU at 15 °C.

stants only if they differ by significantly more than a factor of 2.

We found that in all cases the experimental data could be easily fit by a pseudo first-order rate equation. The second order rate constants obtained after fitting of the experimental data are presented in Table 1, and show that the reduction of the tyrosyl radical by HU in the mutant proteins occurred with similar rates as in the native protein.

The similar rate constants of tyrosyl radical reduction in the mutants compared to the native protein indicate that an intact RTP is not required for HU reduction, i.e. the reduction equivalents are not necessarily delivered via this particular pathway. This is in agreement with earlier studies on *E. coli* R2, where the absence of any saturation of the rate of HU reduction was interpreted in terms of unspecific delivery without any specific interaction site [36].

3.3.2. Reduction by DT and $FADH_2$

The studies on the reduction in mouse R2 protein and its mutants by DT and dihydroflavins were carried

Table 1
The second-order rate constants (M^{-1} s^{-1}) of tyrosyl radical reduction in native, D266A, Y370W and W103Y mouse R2 proteins by HU, DT and $FADH_2$ at 15 °C

Reductant	Native [a,d]	D266A [b,d]	W103Y [c,d]	Y370W [b,d]
HU	0.5	0.7	1	0.5
DT	2	20	0.8	8
$FADH_2$	1300	4000	500	1900

[a] Average value of two measurements by light absorption (414 nm) and one measurement by EPR at 77 K.
[b] Monitored by light absorption at 414 nm.
[c] Monitored by EPR at 77 K.
[d] The uncertainty is about a factor of 2, estimated by comparison of results from repeated experiments.

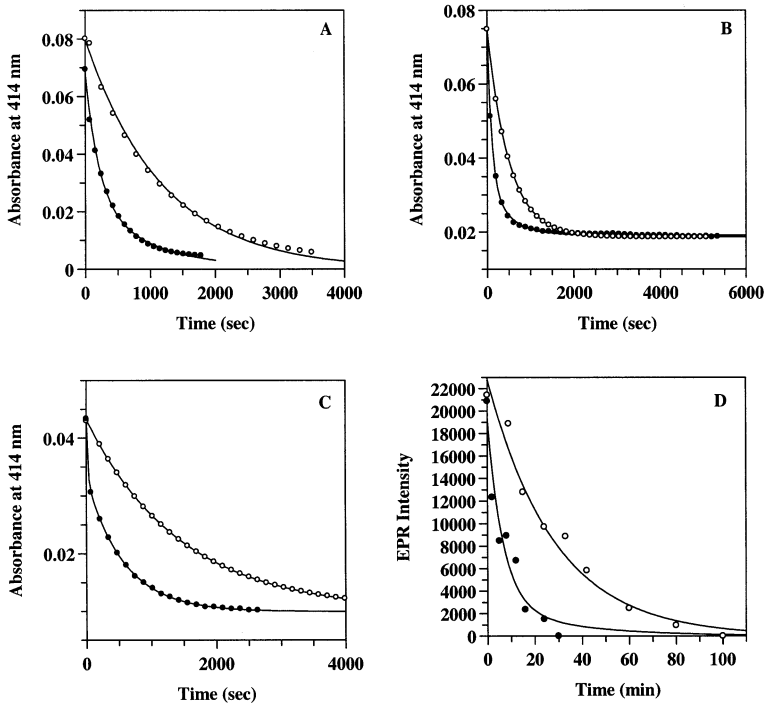


Fig. 4. Time dependence of the tyrosyl radical decay measured by light absorption spectroscopy at 414 nm at 15 °C (A–C) and EPR spectroscopy at 77 K (D) in the reaction of DT (○) and DT with FAD as mediator (●). Native mouse R2 protein; (B) D266A mouse R2 protein mutant; (C) W370Y mouse R2 protein mutant; (D) W103Y mouse R2 protein mutant. The conditions are the following: (A) 9 μM protein, 2 μM FAD, 200 μM DT; (B) 13 μM protein, 2.43 μM FAD and 100 μM DT; (C) 8 μM protein, 2.77 μM FAD, 100 μM DT; (D) 15 μM protein, 6 μM FAD, 600 μM DT. (—) A theoretical curve generated by non-linear regression fit to the experimental data using Eqs. (1) and (2).

out under anaerobic conditions on samples containing 10–15 μM protein, 0.1–0.65 mM DT and 2–10 μM flavin (FAD) as mediator when the reduction by flavin was studied. Since there was a large excess of DT and the reduction of flavins by this agent is thermodynamically favorable, the flavin was maintained in dihydroform during the reaction. The reduction of the iron/radical site in D266A, Y370W and native mouse R2 protein was followed by monitoring the decay of light absorption at 375 and 414 nm for the iron center and the tyrosyl radical, respectively. The reduction of tyrosyl radical in W103Y was followed by monitoring the decay of intensity in the EPR signal.

In native and mutant mouse R2 proteins D266A, Y370W we observed reduction of both the iron center and the free radical by dihydroflavin as well as by DT (Fig. 4(A–C)). The data presented in Fig. 4, representing the reduction of the proteins by DT alone, can easily be fit by a pseudo first-order rate equation:

$$A(t) = A_0 e^{-k_1 t} + A_\infty \quad (1)$$

where A_0 is the initial absorbance originated from the tyrosyl radical or Fe(III)Fe(III) iron center, A_8 is the remaining absorbance after the end of reduction and $A(t)$ is the absorbance at time t after the start of the reaction; k_1 is a pseudo first-order rate constant.

In the case of reduction where dihydroflavin was employed as mediator together with DT we observed kinetics which could be better fit by a two component equation:

$$A(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_\infty \quad (2)$$

where k_1 is the known rate constant for the reduction of the iron/radical site by DT alone and k_2 is the rate constant for the reduction of the same site by dihydroflavins. k_1 was found by fitting the experimental data of Fig. 4 (marked by ○) with Eq. (1). k_2 for $FADH_2$ was obtained by fitting the data of Fig. 4 (marked by ●) with Eq. (2) taking into account the predetermined rate constant k_1. The rate constants obtained by fitting the experimental data are shown in Table 1.

Like in the case with HU, the tyrosyl radical in W103Y and Y370W is reduced by DT and $FADH_2$ with similar rates as in the native protein. Only the mutant D266A appears to be significantly different from the native protein. Table 1 shows that the tyrosyl radical in D266A is more rapidly reduced, particularly by DT. The higher, or similar, rates of radical reduction in D266A, W103Y and Y370W indicate that none of the reductants studied here require an intact RTP to reduce the radical in mouse R2 protein.

From Table 1 it can also be concluded that particularly $FADH_2$ reduces tyrosyl radical in mouse R2 by high and similar efficiency. Despite its being a larger molecule dihydroflavin seems to be much more potent reductant than DT alone. The reason for its high efficiency may be a combination of accessibility and a suitable redox potential. We also observed by the light absorption measurements at 375 nm that DT and $FADH_2$ reduce both the iron site and the tyrosyl radical with similar rate constants (data not shown).

4. Concluding remarks

Our modeling studies and reduction experiments on two point mutants, D266A and W103Y, suggest that even relatively distant mutations within the protein globule may significantly affect the structure and properties of the diferric/radical site in mouse R2 protein.

The data on reduction of native mouse R2 and the three point mutants, D266A, W103Y and Y370W, by different reductants indicate that an intact RTP is not required for the reduction equivalents to be efficiently delivered to the iron/radical site. It is now clear that the exogenous reductants, such as HU, DT, or even the larger FAD, etc. deliver reducing equivalents to the active site of mouse R2 through unspecific pathways. The D266A mutant appears to be more easily reduced than the native protein. This is in contrast to the situation with the radical reconstitution reaction, where one reducing equivalent is transferred preferentially via the specific pathway which is also involved in the catalytic reaction [29].

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