Redox Intermediates of the Mn-Fe Site in Subunit R2 of Chlamydia trachomatis Ribonucleotide Reductase
AN X-RAY ABSORPTION AND EPR STUDY

The R2 protein of class I ribonucleotide reductase (RNR) from Chlamydia trachomatis (Ct) can contain a Mn-Fe instead of the standard Fe-Fe cofactor. Ct R2 has a redox-inert phenylalanine replacing the radical-forming tyrosine of classic RNRs, which implies a different mechanism of O₂ activation. We studied the Mn-Fe site by x-ray absorption spectroscopy (XAS) and EPR. Reduced R2 in the R1R2 complex (R2²⁻) showed an isotropic six-line EPR signal at g ≈ 2 of the Mn(II)Fe(II) state. In oxidized R2 (R2³⁺), the Mn(III)Fe(III) state exhibited EPR g values of 2.013, 2.009, and 2.015. By XAS, Mn-Fe distances and oxidation states of intermediates were determined and assigned as follows: 2.41 Å, Mn(II)Fe(II); 2.35 Å, Mn(III)Fe(II); and 2.75 Å, Mn(IV)Fe(III). Shortening of the Mn/Fe-ligand bond lengths indicated formation of additional metal bridges, i.e. μO(H) and/or peroxodic species, upon O₂ activation at the site. The structural parameters suggest overall configurations of the Mn-Fe site similar to those of homo-metallic sites in other R2 proteins. However, the ~2.6 Å Mn-Fe distances, typical for di-μO(H) metal bridging, are shorter than inter-metal distances in any R2 crystal structure. In diffraction data collection, such bridges may be lost due to rapid x-ray photoreduction of high-valent metal ions, as demonstrated here for Fe(III) by XAS.

Ribonucleotide reductases (RNRs) are the only enzymes that, in all organisms, catalyze the reduction of ribonucleotides to their deoxy forms essential for DNA synthesis (1–3). RNRs also are important targets in cancer and antiviral therapy (4, 5). Class I RNRs found in eukaryotes and microorganisms (6) are heterotetrameric enzymes of R12R2₂ organization. The R1 protein contains the nucleotide binding site and R2 houses a dinuclear metal center, which is the site of dioxygen (O₂) activation and, in conventional RNRs, is of the Fe-Fe type (7).

Extensive investigations on Fe-Fe RNRs from, e.g. Escherichia coli, Saccharomyces cerevisiae, Mus musculus, and Homo sapiens have established that the catalytic reactions involve activation of an O₂ molecule at the di-metal cluster to generate a high potential site, which oxidizes a nearby tyrosine residue to a tyrosyl radical, Y* (8–10). In E. coli R2 this Tyr-122 is at ~6 Å distance to the nearest iron (11, 12). Subsequent proton-coupled electron transfer (13) leads to the re-reduction of Y* and to the oxidation of a cysteine at the substrate binding site in R1 to a radical (C⁻) (14, 15). C⁻ initiates ribonucleotide reduction involving disulfide formation by two additional cysteines (16). Regeneration of reduced cysteines requires electron input from external thio- or glutaredoxins and ultimately from NADPH (17).

At least the Fe(II)₂, Fe(III)₂, Fe(IV)Fe(III), and Fe(IV)₂ oxidation states of the metal center seem to be involved in the electron transfer reactions (18, 19) of classic RNRs. The Fe(III)-Fe(IV) state, termed “intermediate X” (20, 21), is crucial because it oxidizes the tyrosine to Y*, leaving the di-iron site in the Fe(III)₂ state. Y* usually survives a large number of catalytic cycles, but when it is lost, the inactive Fe(III)₂-Met form remains (22). Reactivation of the enzyme first requires reduction of the metal site to Fe(II)₂, which then must react with O₂, leading to the cleavage of the O–O bond and again to the formation of Fe(III)₂ and Y* (22, 23).

According to the above reaction sequences, a tyrosine radical and the Fe(V)Fe(III) state (X) have been anticipated to be decisive for RNR function. This view has been challenged recently because R2 proteins of RNRs in several species have been discovered (24, 25), containing a redox-inert phenylalanine instead of the tyrosine. One enzyme is found in the important human pathogenic bacterium Chlamydia trachomatis (Ct) (25, 26). It is a fully functional RNR and the only RNR encoded in the genome of this organism (27, 28). In E. coli, however, the Tyr → Phe exchange abolishes RNR activity (20, 30). A tyrosyl radical still is not observed in the Phe → Tyr mutant of Ct RNR (31).

Ct R2 can be reconstituted with Fe(II) ions so that a typical Fe-Fe cofactor is formed, but in this case, RNR activity is low...
Mn-Fe Site Structure of C. trachomatis RNR

(28). More recent studies have revealed that a much higher enzyme activity (at least ten times higher) is obtained in the presence of stoichiometric amounts of manganese and iron (27, 32). Thus, the Ct enzyme now is believed to represent the first RNR that contains a hetero-bimetallic Mn-Fe cluster in its native state. Presumably closely related Mn-Fe sites have been found in purple acid phosphatase from sweet potato (33) and in an N-oxygenase of Streptomyces thioluteus (34). These proteins seem to belong to a growing family of O2-activating Mn-Fe enzymes.

A large number of crystal structures of Fe-Fe-containing R2 proteins is available in the PDB data base. Consistently, the metal ions are coordinated by conserved amino acids, i.e. four glutamates and two histidines (9, 35). In addition, a variable number of metal-bound oxygen species (H2O or OH) was detected. In all structures at least one carboxylate group bridges between the metal ions and furthermore, bridging oxo (μO) or hydroxo (μOH) species were observed, their number likely depending on the iron oxidation state. Similar structural features were observed in R2 proteins reconstituted with a Mn-Mn site (36, 37).

Crystal structures of Ct R2 containing an Fe-Fe site recently have been reported (26, 31). Overall, the metal coordination seems to be similar to, e.g. Escherichia coli R2; the significance of certain structural differences is unclear. In previous studies on Ct Mn-Fe RNR, high valent states, i.e. Mn(IV)Fe(III) and Mn(IV)Fe(V), have been proposed to be involved in catalysis (27, 32, 38). Direct structural information on the Mn-Fe site of Ct R2, in particular in its high valent states, is indispensable to unravel the oxygen activation and ribonucleotide-reduction mechanisms of the tyrosine-less Mn-Fe RNRs. It also may serve as a benchmark for quantum-chemical calculations aiming at optimized structures of the Mn-Fe site.

In the present investigation, we use x-ray absorption spectroscopy (39, 40) at the manganese and iron K-edges and EPR to characterize the Mn-Fe site of Ct RNR in the oxidized R2 protein (R2ox) and in reduced R2 in the R1R2 complex (R2red). The first information on the atomic structure (metal-ligand and metal-metal distances) and electronic configuration of several oxidation states is reported, individually for the manganese and iron ions. Possible structures of the Mn-Fe site are discussed.

MATERIALS AND METHODS

Protein Sample Preparation—Ct RNR expression vectors for truncated wild-type R1Δ1–248 and wild type R2 proteins were a kind gift of G. McClarty (University of Manitoba, Canada). Recombinant proteins were expressed and purified as described previously (28, 31, 32). To obtain Mn-Fe R2 protein, Mn(II) was added as 40 μM MnCl2 to the LB growth medium at the moment of R2 overexpression in the Ct cells, and the Mn(II) level in the medium (30–50 μM) subsequently was controlled by monitoring its six-line EPR signal in the supernatant of the bacterial suspension and by the addition of MnCl2. Protein R2 and R1Δ1–248 concentrations were determined photometrically using extinction coefficients of 116 mM−1 cm−1 and 274 mM−1 cm−1 at 280 nm. The total amount of Mn-Fe sites in R2 was quantified by recording EPR spectra (20 K, 0.8 milliwatt) of the Mn(III)Fe(III) state in a catalytic mixture (50 μM R2, 100 μM R1, 0.3 mM ATP, 1.8 mM MgCl2, 2 mM CDP, 10 mM DTT; incubated for 20 min at room temperature with 0.1 mM hydroxyurea) and signal comparison to a standard solution of 1 mM CuSO4 in 10 mM EDTA (32). Reduction of as-isolated (oxidized) Mn-Fe R2 was achieved by incubation of R2 (~50 μM and ~16 ml) with ~100 μM of R1, 0.3 mM ATP, 1.8 mM MgCl2, and 10 mM DTT at 0 °C overnight under argon. Protein samples were concentrated (Amicon), filled into EPR and XAS sample holders, and frozen in liquid nitrogen.

Metal Content Quantification—Metal contents of protein samples were assayed by total-reflection x-ray fluorescence detection (TXRF) (41) on a PicoFox spectrometer (Bruker). Protein samples were mixed with a gallium standard (Sigma, 5 mg/liter), 5 μl of the samples were dried on quartz disks for ~1 h at ~50 °C, and disks were mounted on the sample changer of the spectrometer. The excited x-ray fluorescence was recorded for 10 min for five samples each of R2red and R2ox. Iron and manganese concentrations were determined using the routines of the spectrometer and molecular weights of manganese of 54.93 g/mol and iron of 55.84 g/mol.

EPR Spectroscopy—9.5-GHz X-band EPR spectroscopy was carried out on a Bruker ESP300E spectrometer equipped with a rectangular microwave cavity. Samples were kept in quartz tubes in an Oxford ESR900 helium cryostat. For further spectrometer settings see the figure legends. For determination of g values the magnetic field was calibrated with a LiF standard (42). Spin quantification was performed by comparison of the double-integrated signal of RNR samples with that of a CuSO4 standard.

X-ray Absorption Spectroscopy—Kα X-ray absorption spectra were averaged (11–28 scans) after energy calibration of each scan (for iron spectra using the peak at 7112 eV in the first derivative of the absorption spectrum of an Fe-foil and for manganese spectra using the pre-edge peak at 6543.3 eV in the absorption spectrum of KMnO4 as energy standards (45); estimated accuracy ± 0.1 eV). The respective scan ranges and durations were 6900–8200 eV (up to k = 16 Å−1) and ~1 h for iron spectra and 6400–7100 eV (up to k = 12 Å−1) and ~30 min for manganese spectra. One to two scans were performed per sample spot (x-ray spot size of 5 × 1 mm2 set by slits). XAS spectra were normalized, and EXAFS oscillations were extracted as described before (46). The energy scale of EXAFS spectra was converted to the wavevector scale (k scale) using E0 values of 7112 eV (iron) and 6540 eV (manganese). Unfiltered k3-weighted spectra were used for least-squares curve-fitting with the in-house software SimX (46) using phase functions calculated by using FEFF-7 (47) and for calculation of Fourier transforms (FTs). An amplitude reduction factor, S0, of 0.9 was used in the EXAFS fits. From experimental XANES spectra the pre-edge peak region was extracted by subtraction of a polyno-
results from TXRF were consistent with the ratio Mn:Fe of 1.54 ± 0.75. The TXRF data, Mn-Fe sites per R2-dimer was calculated assuming that manganese only was bound to sites that already contained one iron and neglecting single-Fe/Mn sites. The presented metal concentrations are generally determined from TXRF as the average of five samples each; error values represent ± S.D.

| R2red | R2ox |
|-------|------|
| R2-dimer, μM | 750 ± 100 | 850 ± 100 |
| Fe, μM | 960 ± 30 | 1310 ± 90 |
| Mn, μM | 660 ± 20 | 850 ± 60 |
| Fe:Mn | 1.45: 1 | 1.54: 1 |
| Fe per R2-dimer | 1.28 | 1.54/1.60a |
| Mn per R2-dimer | 0.88 | 1.00/0.86a |
| Mn-Fe sites per R2-dimera | NDb | 1.00/0.75a |

a These values were determined by optical (Fe) and EPR (Mn) spectroscopy in denatured protein.
b ND, not determined.

† This value was determined from the Mn(III)Fe(III) EPR signal in hydroxyurea-treated R2 protein as in Ref. 32.

‡ From the TXRF data, Mn-Fe sites per R2-dimer were calculated assuming that manganese only was bound to sites that already contained one iron and neglecting single-Fe/Mn sites.

†† These values were determined by optical (Fe) and EPR (Mn) spectroscopy in denatured protein.

‡‡ ND, not determined.

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RESULTS

Metal Content and Site Occupancy—Two types of C. trachomatis RNR samples were used in this spectroscopic study: (a) as-isolated, oxidized R2 protein, denoted R2ox, and (b) the same R2 protein extensively reduced with DTT in an R1R2 complex, R2red. The final R2ox protein concentration was ~0.85 mM R2-dimer. The R2red sample was prepared using the same R2 preparation as for R2ox. After mixing with protein R1 and reduction with DTT, the final R2red protein concentration was ~0.75 mM R2-dimer. The metal contents of samples were quantified by TXRF and also by previously described spectroscopy methods (32); briefly, the manganese content was determined by EPR in an acid-denatured protein sample, and the iron content was spectrophotometrically determined using an iron complex assay. Table 1 summarizes the results. The TXRF and spectroscopy methods to determine the total manganese and iron concentrations were in good agreement, as seen for the data on R2ox. In the estimations shown below we have used the data from the spectroscopy methods. The as-isolated R2 protein (R2ox) used in the XAS experiments was produced under conditions of constant and moderate manganese concentration (40 μM) in the overexpression medium; the iron concentration in the medium was ~10 μM (supplemental Table S1). This resulted in R2 protein with ~0.86 manganese per R2-dimer. The amount of Mn-Fe sites in the protein was estimated from the specific EPR signal of the Fe(III)-Mn(III) state (not shown) in the enzymatic mixture after incubation with hydroxyurea (32). The results showed that the R2ox protein contained ~0.75 Mn-Fe sites per R2-dimer. A slightly larger value can be calculated from the TXRF data (Table 1). We therefore concluded that almost all (close to 90%) of manganese was incorporated in the Mn-Fe sites. The excess iron in the samples (total ~1.6 iron per R2-dimer) was estimated to ~0.75 per R2-dimer. At this stage we cannot judge whether the iron not present in the Mn-Fe sites is bound to specific (i.e. dimetal) or unspecific sites.

We found that a lower manganese concentration in the overexpression medium resulted in lower overall manganese concentrations in the protein, whereas higher manganese concentrations led to Mn-Mn clusters with a broad EPR signal at g ~ 2.5 (data not shown, see Ref. 50). Supplemental Table S1 shows the characteristic properties of varying preparation procedures. The dominance of the Mn-Fe site and sufficient metal concentrations in the R2ox and R2red samples allowed for x-ray absorption experiments both at the iron and manganese K-edges to assess the individual metal site structures.

EPR Characterization of Samples—Fig. 1A shows EPR spectra at X-bands of R2ox and R2red, recorded under non-saturating conditions. The signal of R2ox (at high microwave power) is similar, but not identical to the one previously assigned to the Mn(III)Fe(III) state in the R1R2 samples (32). It reflects antiferromagnetic coupling of the high spin Mn(III) (S = 2) and Fe(III) (S = 5/2) ions to produce a ground-state S = 1/2 system (32). Line splittings reflect hyperfine interactions of the 55Mn nucleus (nuclear spin of 5/2). Simulation of the spectrum (Fig. 1B) yielded the g values and hyperfine (hf) tensors given in Table 2.

In a previous study (32), the Mn(III)Fe(III) state has been obtained in a mixture containing, besides of R2ox protein, also R1 protein, reductant, and nucleotides, so that enzymatic turnover occurred until hydroxyurea was added as an inhibitor to stop the reaction and accumulate the inactive Mn(III)Fe(III) state. Thus, this Mn(III)Fe(III) state was generated after several catalytic cycles. Here, the Mn(III)Fe(III) state in the as-isolated oxidized R2 protein was studied, which was never in a complex with R1 or reacted with hydroxyurea. Interestingly, there are subtle differences in the EPR g- and hf-parameters of the respective Mn(III)Fe(III) states (Table 2). In addition, weak features on the high field side of the EPR spectrum (Fig. 1B) seem to indicate contributions from a second Mn(III)Fe(III) species. We did not attempt to simulate these spectral contributions because of their comparably small magnitude. Evidence for a second Mn(III)Fe(III) species, however, also was obtained from XAS (see below).

R2ox samples showed an EPR spectrum of manganese (at low microwave power) with a different shape of its six main lines (Fig. 1, A and C). Such a spectrum was expected for a Mn(II) site with significant zero-field splitting D (Table 2, legend), indicat-
ing binding of the Mn(II) to the protein. Remarkably, there is no indication in the spectrum for a coupling between Mn(II) (S = H11005 5/2) and a high spin Fe(II) (S = H11005 2). However, the clearly visible “forbidden” transitions in the EPR spectrum of Mn(II) (Fig. 1C) show that the Mn(II) is high spin. In line with this observation, the spectrum readily was simulated using an isotropic g-value, an isotropic 55Mn hf-value, and most importantly, a D-value (including D-strain) typical for high spin (hs) Mn(II) (Table 2). Strong coupling within a Mn(II)hsFe(II)hs center is expected to lead to a low spin S = 1/2 state, which has no D-interaction. Accordingly, either the Fe(II)hs ion is absent or low spin Fe(II) is present (both less likely according to the XAS results) or the coupling to the Fe(II)hs is so weak that it does not significantly affect the EPR spectrum of the Mn(II) (in agreement with the large Mn-Fe distance determined by XAS below).

Spin quantification of contributions to the EPR spectra from Mn(III)Fe(III) and Mn(II)Fe(II) states revealed the presence of mixtures of redox states in all samples (Table 3). The fraction of EPR-silent R2 protein was obtained as the difference between total Mn-Fe clusters (Table 1) and those observed by EPR. For R2ox samples, the fraction of EPR-visible species is 0.69, comprising ~75% Mn(III)Fe(III) and ~25% Mn(II)Fe(II). For R2red, the fraction of EPR-visible species is smaller, 0.48, and comprised ~85% Mn(II)Fe(II) and ~15% Mn(III)Fe(II). The EPR-invisible states are suggested to be the high potential Mn(IV)Fe(III) in R2ox and the low potential Mn(III)Fe(II) in R2red.

Electronic Features and Nuclear Geometry of the Manganese and Iron Sites from XANES—The XANES region of XAS spectra is sensitive to the number of metal ligands, the site geometry, and to metal oxidation and spin state. Fig. 2 shows XANES spectra of R2red and R2ox collected at the iron (top) and manganese (bottom) K-edges.

Iron K-edge—The iron edge energy in R2ox (7123.6 eV) and its downshift by ~1.5 eV in R2red (to 7122.1 eV), compared with iron compounds with known oxidation state (51–53), indicate Fe(III) in 80–100% of R2ox and Fe(II) in 70–90% of R2red, in line with the EPR data. Thus, the reductive treatment in the presence of R1 induced a near-quantitative single-electron iron reduction in R2.

The pre-edge feature of the XANES (Fig. 2, insets) reflects formally dipole-forbidden 1s→3d electronic transitions, which may gain intensity, e.g. by 3dap orbital mixing. The small iron pre-edge amplitude in R2red and R2ox suggested a relatively centro-symmetric coordination of Fe(II) and Fe(III) (54). Fig. 3 shows the isolated iron pre-edges. In R2red, curve-fitting

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**TABLE 2**

|                  | g-tensor;  g, g,  g (±0.002) | 55Mn hf-tensor (±0.2) |
|------------------|------------------------------|----------------------|
| Mn(II)Fe(II)     | 2.0 (isotropic)              | 9.56 (isotropic)     |
| Mn(III)Fe(III)   | 2.013, 2.009, 2.015           | 13.3, 10.9, 9.0      |
|                  | [2.015, 2.009, 2.024]         | [14.0, 11.2, 9.6]    |

* Values from Ref. 32 for the Mn(III)Fe(III) state in the hydroxyurea-treated R1R2 complex.
revealed the presence of three spectral contributions (black lines). Their center energies (7111.2, 7112.1, and 7113.3 eV) and relative intensities (1:0.35:0.51) compare well with values for high spin Fe(II) in a near-octahedral ($O_h$) first-sphere coordination with ionic bonding to O/N ligands (54). In R$_2^{ox}$, two contributions (at 7111.9 and 7113.8 eV, relative intensities of 1:0.57; Fig. 3, bottom) are consistent with high spin Fe(III) in $O_h$ geometry in the majority of protein molecules (54). The apparently slightly lower transition energies and smaller energy splitting than in comparable Fe(III) compounds (54) of roughly equivalent to the 3d-orbital energy splitting, 10Dq) likely are due to the minor Fe(II) admixture in R$_2^{ox}$.

**Manganese K-edge**—The manganese edge energy of 6549.4 eV of R$_2^{ox}$ (Fig. 2, bottom) is well compatible with Mn(III), compared with model compounds (Fig. 2, bottom, upper inset) (39, 55). However, the dip in the edge rise (arrow) points to a significant Mn(IV) fraction in R$_2^{ox}$ (Ref. 56 and see below). The edge energy of 6548.0 eV in R$_2^{red}$ suggests a mean oxidation state of 2.5 (39), due to the presence of 50% of Mn(II) and Mn(III). Thus, R$_2^{red}$ mainly contained Mn(II)Fe(II) and Mn(III)Fe(II) sites, in agreement with the EPR data. The small pre-edge magnitudes (Fig. 2) are explained by Mn(II/III) ions in near-octahedral geometries (39, 57, 58), but the flat edge maxima suggest marked Mn-ligand distance heterogeneity.

The Mn(IV)Fe(III) state is EPR-silent due to its total integer spin of 1 (interaction of high spin Fe(III) with $S_{Fe}$ = 5/2 and Mn(IV) with $S_{Mn}$ = 3/2) (27, 32). We searched for Mn(IV) by comparison of the pre-edge features (Fig. 4) and XANES (Fig. 4B) of R$_2^{ox}$ and comparable Mn(II, III, IV) compounds (58, 59). Indeed, the pre-edge spectrum of R$_2^{ox}$ was well reproduced by summation of the Mn(II, III, IV) spectra with relative weight-
ings of 0.14, 0.62, and 0.24 (Fig. 4A). A similar Mn(IV) contribution also clearly was discernable in the first derivative of the R2ox manganese edge spectrum (Fig. 4B). Thus, the pre-edge data suggest surplus Mn(III, IV)-oxide interactions in R2ox.

X-ray Photoreduction—Modification of high valent metal sites due to photoreduction is a concern when using intense X-rays (62, 63). The rate of Fe(III) reduction was estimated by recording series of XAS scans on the same R2ox sample spot (not shown) and determination of the edge energies of the resulting spectra. Surprisingly rapid Fe(III) reduction was observed (Fig. 5), suggesting that the Fe(II) level was reached after ~10 h of x-ray exposure. On this basis and using an x-ray flux of ~10^9 photons s^{-1} mm^{-2}, a dose for the Fe(III)→Fe(II) transition of ~10^{13} photons mm^{-2} (at 20 K) is calculated. The x-ray dose applied in crystallography (46, 62) typically is at least an order of magnitude higher and thus may result in significant iron reduction. Reduction of high valent manganese may be similarly rapid and will be assessed in a forthcoming study (see Ref. 48). At least during the first two XAS scans, there was no evidence for a change in the manganese oxidation state due to the x-ray exposure in R2ox and R2red samples (supplemental Fig. S1). The shown XAS spectra of Fe(Mn) were obtained after ~2 h (~1 h) of x-ray irradiation where photoreduction was negligible.

Interatomic Distances from EXAFS—Simulation (curve fitting) of EXAFS spectra was employed to investigate the atomic structure of the manganese and iron sites (numbers of metal ligands, metal-metal/ligand distances). Fig. 6 shows iron and manganese EXAFS spectra of R2red and R2ox (black lines).

Iron EXAFS—Two resolved FT peaks at reduced distances of ~1.5 Å and ~1.8 Å in R2red (the reduced distance is the true absorber-backscatterer distance minus ~0.4 Å due to a phase shift) indicate at least two significantly different Fe-ligand distances, likely due to longer terminal and shorter metal-bridging Fe-ligand interactions. In R2ox, both FT peaks were at smaller distances, due to bond-shortening at Fe(III). Additional clear FT peaks were observed in the range of ~2.3–3.0 Å (Fig. 6, asterisks), corresponding to Fe-Mn interactions as revealed by the EXAFS simulations (below). These vectors also were
shorter in R2ox, suggesting changes in the Fe-Mn bridging motif.

Manganese EXAFS—Several FT peaks due to first-sphere ligands at manganese were observed. Their comparably small magnitudes indicate interference of EXAFS oscillations from Mn-ligand vectors with significantly different lengths. In R2ox, the enhancement of the peaks at <1.8 Å suggests formation of additional short Mn-ligand bonds in the presence of Mn(III) and Mn(IV). Further FT peaks in the range of 2.3–4 Å exist (Fig. 6, asterisks), reflecting Mn-Fe distances as revealed by the EXAFS simulations (below). The FT peak proportions indicate that shorter Mn-Fe distances prevail in R2ox, again suggesting changes in the metal coordination and bridging motif.

EXAFS Simulations—The spectra in Fig. 6 were analyzed using (in part) a knowledge-based simulation approach. Best fits in Fig. 6 (colored lines) represent parameters in Table 4. The crystal structure of Ct R2 (1SYY (26, 31)), containing an Fe-Fe site, reveals the coordination of one nitrogen from histidine and of three oxygen atoms from carboxylate groups of glutamates or water molecules to each iron, two oxygens in metal-bridging positions, and thus two six-coordinated iron ions. Near-octahedral iron and manganese ions in R2red and R2ox are in agreement with the XANES analysis. Accordingly, the simulations were based on a similar coordination as in the crystal, including Fe/Mn binding to two O-shells (the shorter one accounting for μO-species) and one N-shell (Table 4), to yield a total coordination number, N_{O,N} of six. Notably, in all cases N_{O,N} was in the range 5–6 if it was allowed to vary in the curve fitting.

The XANES and EPR data suggested ~50% Mn(II)Fe(II) and Mn(III)Fe(II) in R2red and Mn(III)Fe(II) (dominant) and Mn(IV)Fe(III) (~25%) in R2ox, plus minor amounts of other species in both cases. Each valence couple could exhibit a different Mn-Fe distance, contributing to the EXAFS. In the EXAFS fits, the Mn-Fe distances of the dominant species (from spectral features above noise level, Fig. 6) were assessed (Table 4).

Primary Ligands to Manganese and Iron—(i) Several significantly different Mn/Fe/O-N bond lengths were determined, which are in the expected range for terminal and metal-bridging Mn(II, III, IV) and Fe(II, III) ligands; the Mn/Fe-N(His) bond may be particularly long. (ii) Considerable shortening of Mn/Fe-O/N distances (up to 0.13 Å) and an increased number of short vectors in R2ox indicate additional Mn-μO(H)-Fe motifs at least in the Mn(III)Fe(II) and Mn(IV)Fe(III) states. Such motifs may be absent in the Mn(II)Fe(II) state, because the number of Mn-O bonds of <1.9 Å was very small in R2red. (iii) The particularly short Mn-O distance of ~1.65 Å in R2ox (Table

TABLE 4
Simulation parameters of EXAFS spectra
Each simulation component (absorber-backscatterer interaction) is represented by three numbers: N_i, coordination number (per metal atom); R_i, absorber-backscatterer distance (in Å); and 2σ_i, Debye-Waller factor (×10^2 Å²). The quality of each simulation is characterized by the error sum R_i (80). R_i was calculated over reduced distances of 0.5–4.5 Å (manganese spectra) and 1–3.5 Å (iron spectra). For the simulations of the manganese spectra: (a) represents simulations with three metal-metal distances, and (b) represents simulations with four metal-metal distances. Restraints used in the simulations: The sum of the N_i-values of the metal-O,N shells was 6; the sum of the N_i-values of the Fe-Mn(Fe)/Mn-Fe interactions was 1; 2σ_low of the first O-shell, the N-shell, and of the metal-metal interactions were fixed to the given values.

|        | Fe EXAFS | Mn EXAFS |
|--------|----------|----------|
|        | Fe-O/N   | Mn-O/N   |
|        | N/R/2σ_i | N/R/2σ_i |
|        |          |          |
| R2red  | 1.18/1.93/2 | 0.35/2.99/2 | 26.9 |
|        | 3.97/2.08/6 | 0.65/3.25/2 |          |
|        | 0.85/2.47/2 |          |          |
| R2ox   | 1.31/1.88/2 | 0.77/2.91/2 | 24.6 |
|        | 4.23/2.02/15 | 0.23/3.31/2 |          |
|        | 0.46/2.48/2 |          |          |

* The Mn-Fe distance of 2.64 Å is not reliably determined.
Mn-Fe Site Structure of C. trachomatis RNR

4.15 Å observed only in R2red is beyond the upper limit of metal-metal distances: (i) The longest Mn-Fe distances were only discernable in the manganese data. This result and the slightly longer metal distances in the iron data may be caused by contributions of Fe-Fe sites; in addition, single occupancy iron sites may diminish the relative contributions of metal-metal distances to the iron EXAFS, rendering minor contributions invisible.

Below we summarize the immediate conclusions on the EXAFS-derived metal-metal distances: (i) The longest Mn-Fe distance of 4.15 Å observed only in R2red is beyond the upper limit of inter-metal distances of divalent ions in R2 crystal structures (Fig. 7). For this long distance, metal-bridging μO-species are not expected. Thus, it presumably reflects the Mn(II)Fe(II) state giving rise to magnetically uncoupled metal ions. (ii) The ~3.7 Å Mn-Fe distance in R2ox is close to those observed in crystal structures containing a bridging-chelating carboxylate or two carboxylate bridges (Fig. 7). (iii) The ~3.25 Å Mn-Fe distance is similar to that of Fe-Fe in the structure of wild-type Ct R2 (15YY). It also is at the lowest limit of distances in any R2 structure (Fig. 7) and compatible with a mono-μO(H) bridge between the manganese and iron ions (65, 66). (iv) The ~2.9 Å and ~2.75 Å Mn-Fe distances are shorter than inter-metal distances in any R2 crystal structure (Fig. 7). The ~2.9 Å was prominent both in the manganese and iron EXAFS of R2ox. The 2.75-Å distance was only derived from the manganese EXAFS of R2ox when four Mn-Fe distances were employed in the simulations. However, the fit quality (as judged by the almost two times lower error sum, \( R_p \), of this R2ox simulation) was significantly better than for the fit with three distances. A similar improvement was not observed for R2red (Table 4, manganese fits a and b; the Mn-Fe distance for R2red of fit b of 2.64 Å refers to a very minor component, which is not reliably determined and hence not further discussed). Mn-Fe distances <3 Å are found for di-μO(H) metal bridging in other proteins (Fig. 7) and models (46, 66–69). The ~25% contribution of the ~2.75-Å distance in R2ox relates it to Mn(IV).

On the basis of their relative contributions to the EXAFS and taking the above considerations into account, the observed Mn-Fe distances (Table 4) are assignable to oxidation states; in R2red, ~4.15 Å, Mn(II)Fe(II) and ~3.25 Å, Mn(III)Fe(II), and in R2ox: ~2.9 Å, Mn(III)Fe(III) and ~2.75 Å, Mn(IV)Fe(III). The ~3.7-Å distance may belong to Mn(II)Fe(II) with a bridging-chelating carboxylate, i.e. to a second Mn(III)Fe(III) species (evidence for such a species comes from EPR) or to a second Mn(II)Fe(II) state found only in R2ox.

BVS Calculations—The consistency of the EXAFS-determined metal-ligand bond lengths with the metal oxidation states was verified by BVS calculations (49) using Equation 2. For the iron data the parameters in Table 4 yielded mean iron oxidation states (BVS values) of 2.18 for R2red (assuming Fe(II) in the calculation) and 3.16 for R2ox (assuming Fe(III)), in agreement with near-quantitative amounts of Fe(II) in R2red and Fe(III) in R2ox. (A match of BVS and experimental oxidation state within ±15% is considered as a reasonable result.) From the manganese parameters (fits a), BVS values of 2.62 for R2red (assuming Mn(II)) and neglecting the shortest Mn-O distance due to Mn(III)) and of 3.06 for R2ox (neglecting the shortest Mn-O distance due to Mn(IV)) and assuming...
Mn(III) were calculated, also in agreement with the manganese oxidation levels from the XANES analysis. Interestingly, for the metal-ligand distances in the Ct R2 crystal structures (26, 31) we calculate mean iron oxidation states of −2.5 (ISSY) and −1.9 (2ANI). The structures were determined from protein initially containing Fe(III). Thus, x-ray photoelectron spectroscopy of Fe(II) may have occurred during diffraction data collection, comparable to the rapid Fe(III) reduction observed in the present XAS study.

**DISCUSSION**

*Mn-Fe Electronic Features*—EPR signals were detected of Mn(II)Fe(II) and Mn(III)Fe(III), as previously observed (27, 32, 71). The Mn(IV)Fe(IV) state attributed to a precursor of Mn(III)Fe(III) in Refs. 71 and 72 was not detected in the present study. This state is not accumulated under our aerobic purification conditions of R2 protein. The g and hyperfine tensors of the Mn(III)Fe(III) state in as-isolated oxidized R2 differ to some extent from that formed in the R1R2 complex during catalytic turnover. Formation of the holoenzyme complex and substrate binding to R1 during catalytic turnover seems to influence the electronic properties of the metal site, as previously proposed (27). The two Mn(III)Fe(III) states may differ in their metal-bridging motifs.

XAS suggested high spin (hs) Fe(II) and Fe(III) ions. This result is in agreement with the previously observed antiferromagnetic coupling of Fe(III)hs and Mn(III)hs, yielding a ground-state S = 1/2 system (27, 32, 48). Magnetic uncoupling of Mn(II)hs from Fe(II)hs and the large metal distance (−4.15 Å) imply the absence of metal-bridging μO-species in the Mn(II)Fe(II) state.

Apparently, the Fe(III) is more rapidly reduced than the Mn(III). Thus, the midpoint potential of the Fe(III) seems to be more positive. The velocity of x-ray photoelectron spectroscopy suggests a value close to 1 V (62). Interestingly, Mn(IV) is more rapidly reduced than Fe(III) (27, 48). These results point to a Mn(IV) > Fe(III) > Mn(III) order of the oxidizing potentials. The Mn(IV) ion hence is the primary target of electron transfer from R1.

**Structure of the Mn-Fe Site**—Overall, the XAS-derived metal-metal distances and metal-ligand bond lengths and the metal ligation by histidines and glutamates observed in the available crystal structures of Ct R2 (26, 31) are compatible with structural motifs of the hetero-metallic site similar to those of homo-metallic sites in other R2 proteins and related enzymes. Four basic motifs are shown in Fig. 7B. Deviations from these motifs with respect to the presence of certain non-amino acid ligands, overall site geometry, and bonding mode of carboxyl groups were observed in crystals and, e.g. may be due to site heterogeneity resulting from x-ray photoelectron spectroscopy of metal ions.

In the reduced R2 sample studied here, two main oxidation states were present, Mn(II)Fe(II) and Mn(III)Fe(II). In the Mn(II)Fe(II) state, μO(H) metal bridges seemed to be absent, in line with Fe(II)Fe(II) crystal structures in other R2 proteins showing only carboxylate bridges (Fig. 7). However, some structures reveal metal ions coordinated only by four ligands (teta-coordinated ions). The XAS data argues against teta-coordinated Mn(II) and Fe(II) in Ct R2, but suggests penta- to hexa-coordinated ions. Thus, there may be metal-bound water species, which are replaced by O₂ and its cleavage products upon the metal site oxidation.

The largely reduced Mn-Fe distance (~3.25 Å) in the Mn(III)Fe(II) state is compatible with a mono-carboxylato, μO(H)-bridging motif (66, 67, 74) (Fig. 7). For a similar Fe-Fe distance (~3.3 Å) in the structure of wild-type Ct R2 (31), two O atoms in bridging positions were found, but two Fe(III) ions were anticipated. Due to limited resolution of crystal structures the assignment of such O-species to true μO(H) bonds often is ambiguous (31, 78). The BVS calculations suggest Fe(III)Fe(II) in the structure, i.e. one iron may have become singly reduced during data collection. In future studies x-ray photoelectron spectroscopy should be quantified under crystallography conditions.

In R2 oxidized at ambient O₂, two main oxidation states were observed, Mn(III)Fe(III) and Mn(IV)Fe(III); only a small fraction was in the Mn(II)Fe(II) state. The metal distance of ~3.7 Å is similar to that of ~3.5 Å in the structure of the Phe-127 → Tyr mutant of Ct R2 (31) where a bridging-chelating carboxylate was observed, but also similar to metal(II)₃ sites (Fig. 7). It thus may represent a Mn(III)Fe(III) site similar to the Ct mutant or a bis-carboxylato bridged Mn(II)Fe(II). Further studies are required to clarify this issue.

In the main Mn(III)Fe(III) and Mn(IV)Fe(III) species, metal-metal distances (~2.9 and ~2.75 Å) are shorter than those in any R2 crystal structure (Fig. 7). An even shorter Fe-Fe distance of ~2.5 Å has been reported for *E. coli* R2 (65). We did not observe such a short metal distance for Ct R2. According to our results it could rather belong to a metal–N(His) bond. The ~2.9 Å distance is similar, e.g. to that of the Fe(III), state in methane monoxygenase (73). Metal distances of ~2.65–2.80 Å are typical for mono-carboxylato, di-μO(H)-bridged metal pairs (46, 66, 67, 74). Density functional theory studies on the Fe(IV)Fe(III) state yielded metal distances of ~2.7 Å (76, 77). A distance of 2.63 Å for a mono-carboxylato, di-μO-bridged site, but of 2.82 Å for a mono-carboxylato, (μO)(μOH)-bridged site was found in (21), similar to the ~2.75–Å distance here assigned to Mn(IV)Fe(III). In summary, these results likely imply di-μO(H) metal bridging at least in the Mn(IV)Fe(III) state of Ct R2 ("diamond core"). Mn(IV)Fe(III) formation may lead to deprotonation of μOH bridges (74, 75) or reduction of a peroxide (OOH) in the Mn(III)Fe(III) state.

Recently, an XAS study on the Mn(IV)Fe(III) state reported a longer Mn-Fe distance of ~2.9 Å (48), similar to the one here observed for Mn(III)Fe(III). In Ref. 48, the Mn(IV)Fe(III) state was populated after reconstitution of apo-R2 with Mn(II) and Fe(II) ions, whereas in the present study R2 was purified, which already contained the Mn(IV) and Fe(III) ions. A possible explanation for the two Mn-Fe distances is the formation of several different Mn(IV)Fe(III) intermediates in the O₂ activation path (Fig. 8), which differ, e.g. with respect to the protonation of μO species. One additional protonated bridge (μOH) in Mn(IV)Fe(III) can explain the elongation of the Mn-Fe distance from ~2.75 Å (this study) to ~2.9 Å (48). The formation of several Mn(IV)Fe(III) intermediates also has been suggested in a recent Mössbauer investigation on Ct R2 (29) and related to redox transitions of amino acids (Trp-51 and Tyr-222) and protolytic reactions. These results point to the presence of several
Mn-Fe Site Structure of C. trachomatis RNR

![Mn-Fe Site Structure](image)

FIGURE 8. Structural models of the Mn-Fe site. A, the Mn(IV)Fe(III) state; amino acid ligation of metal ions is as in the crystal structure of Fe–Fe-containing C. trachomatis R2 (31); metal bonding by O-species is in agreement with the XAS data. B, tentative sequence of intermediates in O2 activation and reduction at the Mn-Fe site based on the XAS and EPR data and previous studies (26, 27, 29, 31, 38, 72). We propose that an Mn(III)Fe(III) state is an intermediate both in formation and reduction of Mn(IV)Fe(III); the main Mn(III)Fe(III) species observed in this study tentatively is placed in the oxidation pathway; binding of a peroxide is uncertain, but plausible. The Mn(II)Fe(II) state may contain a second bridging carboxylate; manganese and iron positions are tentative; protonation of bridging ligands is not assigned; numbers of electrons and protons are not meant to be stoichiometric; further, but omitted metal ligands may be as in A. Broken arrows between Mn(III)O2(Hx)Fe(III) and Mn(IV)O(Hx)Fe(III) denote the possible formation of further intermediates, i.e. the Mn(IV)Fe(III) state(s) described in Refs. 29, 48, one of which may contain a surplus proton on a μ-OH bridge leading to a longer Mn-Fe distance compared with our Mn(IV)Fe(III) state.

configurations of the active Mn(IV)Fe(III) state, which all could be of functional relevance.

Particularly short Mn-O bonds observed in R2ox are expected for metal-μO(H) bonds, but also are compatible with a Mn(IV)=O motif, i.e. due to a terminal oxo/peroxo group or a (semi)bridging peroxide. A metal-bridging peroxide has been proposed for the Fe(III)2 state of classic R2 proteins (Ref. 71 and references therein). Bound O2 reduction products are expected for Mn(III)Fe(III), Mn(IV)Fe(III), and Mn(IV)Fe(IV).

Construction of models of the Mn-Fe site requires knowledge on the manganese and iron binding positions. The metal-to-protein stoichiometries suggest that manganese preferentially is bound to a site already containing one iron. The ion ligated by, e.g. His-230, may be bound more strongly, because it is coordinated by four amino acids and hence may be iron. Accordingly, at present we favor manganese binding to, e.g. His-123, placing it closest, at ~7 Å, to Phe-127, which replaces the Tyr in Ct R2.

Reaction Sequence of O2 Activation—In this investigation, starting from aerobically prepared Ct R2 protein grown in the presence of manganese and iron (31) and found to adopt two main states (Mn(III)Fe(III) and Mn(IV)Fe(III)), two reduced states of R2 (Mn(III)Fe(II) and Mn(II)Fe(II)) were produced by the addition of R1, nucleotides, and reductant (DTT). The reverse process, formation of states after the addition of O2 to R2 apo-protein reconstituted with Mn(II) and Fe(II) ions, also has been studied (27, 38, 72). Consistently, it was found that the active state, Mn(IV)Fe(III), is formed in R2 in the presence of O2 only and does not require the input of electrons, e.g. from the R1 subunit. Mn(III)Fe(III) formation was observed after single electron donation to Mn(IV)Fe(III) from R1, exogenous peroxide, or dithionite (27, 38, 72). Here, the Mn(III)Fe(III) state was dominant in as-isolated R2, in the absence of R1 and reductants. Thus, this Mn(III)Fe(III) state may be a precursor of the Mn(IV)Fe(III) state and the primary product of O2 binding to Mn(II)Fe(II) (Fig. 8). It thus may carry a bridging peroxide. Whether the second minor Mn(III)Fe(II) state also is an intermediate in O2 activation is unclear. It may also result from the formation of an inactive species (22, 81) or from unspecific reduction of Mn(IV)Fe(III). In any event, the differences of the Mn(III)Fe(III) states produced by various treatments may reflect subtle, but functionally important changes at the metal site.

Apparently, the Mn(II)Fe(II) state of Ct R2 is hard to reach even by prolonged R2ox reduction. Mn(III)Fe(II) is formed more rapidly. This state represents a novel intermediate in the reduction pathway. Effective Mn/Fe reduction presumably involves electron transfer from R1. It is an option that the Mn(II)Fe(II) state is not involved in catalysis in vivo and instead, Mn(III)Fe(II) is the starting state of O2 activation, preformed to adopt a bridging peroxide by its shorter Mn-Fe distance.

In Fig. 8, we tentatively summarize the information from our XAS and EPR data, also taking into account previous crystallographic (26, 31) and spectroscopic results (27, 29, 38, 48, 72). A potential role of the Mn(III)Fe(III) state in oxidation as well as reduction of the metal cluster is suggested. Future investigations will aim at determination of the structural basis for preferential iron or manganese binding, of the resistance of Ct R2 toward reactive oxygen species (26, 72), and on the complete sequence of redox intermediates in O2 activation and reduction of the unusual Mn-Fe site in the tyrosine-less Ct RNR.

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Mn-Fe Site Structure of C. trachomatis RNR

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