Autocatalytic Generation of Dopa in the Engineered Protein R2 F208Y from *Escherichia coli* Ribonucleotide Reductase and Crystal Structure of the Dopa-208 Protein[†,‡]

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ABSTRACT: The mutant form Phe-208 → Tyr of the R2 protein of *Escherichia coli* ribonucleotide reductase contains an intrinsic ferric–Dopa cofactor with characteristic absorption bands at 460 and ca. 700 nm [Ormö, M., de Maré, F., Regnström, K., Aberg, A., Sahlin, M., Ling, J., Loehr, T. M., Sanders-Loehr, J., & Sjöberg, B. M. (1992) *J. Biol. Chem. 267*, 8711–8714]. The three-dimensional structure of the mutant protein, solved to 2.5-Å resolution, shows that the Dopa is localized to residue 208 and that it is a bidentate ligand of Fe1 of the binuclear iron center of protein R2. Nascent apoR2 F208Y, lacking metal ions, can be purified from overproducing cells grown in iron-depleted medium. ApoR2 F208Y is rapidly and quantitatively converted to the Dopa-208 form *in vitro* by addition of ferrous iron in the presence of oxygen. Other metal ions (Cu^{2+}, Mn^{2+}, Co^{2+}) known to bind to the metal site of wild-type apoR2 do not generate a Dopa in apoR2 F208Y. The autocatalytic generation of Dopa does not require the presence of a tyrosine residue at position 122, the tyrosine which in a wild-type R2 protein acquires the catalytically essential tyrosyl radical. It is proposed that generation of Dopa initially follows the suggested reaction mechanism for tyrosyl radical generation in the wild-type protein and involves a ferryl intermediate, which in the case of the mutant R2 protein oxygenates Tyr 208. This autocatalytic metal-mediated reaction in the engineered R2 F208Y protein may serve as a model for formation of covalently bound quinones in other proteins.

Quinone cofactors have been found in many proteins. Their structure and origin have in many cases been difficult to assign. Copper amine oxidase was recently shown to contain a covalently bound Topa[1] at the position of a tyrosine codon in the gene coding for the protein (Janes et al., 1990; Mu et al., 1992). In methylamine hydroxylase the cofactor has been shown to be a covalently bound tryptophan quinone (McIntire et al., 1991). Recently a covalently bound Dopa was found in a site-directed mutant R2 protein of ribonucleotide reductase from *Escherichia coli* (Ormö et al., 1992). The mutation in this protein changes a phenylalanine into a tyrosine.

Several mechanisms have been suggested for the formation of these atypical amino acid residues, such as the involvement of unique tRNAs, specific enzymatic oxidations of tyrosine and tryptophan residues, of self-generation through involvement of an adjacent protein-bound metal capable of an oxidative reaction (Mu et al., 1992). In addition, generation of Dopa from tyrosine is catalyzed by tyrosine hydroxylase and tyrosinase. These two enzymes work on free tyrosine: tyrosinase in melanin biosynthesis and tyrosine hydroxylase in catecholamine biosynthesis (Haavik et al., 1991; Hearing & Tsukamoto, 1991).

Ribonucleotide reductase is a key enzyme in DNA synthesis and catalyzes the *de novo* production of deoxyribonucleotide precursors. It is composed of two different dimeric proteins, R1 and R2, both of which are needed to form the active enzyme [for reviews see Eriksson and Sjöberg (1989), Stubbe (1989), and Fontecave et al. (1992)]. The three-dimensional structure of the R2 protein (Nordlund et al., 1990) shows that it contains a dinuclear ferric iron center per monomer, which is coordinated by two histidines and one aspartic and three glutamic acid residues. The two irons are antiferromagnetically coupled via a μ-oxo bridge. The R2 protein lacking iron, apoR2, is transformed into active R2 by ferrous ions and molecular oxygen generating at Tyr 122 a stable free radical which is essential for catalysis.

The mutant protein R2 F208Y, constructed to test the significance of a hydrophobic patch close to the radical–iron cofactor, was unexpectedly found to contain a protein-derived Dopa (Ormö et al., 1992). We here report the kinetics of the autocatalytic Dopa formation in R2 F208Y by addition of ferrous iron and dioxygen to the nascent apoprotein. We have also crystallized the mutant protein Dopa-208 R2 and determined its three-dimensional structure to a resolution of 2.5 Å. The structure shows that Tyr 208 is converted into a Dopa that is ligated bidentate to one of the iron atoms in the iron center. Formation of Dopa in the engineered mutant protein R2 F208Y could serve as a model for the formation of covalently bound quinones in other proteins.

MATERIALS AND METHODS

Strains and Plasmids. The mutant proteins R2 F208Y and R2 Y122F,F208Y were overexposed in *E. coli* MC1009 (Δ(*lacIPOZYA*)X74, *galE*, *galK*, *strA*, Δ(*ara-leu*)7697, *araD*139, *recA*, *srl*::Tn10) cells from the plasmids pTB2F208Y

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[‡] The atomic coordinates in this paper have been submitted to Protein Data Bank under filename 1RNR.

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[1] Abbreviations: Dopa, dihydroxyphenylalanine; Topa, trihydroxyphenylalanine.

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(Ormö et al., 1992) and pMK5F208Y, respectively. The plasmid pMK5F208Y, coding for the double-mutant R2 Y122F,F208Y, was constructed by subcloning of a 992 bp *Bam*HI–*Asp*718I fragment from pTB2F208Y into pMK5, which contains the *nrdB* gene with the Y122F mutation in pTZ18R (Karlsson et al., 1992). Both plasmids are pTZ18R derivatives where the mutated *nrdB* gene is under the control of a T7 promoter. T7 RNA polymerase was introduced via a second plasmid, pGP1-2, that carries the T7 RNA polymerase gene under the control of a heat-inducible promoter (Tabor & Richardson, 1985).

Preparation of Iron-Free Mutant Proteins. *E. coli* MC1009 cells containing pGP1-2 and pTB2F208Y or pMK5F208Y were grown in low-iron medium which contained 45.4 mM phosphate buffer, pH 7.3, 7.57 mM $(NH_4)_2SO_4$, 0.4% glucose, 610 μM L-leucine, 406 μM $MgSO_4$, 50 μM EDTA, 5 μM $CaCl_2$, 0.6 μM $ZnCl_2$, 60 μM $CuSO_4$, 0.6 μM $MnCl_2$, 0.75 μM $CoCl_2$, 5.9 μM thiamin dichloride, 50 μg/mL carbenicillin, and 50 μg/mL kanamycin. The phosphate buffer, ammonium sulfate, glucose, and leucine solutions were all filtered through Chelex chelating ion-exchange membranes (Bio-Rex ion-exchange membrane, Bio-Rad) prior to media preparation. All glassware was washed with 0.1 M sulfuric acid to remove traces of iron. In this medium the cells listed above grew with a doubling time of 2–3 h at 30 °C up to an optical density of A_{600} = 1.1 and, 2.5 h after induction at 42 °C, reached A_{600} = 1.4. No specific precautions were used in the downstream purification steps to remove iron from buffers since the R2 protein does not incorporate Fe^{3+}. Protein prepared in this way was typically more than 90% pure and contained 0.2–0.3 equiv of Fe per R2 as determined by iron analysis (Atkin et al., 1973; Sahlin et al., 1990), compared to 2.7–2.8 equiv of Fe per R2 for protein prepared in the presence of iron. A molar absorption index of 120 000 M^{-1} cm^{-1} (ϵ_{280}–ϵ_{310}) was used for protein R2.

Quinone-Specific Staining. Detection of covalently bound Dopa was carried out as described earlier (Ormö et al., 1992) according to the method of Paz et al. (1991).

Kinetic Measurements. Stopped-flow experiments were carried out with a rapid kinetics spectrophtometer accessory (Model RX 1000, Applied Photophysics). Spectroscopic data were collected on a Perkin-Elmer λ2 or on a Hewlett-Packard 8452 A diode array spectrophotometer. The mixing ratio in the cuvette was 1:1 between one syringe containing 24–75 μM apoR2 protein and the other containing freshly made 24–240 μM $(NH_4)_2Fe(SO_4)_2$ solution, giving additions of 1–10 equiv of Fe^{2+} per R2. The buffer was 50 mM Tris-HCl, pH 7.6. All buffers to contain iron were pretreated by Ar bubbling, and before introduction of iron solution into the syringe, the system was flushed with Ar bubbled buffer. The oxygen needed for the reaction was supplied by the protein solution to which no specific treatment was made other than subjection to atmospheric air. Experiments were carried out at 22 °C.

Addition of iron, copper, cobalt, and manganese to apoR2 F208Y for the quinone staining experiment was carried out by pipetting the metal solution directly into a cuvette containing 12.5 μM apoR2 F208Y protein. The metal ions were added as $(NH_4)_2Fe(SO_4)_2$, $CuCl_2$, $CoCl_2$, and $MnCl_2$ solutions, respectively, at 4 times excess, giving a ratio of 16 equiv of Fe^{2+}, Cu^{2+}, Co^{2+}, or Mn^{2+} per R2. The 50 mM Tris-HCl, pH 7.6, buffer to contain iron metals was pretreated with Ar bubbling. The Fe^{2+} solution also contained ascorbic acid at 2 times excess over iron.

Crystallization and Structure Determination. Mutant protein Dopa-208 R2 was crystallized under the same conditions as have been used for wild-type R2 protein (Nordlund et al., 1989). Crystals obtained were of space group $P2_12_12_1$ with cell axes a = 74.1 Å, b = 85.3 Å, and c = 115.7 Å, which is close to isomorphous with wild-type R2 crystals. A data set, to 2.5-Å resolution, was collected using a MAR-system imaging plate at the Daresbury synchrotron radiation source, station 9.5. Data were evaluated using the MOSFILM program (Leslie, 1992). Map inspection and model building were done using the program O (Jones et al., 1990). Crystallographic refinement was done using the program X-plor (Brünger et al., 1987). The refined R2 wild-type structure (Nordlund et al., 1990) could be used as a starting model for the Dopa-R2 structure since the two crystal forms are practically isomorphous. The structure was refined using a simulated annealing protocol followed by 40 cycles of Jack–Levitt-type refinement without any previous model building. The residue at position 208 was excluded from the model. The Fe atoms were not attributed any electrostatic or van der Waal energies in their interaction with other atoms. The refinement was finished by another four rounds of 40 cycles of Jack–Levitt-type refinement by a restrained B value refinement and model building giving a final R value of 18.6%.

Table I: Data Collection and Refinement

statistics of data collection	
resolution (Å)	60–2.5
N_{obs}	67 726
N_{unique}	24 658
R_{merge}[a] (%)	9.0
completeness (%)	94.5
initial refinement parameters	
resolution (Å)	10–2.5
R factor[b]	0.32
final refinement parameters	
no. of non-hydrogen atoms	5 854
no. of water molecules	263
R factor[b] (all reflections 10–2.5 Å)	0.186
RMS deviations from ideal geometry of the final model	
bond distance (Å)	0.014
bond angle (deg)	2.8
dihedral angles (deg)	21.2

[a] $(\Sigma|I - \langle I\rangle|/\Sigma\langle I\rangle)$. [b] R factor $(\Sigma||F_{obs} - F_{calc}|/\Sigma F_{obs})$.

RESULTS

Structure Determination Identifies Mutant Protein as Dopa-208 R2. The structure of the Dopa-208 R2 protein has been determined at 2.5-Å resolution and refined to a crystallographic R value of 18.6% with good stereochemistry (Table I). The overall structure of the mutant protein Dopa-208 R2 is very similar to the wild-type R2 structure. The RMS difference between the two structures is 0.43 Å for alpha carbons. The differences found are only local and restricted to the place of the mutation and its immediate surroundings including the dinuclear iron center.

As is also the case with the wild-type R2 structure, the 35 C-terminal residues of Dopa-208 R2 cannot be resolved in the structure, and the structure presented here ends at residue 340 at the top of the heart-shaped molecule.

The Dopa that is generated in the mutant protein is located at position 208, which is clearly shown in an "omit" map (Figure 1). It is ligated bidentate to Fe1 with distances of 2.0 and 2.3 Å between the iron and O-3′ and O-4′ of Dopa-208, respectively. In addition, Fe1 is liganded by Asp 84 and His 118. The other iron ion, Fe2, is liganded by Glu 204 and His 241 and bidentate by Glu 238. Glu 115 bridges the two irons. There is no additional electron density corresponding to coordinated water, hydroxo, or oxo groups between or in the

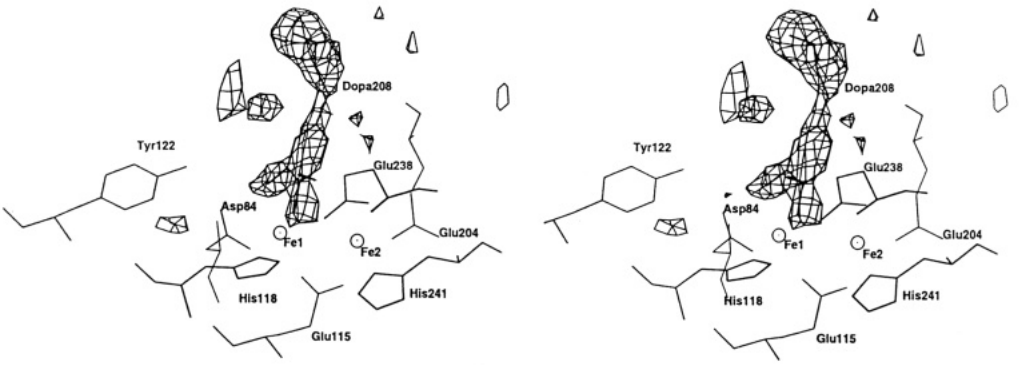

FIGURE 1: "Omit" $|F_o| - |F_c|$ map (Brünger et al., 1987) of the metal center in the Dopa-208 R2 protein. Residue 208 was removed from the structure, and 40 cycles of refinement were performed to relax the structure. The $|F_o| - |F_c|$ map contoured at 2.5σ clearly shows the bidentate Dopa at position 208.

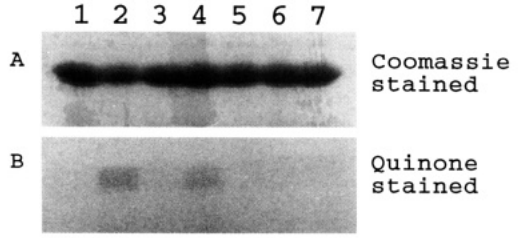

FIGURE 2: Quinone-specific staining of R2 F208Y proteins. (A) Coomassie stained sodium dodecyl sulfate–polyacrylamide gel with 20-μg loadings of the following proteins: lane 1, wild-type R2; lane 2, Dopa-208 R2; lane 3, apoR2 F208Y; lanes 4–7, apoR2 F208Y to which metal solutions were added in a ratio of 16 equiv of metal ions per R2 in the presence of oxygen (lane 4, Fe^{2+}; lane 5, Cu^{2+}; lane 6, Co^{2+}; lane 7, Mn^{2+}). (B) Same as (A) but the proteins were electroblotted onto a NC membrane which was subjected to quinone-specific staining according to the method of Paz et al. (1991).

Table II: Distances between the Fe Ions and Their Ligands in Molecule B

atom	ligand–atom	distance (Å)
Fe1	Fe2	3.5
Fe1	Asp 84–Oδ	1.9
	His 118–Nδ	2.3
	Glu 115–Oε2	2.3
	Dopa-208–Oε	2.0
	Dopa-208–Oζ	2.1
	Glu 238–Oε2	2.5
Fe2	Glu 115–Oε1	2.3
	Glu 204–Oε	1.8
	Dopa-208–Oζ	3.1
	Glu 238–Oε1	1.9
	Glu 238–Oε2	2.1
	His 241–Nδ	2.3

vicinity of the irons. The iron center of the Dopa-208 protein is thus symmetric, and each iron ion is five coordinate with one bidentate ligand, one histidine ligand, and one terminal and one bridging carboxylate ligand. Distances between the irons and their ligands are given in Table II.

Nascent ApoR2 F208Y Does Not Contain Dopa. Nascent apoR2 F208Y was purified to homogeneity from overproducing cells grown in iron-depleted medium. The apoR2 F208Y protein contains only 0.3 equiv of iron per R2 protein, whereas the iron content of Dopa-208 R2 grown in iron-containing medium is 2.8 equiv per protein. The presence of Dopa in the mutated proteins was tested by quinone-specific staining (Paz et al., 1991). Figure 2 shows a stained nitrocellulose filter onto which apoR F208Y has been blotted with wild-type R2 and Dopa-208 R2 as controls. As shown earlier, wild-type R2 protein does not stain for quinone, whereas

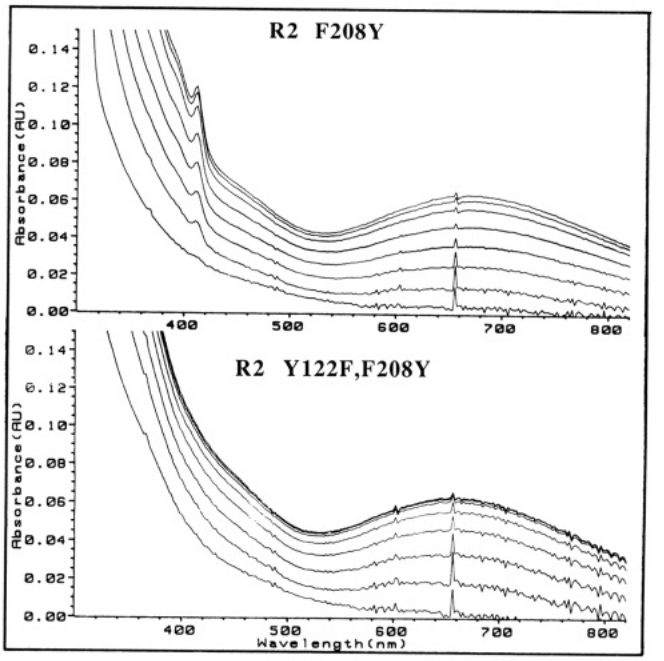

FIGURE 3: Rapid-mixing spectroscopy of the mutant R2 F208Y and R2 Y122F,F208Y. (Top) Spectra of the mutant protein R2 F208Y at times 0, 2.4, 4.8, 8.4, 14.4, 26.4, 40.8, and 57.6 s after addition of 4 equiv of Fe^{2+} per R2 protein. The protein concentration was 37.5 μM. (Bottom) Same as above with the double-mutant R2 Y122F,F208Y.

the Dopa present in Dopa-208 R2 promotes the color reaction (Ormö et al., 1992). The apoR2 F208Y protein shows in the same experiment only a very weak staining that can be attributed to the fact that the apoR2 F208Y protein contains a small amount of iron. It is thus clear that the nascent iron-free form of R2 F208Y does not contain the Dopa side chain.

Ferrous Iron and Oxygen, but Not Tyr 122 and Tyrosyl Radical, Are Needed for Dopa Formation. The absence of Dopa in the apoR2 F208Y protein forms the basis for a mechanistic study of Dopa formation *in vitro*. Addition of ferrous iron in the presence of oxygen to iron-free R2 F208Y protein was followed by the appearance of the Dopa characteristic light absorption bands at 460 and ca. 700 nm (Figure 3). Both bands appear simultaneously, and after 1 min, the reaction is essentially complete. Thus, formation of Dopa is linked to the introduction of iron and oxygen into the metal center.

Concomitant with the appearance of the ferric catecholate band, a light absorption at 410 nm was also seen (Figure 3). This absorption band is indicative of a tyrosyl radical of the same nature as in wild-type R2 protein but accounts for only

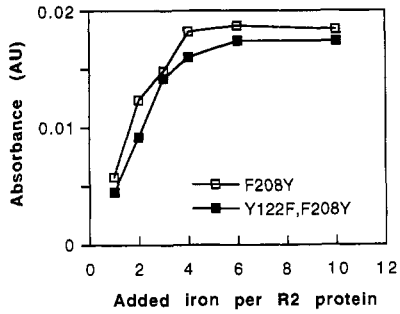

FIGURE 4: Addition of increasing amounts of ferrous iron to R2 F208Y and R2 F122F,F208Y. Protein concentrations were 12 μM, and absorbances were recorded at 700 nm 50 s after iron addition.

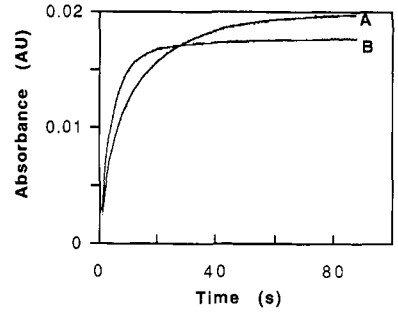

FIGURE 5: Dopa–iron complex formation in the proteins R2 F208Y and R2 Y122F,F208Y followed at 700 nm after rapid mixing of protein and ferrous iron. Four equivalents of Fe^{2+} per R2 protein was added at a protein concentration of 12 μM. The reactions have been fitted to two parallel first-order reactions (Table II). (A) R2 F208Y. (B) R2 Y122F,F208Y.

10% of what is found in wild-type R2. Whereas in the wild-type protein this tyrosyl radical at position 122 is stable for days at room temperature (Atkin et al., 1973; Eriksson et al., 1977), the mutant protein radical is transient and was lost during a period of 20 min. The decreased stability of the radical is most likely due to the structural effects of the mutation on the iron center. The disappearance of the radical was not followed by any gain of absorbance at 700 nm (data not shown).

The possible involvement of a radical at Tyr 122 in the Dopa-generating reaction was tested by construction of the double-mutant protein R2 Y122F,F208Y, where Tyr 122 has been changed to a phenylalanine. It was earlier shown that the R2 Y122F protein, apart from lacking the stable tyrosyl free radical, is similar to wild-type protein with regard to the ferric iron center (Larsson & Sjöberg, 1986). In Figure 3 spectra collected after iron addition to R2 Y122F,F208Y show the generation of the Dopa–iron absorption bands at 460 and ca. 700 nm. The same amount of ferric–Dopa complex is formed in the double mutant as in the F208Y protein. However, no radical-related absorbance in the 410-nm region is detected, thus supporting the idea that the transient radical seen in Dopa-208 R2 is located at Tyr 122 and that it is not required for Dopa generation.

Since wild-type apoR2 protein can bind a variety of divalent cations at the site of the iron center (Atta et al., 1992; Aberg and Nordlund, unpublished results), we also tested if cations other than Fe^{2+} can generate a Dopa side chain in the mutant protein. Addition of Cu^{2+}, Co^{2+}, or Mn^{2+} to apoR2 F208Y did not give rise to any new light absorbances in the region 250–900 nm (data not shown) that differ from additions of the corresponding metal to wild-type R2 protein. Dopa formation upon addition of Cu^{2+}, Co^{2+}, or Mn^{2+} was also tested by quinone-specific staining. None of these metals promoted a Dopa formation that could be detected by the color reaction in the quinone staining experiment (Figure 2), indicating that metal-mediated redox chemistry is involved in the case of the iron addition.

Kinetics of Dopa Formation. Titrating the apoR2 F208Y or apoR2 Y122F,F208Y with increasing amounts of iron showed that the amount of catechol formed increased up to 4 equiv of iron. Addition of excess iron, i.e., 6 and 10 equiv of Fe^{2+} per R2, did not increase the amount of formed catechol (Figure 4). The kinetics of iron-dependent Dopa formation was studied by rapid-mixing spectrophotometry. The increases in 700-nm absorption have poor fits to a single exponential. In the case of addition of four or more irons per R2 protein, the increase in 700-nm absorption can be fitted to two parallel first-order reactions: $f(t) = L_a(1 = e^{-k_a t}) + L_b(1 - e^{-k_b t}) + C$, where k_a and k_b are first-order rate constants with the corresponding limits L_a and L_b and C is a constant (Figure 5). The overall reaction in the mutant protein Dopa-208 R2 takes place with rate constants of 0.24 and 0.05 s^{-1} with approximately half of the reaction following each of the two rates. In the double-mutant protein R2 Y122F,F208Y the rate constants are the same but 90% of the formation follows the faster of the two reactions (Table III).

Table III: Catechol–Iron Complex Formation Measured as Generation of Light Absorption at 700 nm after Addition of Excess Ferrous Iron (6 and 10 equiv of Fe^{2+} per R2) in the Presence of Oxygen[a]

protein	k_a (s^{-1})	k_b (s^{-1})	L_a/L_{a+b}
R2 F208Y	0.24 ± 0.019	0.049 ± 0.002	0.52 ± 0.034
R2 Y122F,F208Y	0.24 ± 0.008	0.047 ± 0.012	0.91 ± 0.015

[a] A curve was fitted to the data according to the function $f(t) = L_a(1 - e^{-k_a t}) + L_b(1 - e^{-k_b t}) + C$, where k_a and k_b are first-order rate constants, with the corresponding limits L_a and L_b, and C is a constant.

DISCUSSION

The structure of the mutant R2 protein shows that the tyrosine residue at position 208 has been modified to a Dopa which is a bidentate ligand of Fe1. In comparison to Phe 208 in the wild-type structure the aromatic ring of the Dopa-208 has moved about 1.5 Å to be a ligand to Fe1. The liganding system around the iron center of the mutant protein Dopa-208 R2 differs from that of wild-type R2 in six respects (Figure 1): (i) the bidentate Dopa at position 208, (ii) the amino acid Asp 84 is a monodentate ligand to Fe1, (iii) Glu 238 is a bidentate ligand to Fe2, (iv) there is no electron density between the irons corresponding to a μ-oxo bridge (also supported by the lack of μ-oxo-specific absorbances in the 330–370-nm area), (v) there is no electron density corresponding to liganding water molecules, and (vi) the distance between the two irons is 0.3 Å longer in the Dopa-208 R2 protein.

The geometry of the two iron ions is octahedral; however, that of Fe2 is somewhat distorted due to the bidentate Glu 238. There are only five ligands to each metal. The distance between O-4 of the Dopa residue and Fe2 is about 3 Å, and it is possible that this atom bridges the two irons. Also, the distance between one of the Fe2 liganding oxygens of Glu 238 and Fe1 is short, about 2.5 Å, and this oxygen might also be bridging the two irons.

The atoms of the side chain of the Dopa residue 208 have unusually high B values. It is unlikely that this is due to a high mobility of the Dopa side chain since it is ligated to Fe1. A more probable explanation is that not all tyrosine 208 has been converted to Dopa and that there is a tyrosine residue in some of the molecules at position 208.

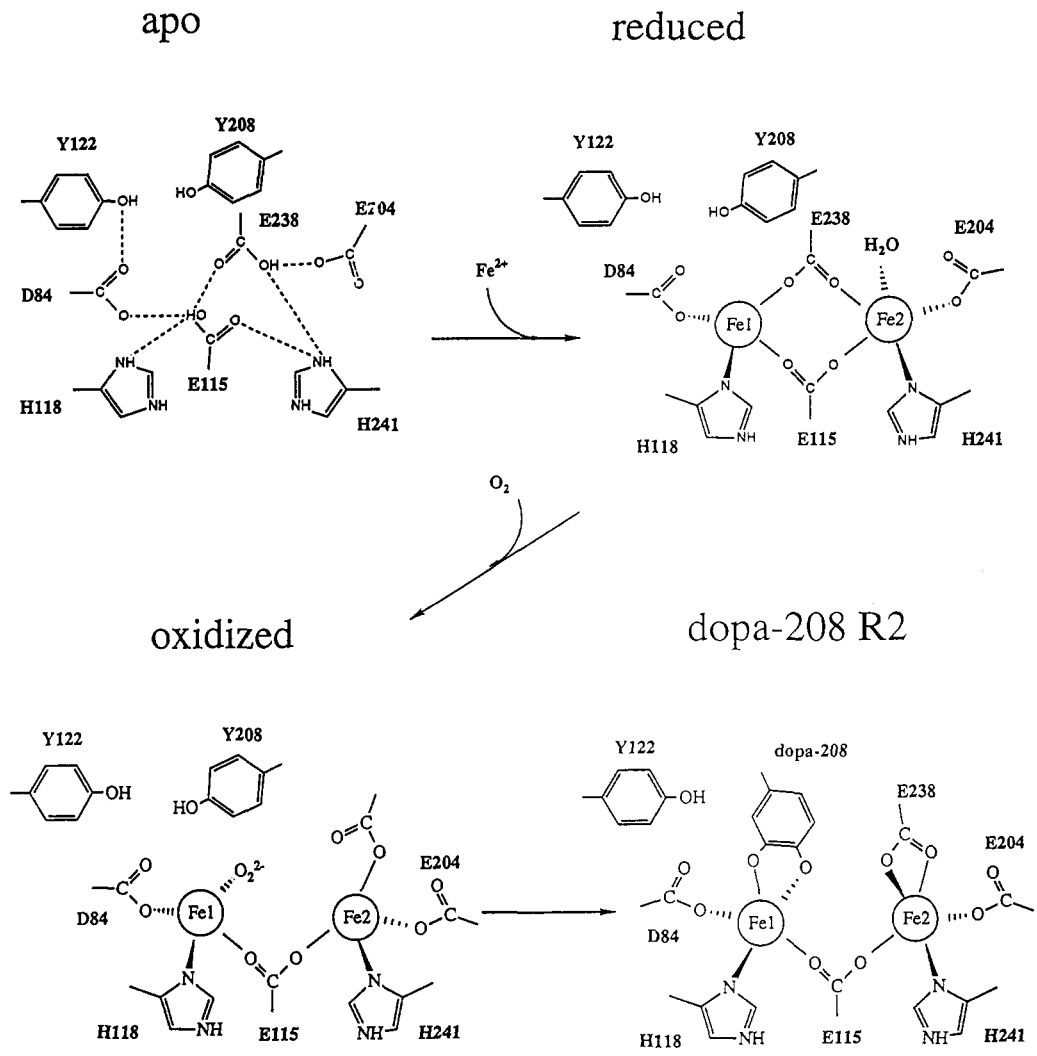

FIGURE 6: Proposed model for the Dopa-generating reaction in the mutant R2 protein. The schematic drawings have been done with the wild-type apoR2 structure, solved at 2.5 Å (Aberg et al., 1993), as a model for the apoR2 F208Y structure. The structure of the manganese-containing protein, solved at 2.5 Å (Atta et al., 1992), has been used as a model for the reduced form of the R2 F208Y protein. The oxidized ferric peroxide form is hypothetical. The structure of the Dopa-208 R2 is the crystallographically determined structure reported here.

In wild-type R2 the metal center is neutral; the six positive charges of the iron ions are compensated for by the μ-oxo bridge, which carries two negative charges, and the four carboxylate ligands. In the mutant structure the loss of the two negative charges from the μ-oxo bridge is compensated for by the bidentate Dopa, which carries two negative charges.

The nascent metal-free form of the mutant protein, apoR2 F208Y, does not contain Dopa. Upon addition of Fe^{2+} in the presence of oxygen to the homogeneous apoR2 F208Y, Dopa is generated in a metal-catalyzed reaction. It was earlier suggested that Dopa formation resembled tyrosine radical formation in wild-type R2 with the involvement of a ferryl-oxygen species (Ormö et al., 1992). Incubation of the wild-type metal-free protein, apoR2, with Fe^{2+} in the presence of oxygen generates the dinuclear ferric iron center and a stable free radical at Tyr 122. This reaction starts with the binding of Fe^{2+} to the metal center (Sahlin et al., 1989). After oxygen binding to the metal center, the irons are oxidized, and it has been suggested that an intermediate ferric peroxide is formed (Sahlin et al., 1990; Fontecave et al., 1990; Bollinger et al., 1991; Que, 1991). Heterolytic cleavage of the peroxide would yield a ferryl oxide capable of abstracting an electron from Tyr 122 and thereby forming the stable free radical. There is also the generation of a transient tyrosine radical in the mutant protein R2 F208Y upon iron addition as seen by the absorption at 410 nm (Figure 4), which indicates that a ferric peroxide can also form in the mutant protein. However, generation of Dopa in R2 F208Y is not dependent on this radical since the double-mutant R2 Y122F,F208Y does acquire a Dopa even though it cannot form a tyrosine radical at position 122.

The data from the rapid-mixing spectrophotometry of the two proteins apoR2 F208Y and apoR2 Y122F,F208Y with iron could not be fitted to a single first-order reaction. Fitting the data to two parallel first-order reactions gave good agreement (Figure 5). The two reactions differ 5-fold in rate constant. Both of these reactions, which monitor the formation of the ferric–Dopa complex, are much slower than the formation of the ferric iron center and the tyrosine radical in wild-type R2. For instance, radical formation in wild-type R2 at 5 °C (Bollinger et al., 1991) is about 4 times faster than the fastest Dopa-generating reaction at 22 °C. One of the reasons for the differences in the reaction rates is probably the need for local conformational changes upon formation of the ferric–catechol complex. In the apoR2 F208Y protein, half of the Dopa formation follows the slower reaction, while in the apoR2 Y122F,F208Y protein, only 10% of the Dopa is formed via the slower reaction. The only difference in these two proteins is the hydroxyl group of Tyr 122. In the wild-type apoR2 protein Asp 84 is at hydrogen-bonding distance

from the hydroxyl group of Tyr 122 (Aberg et al., 1992); such a hydrogen bond in the R2 F208Y protein may favor the slower of the two reactions leading to Dopa formation at position 208.

To explain in structural terms the Dopa formation in R2 F208Y, we assume that the apo and reduced forms of the mutant protein are similar to the wild-type structures. The structure of the metal-free form of ribonucleotide reductase R2 has been solved (Aberg et al., 1993). There is no high-resolution structure for the reduced R2 protein yet, but the structure of the manganese-containing protein, where Mn^{2+} is bound to the metal center, has been proposed to be a model of the reduced form of the R2 protein (Atta et al., 1992). Both the apo and the Mn overall structures resemble the ferric R2 structure. The differences that are found are local and restricted to the area around the metal center. In the apo structure the two carboxyl groups Glu 115 and Glu 238 form hydrogen bonds to each other as well as to Asp 84 and Glu 204. In the Mn structure both residues Glu 115 and Glu 238 are bridging the two Mn(II) ions. Asp 84, which is bidentate in the Fe(III) structure, is monodentate in the Mn(II) structure.

Using the apoR2 and Mn-containing structures together with the Dopa-208 R2 structure, we propose a model for the Dopa formation in the mutant ribonucleotide reductase Dopa-208 R2 (Figure 6). Addition of ferrous iron to the apoprotein leads to binding of the Fe^{2+} to the metal center. Upon binding, the carboxyl groups in the apoprotein have to be deprotonated. The irons are then oxidized by molecular oxygen to ferric iron, and the resulting ferric peroxide decomposes to a ferryl intermediate. Up to this point we believe that the reaction in the mutant protein does not differ from the reaction of the wild-type R2. The ferryl intermediate can then either directly oxygenate Tyr 208 or activate Tyr 208 for oxidation by abstracting an electron from the side chain of 208 and thereby forming a tyrosyl radical which would join with a hydroxyl group. The latter mechanism would be similar to the formation of catechol from phenol in horseradish peroxidase (Dordick et al., 1986).

The formation of the ferric–Dopa complex would also require local conformational changes in the vicinity of the iron center. The formed Dopa ligates to Fe1, and Glu 238 changes from a bridging ligand to a bidentate ligand of Fe2. A similar rearrangement of Glu 238 is expected also for the oxidation of the iron center in wild-type R2. In this case the concomitant change at Fe1 would be Asp 84 going from monodentate to bidentate. These rearrangements in the wild-type as well as the mutant protein are experimental macromolecular examples of the so-called "carboxylate shift" rearrangement, recently suggested to occur quite readily in ligands of polynuclear centers in metalloproteins (Rardin et al., 1990).

We have shown that a mutant ribonucleotide reductase in which Phe 208 has been changed to a tyrosine has the ability to modify the introduced tyrosine to a Dopa by addition of Fe^{2+} and molecular oxygen to the protein. In amine oxidase it has been suggested that the Topa cofactor is generated through the involvement of a hydroperoxide bound to copper in the unmodified enzyme (Mu et al., 1992). In the case of the R2 F208Y protein no metal ion tested, other than ferrous, was able to generate a Dopa. Even though the Dopa generated in R2 F208Y is not a cofactor, its formation demonstrates that proteins have the ability to generate their own cofactors without the need of external enzymatic systems.

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