A heterobimetallic Mn/Fe cofactor is present in the R2 subunit of class Ic ribonucleotide reductases (R2c) and in R2-like ligand-binding oxidases (R2lox). Although the protein-derived metal ligands are the same in both groups of proteins, the connectivity of the two metal ions and the chemistry each cofactor performs are different: in R2c, a one-electron oxidant, the Mn/Fe dimer is linked by two oxygen bridges (µ-oxo/µ-hydroxo), whereas in R2lox, a two-electron oxidant, it is linked by a single oxygen bridge (µ-hydroxo) and a fatty acid ligand. Here, we identified a second coordination sphere residue that directs the divergent reactivity of the protein scaffold. We found that the residue that directly precedes the N-terminal carboxylate metal ligand is conserved as a glycine within the R2lox group, but not in R2c. Substitution of the glycine with leucine converted the resting-state R2lox cofactor to an R2c-like cofactor, a µ-oxo/µ-hydroxo-bridged MnIII/FeIII dimer. This species has recently been observed as an intermediate of the oxygen activation reaction in wild-type R2lox, indicating that it is physiologically relevant. Cofactor maturation in R2c and R2lox therefore follows the same pathway, with structural and functional divergence of the two cofactor forms following oxygen activation. We also show that the leucine-substituted variant no longer functions as a two-electron oxidant. Our results reveal that the residue preceding the N-terminal metal ligand directs the cofactor’s reactivity toward one- or two-electron redox chemistry, presumably by setting the protonation state of the bridging oxygens and thereby perturbing the redox potential of the Mn ion.

Three different native binuclear metal cofactors exist within the ferritin-like superfamily. In most groups, the cofactor consists of a diiron complex,
while other groups contain a dimanganese complex or a mixed manganese/iron complex (1–7). The latter has been identified in two groups: the R2 subunits of subclass Ic ribonucleotide reductases (R2c) and the R2-like ligand-binding oxidases (R2lox) (4, 5, 7). Despite showing a high degree of similarity, these two proteins perform very different redox chemistry.

Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides to deoxyribonucleotides. In most subclasses of the aerobic class I RNRs, a binuclear metal cofactor in the R2 (NrdB) subunit generates a catalytic radical which is reversibly transferred to the active site in the R1 (NrdA) subunit (2, 3, 8). Subclass Ia contains a diiron cofactor which oxidizes a nearby tyrosine residue (2, 3, 8), while in subclass Ib, a tyrosyl radical is generated by a dimanganese complex (9–14). In subclass Ic (R2c), which contains a mixed manganese/iron complex (15–17), and probably also in the recently proposed dimanganese subclass Id (18, 19), the metal complex itself acts as the catalytic radical, with the Mn ion shuttling between the III and IV oxidation states. A metal-free subclass Ie was also recently discovered (20–22).

In contrast, the Mn/Fe cofactor in R2lox performs two-electron redox chemistry, catalyzing the formation of a tyrosine-valine ether cross-link in the protein scaffold during cofactor maturation (23, 24). The physiological function of R2lox is unknown. The name ligand-binding oxidase is derived from the observation that in R2lox proteins the R2 scaffold is remodeled to contain a hydrophobic channel leading from the protein surface to the active site in which a long-chain fatty acid ligand is bound that co-purifies with the protein from heterologous expression hosts (23, 25). While they are structurally more closely related to RNR R2 proteins (26), the function of R2lox proteins appears to resemble bacterial multicomponent monoxygenases (BMMs), which catalyze two-electron oxidations on a variety of hydrocarbon substrates (1, 24).

In both R2c and R2lox, cofactor assembly begins with the binding of MnII and FeII to the apo-protein scaffold, although each system achieves selective metallation by different strategies (27, 28). The two metal ions are bound in near-octahedral geometry, with the manganese ion occupying the N-terminal binding site (site 1) (Figure 1A, B) (23, 25, 29, 30). In the reduced state, each metal ion is coordinated by one histidine and one monodentate glutamate ligand, while two further glutamate residues bridge the metal ions (23, 24, 28). An oxo species, likely a water molecule, is bound at the open coordination site of the manganese ion. In R2lox, the fatty acid ligand also bridges both metal ions, whereas another oxo species, likely also a water molecule, occupies the corresponding position in R2c.

Cofactor activation proceeds via reaction with O2 (Figure 1C). For both systems, the initial bound O2 intermediate has been modelled as bridging the two metal ions as a side-on μ-η2-η2-peroxo species (31). To achieve side-on O2 binding, the C-terminal bridging/chelating glutamate, and in the case of R2lox also the lipid head group, need to shift away from their bridging coordination. Crystallographic data demonstrates that this does occur for the glutamate, which shifts to a monodentate coordination at the Fe ion following O2 activation in both R2lox and R2c (16, 23, 24). It is less clear that the lipid headgroup of R2lox is also displaced, as it is seen in the same position following activation. It is thus currently unclear whether cofactor activation in the two systems follows the same trajectory.

In R2c, O2 reduction generates a bis-μ-oxo MnIV/FeIV intermediate with a terminal hydroxo ligand on the Mn ion, and a similar intermediate is expected to be formed in R2lox (23, 31–34). Subsequent one- or two-electron/proton transfer events lead to the final, resting state cofactor structure. In the case of R2c the MnIV and FeIII ions are linked by two oxo (μ-oxo/μ-hydroxo) bridges with a terminal hydroxo ligand on the Mn ion, whereas in R2lox the two metal(III) ions are linked by a single oxygen (μ-hydroxo) bridge, with the fatty acid ligand occupying the position of one of the oxygen bridges of R2c, and a terminal water ligand on the Mn ion (Figure 1A, B) (16, 23, 35–37). In R2lox, concomitant with cofactor activation, an ether cross-link is formed between the Cβ of a valine and the hydroxyl oxygen of a tyrosine close to the active site. Prior to cross-link formation the tyrosine forms a hydrogen bond with the carbonyl oxygen of the valine (Figure 1A) (23, 24). These two residues are conserved in the R2lox group, but not conserved in R2c (5), which does not form such a covalent link in the protein scaffold.

Here we introduced two different single point mutations into the R2lox scaffold to clarify whether
cofactor activation in R2lox and R2c occurs by the same mechanism or at what point cofactor assembly diverges. These substitutions are targeted towards blocking the binding of the fatty acid ligand that co-purifies with the protein, the only structural difference in the first coordination sphere between the two homologues. Substitution of one specific residue in the second coordination sphere was found to convert the activated R2lox cofactor into an R2c-like cofactor: the glycine residue directly preceding the N-terminal glutamate ligand, G68. This position is completely conserved as a glycine in the R2lox group, whereas it is not conserved in the characterized RNR R2 subclasses, where it is generally occupied by a small hydrophobic residue (Figure 1D). We examined the substitution of G68 with a leucine or a phenylalanine (G68L/F). We found that the G68F substitution had no significant effects on cofactor reactivity, whereas the G68L variant stabilized an R2c-like cofactor and prevented cross-link formation. The EPR signature of the R2c-like cofactor matches that of an intermediate observed during oxygen activation of the wild-type protein (33), demonstrating that it is a physiologically relevant species.

Results and discussion

In analogy to our previous experiments with wt-R2lox (23), we crystallized the G68L/F-R2lox point mutants in metal-free form and soaked the crystals with equal concentrations of Mn II and FeII in large excess under anoxic or aerobic conditions to obtain structures of the non-activated reduced and oxidized resting state of the proteins, respectively. X-ray anomalous dispersion was used to quantify the relative amounts of Mn and Fe in each binding site in the crystals.

The G68L substitution blocks binding of the fatty acid ligand to the metal cofactor

The G68L substitution excludes the fatty acid from the active site, although electron density for a hydrophobic ligand (modelled as caprylic acid in the crystal structures) is observed further up in the ligand-binding tunnel. Since the absence of the fatty acid ligand leaves an open coordination site on both metal ions, it causes some rearrangements in the reduced state active site of G68L-R2lox as compared to the wild-type (Figure 2A). E167 acts as a bidentate ligand to Fe2 instead of its usual monodentate coordination, such that Fe2 remains near-octahedral. Mn1 retains a free coordination site, although this may be occupied by an additional water molecule that is not observed in the electron density (see XAS characterization below). The second-sphere residue Y175 is disordered, but best modelled in two alternate conformations: its usual conformation as a hydrogen bonding partner to the terminal water ligand of the site 1 metal ion, and a rotated conformation in which it may form a hydrogen bond with the non-binding carboxyl oxygen of E69 instead, otherwise the other hydrogen bonding partner of the water molecule (37). Y175 is somewhat disordered in reduced state wt-R2lox as well and may always be present in both conformations, just to different degrees (23, 24).

G68L-R2lox accumulates more Mn in site 2 than the wild-type

Following soaking of apo-protein crystals with equal concentrations of MnII and FeII under anoxic conditions, the metal distribution in G68L-R2lox was (within error) identical to the wild-type (23), with roughly 60% Mn in site 1 and 80% Fe in site 2 (Figure S1). Soaking with MnII and FeII under aerobic conditions led to drastically different results, however. Under these conditions, wt-R2lox accumulates predominantly Mn in site 1 and Fe in site 2 (23), whereas aerobically soaked G68L-R2lox crystals contained mainly Mn in both sites (Figure S1). The active site in aerobically soaked G68L-R2lox is in a reduced state conformation that is practically identical to that obtained by soaking under anoxic conditions, and the V72-Y162 ether cross-link is not observed (Figure 2B). As in wt-R2lox (24), a Mn/Mn cofactor in G68L-R2lox therefore seems to be unable to react with O2 and consequently does not support cross-link formation.

In contrast, in solution reconstitution experiments Mn/Fe cofactors were efficiently assembled in G68L-R2lox, although it also retained more Mn than the wild-type under the same conditions and, unlike wt-R2lox, assembled MnIII complexes if only Mn was present, thus following an R2c-like metallation pathway (see SI S2 and S3.4, Figures S2 and S6) (27, 28). The large amount of Mn observed in site 2 in aerobically soaked crystals might therefore be caused by a combination of increased Mn affinity and the progressive depletion of bulk Fe by its reaction with O2, which
would drive equilibrium protein metallation towards a Mn/Mn cofactor, as Mn\textsuperscript{II} does not react with O\textsubscript{2} and thus remains available for protein binding. This would require the rate of cofactor maturation in G68L-R2lox to be slower than that of bound/free metal exchange. It has previously been inferred that these two rates are similar in wt-R2lox if a large excess of metal ions over protein is used, to rationalize the enrichment of Mn in site 1 which is observed in aerobic crystal soaking experiments, but not in solution experiments in which near-stoichiometric amounts of metal ions are used (23, 27). Thus the observation that in the presence of oxygen more Mn accumulates in both sites in the mutant suggests that cofactor maturation in G68L-R2lox is significantly slower than in the wild-type.

**The G68L substitution does not prevent oxygen activation of the R2lox cofactor**

Crystals were also soaked with an excess of Fe\textsuperscript{II} under aerobic conditions, forcing the protein to assemble a diiron cofactor. In this structure, Fe is bound in both sites, and the active site is in an oxidized state conformation with two oxo-type species (i.e. bis-oxo or oxo/hydroxo) bridging the metal ions, instead of the single $\mu$-hydroxo bridge and the bridging fatty acid ligand seen in the wild-type (23). This arrangement leads to an active site geometry that more closely resembles that of oxidized R2c than that of wt-R2lox (Figure 2C, F) (16). The G68L substitution thus does not prevent oxygen activation of the cofactor. However, electron density for the tyrosine-valine ether cross-link, though observed, was too weak to model the link, suggesting that the leucine substitution causes catalytic impairment.

**Other second-sphere substitutions that block ligand binding do not cause any noticeable defects**

In contrast to the G68L mutation, substitution of G68 with a phenylalanine does not cause any visible defects in cofactor assembly, oxygen activation or cross-link formation (Figure 2E and SI S2). F68 blocks the ligand-binding tunnel in the reduced state, but does not exclude the fatty acid ligand from the metal site in the oxidized state, where it adopts a different rotamer (Figure 2D and E). The active site geometry is the same as in the wild-type in the oxidized state of G68F-R2lox, whereas in the reduced state the active site looks very similar to G68L-R2lox. It must be presumed that a fatty acid is bound higher up in the tunnel in G68F-R2lox in the reduced state as well, but electron density for it was not observed.

Another second-sphere substitution roughly opposite to G68 in the ligand-binding tunnel, A171F (see Figure 2F), demonstrates that the different effects of the G68L and G68F substitutions are not a consequence of the absence or presence of the fatty acid. The A171F substitution completely blocks ligand binding to the active site, but has no discernible effect on cofactor assembly or cross-link formation (38), indicating that the phenotype of G68L-R2lox is caused specifically by this mutation. We further characterized the interesting effects of the G68L mutation using EPR and X-ray spectroscopy as well as mass spectrometry.

**G68L-R2lox predominantly assembles an R2c-like Mn\textsuperscript{III}/Fe\textsuperscript{III} cofactor species in solution**

To elucidate the cofactor structure of the G68 mutants in solution, CW X-band EPR measurements were performed on aerobically Mn/Fe-reconstituted G68L- and G68F-R2lox. In Mn/Fe cofactors described in the literature the two metal ions, in the III oxidation state, magnetically couple to form an effective spin $\frac{3}{2}$ ground state, which gives rise to an EPR signal centered at approximately $g = 2.0$ (23, 36). The EPR spectrum is highly structured, a consequence of the hyperfine interaction of the $^{55}$Mn ($I = 5/2$) nucleus with the unpaired electron spin, which should split the EPR line into a sextet. The structure of the signal is a fingerprint of the two cofactor types. For R2lox a simple six line pattern is observed with an additional small line on the low field edge, whereas for R2c a six line pattern is observed, but several of the major lines display hyperfine structure (15, 17, 23, 27, 36).

Deviations from the six line pattern come about because the cofactor’s EPR signal changes depending upon how it is oriented in the applied magnetic field. As we measure samples in the frozen state, we probe all possible orientations the cofactor can take, with the resultant EPR spectrum thus representing the superposition of multiple hyperfine patterns (see ref. (36) for more details).

The EPR line shape of G68L-R2lox was found to strongly depend on temperature and microwave power (Figure S3). The dominant cofactor signal is best observed at higher temperatures ($T = 30$ K),
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similar to R2c (27). It also bears a strong resemblance to the cofactor signal of the R2c protein (Figure 3A) (15, 17, 27, 36). The overall hyperfine structure matches that of the R2c cofactor signal, resolving approximately six lines with additional superstructure best resolved on the first, fifth and sixth line. The main difference between the two signals is that the homogeneous linewidth of the R2c spectrum is somewhat narrower than that of the G68L-R2lox spectrum. If instead the absorption line shapes are compared, the similarities between the two spectra are more easily observed (see SI S3.3, Figure S5).

In addition to this R2c-like cofactor signal, at lower temperatures and higher powers (T = 10 K, P = 20 mW) a small wt-R2lox signal is observed in G68L-R2lox (Figure 3A). Under these conditions the R2c-like cofactor signal is completely suppressed due to slower relaxation properties, allowing the wt-like population to be quantified. It accounts for ~30-40% of MnIII/FeIII centers (SI S5, Table S3). At higher temperatures the wt-like contribution to the overall line shape is further suppressed due to a smaller exchange coupling compared to the R2c-like species, leading to thermal population of higher spin states (see SI S4). In contrast, in G68F-R2lox only the wt-like cofactor form was observed (Figure S4).

To uniquely constrain the set of field-dependent and field-independent spin Hamiltonian terms that describe the EPR spectrum of the R2c-like G68L-R2lox cofactor signal, higher frequency (Q-band) measurements were performed (Figure 3B). The fitted spin Hamiltonian parameters match those seen earlier for R2c (Table 1) (15, 17, 36). In particular the fitted 55Mn hyperfine tensor is very similar, albeit 10% smaller in magnitude, to that previously reported for the R2c cofactor, demonstrating that the Mn coordination environment of both cofactors is similar. Likewise, the 57Fe (I = ½) hyperfine tensor, which can be interrogated by labelling the cofactor with 57Fe, demonstrates that the Fe coordination environment of both cofactors is also similar (Figure S9). Taken together, these observations strongly suggest that the two cofactor forms are structurally equivalent, meaning that the G68L-R2lox protein harbors an R2c-like MnIII/FeIII cofactor.

We note that the R2c-like cofactor species is also present in wt-R2lox in a fraction of centers. We had previously assigned this fraction to an incorrectly metallated population (36). However, the characteristics of this fraction match those seen for R2c, indicating that it represents a physiologically relevant cofactor state.

Additional cofactor signals could be observed for G68L-R2lox samples prepared with excess MnII and substoichiometric amounts of FeIII following the initial incubation time of several hours. These additional signals decayed after approximately 24 hours of additional incubation at room temperature, leading to an increase in the EPR signals of both MnIII/FeIII cofactor signals (SI S6, Figures S11 and S12). The additional signal bears a strong resemblance to the recently postulated MnIV/MnIII cofactor that constitutes class Id RNR R2 proteins (18, 19) as well as to related MnIV/MnIII model complexes (39–43). It is important to note that the reduced MnIV/MnII cofactor of class 1b and Id R2 proteins does not directly react with O2 (9, 13, 19). Instead, superoxide is thought to be the in vivo oxidant (14, 19, 44). In the G68L-R2lox system, Fenton-like chemistry of free FeII in solution could instead be the source of superoxide. Thus, the scaffold is able to assemble a MnIV/MnIII cofactor in a small fraction of centers under the condition of preloading both sites with Mn in addition to having a source of reactive oxygen species.

The EPR-visible form of the dimanganese cofactor (MnIV/MnIII) is unstable in G68L-R2lox (t1/2 <12 hours), presumably reflecting the slow rate of reduction of the cofactor to the III/III level, which is EPR-silent. Consistent with this notion is the progressive increase of the MnIII/FeIII signal in which we observe the product of the reduction, i.e. the slow reduction of the initially formed, EPR-silent MnIV/FeIII state to the EPR-observable MnIII/FeIII state. Thus both results point to the G68L-R2lox cofactor having redox properties more similar to those of R2c, whose resting state is the IV/III oxidation level.

The R2c-like MnIII/FeIII cofactor species in G68L-R2lox contains two oxygen (µ-oxo/µ-hydroxo) bridges

The nature of the bridging ligand between the two metal sites strongly influences the magnetic properties of the cofactor and thus its EPR spectrum. The (super)exchange interaction (J) is mediated by the overlap of the orbitals of the bridging oxygen(s) with those of the metal ions. In the MnIII/FeIII cofactor of wt-R2lox, which has a
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The superexchange interaction can be estimated from the temperature dependence of the EPR spectrum. For the G68L-R2lox cofactor, J coupling is estimated to be 33 cm\(^{-1}\) (SI S4, Figure S10), significantly larger than in wt-R2lox, suggesting that the G68L-R2lox cofactor has a more extensive oxygen bridging network, i.e. at least one oxo bridge. The large exchange interaction probably precludes other connectivities such as a bridging peroxo, which is expected to have a smaller superexchange interaction owing to the longer metal-metal distance and more extended through-bond superexchange pathway.

The structure of the EPR signal also indicates that the G68L-R2lox cofactor contains a µ-oxo bridge. The structure is primarily due to the \(^{55}\text{Mn}\) hyperfine interaction, as described above. The \(^{55}\text{Mn}\) hyperfine tensor provides detailed information on the local coordination of the Mn ion. In an earlier EPR and DFT analysis of the \(^{55}\text{Mn}\) hyperfine tensor of both the R2lox and the R2c cofactor, based on an extensive set of model complex data, it was shown that the local ligand field of the Mn\(^{III}\) ion is different for the two cofactor forms (36). In R2lox, the Mn\(^{III}\) site is tetragonally elongated, with the unique Jahn-Teller axis perpendicular to a vector connecting the Mn-Fe sites, whereas in R2c the Mn\(^{III}\) site is instead tetragonally compressed, with the unique axis in a plane containing the Mn-Fe vector. Importantly, this rotation of the local ligand field axis is caused by the different protonation states of the bridging oxygen ligand in the two cofactors. Thus, the observation that the cofactor of G68L-R2lox displays approximately the same \(^{55}\text{Mn}\) hyperfine tensor as the R2c cofactor (Table 1) indicates that both cofactors contain a tetragonally compressed Mn\(^{III}\) ion, and therefore at least one oxo bridge (36).

To test whether the G68L-R2lox cofactor also has a second, protonated bridge, double resonance measurements were performed, i.e. \(^{2}\text{H}\)-HYSCORE at Q-band (Figure 4). In our previous study on wt-R2lox we showed that this was the method of choice for detecting one or more protonated oxygen bridges. For the wt-R2lox cofactor, the single µ-OH bridge gives rise to a large \(^{2}\text{H}\) coupling of 3.4 MHz (36). This manifests in the HYSCORE spectrum as a doublet split by 3.4 MHz and centered about the \(^{2}\text{H}\) Larmor frequency [\(\nu_{\text{Larmor}} \approx 8\text{ MHz at 1200 mT}\)]. For the R2c-like cofactor of G68L-R2lox, a new \(^{2}\text{H}\) coupling is observed at the expense of the wt signal. This coupling is also large (4.5 MHz), indicative of a µ-OH bridge, but clearly different from that of the wt.

In summary, multifrequency, multiresonance EPR data suggest that the dominant Mn\(^{III}/Fe^{III}\) cofactor seen in G68L-R2lox contains both a µ-oxo and µ-hydroxo bridge. Its geometric structure most likely resembles that observed in the crystal structure of Fe-only soaked G68L-R2lox (Figure 2C). We used XAS to further assess the cofactor species formed in G68L-R2lox.

X-ray absorption spectroscopy confirms that G68L-R2lox stabilizes a µ-O, µ-OH-bridged Mn\(^{III}/Fe^{III}\) cofactor

G68L-R2lox was aerobically reconstituted with either Mn and Fe or only Fe for XAS analysis. Both were examined because solution maturation of the R2lox protein in the presence of Mn\(^{II}\) and Fe\(^{II}\) leads to significant populations of both the Mn\(^{III}/Fe^{III}\) and Fe\(^{III}/Fe^{III}\) forms. Thus Mn/Fe and Fe-only samples were characterized to derive complementary information on both cofactor types.

The Fe-only G68L samples show XANES spectra at the Fe K-edge that are very similar to the wild-type, suggesting a similar near-octahedral coordination of iron (Figure 5A). The slightly larger pre-edge peak feature due to \(1s \rightarrow 3d\) electronic excitation transitions for G68L-R2lox compared to wt-R2lox implies lowered symmetry of the iron sites in this mutant. The K-edge energy of ~7124.7 eV indicates the near-quantitative presence of Fe\(^{III}\) (Table 2) (37), demonstrating that initial Fe\(^{II}\) centers activate oxygen to yield Fe\(^{III}\) cofactors in both wild-type and G68L-R2lox.

XANES spectra at the Mn K-edge collected for G68L-R2lox reconstituted with both Mn and Fe are more difficult to interpret due to the presence of both Mn\(^{III}/Fe^{III}\) cofactors and Mn\(^{II}\) cofactors in G68L-R2lox (see above, SI S2 and S3.4). For G68L-R2lox samples prepared for XAS the Mn\(^{II}\) fraction was significant, as indicated by a ~1.4 eV lower K-edge energy (Table 2), larger edge maximum, and increased pre-edge peak amplitude.
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compared to wt-R2lox. Assuming that MnII and MnIII are present only in MnII2 or MnIII/FeIII cofactors, respectively, the results suggest that the Mn/Fe-reconstituted G68L-R2lox XAS samples contained ~50% FeIII2, ~20% MnIII/FeIII, and ~30% MnII2 cofactors.

The Fe EXAFS of G68L-R2lox reveals increased numbers of short iron-ligand bonds and the predominance of a ~0.5 Å shorter Fe-Fe distance than in wt-R2lox (~3.05 Å) (Figure 5D). The same short distance was observed in the fraction of Mn/Fe-reconstituted G68L-R2lox assigned to the MnIII/FeIII population (Table 3). The metal-metal distance of ~3.05 Å in G68L-R2lox is almost identical to the distance in the (Mn/Fe)III/FeIII cofactors in R2c and mouse R2a proteins, for which such a distance has been assigned to the presence of a µ-O and a µ-OH bridge between the trivalent metal ions (49, 50). These findings suggest that an R2a/c-like µ-O, µ-OH-bridged (Mn/Fe)III/FeIII cofactor is formed in G68L-R2lox instead of the wild-type cofactor with a single µ-OH bridge, in agreement with the crystallographic and EPR data (Figure S13).

Mn EXAFS allows the MnII2 homodimer population to be characterized. It has overall ~0.1 Å longer manganese-ligand bonds typical for MnII and likely no short metal-ligand bonds compared to wt-R2lox, as well as a larger metal-metal distance (~4.1 Å), similar to metal(II) cofactors in R2 proteins lacking bridging oxides and holding a single carboxylate bridge (Figure 5C, Table 3) (50, 51). In the crystal structure of aerobically Mn/Fe-soaked G68L-R2lox (Figure 2B), E202 adopts a semi-bridging position as in the reduced state conformation, which may account for the ~3.55 Å Mn-Mn distance also detectable by EXAFS, as supported by similar structures of R2 proteins (51). Mn/Mn-G68L-R2lox in solution may contain E202 predominantly in a non-bridging position, which would explain the prevalence of the ~4.1 Å distance in the EXAFS.

Finally, photoreduction of the metal ions was examined by extended X-ray exposure during successive monochromator scans on the same sample spot. This caused shifts of the Mn and Fe K-edge spectra to lower energies (37, 49, 52). The respective K-edge energies decreased exponentially due to MnII→MnI or FeIII→FeII transitions (Figure 5A and B, insets). The magnitudes of the energy shifts reveal formation of FeII/FeIII centers after ~80 min of X-ray exposure and about two-fold faster formation of MnII (Table 2), as previously observed in wt-R2lox (24, 37). The much smaller K-edge energy shift in G68L-R2lox vs. wt-R2lox corroborates that only ~20% MnIII was initially present in the former variant.

The G68L substitution inhibits cross-link formation

To firmly establish if the G68L substitution causes a catalytic impairment, we used mass spectrometry to quantify the tyrosine-valine ether cross-link in Mn/Fe- and Fe/Fe-G68L-R2lox in comparison to the wild-type. We have recently shown that the cross-link is formed far more efficiently by a Mn/Fe cofactor than a diiron center in wt-R2lox (54). In agreement with our previous results, Mn/Fe-wt-R2lox contained roughly five times more cross-linked peptide than the iron-only form. In contrast, both Mn/Fe- and Fe/Fe-G68L-R2lox samples contained nearly four times less cross-link than Mn/Fe-wt-R2lox, but slightly more than Fe/Fe-wt-R2lox (Figure S14). Thus, although G68L-R2lox efficiently assembles Mn/Fe cofactors in solution which reduce O2, the leucine substitution does impair the catalytic process coupled to cofactor assembly, i.e. cross-link formation (38). It is not possible to determine the total amount of cross-link per protein, as only relative amounts of the same peptide across different samples can be determined. It appears likely, however, that the fraction of G68L-R2lox which forms the cross-link corresponds to the wt-like population observed by EPR, which would also suggest that the cross-link is formed near-quantitatively in Mn/Fe-wt-R2lox.

The G68L variant stabilizes an intermediate of the R2lox maturation cycle

A recent study by Miller et al. characterized a series of assembly and O2 activation intermediates of R2lox (33). Transient absorption spectroscopy identified relatively intense features in the visible region assigned as charge transfer bands associated with two early, bridging peroxy-type MnIII/FeIII
Cofactor intermediates, termed I1 and I2. The yield of these states was estimated to be very small (<5%), which would hamper their detection using other spectroscopies such as EPR. Interestingly though, two EPR signals were observed in the same study and tentatively assigned to the same two intermediates, although the same kinetic information could not be obtained from the EPR experiment. The first EPR signal, assigned to the I1 state, matches the EPR signal reported here assigned as a \(\mu\)-O, \(\mu\)-OH-bridged Mn \(\text{III}/\text{Fe}^{\text{III}}\) cofactor (Figure 3A), demonstrating that the G68L variant stabilizes a physiologically relevant intermediate of the R2lox cofactor assembly pathway.

We would therefore propose that the two EPR intermediates observed in the study of Miller et al. represent intermediates following O-O bond cleavage, as opposed to \(\text{O}_2\) binding/peroxo type intermediates. The first is the R2c-like intermediate described in this study, while the second presumably represents an intermediate involved in the re-binding of the fatty acid to the cofactor, e.g. a \(\mu\)-O, \(\mu\)-OH\(_2\)-bridged Mn \(\text{III}/\text{Fe}^{\text{III}}\) cofactor (Figure 6). We note that these two states are not expected to display an intense optical signature and would therefore not be observed in the transient absorption kinetics.

Thus our study, together with Miller et al. (33), suggests that lipid rebinding represents the rate-limiting step of maturation of the R2lox cofactor, with all preceding chemical steps, i.e. O-O bond cleavage and cross-link formation, occurring much faster. We suspect that the same process explains the very low yields of \(\text{O}_2\) activation intermediates seen in the Miller et al. study because the detachment of the fatty acid from the reduced cofactor, which is needed for \(\text{O}_2\) binding to initiate cofactor activation (23, 24), is also a slow chemical event. This would then explain why R2lox cofactor maturation is inefficient in vitro, i.e. orders of magnitude slower than the kinetics measured for R2c (33, 34).

**Conclusions**

The results described here shed further light on the chemical activity of manganese/iron cofactors. Our data demonstrate that \(\text{O}_2\) activation and maturation of the R2c and R2lox cofactors proceed along the same pathway, only diverging following formation of the Mn \(\text{IV}/\text{Fe}^{\text{IV}}\) state (Figure 6). We further show that a small second sphere perturbation of the protein scaffold can redirect their activity. Exchange of a single, uncharged, non-polar amino acid for a larger hydrophobic residue is sufficient to convert one cofactor form into another, namely convert the R2lox cofactor into an R2c-like cofactor. This conversion is not limited to the structure the cofactor adopts, but also extends to its chemical properties. Photoreduction kinetics, EPR parameters and the observed inhibition of cross-link formation demonstrate that this single second-sphere mutation alters the redox potential of the R2lox cofactor, making it a less potent oxidant. In contrast, other changes to the second coordination sphere (G68F, A171F) affect the cofactor structure to varying degrees, but not its reactivity (38).

When comparing the structures of the G68L and other R2lox variants to those of R2c, it appears that the leucine preceding the N-terminal glutamate ligand in G68L-R2lox exerts its effects on the structure and redox potential of the cofactor through a combination of increased hydrophobicity and decreased solvent accessibility of metal site 1 and steric exclusion of the fatty acid ligand (see Figure 2). These structural changes explain the change in chemical properties of the two cofactor forms. We have previously shown that the different electronic structures of the R2lox and R2c cofactors are caused by the different protonation states of their bridging oxo ligands, with the ground electronic state (HOMO) of the Mn ion switching from \(d_\sigma\) to \(d_{\sigma^2\gamma^2}\), with concomitant reduction in its redox potential (36). The introduction of a leucine must therefore stabilize a different equilibrium bridge protonation state by remodelling the local H-bonding environment in the vicinity of the \(\text{O}_2\)-derived bridge, presumably through solvent water exclusion.

In conclusion, we show that small perturbations of the second ligand sphere tune the structure and redox potential of Mn/Fe cofactors, opening up possibilities for the design of novel catalysts. The apparent unpredictability of the effects of these small perturbations suggests that site-saturation mutagenesis of the second sphere followed by screening is the most promising approach to identify cofactors with improved or novel reactivities.
Experimental procedures

Site-directed mutagenesis, protein production and purification

The G68L and G68F point mutations were introduced into a construct encoding full-length *Geobacillus kaustophilus* R2loxI (accession number WP_011232245) inserted into pET-46 Ek/LIC (Novagen) (23) by site-directed mutagenesis using the QuikChange Lightning kit (Agilent) and verified by DNA sequencing. Both point mutants were produced and purified in metal-free form according to the same protocol as wild-type R2lox (23). Briefly, protein was produced recombinantly in *E. coli* BL21(DE3) (Novagen) grown in terrific broth (ForMedium). To obtain metal-free protein, 0.5 mM EDTA was added to the cultures immediately before induction with 0.5 mM IPTG. Apo-protein was purified via heat denaturation of contaminating proteins and nickel chelate affinity chromatography. Cells were disrupted by high-pressure homogenization in lysis buffer (25 mM HEPES-Na, pH 7.0, 300 mM NaCl, 20 mM imidazole, 0.5 mM EDTA). The lysate was cleared by centrifugation, incubated at 60°C for 10 min, and again cleared by centrifugation. The supernatant was applied to a Ni-NTA agarose (Protino, Macherey-Nagel) gravity flow column. The beads were washed with lysis buffer containing 40 mM imidazole, followed by the same buffer without EDTA. Protein was then eluted using lysis buffer containing 250 mM imidazole and without EDTA. The eluate was exchanged into storage buffer (25 mM HEPES-Na, pH 7.0, 50 mM NaCl) using a HiTrap Desalting column (GE Healthcare), concentrated to ~1 mM, aliquoted, flash frozen in liquid nitrogen and stored at -80°C. Protein concentration was determined using the extinction coefficients at 280 nm determined for wild-type R2lox, 47.8 mM⁻¹ cm⁻¹ and 50.6 mM⁻¹ cm⁻¹ for metal-free and metal-bound protein, respectively (36). The metal contents of purified apo-protein batches were quantified by TXRF (see below). The only metal that is generally present in significant amounts is Fe, occupying ≤10% of the cofactor sites.

Crystallization and diffraction data collection

Both R2lox variants were crystallized in metal-free form by vapor diffusion in hanging drops at 22°C in the same condition as the wild-type protein, 25 – 32.5% (w/v) PEG 1500, 100 mM HEPES-Na, pH 7.2 – 7.5. To reconstitute the oxidized resting state Mn/Fe cofactor, crystals of metal-free protein were removed from their drop and soaked in mother liquor additionally containing 5 mM each MnCl₂ and (NH₄)₂Fe(SO₄)₂ for 1-2 h under aerobic conditions and then briefly washed in cryoprotectant solution (40% (w/v) PEG 1500, 100 mM HEPES-Na at the pH of the mother liquor) before flash-cooling in liquid nitrogen. Crystals of G68L-R2lox were also soaked aerobically with only (NH₄)₂Fe(SO₄)₂ in the same way. To obtain the non-activated reduced Mn/Fe cofactor, apo-protein crystals were soaked in 1 ml of 40% (w/v) PEG 1500, 100 mM HEPES-Na (at the pH of the mother liquor), 5 mM (NH₄)₂Fe(SO₄)₂, 5 mM MnCl₂, 0.5% (w/v) sodium dithionite, 0.5 mM phenosafranin, and 0.05% (v/v) Tween 20 for 1-2 h and flash-cooled directly without washing. Soaking solutions were always freshly prepared immediately before use, using freshly dissolved (NH₄)₂Fe(SO₄)₂ and dithionite to ensure that the Fe was ferrous, and that oxygen was effectively removed from soaking solutions used to obtain reduced states, with phenosafranin serving as redox indicator. Data were collected at 100 K at beamlines X06SA and X10SA at the Swiss Light Source (Villigen, Switzerland) and I911-3 at Max II (Lund, Sweden). For the purpose of metal quantification, data collection proceeded in the order Fe edge – Mn edge on the same crystal. High resolution “native” data were collected on separate crystals or after the anomalous datasets.

Structure determination, model building and refinement

Diffraction data were processed with XDS (55). Both R2lox point mutants crystallized in the same space group as wild-type R2lox, I222, with one molecule in the asymmetric unit (see Table S1). The structures were solved by Fourier synthesis using the structure of the wild-type protein in the same redox state (23) not containing any ligands as a starting model. Refinement was carried out with phenix.refine (56, 57) and iterated with rebuilding in Coot (58). Refinement included bulk solvent corrections, individual atomic coordinate and isotropic B factor refinement, and occupancy refinement for alternate conformations and metal ions bound on the protein surface, but not the active site metal ions. Metal-ligand bond lengths were restrained. Solvent molecules were added with
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phenix.refine and manually. Hydrogens were added to the models in the later stages of refinement. Structures were validated using MolProbity (59). Data and refinement statistics are given in Tables S1 and S2. All figures were prepared with PyMOL (version 1.8.6.2, Schrodinger, LLC).

Analysis of anomalous diffraction data

All anomalous datasets were integrated over the same resolution range (50.0–3.0 Å) with XDS (55) (Table S1). The Fe and Mn edge datasets from one crystal were placed on a common scale with XSCALE (55). Both scaled and unscaled datasets were analyzed. Anomalous difference maps were calculated with PHENIX (57) using the phases from a ligand-free model, and the relative amounts of Fe and Mn in each metal site were calculated as previously described (60). The intensities of the anomalous difference density peaks in spheres of 1.9 Å radius around the center of the peaks were integrated using MAPMAN (61). The relative amounts of Fe and Mn in each site were estimated from the integrated intensities at the Fe and Mn edges by taking into account the different contributions of both elements to the anomalous signal at the two wavelengths, assuming a total occupancy of each site of 1, as described (60). Since only relative amounts are calculated, the actual occupancy is irrelevant as long as it is high enough to yield a significant anomalous peak, which was the case in all datasets. The quantification results for scaled and unscaled datasets matched within 10%.

Sample preparation for XAS, EPR and TXRF analysis

To obtain the oxidized resting state of the Mn/Fe cofactor for EPR analysis, 100–200 µM apo-protein (monomer concentration) was incubated with 2 equivalents of MnCl₂ and 1 equivalent of (NH₄)₂Fe(SO₄)₂ in reconstitution buffer (100 mM HEPES-Na, pH 7.0, 50 mM NaCl) for 1 h at room temperature under aerobic conditions. To label the Mn/Fe cofactor with ⁵⁷Fe, ⁵⁷FeCl₂ was used instead of natural abundance (NH₄)₂Fe(SO₄)₂. For XAS samples, 250 – 300 µM apo-protein was reconstituted in the same way at a protein:Mn ratio of 1:2:1 or 1:2.4:1.2. Fe-only loaded XAS samples were prepared using a 3- or 4-fold molar excess of (NH₄)₂Fe(SO₄)₂ over polypeptide chains. Excess metal ions were removed by passing the sample through a HiTrap Desalting column (GE Healthcare) equilibrated in storage buffer. Mn/Fe-reconstituted EPR samples were exchanged into 50% ²H₂O-based buffer (25 mM HEPES-Na, pH 7.0, 50 mM NaCl in 50% ²H₂O) by four rounds of dilution and concentration. Mn-only loaded EPR samples were prepared analogously at a protein:Mn ratio of 1:1 or 1:2, both with and without removal of the excess Mn²⁺ and following concentration. For EPR spectroscopy, the reconstituted protein was concentrated to 0.3–0.4 mM (X-band samples) or ~1 mM (Q-band samples), and 50% (v/v) glycerol was added before transfer into EPR tubes and flash-cooling in liquid nitrogen. For XAS samples, the reconstituted protein was concentrated to 1.5–3.5 mM. 10% (v/v) glycerol was added to one batch of samples, while the other was directly transferred into sample holders and flash-cooled in liquid nitrogen.

In order to assess the cofactor assembly mechanism of the R2lox variants using EPR and TXRF, 100 µM apo-protein (monomer concentration) was incubated with varying equivalents of MnCl₂ and (NH₄)₂Fe(SO₄)₂ in reconstitution buffer for 1 h at room temperature under aerobic conditions. TXRF samples were prepared in duplicates. Fe titration series for EPR were prepared by adding 4 equivalents (per monomer) of MnCl₂ to 100 µM apo-protein. Then, 0.2 equivalents of (NH₄)₂Fe(SO₄)₂ were added from a 10 mM stock solution. After 10 min incubation at room temperature, an aliquot was removed, and another 0.2 eq. of (NH₄)₂Fe(SO₄)₂ were added to the remaining solution. These steps were repeated until 1 equivalent of (NH₄)₂Fe(SO₄)₂ had been added. For TXRF measurements, only three titration points were sampled, with final protein:Mn:Fe ratios of 1:4:0.2, 1:4:0.6, and 1:4:1. All samples were incubated at room temperature for 1 h after the last addition of (NH₄)₂Fe(SO₄)₂; i.e. for a total of 1:40 h. Following the reconstitution procedure, excess metal ions were removed as above, and the reconstituted protein was concentrated to 0.2–0.3 mM (EPR) or ~0.5 mM (TXRF). 50% (v/v) glycerol was added to EPR samples before transfer into X-band tubes and flash-cooling in liquid nitrogen.

X-ray Absorption Spectroscopy (XAS)

XAS spectra at Mn and Fe K-edges were collected in fluorescence detection mode at the
SuperXAS beamline of SLS (Villigen, Switzerland) and at the Samba beamline of SOLEIL (Paris, France) using standard set-ups as previously described (Si[111] or Si[220] double-crystal monochromator, energy-resolving 5-element silicon-drift or 36-element Ge detector, samples held in a liquid-He cryostat at 20 K) (24, 37). X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) spectra were collected with scan durations of about 1 min or 15 min (up to 20 scans on separate sample spots averaged for signal-to-noise ratio improvement, incident beam appropriately attenuated by Al filters). XAS spectra were processed using in-house software (62) and EXAFS spectra were simulated using phase functions calculated with FEFF9 (63).

Total-reflection X-ray fluorescence (TXRF)

Metal contents of apo-protein preparations, metal titration and selected EPR samples as well as X-ray spectroscopy samples were quantified using TXRF analysis on a Bruker PicoFox instrument (64). A gallium standard (Sigma) was added to the samples (v/v 1:1) prior to the measurements. Technical duplicates were prepared of each sample. TXRF spectra were analyzed using the routines provided with the spectrometer.

EPR spectroscopy

X-band CW EPR measurements were performed in the temperature range of 10 to 30 K using a Bruker E500 spectrometer equipped with a Bruker ER 4116DM resonator, Oxford Instruments ESR 935 cryostat and ITC503 temperature controller. Magnetic field modulation amplitude was 7.5 G, the microwave (mw) frequency was around 9.63 GHz. Q-band pulse EPR and HYSCORE measurements were performed between 4 and 20 K using a Bruker ELEXSYS E580 Q-band pulse EPR spectrometer equipped with a home-built TE011 microwave resonator (65) and cryogen free variable temperature cryostat (66). Field-swept EPR spectra were obtained via integration of the electron spin echo (ESE) signal, produced by two microwave pulses of 156 ns with an inter-pulse delay of 348 ns and shot repetition time (SRT) of 20 µs. Long mw pulses combined with a narrow integration window of 20 ns were used in order to isolate the S=1/2 species from the S=5/2 MnII hexaaquo and other background EPR signals (66). The mw frequency was around 34.12 GHz. HYSCORE spectra were measured using the pulse sequence $t_{n/2}$–$\tau$–$t_{n/2}$–$\tau$–$t_{n/2}$–$\tau$–echo with SRT = 500 µs. A pulse length of $t_{n/2} = 16$ ns and inter-pulse delay time of $\tau = 240$ ns were used. The inter-pulse distances $t_1$ and $t_2$ were sampled in the range of 200–1736 ns with an increment of 12 ns. Simulations of EPR spectra were performed using the EasySpin toolbox (67) for MATLAB®.

Mass spectrometric analysis of cross-link formation

Samples were kept dark during processing. Three samples each of 100 µM apo-wt-R2lox or apo-G68L-R2lox were incubated with either 4 equivalents (per monomer) of MnCl₂ and 1 equivalent of (NH₄)₂Fe(SO₄)₂, with the Fe salt added in steps of 0.2 equivalents every 10 min to maximize Mn/Fe cofactor formation (27), or 3 equivalents of (NH₄)₂Fe(SO₄)₂ only in reconstitution buffer under aerobic conditions for 1 h at room temperature. Excess metal ions were removed by passing the samples through a HiTrap Desalting column (GE Healthcare) equilibrated in storage buffer. The reconstituted protein was concentrated to 0.3 – 0.5 mM. From each assay replicate, 80 µg of protein were subjected to proteolytic digestion by Glu-C (Promega, enzyme:substrate ratio 1:40) in phosphate buffer (50 mM, pH 7.6) using the SP3 sample preparation method (68). Glu-C in phosphate buffer shows a high rate of cleavage at the C-terminal side of glutamate and aspartate residues. From each replicate, 300 µl of digested sample was collected and acidified by addition of 100 µl 10% formic acid (FA) prior to LC-MS. The auto sampler of a HPLC 1200 system (Agilent Technologies) injected 1 µl (approximately 200 ng of peptides) into a C18 guard desalting column (Zorbax 300SB-C18, 5 x 0.3 mm, 5 µm bead size, Agilent). Then a 15 cm long C18 picofrit column (100 µm internal diameter, 5 µm bead size, Nikkyo Technos Co., Tokyo, Japan) installed on to the nano electrospray ionization (NSI) source was used. Solvent A was 97% water, 3% acetonitrile (ACN), 0.1% FA; and solvent B was 5% water, 95% ACN, 0.1% FA. At a constant flow of 0.4 µl/min, a linear gradient went from 2% B up to 40% B in 45 min, followed by a steep increase to 100% B in 5 min, plateau at 100%
B for 5 min, and subsequent re-equilibration with 2% B. Online LC-MS was performed using a LTQ Orbitrap Velos Pro mass spectrometer (Thermo Scientific). FTMS master scans (AGC target of 1e6) were acquired with a resolution of 30,000 and were followed by data-dependent MS/MS (AGC target of 1e5) at a resolution of 7,500. In d-d MS/MS, the top 2 ions from the master scan were selected first for collision induced dissociation (at 35% energy) and afterwards for higher energy collision dissociation (at 30% energy). Precursors were isolated with a 2 m/z window. Dynamic exclusion was used with 60 s duration. Each sample was analyzed in technical triplicates. MS raw files were then searched against a FASTA database containing only the R2lox protein sequence (but including both wt and G68L variants) using Sequest HT under Proteome Discoverer (PD) 1.4.0.288 (Thermo Scientific). No-enzyme was used as cleavage setting, oxidation of methionine as variable modification and carbamidomethylation of cysteine as fixed modification (not relevant, however, because there are no cysteine residues in R2lox). Precursor mass tolerance was 15 ppm and product mass tolerance was 0.02 Da. PSMs with Xcorr>1.2 passed the Fixed value PSM validator. PD 1.4 can calculate protein areas based on the MS1 peak area integration of the three most abundant identified peptides. The MS1 peak area in each LCMS run of m/z 689.038, z=3 (which corresponds to the cross-linked peptide AVIRAATVYNMIVE-AVTLD), normalized to the protein area of the same run, was used as surrogate marker for the amount of cross-link in the samples. For an annotated spectrum of the cross-linked peptide, see Figure S7 in Griese et al. (23).

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Data availability: The atomic coordinates and structure factors have been deposited in the Protein Data Bank (http://wwpdb.org/) with accession codes 6I92 (anoxically Mn+Fe soaked, non-activated reduced state G68F-R2lox), 6I90 (aerobically Mn+Fe soaked, oxidized resting state G68F-R2lox), 6I95 (anoxically Mn+Fe soaked, non-activated reduced state G68L-R2lox), 6I94 (aerobically Mn+Fe soaked, non-activated reduced state G68L-R2lox), and 6I93 (aerobically Fe soaked, oxidized resting state G68L-R2lox). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the PRIDE partner repository with the dataset identifier PXD015549.

Supporting Information: S1, Crystallographic data; S2, Metallation of R2lox variants; S3, CW EPR characterization of the G68L- and G68F-R2lox point mutants; S4, Temperature dependence of the stabilized intermediate’s EPR intensity in G68L-R2lox; S5, EPR quantification of the two MnIII/FeIII and the MnII/MnII paramagnetic species in G68L-R2lox; S6, Evidence of an earlier intermediate state in G68L-R2lox; S7, Cofactor structures in R2lox mutants compared to wild-type; S8, Mass spectrometric quantification of the cross-link in G68L-R2lox compared to the wild-type.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: Y.K. collected and analyzed EPR data. R.K. and M.Ha. collected and analyzed XAS and TXRF data. R.M.M.B. performed MS analysis of the cross-link. V.S. prepared samples for MS. D.L. performed sequence classification and analysis. J.J.G. performed protein production, purification and crystallization, collected and analyzed X-ray crystallographic data, and prepared samples for EPR, XAS and TXRF. M.Hö., N.C. and J.J.G. designed the study. N.C. and J.J.G. wrote the paper with contributions from all authors. All authors reviewed the results and approved the final version of the manuscript.
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**FOOTNOTES**

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The abbreviations used are: ACN, acetonitrile; FA, formic acid; RNR, ribonucleotide reductase; R2c, class Ic RNR R2 protein; R2lox, R2-like ligand-binding oxidase; wt, wild-type.
TABLES

Table 1. Spin Hamiltonian parameters for EPR simulations of wt-, G68F- and G68L-R2lox cofactor signals as compared to R2c (15, 17, 36). Signal processing and EPR simulations are described in SI S3.5 – S3.7.

|     | g-tensor | $^{55}$Mn hyperfine tensor | $^{57}$Fe hyperfine tensor |
|-----|----------|--------------------------|--------------------------|
|     | $g_x$    | $g_y$ | $g_z$ | $A_x$ | $A_y$ | $A_z$ | $A_x$ | $A_y$ | $A_z$ |
| wt-R2lox | 1.953    | 1.968  | 2.034 | 257  | 249  | 282  | 62.5 | 51.6 | 93.9 |
| G68F*    | 1.954    | 1.965  | 2.033 | 256  | 251  | 286  | -    | -    | -    |
| G68L, product* | 1.955    | 1.968  | 2.033 | 258  | 253  | 286  | -    | -    | -    |
| G68L, intermediate** | 2.007    | 2.025  | 2.039 | 281  | 365  | 246  | 59.2 | 60.1 | 65.9 |
| R2c      | 2.009    | 2.015  | 2.024 | 314  | 392  | 269  | 50.2 | 48.4 | 44.5 |

*Based on single-frequency X-band simulations. **For the dominant, high-temperature EPR signal of G68L, the g-tensor and $^{55}$Mn A-tensor are rotated relative to each other by the Euler angles $[\alpha, \beta, \gamma] = [-80.1^\circ, 69.2^\circ, 41.7^\circ]$ (in the z’yz’’ convention).

Table 2. K-edge energy and X-ray photoreduction rate in G68L-R2lox compared to wt-R2lox. Data represent mean values for two preparations of each R2lox variant (full parameter variation ranges in parentheses). K-edge energies and X-ray photoreduction parameters are for proteins reconstituted with Mn and Fe (left, Mn data) or Fe only (right, Fe data).

| R2lox variant | Mn/Fe (Mn) | Fe/Fe (Fe) |
|---------------|------------|------------|
|               | $E_{K\text{-edge}}$ [eV] | $E_{K\text{-edge}}$ [eV] |
| wt            | 6549.1(1)  | 7124.6(1)  |
| G68L          | 6547.7(1)  | 7124.8(1)  |
|               | X-ray photoreduction rate $[h^{-1}] / \Delta E_{K\text{-edge}}$ [eV] |
| wt            | 4.9(2) / 1.9(2) | 2.7(2) / 1.3(1) |
| G68L          | 4.5(3) / 0.4(1) | 2.1(4) / 1.4(2) |
Table 3. EXAFS simulation parameters.

| R2lox variant | Fe/Mn-N/O | Fe/Mn-Fe/Mn | Reconst. (XAS) | N [per metal] | R [Å] | 2σ² x10³ [Å²] | N [per metal] | R [Å] | 2σ² x10³ [Å²] | RF [%] |
|---------------|-----------|-------------|----------------|-------------|-------|----------------|-------------|-------|----------------|-------|
| wt            |           |             | wt Fe/Fe (Fe)  | 1.1(3)     | 1.89(1)| 11(1)         | 0.3(1)     | 3.03(1)| 5'             |       |
|               |           |             |                | 3.9(3)     | 2.04(1)| 11(1)         | 0.7(1)     | 3.48(1)| 5'             | 13(2) |
|               |           |             |                | 1.0         | 2.53(1)| 5'             |            |       | 5              |       |
|               |           |             |                | 1.2(1)     | 2.95(1)| 5'             |            |       |                |       |
| G68L          |           |             | wt Fe/Fe (Mn)  | 2.1(2)     | 1.94(1)| 8'             | 0.7(1)     | 3.02(1)| 5'             |       |
|               |           |             |                | 3.9(2)     | 2.06(1)| 8'             | 0.3(1)     | 3.47(1)| 5'             | 13(1) |
|               |           |             |                | 1.0         | 2.56(2)| 5'             |            |       | 5              |       |
|               |           |             |                | 1.3(1)     | 2.89(1)| 5'             |            |       |                |       |
|               |           |             | G68L Mn/Fe (Mn)| 1.4(2)     | 1.88(1)| 12(1)         | 0.1(1)     | 3.26(2)| 5'             |       |
|               |           |             |                | 3.6(2)     | 2.11(1)| 12(1)         | 0.9(1)     | 3.53(1)| 5'             | 17(2) |
|               |           |             |                | 1.0         | 2.40(3)| 5'             |            |       | 5              |       |
|               |           |             |                | 1.3(2)     | 3.07(2)| 5'             |            |       |                |       |
|               |           |             | G68L Mn/Fe (Mn)| 0.9(2)     | 1.90(1)| 11(1)         | 0.6(1)     | 4.08(2)| 5'             |       |
|               |           |             |                | 4.1(2)     | 2.13(1)| 11(1)         | 0.4(1)     | 3.54(1)| 5'             | 14(2) |
|               |           |             |                | 1.0         | 2.61(2)| 5'             |            |       | 5              |       |
|               |           |             |                | 1.3(2)     | 3.05(2)| 5'             |            |       |                |       |

Data correspond to spectra at the Fe or Mn K-edges in Figure 5 of two Fe-only or Mn/Fe reconstituted preparations each of the wt- and G68L-R2lox variants. Full parameter ranges from individual fitting of the two data sets are given in parentheses. N, coordination number; R, interatomic distance; 2σ², Debye-Waller factor; RF, fit error sum calculated for reduced distances of 1-3.5 Å. Fit restraints: *fixed parameters, &coupled to yield the same 2σ² value, §coupled to yield a sum of 5, §coupled to yield a sum of 1.
Figure 1. Active site structures of (A) R2lox and (B) R2c in the (left) non-activated, reduced state (PDB IDs: R2lox, 4HR4; R2c, 4M1I) and (right) oxidized resting state (PDB IDs: R2lox, 4HR0; R2c, 1SYY) (16, 23, 28). (Only a Fe/Fe-bound oxidized state structure of R2c is available.) (C) Proposed activation pathway for R2lox. Both E202 and the fatty acid need to shift from their bridging positions to allow O2 binding for R2lox, whereas for R2c only E227 has to detach. (D) Sequence logos of R2lox and the biochemically characterized NrdB subclasses, centered on the N-terminal metal ligand following the conserved Gly in R2lox (marked with a red box). The biochemical R2 subclass Ia consists of several phylogenetically distinct subclasses and is represented here by subclasses NrdBe and NrdBg. The logos were generated from the RNRdb (http://rnrdb.pfitmap.org) HMMER (69) search profiles using the Skylign server (70).
Figure 2. Active site structures of (A-C) G68L- and (D, E) G68F-R2lox after soaking crystals with equal amounts of Mn$^{II}$ and Fe$^{II}$ under (A, D) anoxic or (B, E) aerobic conditions, or (C) with only Fe$^{II}$ under aerobic conditions. All structures are shown in roughly the same orientation, with site 1 on the left. The substituted residue is highlighted in orange. Metal-ligand bonds are indicated by grey lines, hydrogen bonds by dashed blue lines. Residues in alternate conformations are labeled in red. The insets show $mF_o - DF_c$ refined omit electron density contoured at 3.0 σ for residues V72 and Y162. (F) Superposition of Mn/Fe-wt-R2lox (grey, PDB ID 4HR0) (23), Fe/Fe-G68L-R2lox (orange) and Fe/Fe-R2c (green, PDB ID 1SYY) (16) in the oxidized resting state, highlighting the similarity of the metal coordination and the residues that allow or block access of the fatty acid to the metal cofactor, G68 and A171 in R2lox, or A88 and F197 in R2c, respectively. The latter position is conserved as alanine in R2lox proteins and as phenylalanine in all RNR R2 proteins (5), while the former position is conserved as glycine in R2lox and most commonly an alanine in R2c (see Figure 1D).
Figure 3. (A) EPR spectra of the Mn$^{III}$/Fe$^{III}$ cofactor of wt- and G68L-R2lox measured under different conditions. The EPR spectra of an O$_2$ activation intermediate seen for wt-R2lox (11) (33) and the Mn$^{III}$/Fe$^{III}$ (one-electron reduced) state of the R2c cofactor (adapted from Voevodskaya et al. (17)) are shown for comparison. (B) Multifrequency simulations of the dominant G68L cofactor signal recorded at X- (T = 15 K) and Q-band (T = 19 K). Simulation parameters are given in Table 1. Signal processing and EPR simulations are described in SI S3.5 – S3.7.

Figure 4. Q-band $^2$H-HYSCORE measurements of the G68L-R2lox Mn$^{III}$/Fe$^{III}$ cofactors at (A) low temperature, where the wt-like cofactor signal is dominant, and (B) high temperature, where the R2c-like cofactor signal is dominant. Red dashed circles indicate the cross peaks of the µ-OH bridge signal for both cofactors.
Figure 5. XAS analysis of wt and G68L variants of R2lox. Data for two preparations of each variant are shown. Left: spectra at the Mn K-edge of Mn/Fe-reconstituted variants. Right: spectra at the Fe K-edge of Fe-only reconstituted variants. XANES spectra in A and B and K-edge shifts due to X-ray photoreduction in the insets with fit curves for merged data sets (red and blue lines; time axes for data collected at Soleil (dark-blue and orange data) were expanded by a factor of 1.2 vs. data collected at SLS (dark-cyan and dark-red data) for comparison; see Table 2). Fourier-transforms (FTs) in C and D of EXAFS spectra in the insets (vertically shifted for comparison; black lines, experimental data; colored lines, fit curves with parameters in Table 3). FTs were calculated for $k$-values of 3.8-12.3 Å$^{-1}$ (cos-windows over 10% of both $k$-range ends) to emphasize metal-metal distances (asterisks in C and D).
Figure 6. Summary of cofactor maturation steps for R2lox and R2c. Although the two cofactors have divergent metallation (23, 27, 28) and catalytic chemistry (15–17, 23, 24), their maturation in the course of the reaction with O₂ is likely to follow the same pathway (23, 31–37). The G68L-R2lox mutant traps an intermediate of the reaction following O-O bond cleavage (MnIV/FeIV), but preceding the loss of a bridging hydroxide and the rebinding of the lipid headgroup. While cofactor maturation is coupled to cross-link formation in the wild-type, it is likely that redox equivalents are instead lost to the solution environment in G68L-R2lox.
Chemical flexibility of heterobimetallic Mn/Fe cofactors: R2lox and R2c proteins
Yury Kutin, Ramona Kositzki, Rui M. M. Branca, Vivek Srinivas, Daniel Lundin, Michael Haumann, Martin Högbom, Nicholas Cox and Julia J. Griese

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