Paramagnetic states
of diiron carboxylate proteins

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ABSTRACT

Diiron carboxylate proteins constitute an important class of metall-containing enzymes. These proteins perform a multitude of reactions in biological systems that normally involve activation of molecular oxygen at the diiron site. During activation and functioning of these proteins their diiron sites undergo redox changes in a rather wide range: from diferrous (FeII-FeII) to high potential intermediate Q(FeIV-FeIV). Two of these redox states are paramagnetic: (FeIV-FeIII), called high potential intermediate $X$, and (FeII-FeIII), called mixed-valent state of the diiron carboxylate proteins. In the present work it has been shown that these redox states are of functional relevance in two proteins with different functions.

Ribonucleotide reductase (RNR) from the human parasite *Chlamydia trachomatis* is a class I RNR. It is typical for class I RNR to initiate the enzymatic reaction on its large subunit, protein R1, by activation from a stable tyrosyl free radical in its small subunit, protein R2. This radical, in its turn, is formed through oxygen activation by the diiron center. In *C. trachomatis* the tyrosine residue is replaced by phenylalanine, which cannot form a radical. We have shown in the present work, that active *C. trachomatis* RNR uses the FeIII-FeIV state of the diiron carboxylate cluster in R2 instead of a tyrosyl radical to initiate the catalytic reaction.

The alternative oxidase (AOX) is a ubiquinol oxidase found in the mitochondrial respiratory chain of plants. The existence of the diiron carboxylate center in this protein was predicted on the basis of a conserved sequence motif consisting of the proposed iron ligands, four glutamate and two histidine residues. In experiments modeling the conditions of the enzyme catalytic cycle, i.e. reduction and reoxygenation of the overexpressed AOX in *Escherichia coli* membranes we were able to generate an EPR signal characteristic of a mixed-valent Fe(II)/Fe(III) binuclear iron center. The alternative oxidase is the first membrane protein where the existence of the diiron carboxylate center has been shown experimentally.
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Paper IV. Nina Voevodskaya, Marcus Galander, Grant McClarty, Astrid Gräslund, Friedhelm Lendzian. EPR and ENDOR characterization of the high-valent diiron species X in ribonucleotide reductase protein R2 of *Chlamydia trachomatis.* 2005, manuscript.


*Papers not included in the thesis:*

Guittet, Olivier; Hakansson, Pelle; Voevodskaya, Nina; Fridd, Susan; Graslund, Astrid; Arakawa, Hirofumi; Nakamura, Yusuke; Thelander, Lars. Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. *Journal of Biological Chemistry*, 2001, 276, 40647-40651

Schroeder, Peter; Voevodskaya, Nina; Klotz, Lars-Oliver; Brenneisen, Peter; Gräslund, Astrid; Sies, Helmut. Loss of the tyrosyl radical in mouse ribonucleotide reductase by (-)-epicatechin. *Biochemical and Biophysical Research Communications*, 2005, 326, 614-617.

List of common abbreviations

RNR, ribonucleotide reductase;
R1, the large protein subunit of RNR;
R2, the small protein subunit of RNR;
AOX, alternative oxidase;
*C. tm, Chlamydia trachomatis*;
*E. coli, Escherichia coli*;
DTT, dithiothreitol;
EDTA, ethylenediaminetetraacetate;
ENDOR, electron nuclear double resonance;
EPR, electron paramagnetic resonance;
MMO, methane monooxygenase;
MMOH, the hydroxylase protein subunit of MMO;
NADH, β-nicotineamide adenine dinucleotide;
NADPH, β-nicotineamide adenine dinucleotide phosphate;
RFQ, rapid freeze quench;
Tris, Tris(hydroxymethyl)aminomethane;
UV, ultra violet light.

**Standard amino acids:**
A Ala Alanine
C Cys Cysteine
D Asp Aspartic acid
E Glu Glutamic acid
F Phe Phenylalanine
G Gly Glycine
H His Histidine
I Ile Isoleucine
K Lys Lysine
L Leu Leucine
M Met Methionine
N Asn Asparagine
P Pro Proline
Q Gln Glutamine
R Arg Arginine
S Ser Serine
T Thr Threonine
V Val Valine
W Trp Tryptophan
Y Tyr Tyrosine
Introduction

This work is devoted to the investigation of two diiron carboxylate proteins – Ribonucleotide reductase from *Chlamydia trachomatis* (*C. tm* RNR) and Alternative oxidase (AOX) from *Arabidopsis thaliana*. These proteins are, on the one hand, typical examples of their class of enzymes, but on the other hand, they demonstrate some specificity in structures and functions that may help to understand the general properties of the whole class of the diiron carboxylate proteins.

Diiron carboxylate proteins

Metal containing proteins play a key role in many reactions that maintain life on Earth – the water oxidation reaction in photosynthesis releasing molecular oxygen and the opposite one, reductive cleavage of molecular oxygen resulting in water formation. The relatively few diiron carboxylate proteins involved in the process of dioxygen cleavage are of special interest because of their surprisingly different catalytic functions, as listed in Table I. (Solomon, 2001).

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Representative Enzyme</th>
<th>Catalytic Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>reversible dioxygen binding</td>
<td>hemerythrin</td>
<td>[Fe(^{III})Fe(^{III})] ↔ (\text{O}_2) ↔ [Fe(^{III})Fe(^{III})]–OOH</td>
</tr>
<tr>
<td>one e(^{-}) oxidation</td>
<td>ribonucleotide diphasate reductase (RR)</td>
<td>([\text{Fe}^{III}\text{Fe}^{III}]) + Tyr ↔ ([\text{Fe}^{III}\text{Fe}^{III}]) + Tyr*</td>
</tr>
<tr>
<td>hydroxylation</td>
<td>methane monooxygenase (MMO)</td>
<td>([\text{Fe}^{III}\text{Fe}^{III}]) + (\text{O}_2) ↔ ([\text{Fe}^{III}\text{Fe}^{III}]) + (\text{CH}_3\text{OH}/\text{H}_2\text{O})</td>
</tr>
<tr>
<td>desaturation</td>
<td>stearoyl-acyl carrier protein (\Delta^\text{A} \text{desaturase (ACP A}}^\Delta\text{T}))</td>
<td>([\text{Fe}^{III}\text{Fe}^{III}]) + Stearoyl ACP ↔ ([\text{Fe}^{III}\text{Fe}^{III}]) + Cleoyl ACP</td>
</tr>
<tr>
<td>hydrolysis of phosphate ester</td>
<td>purple acid phosphatase (PAP)</td>
<td>([\text{Fe}^{III}\text{Fe}^{III}]) + ROHPO(_4) + (\text{H}_2\text{O}) ↔ ([\text{Fe}^{III}\text{Fe}^{III}]) + (3\text{HPO}_4)</td>
</tr>
<tr>
<td>NADH peroxidation</td>
<td>Rehemythrin (Rb)</td>
<td>([\text{Fe}^{III}\text{Fe}^{III}]) + (\text{H}_2\text{O}_2) ↔ ([\text{Fe}^{III}\text{Fe}^{III}])</td>
</tr>
<tr>
<td>Ferroxidation</td>
<td>Ferritin</td>
<td>([\text{Fe}^{III}\text{Fe}^{III}]) + (\text{O}_2) ↔ ([\text{Fe}^{III}\text{Fe}^{III}])</td>
</tr>
</tbody>
</table>

**Hemerythrin** and **myohemerythrin** play roles of oxygen carriers in marine invertebrates, a function similar to that of the heme proteins hemoglobin and myoglobin in mammals (Que *et al.*, 1990, Cormick *et al.*, 1991; Wilkins, 1992; Solomon *et al.*, 1992).

**Ribonucleotide reductase** (Reichard, 1993; Thelander *et al.*, 1994; Stubbe, 1990; Nordlund *et al.*, 1993; Fontecave *et al.*, 1992) catalyses a highly regulated essential reaction for all living cells, the reduction of all four ribonucleotides to their corresponding deoxyribo nucleotides. The diiron carboxo core in class I RNR resides in its small R2 subunit. It has been found in some bacteria and eukaryotic cells, and certain bacterio phages and eukaryotic viruses also have genes encoding this type of RNR.

**Methane monooxygenase** (Wilkins *et al.*, 1994; Rozenzweig *et al.*, 1993; Lipscomb, 1994; Dalton, 1980) from methanotrophic bacteria carries out one of the most
difficult monooxygenations known, the conversion of the inert methane to methanol at
ambient temperature and pressure.

Soluble **stearol-acyl carrier protein \( \Delta^9 \) desaturase** from higher plants catalyzes
an important step in plant lipid desaturation (Fox *et al.*, 1993; Fox *et al.*, 1994;
Shanklin, *et al.*, 1991; Broadwater *et al.*, 1998) which allows the modification of
membrane properties.

The **purple acid phosphatases** catalyze hydrolysis of phosphate esters at pH
below 7.0 (Vincent *et al.*, 1992; Klabunde *et al.*, 1997; Hayman *et al.*, 1994; Klabunde
*et al.*, 1995; Bozzo *et al.*, 2002). However, this protein does not possess a four-helix
bundle structure and its diiron site does not appear to react with dioxygen during the
primary functional process (Kurtz, 1997).

**Ruberythrin** (Rbr) is a relatively small homodimeric protein, found in many air-sensitive
bacteria and archaea, which contain a unique combination of a rubredoxin-like
\([Fe(Cys)_4]\) site and an diiron carboxylate site (Jin *et al.*, 2002; de Mare *et al.*, 1996;
Coulter *et al.*, 2000). Although the diiron site structure of Rbr resembles those found in
\(O_2\)-activating diiron enzymes, Rbr appears to function as a hydrogen peroxide reductase
(peroxidase).

The iron storage protein **ferritin** (Pereira *et al.*, 1998; Hwang *et al.*, 2000; Bou-
Abdallah *et al.*, 2002) forms its shell with 24 subunits of two types: one having a Fe(II)
oxidizing diiron center and another, which lacks such a center and appears to be largely
involved in Fe(III) mineralization.

We also may include in this list several membrane bound proteins that have been
identified recently as diiron carboxylate proteins on the basis of the conservation of six
amino acids (four carboxylate residues and two histidines) constituting an iron-binding
motif (Berthold *et al.*, 2003). These proteins are: the plant mitochondrial alternative
oxidase (AOX) from *Arabidopsis thaliana* (Siedow *et al.*, 1995; Berthold *et al.*, 2000);
the prokaryotic alternative oxidase (bAOX) from *Novosphingobium aromaticivorans*
(Stenmark *et al.*, 2004); the diiron 5-demethoxyquinone hydroxylase (Coq7),
membrane bound protein involved in ubiquinone biosynthesis (Stenmark *et al.*, 2001);
the plastid terminal oxidase (PTOX) (Wu *et al.*, 1999); and the aerobic Mg-protoporphyrin
IX monomethylester (MME) hydroxylase (Walker *et al.*, 1988). Unlike
previously described soluble diiron carboxylate proteins, they are bound monotopically to
one leaflet of the membrane bilayer. Most of these enzymes utilize a quinol substrate,
with two (AOX and PTOX) oxidizing the quinol and one (MME hydroxylase)
hydroxylating it. MME hydroxylase is involved in the synthesis of the iso cyclic ring of
chlorophyll. Three-dimensional structures for these proteins are not yet available, and the
conclusion that they belong to the class of diiron carboxylate proteins is based on their
amino acid sequences analysis and was spectroscopically confirmed only for AOX (Paper
V).

Almost all of the proteins listed in the Table I contain diiron sites and share the
following structural properties: a four-helix bundle protein fold (except purple acid
phosphatases) surrounding two iron atoms separated by 4 Å or less, one or more bridging
carboxylate ligands, terminal carboxylate and/or histidine ligands, and a bridging oxo,
hydroxo or aqua ligand(s) in at least the diiron(III) oxidation level. These proteins
possess very similar folds despite the absence of any significant sequence similarity.
One of the first described diiron carboxylate protein hemerythrin (Scheriff et al., 1987; Holms et al., 1991; Stenkamp 1994) demonstrates some features that are not typical for the other proteins of this class which were discovered later. First of all, the deoxyHr is the sole O\textsubscript{2} carrier in the group. The Hr diiron site is obviously more histidine rich (HX\textsubscript{29}HX\textsubscript{3}EX\textsubscript{16}HX\textsubscript{5}HX\textsubscript{23/28}HX\textsubscript{3}D) than the (D/E)X\textsubscript{30–37}EX\textsubscript{2}H ligand sequence motifs for MMO, RNR R2 and the others. Its diferrous site already contains hydroxo bridging ligands, and presents only a single open coordination position for O\textsubscript{2} (Figure 1). This is enough for terminal O\textsubscript{2} binding and allows Hr to play the role of oxygen carrier, whereas the diiron sites of the other proteins of this class have open or labile coordination positions on both irons to provide the O\textsubscript{2}-activating mechanism.

Figure 1. Schematic structures of deoxy-, oxy- and met Hemerythrin (adapted from Kurtz, 1997).

Hr is the only diiron carboxylate protein for which the structure of an O\textsubscript{2} adduct is reasonably well established. For the other diiron carboxylate proteins this compound has been suggested as a first step oxygen cleavage intermediate.

In contrast to Hr where the oxygen binding does not lead to any significant conformational changes (for example, the Fe1-Fe2 distances remain almost the same in deoxy (3.32 Å), oxy (3.27 Å) and met (3.25 Å) forms of the protein (Stenkamp, 1994), the proteins with oxygen-activating mechanism undergo dramatic transformations when interacting with oxygen (Figure 2).

Figure 2. Schematic diferrous and diferric structures of the activation sites of the small subunit of Ribonucleotide reductase R2 (RNR-R2) and the hydroxylase component of methane monooxygenase (MMOH) (adapted from Kurtz, 1997).
Conversion of RNR-R2$_{\text{red}}$ to RNR-R2$_{\text{ox}}$ produces a large decrease in Fe1–Fe2 distance (3.9 Å to 3.4 Å), an obvious rearrangement of one of the bridging carboxylates (a manifestation of the “carboxylate shift” (Rardin et al., 1991), which is one important source of the coordinative flexibility) and addition of bridging and terminal solvent ligands (Nordlund et al., 1995). Conversion of the MMOH$_{\text{red}}$ to MMOH$_{\text{ox}}$ diiron site leads to a carboxylate shift, a smaller but still significant decrease in Fe1–Fe2 distance (3.28 Å to 3.1 Å), and introduction of two solvent bridges (Rosenzweig et al., 1995).

Thus relative coordinative flexibility upon oxidation state changes may be a characteristic of the diiron carboxylate sites that activate O$_2$, as opposed to one that reversibly binds O$_2$. The relative lack of coordinative flexibility and limited access of O$_2$ to both irons at the Hr diiron site may be crucial for inhibiting autoxidation of the diiron(II) to the (apparently) physiologically irrelevant metHr diiron(III) site (Figure 1). At room temperature, this autoxidation occurs on the time scale of hours to days for Hr (Wilkins et al., 1987) compared to seconds for the corresponding reactions of the RNR-R2 and MMOH diiron sites (Bollinger et al., 1994; Liu et al., 1994).

The process of oxygen activation and diiron site intermediates is still one of the most fascinating unsolved aspects of diiron protein functions. The scheme in Figure 3 presents a possible set of structures and formal iron oxidation states for various intermediates which have been detected during reactions of the diiron(II) sites with O$_2$.

**Figure 3.** A possible scheme for the reactions of biological diiron carboxylate sites with O$_2$ and substrates. Formal oxidation states, ground spin states, and shorthand letter designations are listed below each diiron species. Intermediate X has been detected only in RNR-R2, and intermediate Q only in MMOH. Except for the tyrosyl radical, the depiction of organic free radicals is hypothetical. (Kurtz, 1997).
Evidence for the (µ-1,2-peroxo)bis(µ-carboxylato)diiron(III) geometry for intermediate P in Figure 3 comes from an X-ray crystallographic study of a synthetic complex with this structure (Kim et al., 1996). The UV/vis absorption spectrum, O–O stretching frequency, and Mössbauer parameters are good matches to those of a trapped intermediate formed upon reaction of MMOH$_{red}$ with O$_2$ (Liu et al., 1995). (A species both kinetically and spectroscopically consistent with intermediate P has been briefly mentioned as observed in RNR-R2, Tong et al., 1996). Bridging peroxo geometry for P is also consistent with the statement about equally probable oxygen access to both iron atoms: according to Mössbauer spectra (Kim et al., 1996), P exhibits only a single type of iron environment consistent with the symmetrical bridged structures shown in Figure 3. Evidence for the next diiron intermediate [Fe$_2$(µ-O)$_2$]$^{4+}$, designated Q, at the formal Fe$_{IV}$Fe$_{IV}$ oxidation level, has been obtained from stopped-flow absorption and freeze-quench Mössbauer experiments on MMOH (Lee et al., 1993; Lee et al., 1993; Liu et al., 1994) and ferritin (Pereira et al., 1998; Hwang et al., 2000). The Mössbauer spectra of Q indicate very similar, if not identical, environments for both irons. According to Que and Dong (Que et al., 1996), the diamond structure is favorable not only for Q but for its redox successor X as well. Remarkably, intermediate Q has not been detected in RNR-R2, whereas, intermediate X has been detected only in RNR-R2. This latter intermediate forms during activation of R2 by aerobic addition of Fe(II) to the apoprotein, which results ultimately in generation of a stable, functionally essential tyrosyl radical from the proximal residue, Tyr122 (Figure 3) (Sjöberg et al., 1978; Ravi et al., 1994; Bollinger et al., 1994). Detailed analysis of the kinetics of formation and decay of X and the buildup of the tyrosyl radical in RNR-R2 following exposure of the diiron(II) protein to O$_2$ strongly suggests that X is the diiron species that oxidizes Tyr122 (Tong et al., 1994). The source of the extra electron needed to reach X has not been identified in vivo (our suggestion will be discussed in paper III) but excess Fe$^{2+}$ can supply this electron in vitro. While there is general agreement that intermediate X is formally at the Fe$_{III}$Fe$_{IV}$ oxidation level, the spectroscopic data were initially interpreted in terms of a diferric site which is spin coupled to an unknown ligand radical. However, spectroscopic comparisons to a synthetic [Fe$_2$(µ-O)$_2$]$^{3+}$ diamond core complex have led Que and Dong (Que et al., 1996) to suggest that antiferromagnetically coupled high-spin Fe$_{III}$Fe$_{IV}$ is a more accurate description of X which however should have a significant spin delocalization on the ligand. A Q-band ENDOR study of X has lent support to the Fe$_{III}$Fe$_{IV}$ description (Sturgeon et al., 1996). However, the structure(s) of the high-valent intermediates corresponding to Q and X, are not still completely defined.

Ribonucleotide reductase

Ribonucleotide reductase (RNR) is the enzyme responsible for the conversion of the four standard ribonucleotides – adenosine, cytidine, guanosine, and uridine – to their deoxyribonucleotide counterparts (Figure 4), and thereby provides the precursors needed for both the synthesis and the repair of DNA.

The reduction of ribonucleotides is the rate-limiting step of DNA synthesis, which makes RNR an important target for cell growth control. Several RNR inhibitors are being used, or have been proposed, as drugs for chemotherapeutic treatment of cancer (Pötsch et al., 1994; Nocentini, 1996) and AIDS (Bianchi et al., 1994). The understanding of the
catalytic mechanism of RNR on a molecular level is therefore important for the
development of new strategies in the treatment of cancer, as well as bacterial and viral
infections, where the inhibition of cell growth is needed.

There are at least three classes of RNR. Each class has its unique composition, but
all contain a metal site and involve radical chemistry to reduce the substrate (Reichard
1993, Stubbe et al., 1998), (see Table II). Many bacteria, e.g. E. coli, can express two or
even three different RNR’s depending on their current environment, but higher organisms
only have class I reductase. The three classes have very little amino acid sequence
similarity. They have a slightly different allosteric regulation pattern, and they differ in
their tolerance for oxygen. While class III is completely inactive in the presence of
oxygen, class I requires oxygen for activation. Based on this fact, it is believed that class
I could only have evolved after the photosynthetic reactions started to produce an
atmosphere with a surplus of oxygen.

Class I enzymes are found in practically all eukaryotic organisms, from yeast and
algae to plants and mammals, and some prokaryotes and viruses also express this type.
This class is divided into two subclasses, Ia and Ib, which differ in their regulation
pattern. Class I has an \( \alpha_2\beta_2 \) quaternary structure with a total molecular mass of about 270
kDa. (Nordlund et al., 1990; Nordlund et al., 1993). Homodimers \( \alpha_2 \) and \( \beta_2 \) are usually
referred to as proteins R1 and R2. Each R1 polypeptide contains one substrate-binding
site and two separate allosteric sites, and each R2 polypeptide contains a binuclear non-
heme iron activation site with a stable tyrosyl radical essential for enzymatic activity
(Brown et al., 1969; Thelander 1974; Stubbe 1990; Uhlin et al., 1994). A distinctly
different example of a class I RNR, *Chlamydia trachomatis* (*C. tm.*) RNR, which lacks
the tyrosine residue in the vicinity of the diiron center in the polypeptide R2, will be
discussed later (papers I-IV).

Class II RNR has an \( \alpha \) or \( \alpha_2 \) homodimer structure and a molecular mass of 82
kDa. The radical required for reactivity is generated by adenosylcobalamin (Licht et al.,
1996; Booker et al., 1994). Enzymes belong to this class function both aerobically and
anaerobically and are found in some microorganisms but not in higher organisms. The
best characterized enzyme is that from *Lactobacillus leichmannii.* (Booker et al., 1993)

Class III RNR only functions anaerobically (Reichard, 1993; Fontecave et al.,
1989; Barlow, 1988; Yang et al., 1990). This type of enzyme is an iron-sulfur protein
with a glycyln radical. These proteins have an \( \alpha_2\beta_2 \) quarternary structure with a molecular
mass of 160 kDa. (Mulliez et al., 1993; Ollagnier et al., 1996). The \( \alpha \) subunits
contain the active site including the substrate-binding site, the allosteric sites, and the glycyl radical, while polypeptides β contain a 4Fe-4S iron-sulfur center.

**Table II. Classes of ribonucleotide reductases.**

<table>
<thead>
<tr>
<th></th>
<th>Class Ia</th>
<th>Class Ib</th>
<th>Class II</th>
<th>Class III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal cofactor</td>
<td>Fe-O-Fe</td>
<td>Fe-O-Fe</td>
<td>Co</td>
<td>4Fe-4S</td>
</tr>
<tr>
<td>Subunit structure</td>
<td>(\alpha_2\beta_2)</td>
<td>(\alpha_2\beta_2)</td>
<td>(\alpha) or (\alpha_2)</td>
<td>(\alpha_2\beta_2)</td>
</tr>
<tr>
<td>Radicals involved in</td>
<td>Tyr, Cys</td>
<td>Tyr, Cys</td>
<td>Cobalamin, Cys</td>
<td>AdoMet, Gly, Cys</td>
</tr>
<tr>
<td>turnover</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen dependence</td>
<td>Aerobic</td>
<td>Aerobic</td>
<td>No dependency</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Substrates (^a)</td>
<td>NDP</td>
<td>NDP</td>
<td>NDP/NTP</td>
<td>NTP</td>
</tr>
<tr>
<td>Reductant</td>
<td>Thioredoxin</td>
<td>NrdH-redoxin</td>
<td>Thioredoxin</td>
<td>Formate</td>
</tr>
<tr>
<td></td>
<td>Glutaredoxin</td>
<td>Glutaredoxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allosteric sites</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Occurrence</td>
<td>Eukaryotes</td>
<td>Eubacteria</td>
<td>Archaebacteria</td>
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<td></td>
<td>Eubacteria, e.g.</td>
<td>Eubacteria, e.g.</td>
<td>Eubacteria, e.g.</td>
<td>Eubacteria, e.g.</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td><em>Lactobacillus leichmannii</em></td>
<td><em>Escherichia coli</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacteriophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Viruses</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) N represents any of the four ribonucleotide bases, A, C, G, or U.

As was mentioned above, class I RNR requires oxygen for activation of its enzymatic activity. The reconstitution reaction (1) modeling the process of oxygen activation in R2 subunit of *E. coli* RNR leads (via several intermediates, see Figure 3) to oxidation of the diferrous center and formation of the stable tyrosyl radical.

\[
\text{R2-apo} + \text{Tyr122-OH} + 2\text{Fe}^{II} + e^- + O_2 + H^+ \rightarrow \text{R2-(Fe}^{III}-\text{O-Fe}^{III}) + \text{Tyr122-O}^- + \text{H}_2\text{O} \tag{1}
\]

The incorporation of iron into the R2 subunit, its reduction and oxygen activation *in vivo* is still poorly understood. Isolated *E. coli* R2 samples, however, have been studied in detail. The X-ray structure of the diferrous form was solved to 1.7 Å resolution (Logan *et al.*, 1996), Figure 2. The ferrous iron atoms have fourfold (or four- and fivefold), according to CD and MCD studies, (Pulver *et al.*, 1995) ligand coordination and they are bridged by two bidentately ligated carboxylates from glutamic acid residues. The two iron atoms are high-spin ferrous ions (Lynch *et al.*, 1989). A spin Hamiltonian analysis of the saturation magnetization behavior indicates that the two iron atoms are weakly antiferromagnetically coupled with a magnitude of the exchange coupling of \(J \approx -0.5 \text{ cm}^{-1}\) and the two sites have unequal zero field splitting (ZFS) parameters (Solomon *et al.*, 2000). The intrinsic electron spins of the two irons result in a net diamagnetic ground state with \(S = 0\), which is not observable by EPR. A very weak
An integral spin signal at g = 14.4, which is considered to derive from a small fraction of molecules having ferromagnetically coupled sites can be registered in a parallel mode EPR experiment (Elgren et al., 1993; Atta et al., 1996).

When molecular oxygen is allowed to react with diferrous R2, it will spontaneously oxidize the diiron site through a series of intermediate states, leading to the diferric iron center (Fe$^{III}$Fe$^{III}$) and a stable tyrosyl radical. The two oxygen atoms are reduced in a four-electron process from formally O$^{0}$ in molecular oxygen, via an O$^{-}$ peroxo state, to O$^{2-}$ as water and the oxide ion in the µ-oxo-bridge, respectively. Not all of these intermediates have been observed, but they have been proposed based on analogous activation reactions in other diiron proteins (Solomon et al., 2000, Andersson et al., 1999). The first step of the oxygen reaction is formation of a short-lived diferrous-oxygen complex (Fe$^{II}$Fe$^{II}$/O$^{0}$=O$^{0}$), which has so far not been observed. The presence of open coordination positions on both iron atoms in the binuclear diferrous active center of R2 allows the binding of dioxygen in a bridging mode (Figure 3). The oxygen atoms immediately abstract one electron from each iron to form a diferricperoxy intermediate (Fe$^{III}$Fe$^{III}$/O$^{-}$/O$^{-}$), which has been observed in MMOH (intermediate P, Figure 3). In the R2 protein, the peroxy intermediate has only been observed in the mutants R2-D84E and R2-W48F/D84E, which have exactly the same iron ligands as MMOH (Bollinger et al., 1998, Moënne-Loccoz et al., 1998; Baldwin et al., 2003). In R2 the next directly observed intermediate "X" is formally described as an Fe$^{III}$Fe$^{IV}$ mixed valence state (Sturgeon et al., 1996) (Intermediate Q, formal Fe$^{IV}$Fe$^{IV}$ oxidation state has not been detected in R2 proteins). At this stage the two oxygen atoms are fully reduced (O$^{2-}$), having accepted one electron from the iron site and one from an external source. From EXAFS studies of intermediate X, it has been suggested that the diiron center forms a diamond core with a very short iron-iron distance, 2.5 Å, compared to 3.2-3.4 Å measured for the diferrous met R2 form, and 3.4-3.7 for the diferrous reduced form (Riggs-Gelasco et al., 1998). Intermediate X is stable in R2 proteins on a few seconds timescale. In mutants where the essential tyrosyl is substituted with non-oxidizable amino acids, such as R2-Y122F, the lifetime of intermediate X is doubled, allowing for freeze-quenching and spectroscopic characterization (Bollinger et al., 1991, Bollinger et al., 1994a,b, Ravi et al., 1994, Tong et al., 1996, Burdi et al., 1996, Burdi et al., 1998, Willems et al., 1997, Veselov & Scholes, 1996). These studies suggest that both irons are six-coordinated in intermediate X, one oxygen atom is forming a terminal hydroxo ligand to one iron, whereas the second oxygen atom is engaged in a µ-oxo bridge between the irons. Due to the mixed valence state, the antiferromagnetically coupled high-spin iron Fe$^{III}$ (S = 5/2) and Fe$^{IV}$ (S =2) form an S = ½ ground state that is paramagnetic and suitable for EPR characterization. Conditions that allow stabilizing the intermediate X in a substrate-enzyme complex of RNR of C. trachomatis for hours are described in Papers II and III of this work and will be discussed in the Discussion Section. In the final step of the activation process, the intermediate X oxidizes the nearby tyrosyl residue Y122 to a stable tyrosyl free radical, and the iron site is left in a µ-oxo bridged diferric form (Fe$^{III}$Fe$^{III}$), with water ligands on both irons. The tyrosyl radical can be detected by optical spectroscopy by a characteristic peak at 410 nm (Pettersson et al., 1980). It can also be observed by EPR, giving rise to a doublet spectrum with an isotropic g-value of 2.0047 (Sjöberg et al., 1978). This is typical for an organic radical, but its microwave saturation behavior is influenced by the nearby diiron site, which leads to faster
relaxation. The antiferromagnetically coupled (\( J \approx -100 \text{ cm}^{-1} \), according to magnetic susceptibility and saturation-recovery EPR studies), diferric iron center itself is EPR silent, but it gives rise to a characteristic optical spectrum with broad maxima at 325 and 370 nm (Petersson et al., 1980; Galli et al., 1995). The hydrophobic environment of Tyr 122 protects the radical from spontaneous reduction leaving, however, the possibility of transferring the radical character from the tyrosine to the cysteine 439 residue at the active site in R1 during the catalytic turnover. There is a conserved pathway of hydrogen bonded side chains leading from this tyrosine residue via Asp 237 (R2), Trp 48 (R2), Tyr 356 (R2), Tyr 731 (R1), Tyr 730 (R1) to a cysteine residue (Cys439) at the active site in R1, about 35-40 Å away according to modeled R1-R2 complex structures (Figure 5). This pathway may be referred to as a radical transfer chain or an electron transfer pathway (ETP). The transfer process can be described as a coupled long-range electron/proton transfer from Cys439 in R1 to Tyr122 in R2 (Ekberg et al., 1998, Siegbahn et al., 1998, Ehrenberg, 1999).

Figure 5. Scheme of the amino acid side chains involved in the radical transfer chain of R1 and R2, which connects the site of the stable tyrosyl radical, Y122, and the substrate binding site with the postulated thiyl radical site C439, a model for R1-R2 complex. (Adapted from Eriksson et al., 1997, Nordlund et al., 1993).

The binding and reduction of ribonucleotides in class I RNR take place in the large subunit, R1. Since RNR is a multi-substrate enzyme responsible for the conversion of all four deoxyribonucleotides necessary for the assembly of DNA, it needs to provide a balanced supply of dNTP's. This is accomplished through a feedback control mechanism of RNR via two different allosteric effector sites that are also situated on the R1 subunit. (Thelander & Reichard, 1979; Thelander, 1974). On the surface of R1 there are also docking sites for both the small subunit R2 and the small hydrogen donor proteins thioredoxin or glutaredoxin, which are needed for the reduction of the active site disulfide bridges which are generated during the turnover. The crystal structure of the R1 subunit has been solved to a resolution of 2.5 Å by Uhlin and Eklund, 1994.

During the substrate turnover, a disulfide bridge that must be reduced before a new substrate can bind, is formed at the active site in R1. The reduction is accomplished by the hydrogen donor proteins, thioredoxin or glutaredoxin, via a second disulfide
bridge, C754 and C759, which is located on the flexible C-terminal tail of R1, and is therefore not resolved in the crystal structure. Another necessary condition for the substrate turnover is generation of the free radical on the Cys439 residue.

Figure 6. A scheme for the catalytic cycle in R1 (adapted from Pelmenschikov et al., 2003)

For radical transfer from the tyrosyl radical Y122* to the cysteine C439 at the active site of R1 (*E. coli* numbering), complex formation between R1 and R2 and binding of the substrate in the reduced form of R1 is essential. A scheme for the catalytic cycle in R1 starting from a thiol radical at Cys439 (Figure 6) is based on experimental data from several biochemical studies, and DFT (density functional theory) calculation assuming Glu441 to be an anion. The thiol radical as a starting point of the catalysis is a central point of the reaction mechanism. It is believed to be a common feature of all the three known classes of RNR (Stubbe et al., 2001), and theoretical studies confirm its importance (Siegbahn, 1998). So far among the class I RNR's there is no spectroscopic evidence for the existence of a catalytic thiol radical at C439.

In the first step of the substrate turnover cycle (Figure 6), the thiol radical C439 will abstract the 3'-hydrogen from the ribose ring of the substrate and thereby generate a substrate radical. The abstraction of the 3'-hydrogen has been verified by 3H-labeling of a substrate analogue, where a 3H label was found on the protein after an interrupted turnover (Stubbe et al., 1980). 3'-Hydrogen abstraction from ribose by thiol radicals has also been mimicked in model compounds (Lenz and Giese, 1997, Robins and Ewing, 1999). The ribose radical makes the 2'-OH-group more nucleophilic, and abstracts one hydrogen atom from C225 and then leaves as a water molecule. The substrate is thereby converted to a 2' ketyl radical. The two cysteine residues C225 and C462 are then oxidized to a disulfide anion radical losing the second hydrogen to the substrate. The identity of the disulfide anion radical was evidenced by high-field pulsed EPR (Lawrence et al., 1999). In the proposed reaction scheme for the wild type enzyme, the electron is then transferred via a chain of hydrogen bonded active site residues.
including E441, to the substrate 2' position, thereby generating a substrate radical which finally abstracts the hydrogen from C439 and leaves as a deoxyribonucleotide product. For a complete turnover, the C439 thyl radical regains its hydrogen and the Y122 in R2 its radical form via the electron transfer pathway. NADPH indirectly reduces the disulfide bridge at C225 and C462 via a cysteine pair at the surface of R1, which in turn is reduced by thioredoxin (or glutaredoxin), (Holmgren, 1988).

**Chlamydia trachomatis RNR**

Chlamydiae are obligate eubacterial parasites classified into four species, two of which, *Chlamydia trachomatis* (*C. tm*) and *Chlamydia pneumoniae*, are pathogenic for humans. Human chlamydial infections are a leading cause of sexually transmitted disease, blindness, and respiratory disease. Epidemiological data also suggest that *C. pneumoniae* may disseminate from the respiratory tract to produce vascular infection and contribute to atherogenesis (Schachter, 1999; Kuo et al., 1995). All chlamydiae share a common biology (Moulder, 1991). The organisms grow only within a specialized vacuole in the post-Golgi exocytic vesicular compartment of the eukaryotic cell. They undergo a distinct developmental cycle that alternates between an extracellular transmission cell, called the elementary body (EB), and an intracellular replicating cell, called the reticulate body (RB). The chlamydiae have evolved close metabolic relationship with their host. Early studies on chlamydial metabolism indicated that they were auxotrophic for amino acids, nucleotides, and many other components of intermediary metabolism that most free living bacteria are capable of synthesizing themselves. It was shown (McClarty, 1994) that chlamydiae are capable of transporting NTPs but not dNTPs directly from the host cell. Recently, the genome of *C. tm* was published and it encodes 894 proteins (Stephens et al., 1998) including only one ribonucleotide reductase typical for class I RNR. While conserving substrate binding site, allosteric regulation sites (in R1), docking sites and putative electron pathway (in both subunits) and iron binding site (in R2), the chlamydial R1 and R2 protein sequences show some unusual characteristics. The R1 protein has a calculated molecular mass of 119,000 Da, which is about 30 kDa larger than the prototype class Ia *E. coli* R1. The R2 protein has a phenylalanine at the position where the essential free radical tyrosine is located in all other class I R2 proteins (Roshick et al., 2000).

Despite the lack of the normally essential tyrosyl residue, chlamydial class I RNR is active. In papers I-IV we show that this enzyme may use the high valent Fe^{III}Fe^{IV} state (“intermediate X”) as a radical analog for triggering of the mechanism of the catalytic reaction.

**Alternative oxidase (AOX) from Arabidopsis thaliana.**

The alternative oxidase is a mitochondrial cyanide-resistant non-haem ubiquinol oxidase. It is found in all plants as well as in some fungi and protozoa (Moore & Siedow, 1991; Day et al., 1995; Day & Wiskich, 1995). In contrast to cytochrome c oxidase, the other mitochondrial terminal oxidase, the alternative oxidase is not involved in the process of energy conservation. It does not pump protons, and a huge free energy release in ubiquinol oxidation dissipates as heat (Figure 7). Sometimes this uncoupled respiration may be useful, because in the thermogenic floral tissue of some plants, the produced
heat volatilizes foul-smelling compounds that attract insect pollinators (Meeuse, 1975). The other possible role of alternative oxidize is to minimize the production of reactive oxygen species in respiring mitochondria (Purvis, 1997; Maxwell et al., 1999). The expression of the alternative oxidize is induced when plants are exposed to a variety of stresses, including chilling and pathogen attack, as well as during senescence and fruit ripening. The reason for this may be related to the metabolic flexibility it provides, enabling the plant to respond to a range of developmental and environmental conditions (Purvis, 1997; Mackenzie & McIntosh, 1999).

The alternative oxidase is a homodimeric membrane protein (Umbach & Siedow, 1993). The mass of the monomer derived from sequence data is about 32 kDa (Tanudji et al., 1999).

The enzyme remains notoriously difficult to purify to homogeneity in a stable, active form despite of more than 20 years of effort (Berthold et al., 2000).

In the alignments of the alternative oxidase sequences, at least two EXXH conserved motifs were found, which were suggested to be involved in forming the diiron carboxylate center (Siedow et al., 1995). The structure model, which shows how AOX could accommodate a diiron carboxylate center within a four-helix bundle protein fold, is typical for diiron carboxylate enzymes. It suggests the possible ways of AOX interaction with the membrane and binding the ubiquinol substrate, and was proposed by Nordlund and Andersson (Andersson & Nordlund, 1999; Figure 8).

In paper V we report high levels of expression of the A. thaliana alternative oxidase AOX1a in E. coli membranes. These membranes were used to obtain the first EPR spectra of AOX, which undoubtedly confirmed the existence of the diiron carboxylate center in this enzyme.
Methods
Spectroscopic techniques: EPR and ENDOR

Electron paramagnetic resonance (EPR) is a powerful, selective, and sensitive tool for studying systems with unpaired electrons, e.g., radicals and paramagnetic transition metals. Since a great number of biological processes go through oxidation/reduction reactions and are accompanied by electron transfers, stable paramagnetic centers and transient radical species play functionally important roles in metabolic processes. With EPR spectroscopy, detailed information about the nature, location, and the electronic structure of the centers with an unpaired spin can be obtained, and thereby contribute to the understanding of the mechanism of the biological functions of proteins.

The subject of this work, diiron carboxylated proteins, during the enzymatic reactions can demonstrate a wide range of oxidation states from extremely oxidized Fe(IV)-Fe(IV) to completely reduced Fe(II)-Fe(II). Among them the oxidation states Fe(II)-Fe(III) and Fe(III)-Fe(IV) are paramagnetic in their ground state and thus become ideal objects for EPR studies.

Electron paramagnetic resonance spectroscopy is based on the interaction of the magnetic dipole moment of an unpaired electron with an external magnetic field $B_0$. This interaction is called the Zeeman-interaction and can be described by the Hamiltonian

$$\hat{H} = -\hat{\mu} \cdot \vec{B}$$  \hspace{1cm} (2)

With the magnetic moment operator written as

$$\hat{\mu} = -\gamma h \hat{S} = -g_\beta \hat{\mathbf{S}}$$  \hspace{1cm} (3)

the Hamiltonian becomes

$$\hat{H} = -g_\beta \hat{S}_z B_z$$  \hspace{1cm} (4)

for a magnetic field oriented in $z$ direction. $\gamma$ and $\beta$ denote the gyromagnetic ratio and Bohr magneton. $g$ is the Landé factor that is characteristic for the paramagnetic species and its surrounding. Solution of the equation (4) for the system with $S=1/2$ results in

$$E = g_\beta B_z m_z$$, \hspace{1cm} where \hspace{0.5cm} $m_z = \pm 1/2$  \hspace{1cm} (5)

The two states obtained are defined by the spin quantum number $m_z$ and can be interpreted as the energy levels splitting of the system of free electrons. If we apply the microwave field $B_1$ perpendicular to the external magnetic field $B$ with the frequency matching the resonance conditions:

$$h \nu = \Delta E = g_\beta B_0$$  \hspace{1cm} (6)
the energy quantum is absorbed causing the transitions between energy levels according
the selection rule for electron transitions $\Delta m_s = 1$ (Figure 9).

![Figure 9. Schematic diagram showing the Zeeman effect. At zero field, the two energy levels of an electron spin have the same energy. External magnetic field $B$ splits the degenerated energy levels according to their projection on the direction of $B$: particles oriented along magnetic field ($m_s = -1/2$) have lower energy level than they had before, and particles oriented against magnetic field ($m_s = +1/2$) – higher. When microwave electromagnetic field $B_1$ is applied to the system in the direction $B_1 \perp B$ spin transitions occur causing the absorption of microwave energy according to equation (6).](image)

Several biological paramagnetic centers have relatively isotropic environments and thus
the value of the $g$-value is close to that of the free electron ($g_e = 2.0023$). When the
paramagnetic species exhibits $g$-anisotropy like it happens in the powder spectra or in the
spectra of frozen solutions of paramagnetic transition metal ions, the spatial dependency
of the $g$-factor is represented by a $3 \times 3$ matrix $g$. Choosing the coordinate system in which
the off-diagonal terms are zero, the three principal values of the $g$-matrix ($g_x$, $g_y$, $g_z$) can
be obtained.

![Figure 10. Energy level diagram of hyperfine interactions on a system with one unpaired electron and one nucleus with spin $I = 1/2$ and $a_{iso} > 0$, $a_{iso}/2 < \nu_N$. In an applied magnetic field, the Zeeman interactions lead to a splitting of the energy levels of both the electron spin and nuclear spin, where $\nu_e >> \nu_N$. The EPR transitions ($\nu_e \pm a/2$) are indicated by double line arrows, and the ENDOR transitions ($\nu_N \pm a/2$) by single line arrows.](image)

In a molecule, the unpaired electron spin occupies an orbital and will interact with spins
from other closely localized electrons and nuclei. These interactions will lead to further
splitting of the energy levels (Figure 10). The different types of interactions can be expressed as distinct terms in the spin Hamiltonian ($\mathcal{H}_s$), from which the spin energy levels can be obtained:
The electron-Zeeman term ($\mathcal{H}_{\text{EZ}}$) describes the interaction between the electron spin operator ($S$) and the applied external magnetic field vector ($B$).

$$\mathcal{H}_{\text{EZ}} = g\beta B \hat{S}$$  

Here, the $g$-value is the observable, and any deviations from $g_e$ results from the so-called spin-orbit coupling between the unpaired spin ($S$) and the orbital angular momentum ($L$). This spin-orbital interaction can be described by the Hamiltonian

$$\mathcal{H}_{\text{LS}} = \lambda \hat{L} \hat{S}$$

where $\lambda$ is called the spin-orbit coupling constant, $\lambda$ is constant for a particular shell in a particular atom, increasing sharply with the atomic mass (Atherton, 1993). For organic radicals on carbon or oxygen the spin-orbit coupling is usually small and therefore the low energy separation between the partly filled $d$-orbitals can lead to large deviations from $g_e$.

**Hyperfine interactions** ($\mathcal{H}_{\text{HF}}$) are the interactions between the magnetic moment of the electron spin and the magnetic moment of a nucleus in the vicinity. For these interactions it is necessary that the nuclear spin ($I$) of these nuclei is different from zero, which is the case for protons ($^1\text{H}$), nitrogen ($^{14}\text{N}$), and phosphorus ($^{31}\text{P}$) in unlabeled biological samples, as well as for certain transition metals like copper ($^{63}\text{Cu}/^{65}\text{Cu}$) and manganese ($^{55}\text{Mn}$), and others. The specific incorporation of stable isotopes, like $^2\text{H}$, $^{13}\text{C}$, $^{15}\text{N}$, $^{17}\text{O}$, and $^{57}\text{Fe}$ is therefore a useful approach in the EPR and ENDOR analysis to identify the nature of a paramagnetic center. In an EPR spectrum, the hyperfine interactions give rise to resolved splitting of the EPR line into $2I + 1$ lines (see Figure 10 for the $I = \frac{1}{2}$ case). However, in cases where the EPR linewidth is large, e.g. due to fast spin relaxation (see below), or if many nuclei contribute to the EPR line, the hyperfine interaction is often not resolved. Here, ENDOR spectroscopy is the preferred method to determine the hyperfine couplings (see ENDOR section).

The hyperfine interactions can be expressed by

$$\mathcal{H}_{\text{HF}} = \hat{S} \hat{A} \hat{I}$$

containing the vector operators $\hat{S}$ for the electron spin and $\hat{I}$ the nuclear spin, and the hyperfine coupling (hfc) tensor $\mathbf{A}$. The hfc tensor can be separated into an isotropic hfc constant ($a_{\text{iso}}$) and the anisotropic hfc tensor ($\mathbf{A}'$)

$$\mathcal{H}_{\text{HF}} = a_{\text{iso}} \hat{S} \hat{I} + \hat{S} \mathbf{A}' \hat{I}$$
The isotropic term, also called the Fermi contact term, accounts for the interaction between the nucleus and the unpaired electron spin density localized on the nucleus, i.e. the s-orbital contribution. The anisotropic term, sums up the orientation dependent interactions, and contains information about the distance and angle between the unpaired electron spin and the nuclear spin.

**Exchange interactions** ($\mathcal{H}_{\text{EXCH}}$) denote the process of coupling of the spins of two nearby ions. It becomes very significant for diiron carboxylate proteins because pairs Fe(III)/Fe(IV) ($S_1=2$, $S_2=3/2$) and Fe(II)/Fe(III) ($S_1=5/2$, $S_2=2$) are usually antiferromagnetically coupled via a $p$-orbital of the $\mu$-oxo(hydroxo) bridging oxygen ion. The theory of the exchange interaction was initially developed by Gibson (Gibson, 1966) for diiron sulfur proteins. Now it is also used for the description of diiron carboxylate proteins and corresponding model systems.

$$\mathcal{H}_{\text{EXCH}} = -J \cdot \hat{S}_1 \cdot \hat{S}_2$$ (12)

Exchange coupling is significant for $\mu$-oxo bridged iron ions ($J \approx -100$-200 cm$^{-1}$) and much weaker for $\mu$-hydroxo bridged iron ions ($J \approx -10$ cm$^{-1}$).

Analogous to the electron spins, **Nuclear-Zeeman interactions** split the energy of nuclear spins ($I \neq 0$) into two levels when a magnetic field is applied, which is also the basis for performing NMR experiments. This interaction can be expressed in a similar fashion as the electron Zeeman interaction by using a nuclear magneton constant $\beta_N$ and a nuclear $g$-tensor for the nuclear spin operator ($\hat{I}$)

$$\mathcal{H}_{\text{NZ}} = -g_N \beta_N \hat{B} \hat{I}$$ (13)

Since the charge of the nucleus is opposite that of the electron, this term is given a negative sign. The nuclear-Zeeman interactions apply for the same elements as the hyperfine interactions, and their contributions are often in the same order of magnitude (Figure 10).

The **nuclear quadrupole** term is only relevant for nuclei with a spin $I \geq 1$, typically $^{14}$N in biological samples, or $^2$H in labeled samples. These nuclei have a non-spherical distribution of their nuclear charge namely a so-called electric quadrupole moment ($Q$) which interacts with the electric field gradient generated by all the electrons and nuclei surrounding the nucleus. The Hamilton term for the quadrupole interaction is

$$\mathcal{H}_Q = \hat{I} Q \hat{I}$$ (14)

where $Q$ is the quadrupole tensor.

A continuous wave (CW) EPR spectrum is obtained by placing a sample in a microwave resonator where it is exposed to constant, instrument specific, microwave radiation, as well as a variable magnetic field, which in most cases is oriented perpendicular to the magnetic microwave field component. A net absorption of microwave energy due to induced spin transitions will occur as long as the spin population in the ground state level is higher than in the excited level. The EPR signal is
recorded using field modulation and phase sensitive detection, which results in a first derivative absorption spectrum.

In an applied magnetic field, the electron populations in the excited and ground energy levels are given by the temperature dependent Boltzman distribution,

\[ \frac{N_{\text{Upper}}}{N_{\text{Lower}}} = e^{-\frac{\Delta E}{kT}} \]  

The population difference that is the reason for net microwave energy absorption and consequently for the EPR signal is upheld by the relaxation mechanisms. There are two distinct mechanisms involved in the spin relaxation, spin-lattice relaxation (longitudinal relaxation) and spin-spin relaxation (transversal relaxation), which are measured by the two relaxation times \( T_1 \) and \( T_2 \), respectively. The magnitude of \( T_1 \) increases with lower temperature; however, the relation between these parameters varies for different spin systems and is most prominent for transition metals. The spin-spin relaxation describes the dephasing of the spins in the system. \( T_1 \) and \( T_2 \) are the characteristics for the environment of the electron, which means that different paramagnetic species can be differentiated not only by their \( g \)-values, but also by their different relaxation behavior. As long as the population in the ground state is higher than in the excited level, a net absorption of microwave energy will occur, proportional to the square root of the applied microwave power. However, as more power is used, the spin system will approach a situation where the populations of the two levels are equal, the system is then said to be saturated. The microwave power needed for halfsaturation, \( P_{1/2} \), is a temperature dependent characteristic quantity for the paramagnetic species. This constant can be determined from a semiempirical formula (Ohnishi, 1987)

\[ \frac{I}{\sqrt{P}} = \frac{I_0/\sqrt{P_0}}{(1 + P/P_{1/2})^{b/2}} \]  

where \( I \) is the EPR signal intensity, \( P \) the power of the applied microwave, and \( b \) an empirical inhomogeneity parameter. \( I_0/(P_0)^{1/2} \) is a the constant ratio at non-saturating conditions. The magnitude of \( b \) depends on the spin relaxation mechanism, which is described by \( T_1 \) and \( T_2 \). In exchange coupled systems, e.g. dinuclear iron centers, the \( T_2 \) can become very small and comparable to \( T_1 \). Therefore, if the power saturation curve can only be fitted using a value of \( b \) that is smaller than 1, this is diagnostic for an exchange coupled system (Galli et al., 1996).

The signal intensity, or the integral of the EPR absorption spectrum, is proportional to the number of spins in the sample, and can therefore be used as estimation for the concentration of the paramagnetic species (Aasa & Vängård, 1975). However, due to baseline errors and sample filling factors, the error of this quantitation method is 5-10 %, or more, when the integral absorption of the unknown sample is compared with that of a sample of known spin concentration, measured under exactly the same, non-saturating, conditions. In this work, 1 mM CuSO\(_4\) in 10 mM EDTA was used as reference sample.
Electron nuclear double resonance (ENDOR) spectroscopy is a combined EPR and NMR experiment, hence double resonance, where the EPR transitions are saturated by microwave power and NMR transitions of the nuclear spins ($I$) are induced by a radio frequency source (Feher, 1956). This method is used to obtain a better resolution of the hyperfine interactions between the unpaired electron in a radical or metal center and the surrounding atomic nuclei that have nuclear spin. By monitoring the intensity of the saturated EPR line at a fixed microwave frequency and magnetic field, and then sweeping the radio frequency to induce nuclear spin transitions, only the nuclear spin excitation that are in coupling distance of the electron spin will be observed, in contrast to an NMR experiment.

The Hamilton operator for an ENDOR experiment is reduced to include only nuclear terms

$$\mathcal{H} = \mathcal{H}_{\text{HF}} + \mathcal{H}_{\text{NZ}} + \mathcal{H}_{\text{Q}} = \hat{S}A\hat{I} - g_n\beta_nB + \hat{I}Q\hat{I}$$

(17)

and since the magnetic field is kept at a constant value, the nuclear-Zeeman term is also constant. The resonance frequency for the nucleus at the given magnetic field strength can thus be calculated. This frequency is called the Zeeman frequency, or Larmor frequency $\nu_N$. The resonance condition, the observed ENDOR frequency, is

$$\nu_{\text{ENDOR}} = |\nu_N \pm A/2|$$

(18)

if we consider a simple case with only one $I = \frac{1}{2}$ nucleus, thus having no quadrupole splitting, and the position of the ENDOR lines depends only on the magnitude of the hyperfine coupling tensor, $A (= |A|)$. Based on the first order perturbation theory, if the Larmor frequency is larger than $A/2$, which is often the case for protons, the ENDOR transitions will appear at

$$\nu_{\text{ENDOR}} = \nu_N + A/2 \quad \text{and} \quad \nu_{\text{ENDOR}} = \nu_N - A/2$$

(19)

If, on the other hand, $A/2$ is larger than the Larmor frequency, which is the case if there is a large coupling on nitrogen or $^{57}\text{Fe}$

$$\nu_{\text{ENDOR}} = A/2 + \nu_N \quad \text{and} \quad \nu_{\text{ENDOR}} = A/2 - \nu_N$$

(20)

Rapid Freeze Quench (RFQ) technique

In order to detect the kinetics of the formation and decay of short-lived species a special reaction method, Rapid Freeze Quench (RFQ), was used to follow the reaction on the millisecond time scale. The principle of the RFQ technique is to mix the reactants quickly, let the reaction develop during a certain time, and then quench the reaction by rapid freezing of the produced sample (Bray et al., 1961; Balou, 1978). A scheme of RFQ apparatus is presented in the Figure 11.
**Figure 11.** The RFQ consists of two syringes for reactants (protein solution and anaerobic iron solution), a ram that drives the plungers in the syringes, a flow system with the mixer, reaction tube, and a nozzle that sprays the mixture into an EPR tube filled with isopentane cooled to –110°C. By changing the length of the reaction tube one can control the reaction time. The protein – iron mixture ejected from the nozzle forms ice flakes, which are packed in the EPR tube as tight as possible to make the sample of 20 mm height that corresponds the usual sample volume of 180 µl. After EPR measurements, samples were thawed and the volume of the liquid was measured to determine the “packing factor” for each sample \( k = \frac{180}{\text{actual volume}} \). The results of signal quantitation were corrected taking into account the possible deviation of packing density.

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**C. tm** RNR. Protein expression, extraction and purification. Sample preparation. Activity assay.

*C. tm* R2s and truncated *C. tm* R1Δ1–248 were expressed and purified as described in papers I-IV. Protein concentration was controlled by UV absorption at 280 nm \( (\varepsilon_{280} = 62000 \text{ M}^{-1}\text{cm}^{-1} \text{ for R2 polypeptide and } \varepsilon_{280} = 137000 \text{ M}^{-1}\text{cm}^{-1} \text{ for R1Δ1-248 polypeptide}).

For reconstitution of the diiron site in iron-depleted *C. tm* proteins anaerobic solutions of (NH\(_4\))\(_2\)Fe(SO\(_4\))\(_2\) (for \( ^{56}\text{Fe} \) reconstitution) or FeCl\(_2\) (for \( ^{57}\text{Fe} \) reconstitution) were used. To keep the iron solution anaerobic before its contact with protein, Hamilton syringes were used for iron administration. By filling and then emptying the syringe we kept iron solution only in the syringe’s needle, which minimized the error associated with taking only a small volume of the reagent. Following the iron solution, the protein solution was taken up in the syringe, and the syringe emptied into the EPR tube. This procedure provides quick and efficient mixing of iron and protein and allows the sample preparation time of 1-2 seconds. Then samples were frozen in cold isopentane and used for EPR measurements. The following expression was used for the calculation of the stock iron solution concentration

\[
C_{\text{iron}} = C_{\text{protein}} \cdot k \cdot \frac{V_{\text{sample}}}{V_{\text{needle}}} \quad (21)
\]

where \( C_{\text{iron}} \) - iron stock solution concentration, \( C_{\text{protein}} \) – initial concentration of the protein, \( k \) – chosen coefficient for reconstitution ratio iron : protein. The final protein concentration which is used for estimation of the EPR signal, becomes

\[
C_{\text{protein}}^{\text{final}} = \frac{C_{\text{protein}} \cdot V_{\text{sample}}}{V_{\text{sample}} + V_{\text{needle}}} \quad (22)
\]
after reconstitution.

*C*.tm activity measurements based on the use of a radioactively labeled substrate and chromatographic separation of the radioactively labeled product are described in [Paper IV](#).

**Redox titration and activity measurements of AOX**

All preparations for alternative oxidase protein were described in [Paper V](#). The potential measurements for different oxidation states of the diiron site of AOX were made using a home made cell (Figure 12) similar to the one described by Dutton (1971).

Figure 12. Redox-titration cell. Membrane suspension (with or without redox- mediators) continuously stirring was flushed with Ar for 40 minutes. Then protein was reduced with freshly prepared dithionite (DT) or NADPH solutions resulting in the lowering of the redox potential of the suspension to -320 mV or -180 mV respectively. Reoxidations of the samples (monitoring their redox state) were done by two different methods: either by careful titration with ferricyanide solution in argon atmosphere or by switching the argon flow to the oxygen flow. To avoid drying of the sample the incoming gas was previously bubbled through water.

**Polarographic system for measurement of dissolved oxygen (Activity assay for AOX)**

The reduction activity of the AOX was measured using a polarographic system equipped with a Clark oxygen electrode. The suspension to be assayed was placed in a sealed chamber that is exposed to the surface of a Clark oxygen electrode. The medium is stirred to ensure homogeneity and to ensure that oxygen can freely diffuse into the electrode and amplifies the current that is directly proportional to the concentration of oxygen in the chamber.
Results and discussion

Papers I-IV. *C. tm* RNR.

Chlamydial RNAs display significant sequence similarity to other class I RNAs (Stephens *et al.*, Read *et al.*, 2000). The R1 subunit conserves the amino acid residues responsible for the enzymatic activity (substrate binding site), allosteric regulation, R2 docking, and also amino acids attributed to the putative electron transport pathway (ETP). All residues belonging to the ETP are also conserved in the R2 subunit, together with amino acids forming the diiron carboxylate site needed for activation of the enzyme (with exception of Glu89, which replaces the chemically similar Asp) (Roshick *et al.* 2000).

The most significant difference in the structure of R2, which makes Chlamydia RNR very specific, is the replacement of the radical carrier tyrosine, conserved in more than 200 sequenced R2s, with a phenylalanine residue (Roshick *et al.* 2000; Högbom *et al.*, 2004). Numerous studies in different laboratories (Ehrenberg & Reichard, 1972; Sjöberg *et al.*, 1978; Bollinger *et al.*, 1991; Nordlund & Eklund 1993; Schmidt *et al.*, 1998; Stubbe, 2003) have proved the crucial role of the radical harboring tyrosine residue in at least two important phases of the RNR catalytic cycle. It functions as an electron donor in the oxygen activation of the diiron carboxylate center and its radical form serves as an electron acceptor in initiating the long range coupled electron/proton transfer, the first step of the enzyme turnover.

The goal of our investigations, reported in Papers I-IV, was to understand how the chlamydial RNR can function without the tyrosine residue in the vicinity of the diiron carboxylate site and what species takes over its function.

The X-ray structural data (Paper I) have confirmed the absence of a tyrosine close to the diiron site and refuted the assumption made in (Roshick *et al.* 2000) that its important catalytic functions can be carried out by Tyr129, located two residues further down the sequence. According to the structure data (Paper I), Tyr129 is located at the surface of the protein and does not have a possibility to contact the diiron center.

![Figure 13. Diferric site structure of the *C. tm* R2 protein.](image)

Our initial EPR experiments did not give any significant results. Neither untreated nor Fe^{2+} reconstituted R2 preparations showed any tyrosyl radical signal in the spectra.

Adding an anaerobic iron solution to the protein gave relatively slow rise to small quantities of a featureless radical signal, resembling the signal H now described in R2 Y122H *E. coli* mutants and attributed to hydroxylated phenylalanine F208 (Kolberg *et al.*, 2005).
Very important information obtained from the X-ray structure analysis was the data about the high iron affinity to specific iron-binding places in the chlamydial R2 protein. These results were confirmed by subsequent iron content analysis, indicating 90-95% iron occupancy of the iron binding sites. That explained the failure of our earlier reconstitution attempts and required that we find a way of obtaining chlamydial R2 as an apoprotein. Unfortunately, the chelation procedures suitable for the E.coli R2 protein (Atkin et al., 1973) led to destruction of the chlamydial R2. The solution was found when we made changes in the growing conditions of recombinant C.tm R2 protein. As the bacteria were made to grow in a regular LB medium, we chelated iron from the media by adding 1 mM EDTA before initiating the expression of C.tm R2 protein with IPTG. This procedure resulted in a significant decrease of iron content in the C.tm R2 protein from 1.8-1.9 to 0.7-0.8 iron per polypeptide. This iron-depleted (sometimes in the text called “apo”) C.tm R2 protein exhibited 10 times less enzymatic activity than the regularly grown one in an assay without added extra iron. The activity could be restored (up to 60-70%) after reconstitution with iron and oxygen. The reconstitution procedure also resulted in generation of a singlet EPR signal with a $g$-value of 1.999 and a linewidth of about 18 G (Figure 2, Paper I). The EPR properties of the paramagnetic species suggested that it is very similar to what in the reconstitution reaction of E. coli R2 is known as intermediate X (Bollinger et al., 1991), the penultimate species directly responsible for oxidation of the tyrosine. Intermediate X is formally an Fe(III)-Fe(IV) state of the diiron site, however large delocalization of the spin density onto the iron ligands (Sturgeon et al., 1996). For this reason it was also referred as an iron-coupled radical. The EPR signal intensity was maximal at about 1 s after mixing at 0°C and corresponded to approximately 30-35% of the polypeptide concentration. The identity of the EPR-observable species was verified by using $^{57}$Fe in the reconstitution reaction. The observed 26 G hyperfine splitting by $^{57}$Fe is typical for intermediate X (Bollinger et al., 1991; Sturgeon et al., 1996). The EPR signal of this species decayed with a rate of 0.2 s$^{-1}$ at 20°C without formation of EPR-visible successor (Paper I).

Assuming that the lifetime of the high potential intermediate X in the isolated R2 subunit could differ from its lifetime in a holoenzyme, we reconstituted the diiron carboxylate site of C.tm R2 protein in the presence of excess of C.tm R1 protein, reductant dithiothreitol (DTT) and necessary effectors (ATP and Mg$^{2+}$) (R1R2 complex). The EPR signal of the species X, generated in the R1R2 complex upon the reconstitution with Fe$^{2+}$/O$_2$ had approximately the same intensity as the one generated in R2 alone. For characterization of the center stability we estimated the time required for decay to one half of the signal intensity, $\tau_{1/2}$. This time for R2 alone is about 4 seconds and increases for an R1R2 complex up to 5 minutes at 0°C (Figure 2C, Paper II). The signal could not be stabilized if ATP and Mg$^{2+}$ were omitted from the reaction mixture. The relation between species X and enzyme activity was investigated in Paper III.

The enzymatic activity of the reconstituted R1R2 complex was close to the activity of the reconstituted R2 alone, i.e. 60-70% of the native enzyme. The intensity of the EPR signal from species X generated in the R1R2 complex was proportional to the content of R1 subunit in the reaction mixture and grew linearly with the increasing of R2:R1 ratio to 1:4 (Paper III). This observation correlates well with the specific activity data obtained in the McClarty laboratory. They showed the linear growth of activity
during the titration of the R2 protein with the increasing amounts of R1 up to a 6 fold excess (Roshick et al. 2000).

To further investigate the relation between the generation of species X and the enzymatic activity we incubated the regularly grown R2 protein with the other components of activity assay (two fold excess of R1, DTT, ATP and Mg$^{2+}$ and CDP as a substrate) in the concentrations suitable for EPR determination during 7 minutes at different temperatures (Figure 1, a-c, Paper III). Indeed, after freezing the samples we were able to detect relatively small, but significant concentrations of species X (up to 5% to R2 polypeptide) in the sample incubated at 36°C. Incubation at 15°C reduced the signal by a factor of about 2, whereas it was virtually absent after incubation at 0°C. This strongly suggested that the dynamic appearance of this EPR signal is related to enzyme activity, which also grows with increasing temperature. (Figure 2, Paper III). The dependence of the specific activity on temperature appeared to be steeper than the dependence of X, suggesting that not only concentration, but also the rate of the enzyme turnover grows with temperature. Control experiments showed that if either R1 or CDP were omitted from the incubation media, no EPR signal could be detected (Figure 1, d,e, Paper III). Our results strongly suggest the species X as the storage place for the protected oxidation equivalent needed to initiate catalysis in the Chlamydial RNR, thereby replacing the usual tyrosyl radical. The results also suggest that the iron site is a partner in the electron transfer process occurring during catalysis also in the tyrosyl radical based class I RNRs.

The following scheme proposes a possible mechanism of the enzyme turnover (Figure 14). The activation center of the C.tm R2 protein (Figure 13) represents a diamond core structure formed by two ferric ions joined by two oxo- and one carboxylate bridge. Assayed together with C.tm R1, Mg$^{2+}$-ATP, DTT and CDT as a substrate this protein demonstrated enzymatic activity similar to that reported before (Roshick et al. 2000). The proposed scheme of catalytic cycle of C.tm RNR (Figure 14) includes oxygen activation of the diiron site in the R2 subunit (I-III) as a first necessary step of the whole process.

I. The excess of the reductant in the incubation media (DTT: protein = 500 in our experiments) leads to a gradual reduction of the diiron site. We suppose that the ETP, the topographically arranged chain of amino acids from both protein subunits, R1 and R2, which have been shown to be conserved in all class I RNRs and necessary for enzymatic activity, is one probable pathway for diffusion reduction by external electrons. The overall role of this chain is to let oxidation/reduction equivalents move forth and back through the protein complex.

II. As all oxygen-activating diiron carboxylate proteins (Kurtz, 1997), C.tm R2 loses its oxo bridges in the diferrous state. Oxygen activation, which follows immediately after reduction converts the diferrous center via a number of intermediates to the state [Fe$_2$(µ-O)$_2$]$^{4+}$ (Solomon, 2001).

III. The extremely high valent state [Fe$_2$(µ-O)$_2$]$^{4+}$ state cannot exist for a long time in a reductant enriched medium and rapidly transforms to Fe$^{III}$Fe$^{IV}$(µ-O)$_2$. 

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Figure 14. A proposed scheme of the Chlamydia RNR turnover.
IV. \( \tau_{1/2} \) for \( \text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2 \) in a R1R2 complex is in the range of minutes, as it was shown in our studies (see above and Paper II). If the concentration of substrate in the medium is high enough to cause enzyme-substrate collision in this time period, the catalytic cycle starts. In the opposite case, further reduction returns enzyme to the resting state \( \text{Fe}_2(\mu\text{-O})_2^{3+} \) and the activation process should be started de novo.

V. Substrate binding leads to conformational changes that result in at least two important consequences. First, facilitation of the electron-proton coupled transport between the active center in R1 subunit (more precisely, Cys672) and diiron site in R2 subunit. This may be achieved, for example, by changing the mutual orientation of the phenol rings of the tyrosine residues, which form in a certain region of the ETP a perfect system for conducting \( \pi \)-electrons. Second, the active site in R1 (and, therefore, the diiron site in R2) becomes efficiently isolated from the external medium (Ehrenberg, 1999; Ehrenberg, 2001)

VI. The relatively high potential state \( \text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2 \) is isolated from external reductant and the potential barrier between diiron site and Cys672 is lowered by rearrangement of the components of the ETP. Cys672 becomes a thyl radical, donating its electron to the diiron center and reducing it to the state \( \text{Fe}^{\text{III}}\text{Fe}^{\text{III}}(\mu\text{-O})_2 \). Once Cys672 becomes a radical, the enzymatic reaction starts and results in dCDP formation and oxidation of Cys679 and Cys458 (Stubbe & van der Donk, 1998, Pelmenschikov et al., 2004).

VII. We propose that the formation of the disulfide bridge between Cys679 and Cys458 may influence the redox equilibrium in the system

\[
\begin{align*}
\text{C}672(439)-\text{SH} + \text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2 & \xrightarrow{k_1} \text{C}672(439)-\text*S + \text{Fe}^{\text{III}}\text{Fe}^{\text{III}}(\mu\text{-O})_2, \\
& \xleftarrow{k_1} \text{C}672(439)-\text{SH} + \text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2
\end{align*}
\]

where \( k_1 \geq k_1 \) for bound substrate and reduced Cys679/Cys458 pair, and \( k_1 \leq k_1 \) for “bound” product and disulfide bridge between Cys679 and Cys458, and move it to the left. Thus, at the end of the turnover the high potential state of the diiron site and the reduced form of the Cys679 are restored.

VIII. Once the state \( \text{C}672-\text{SH} + \text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2 \) is achieved, product release occurs followed by conformational changes which rearrange the ETP back to the conformation of hindered electron transfer.

IX. The reduction of Cys679-Cys458 pair (Holmgren, 1988) and a collision of the enzyme with the next substrate molecule should take place within the next few minutes (the lifetime of \( \text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2 \) in a R1R2 complex). In this case the enzyme starts the new catalytic cycle, returning to V. Otherwise \( \text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2 \) undergoes reduction to the resting state \( \text{Fe}^{\text{III}}\text{Fe}^{\text{III}}(\mu\text{-O})_2 \).

This model of the catalytic cycle correlates with the fact that Chlamydiae are obligate eubacterial parasites and cannot exist outside the host cell. The limited time of existence of the activating state \( \text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2 \) in comparison to the practically infinite lifetime of the tyrosyl radical, for example, in human RNR, should provide preferred conditions for replication of the host cell, preserving thus the proper environment for the parasites. Replication of Chlamydia may take place only in excess of substrate and reductants, otherwise its RNR exists in a resting state, R2 with \( \text{Fe}^{\text{III}}\text{Fe}^{\text{III}}(\mu\text{-O})_2 \). One can
predict that the similar (non-tyrosyl radical) activation mechanisms of RNR might be found in some other obligate parasites and symbiotic bacteria. Sequence analyses (Paper I) show that some eubacteria (*Thermobifida fusca, Tropheryma whipplei, Mycobacterium tuberculosis, M. bovis, and Bacillus thermoleovorans*) also possess the Chlamydia-type RNRs. The difference in activation process may be a reason to define this type of RNR as a new variant subclass, i.e class Ic as was suggested in Paper I.

As can be easily concluded from the scheme above, a crucial condition for the proposed mechanism is a very efficient insolation of the diiron carboxylate site from the medium. Any changes in the redox state of this site can be brought about only through the active site and ETP. Data confirming this high level of protection were obtained from the experiments with Fe^{2+}/O_2 reconstitution of apoR2R1-substrate complex (Paper II).

The preloaded apoR2R1-substrate complex (apoR2:R1= 1:3, ATP, Mg^{2+}, DTT and CDP as a substrate in Tris buffer) was mixed with anaerobic iron solution, which resulted in the formation of an Fe^{III}Fe^{IV} complex with an abnormal stability (Figure 1 and 2 A,B, Paper II) but lacking the enzymatic activity (Paper III).

One possible explanation for this puzzling observation, which is in line, however, with our main hypothesis of the highly protected diiron site, is presented in the following scheme (Figure 15).

*Figure 15. Possible explanation for the stability of species X formed under reconstitution of apoR2R1-substrate complex with Fe^{2+}/O_2*
I. R1 and apoR2 subunits of Chlamydia RNR in the presence of necessary effectors form apoR1R2 complex.

II. The apoR1R2 complex binds the substrate despite the unoccupied diiron site. The process of substrate binding is followed by the same conformational changes as was described in the comments to the previous scheme (step V, Figure 14).

III. Reconstitution of the apoR1R2-substrate complex with Fe$^{2+}$/O$_2$ rapidly results in formation of a high potential intermediate Fe$^{IV}$Fe$^{IV}$($\mu$-O)$_2$. The only available electron in the highly protected environment of the diiron site can be delivered from the reduced cysteine 672 of the active site of R1. The diiron site becomes Fe$^{III}$Fe$^{IV}$($\mu$-O)$_2$, cysteine 672 forms thyl radical.

IV. Substrate turns over, but the initial configuration (reduced cysteine 672) cannot be achieved because the potential of the Fe$^{III}$Fe$^{IV}$($\mu$-O)$_2$ site is too high to donate an electron to the thyl radical and the product cannot be released. The whole system remains locked, which allows detection of X over a time period of hours. The further destiny of the cystein radical is not yet clear. We suppose that it may be involved in some unspecific chemical reaction in its close neighborhood.

The detailed investigation of the Chlamydia high valent species X by $^{57}$Fe-ENDOR in WT R2 as well as in two mutants (Paper IV) has confirmed its identity with intermediate X from the E.coli mutant R2F122Y (Figure 2, Paper IV).

![Figure 16. ENDOR spectra of the species X generated in the apo-holoenzyme-substrate C.tm. complex (upper) and in the apoR2 alone under reconstitution reaction with Fe$^{2+}$/O$_2$. Experimental conditions as for Figure 1, Paper IV.](image-url)
Recent ENDOR experiments have also shown that the stable high valent species $X$, generated in the R1R2-substrate complex has the same iron site structure, as was shown for the R2 protein alone (Figure 16).

**Paper V. Diiron carboxylate center of the alternative oxidase.**

The alternative oxidase (AOX) is a ubiquinol oxidase found in the mitochondrial respiratory chain of plants as well as some fungi and protists (Veiga *et al.*, 2000; Dinant *et al.*). It has been predicted to contain a coupled diiron center on the basis of a conserved sequence motif consisting of the proposed iron ligands, four glutamate and two histidine residues (Siedow *et al.*, 1995). However, this prediction had not been experimentally verified. The difficulties with extraction and purification of this membrane protein did not allow the undertaking of a detailed spectroscopic study of AOX. Paper V reveals the first attempt of the EPR investigation of the *Arabidopsis thaliana* alternative oxidase AOX1a overexpressed in *E. coli*.

The iron dependent activity together with the conserved sequence motifs EXXH indicate the presence of a diiron carboxylate center in AOX, and the functional role of this enzyme in plant metabolism (quinol oxidation leading to oxygen reduction to water) suggests the oxygen-activation mechanism for this process. A dioxygen cleavage reaction on the diferrous site has been proposed for a number of diiron carboxylate proteins. By trying to reproduce the catalytic cycle of alternative oxidase using preparations of bacterial membranes containing AOX in amount of 4-25% of total protein, we were able to detect the EPR signal ($g = 1.86, 1.67, 1.53$) similar to mixed valent Fe(II)-Fe(III) signal previously described for other diiron carboxylate proteins (Figure 2, b-e, Paper V). Using the *E. coli* bacterial membranes for overexpression of the *A. thaliana* alternative oxidase allowed us not only to accumulate the protein of interest in the amounts sufficient for an EPR study, but provided us with a redox monitoring system. This is due to the presence of the respiratory chain in the membranes. The paramagnetic states of iron-sulfur clusters integrated into the NADPH-dehydogenase and Succinate-dehydrogenase complexes of the bacterial respiratory chain are transient and their well characterized EPR signals can directly indicate the redox state of the sample. In addition, probably most important, the bacterial respiratory chain supplies the endogenous reductant, ubiquinol, which is the natural substrate for AOX.

In order to reach the mixed-valent paramagnetic state Fe(II)-Fe(III) (state IV, Figure 17) in the turnover cycle, there is a requirement for reduced ubiquinone as a substrate. The high level of reduction in the membrane preparation was achieved by adding dithionite to an anaerobic membrane suspension. The EPR spectrum of this sample (Figure 2,a, Paper V) shows the signals of iron-sulfur proteins, which are paramagnetic in the reduced state ($E_m$ below -280 mV; Dunham *et al.*, 1971). This redox potential is definitely low enough to keep the ubiquinol pool ($E_m$ $\sim$ -60 mV; Hendler *et al.*, 1985) reduced. The same sample being recorded in parallel mode EPR (Figure 5, a, Paper V) gave rise to a signal, typical for a ferromagnetically coupled diferrous carboxo-bridged diiron carboxylate center. This observation allows us to relate this state to a state I (diferrous non-oxo-bridged state) of the proposed scheme (Figure 17). When the reduced membrane suspension was exposed to air, the oxygen activation process
occurred, followed by diferric peroxide intermediate formation and its fast reduction to the meta diferric-2µ-oxo(hydroxo?)-bridged state, indicated in the scheme as states II and III, which are not paramagnetic. We were not able to detect any high valent paramagnetic intermediate in these samples using EPR.

Figure 17. The proposed scheme for AOX turnover.

Therefore, the explanation of the next stage of AOX turnover – accumulation of an EPR detectable amount of mixed valent Fe(II)-OH-Fe(III) species – requires an additional assumption: The ubiquinol oxidation proceeds in two steps via ubisemiquinone formation, since it is known that the redox potentials of QH2/QH* and QH*/Q are -60 mV and +60 mV (Hendler et al., 1985), respectively. We hypothesize that the redox potential of the ubisemiquinone species formed as a result of one electron reduction of the meta state of AOX to the mixed valent Fe(II)-OH-Fe(III) is too high to provide the further reduction process. To complete the turnover and return the enzyme to the diferrous state “ready for activation” an extra ubiquinol molecule may be required. This chain of events – release of ubisemiquinone and binding another ubiquinol molecule – should slow down the transformation of the state III to the state I in our scheme and make it possible to maintain a dynamic concentration of the mixed valent form of AOX on an EPR detectable level. (We were able to generate 12 µM at 0°C and 18 µM at room temperature in the preparations containing 125 µM of AOX dimer, i.e. 9.6% and 14.4% respectively).

The EPR detection of an ubisemiquinone signal is complicated in the preparations containing the whole respiratory chain: in the redox conditions corresponding to mixed valent state formation, the g=2.00 signal was typically overlapped by the intensive signal of high potential iron sulfur protein S-3 (HIPIP) from succinate dehydrogenase (Figure 2, b-e, Paper V). Nevertheless, increasing the temperature of the EPR measurements to 30 K we were able to register a free radical signal with a linewidth of 8-10 G, typical for ubisemiquinone, distorted, however, by the signal of the Cu2+ containing cytochrome oxidase. The intensity of this free radical signal was significantly lower than the one of the mixed valent AOX species, which is evidence for the absence of free radical species accumulation during the turnover. We may propose several variants of the further ubisemiquinone transformation. First – the trivial dismutation of two ubisemiquinones
resulting in the formation of ubiquinol and ubiquinone. Second – ubisemiquinones consumption in the Q-cycle of the cytochrome $b_{c_1}$ complex, and, finally, ubisemiquinone may be used as a reductant in the previous steps of AOX catalytic turnover where it is “redox-competent”.

When the ubiquinol pool is exhausted the enzyme stops turning over and the concentration of the mixed valent AOX species, according to the proposed scheme, should become stable. This was confirmed with the time dependence of the EPR signal generation. Growing relatively fast during the first 30 minutes, the intensity of the signal then reveals a plateau and remains on the same level for at least another hour. Involvement of dioxygen in the $\mu$-hydroxo bridges formation was confirmed by the experiments with oxidation of the reduced membrane preparations by ferricyanide. The absence of the mixed valent AOX signal in these samples is evidence that oxygen is not only an oxidant, but a crucial participant of the enzyme turnover. The suggestion of the formation of $\mu$-hydroxo bridges rather than $\mu$-oxo bridges was based on the saturation-recovery EPR experiments, which allowed us to determine the exchange coupling constant $J$ as $-5.5 \text{ cm}^{-1}$ (Figure 4, paper V). This is typical for hydroxyl-bridged diiron carboxylate proteins.
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