EPR-and Moessbauer Studies of Benzoyl-CoA Reductase*

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Running title: Redox Clusters of Benzoyl-CoA Reduktase

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Abbreviations:

BCR, benzoyl-CoA reductase; Fd, ferredoxin; AdoPP[NH]P, adenosine 5’-[β,γ-imido]triphosphate; SHE, standard hydrogen electrode.
SUMMARY

Benzoyl-CoA reductase catalyzes the two-electron transfer from a reduced ferredoxin to the aromatic ring of benzoyl-CoA; this reaction is coupled to stoichiometrical ATP-hydrolysis. A very low reduction potential (< -1 V) is required for the first electron transfer to the aromatic ring. In this work the nature of the redox centers of purified benzoyl-CoA reductase from *Thauera aromatica* was studied by EPR- and Moessbauer spectroscopy. The results obtained indicated the presence of three [4Fe-4S] clusters. Redox titration studies revealed that the reduction potentials of all three clusters were below –500 mV. The previously reported S=7/2 state of the enzyme during benzoyl-CoA independent ATPase activity [Boll, M., Albracht, S.J.P., and Fuchs, G., 1997 *Eur. J. Biochem.* 244, 840-851] was confirmed by Moessbauer spectroscopy. Inactivation by oxygen was associated with the irreversible conversion of part of the [4Fe-4S] clusters to [3Fe-4S] clusters. Acetylene stimulated the benzoyl-CoA independent ATPase activity and induced novel EPR-signals with $g_{av}>2$. The presence of simple cubane clusters in benzoyl-CoA reductase as the sole redox-active metal centers demonstrates novel aspects of [4Fe-4S] clusters since they adopt the role of elemental sodium or lithium which are used as electron donors in the analogous chemical Birch reduction of aromatic rings.
INTRODUCTION

For many decades it appeared that the biological metabolism of aromatic compounds was restricted to an aerobic metabolism. However, in recent years an increasing number of anaerobic bacteria, which are able to oxidize various mononuclear aromatic compounds completely as their sole source for energy and cell carbon, has been discovered. The strategy of these bacteria is the conversion of the multitude of aromatic compounds to only a few key intermediates which become dearomatized in a reductive enzymatic process (for recent reviews see 1,2).

Most of the known pathways lead to benzoyl-CoA which represents the substrate for the dearomatizing enzyme benzoyl-CoA reductase (BCR). The biochemistry of this enzyme has only been studied in the denitrifying bacterium _Thauera aromatica_ [3]. It catalyzes the two-electron reduction of benzoyl-CoA to cyclohexa-1,5-diene-1-carbonyl-CoA (Fig.1). This two-electron transfer from reduced ferredoxin to the aromatic ring is stoichiometrically coupled to the hydrolysis of two molecules of MgATP to MgADP [3,4]. A radical mechanism, corresponding to the Birch reduction, of alternate one-electron and one–proton transfer steps to the aromatic has been proposed for the BCR reaction [5,6]. In this case a high redox barrier has to be overcome, since the transfer of the first electron to a thioester of benzoic acid requires a potential of -1.9 V [7].

The 163 kDa enzyme consists of four different subunits with molecular masses of 48, 45, 38 and 32 kDa containing 10-12 mol/mol enzyme of iron and acid-labile sulfur and substoichiometric amounts (0.2-0.3 mol/mol enzyme) of FAD [3,4]. The flavin is assumed to bind unspecifically to the two ATP binding sites of BCR. In a previous study the metal centers of BCR were investigated by electron paramagnetic resonance (EPR-) spectroscopy [8]. In the dithionite-reduced state, EPR signals of two interacting [4Fe-4S]^{+1} centers were detected; additional EPR-signals could not clearly be assigned but were characteristic for [2Fe-2S]^{+1} clusters. BCR also hydrolyses MgATP in the absence of a reducible substrate [3]. This uncoupled ATPase activity was only observed in the dithionite-reduced and not in the thionine-oxidized state of the enzyme.
Novel EPR signals were induced only in the steady state of this ATPase activity and were assigned to an $S=7/2$ high spin system. In parallel, a shift from interacting to non-interacting $[4\text{Fe}-4\text{S}]^{+1}$ clusters was detected. These observations allowed the presentation of a first model for the catalytic cycle of the BCR reaction. This model included an ATP-driven electron activation as a result of conformational changes affecting a reduced, EPR-active iron-sulfur cluster [8].

In this work we investigated the EPR properties of BCR in more detail. These studies included temperature- and redox potential-dependent studies. In addition we studied the effects of oxygen, Ado$PP[NH]P$ and acetylene on the EPR properties of BCR. We also investigated $^{57}\text{Fe}$-enriched BCR by Moessbauer spectroscopy in the reduced and oxidized state in the absence and presence of the individual substrates. The results obtained clarified the nature of the redox centers of BCR which we identified as three $[4\text{Fe}-4\text{S}]$ clusters. The ATP dependent switch from the $S=1/2$ to the $S=7/2$ state was confirmed by Moessbauer spectroscopy and could clearly be assigned to a $[4\text{Fe}-4\text{S}]$ cluster.
EXPERIMENTAL PROCEDURES

Growth of bacterial cells

*T. aromatic* was isolated in our laboratory and has been deposited in the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany; DSM 6984) [9]. It was grown anoxically at 28°C in a mineral salt medium in a 200 l fermenter; 4-OH-benzoate and nitrate in a molar ratio of 1:3.5 served as sole sources of energy and cell carbon. Continuous feeding of the substrates, cell harvesting, storage and preparation of cell extracts were carried out as described previously [10].

$^{57}$Fe-enrichment of BCR was achieved by adding $^{57}$Fe$^{2+}$ as sole source of iron to the medium. For this purpose 175 mg of metallic $^{57}$Fe (95.7% enriched, Advanced Materials and Technology Consulting, New York) was dissolved in 2.2 ml of 12 M HCl overnight at 80°C. After the metal was completely dissolved it was added drop-wise to 100 ml of 0.6 M nitrilotriacetic acid pH 5.2 under continuous stirring. During this procedure the pH was held constant between 3-5 by adding 2 M NaOH. With this amount of $^{57}$Fe *T. aromatic* was grown in a 100 l batch culture under continuous feeding of 4-OH-benzoate and nitrate to OD$_{578}$ ~ 2.6 yielding 250 g of cells (wet mass). Enrichment in $^{57}$Fe was estimated >90 % in purified BCR.

Protein purification, enzyme activity assay, purity control and sample storage

Purification of BCR from extracts of *T. aromatic* was performed under strictly anaerobic conditions in a glove box under a N$_2$/H$_2$ atmosphere (95:5, by vol.) as described earlier [3]. The procedure included three chromatographic steps including anion exchange chromatography on DEAE-Sepharose (Pharmacia), chromatography on ceramic hydroxyapatite (BioRad) and gel filtration using Sephadex G-75 (Pharmacia). Concentration of the protein samples was achieved by centrifugation (8 000 x g) in Microsep Microconcentrators (exclusion limit 50 kDa). The concentration of the enzyme was 175 mg ml$^{-1}$ for Moessbauer studies or 15-50 mg ml$^{-1}$ for EPR spectroscopy. The purity of these enzyme preparations was >90 % as estimated by Coomassie staining of SDS-PAGE gels. In previous experiments we have shown that an additional chromatography step using a Mono Q anion exchange column (Pharmacia) increased the purity up to virtual homogeneity but did not increase the specific activity of the enzyme indicating a
concomitant loss of activity. Since there was no difference in the EPR spectra between the samples of 90 %-100 % purity the Mono Q chromatography step was omitted in routine enzyme preparations. Enzyme activity was determined in a continuous spectrophotometric assay recording the benzoyl-CoA- and MgATP-dependent oxidation of reduced methyl viologen at 730 nm at 37°C [3]. Purified BCR (250-300 mg) was obtained from 200 g of cells (wet mass) with specific activities of 0.40-0.45 µmol benzoyl-CoA min⁻¹ mg⁻¹. Concentrated protein samples were stored anaerobically in tubes sealed with gas-tight stoppers at -80°C for several months.

**Effect of acetylene on BCR activity**

The effect of acetylene on BCR activity was tested in the standard spectrophotometric assay following the oxidation of reduced methyl viologen at 730 nm [3]. A saturated stock solution was prepared at 20°C (diameter 2 cm) by flushing acetylene with an overpressure of 0.2 bar first through wash bottle containing 10 mM dithionite and then through a stoppered 5 ml test tube (2 cm diameter) containing 1 ml of assay buffer with 150 mM Mops/KOH, 10 mM MgCl₂, pH 7.3. Aliquots of this acetylene stock solution (5-40 µl) were added to a standard enzyme assay mixture.

To test the effect of acetylene on benzoyl-CoA-independent ATPase activity, ATP consumption and ADP formation were discontinuously determined at 30°C. Samples (usually 100 µl) were periodically retrieved from a 1 ml assay mixture in stoppered vials containing 150 mM Mops/KOH pH 7.3, 5 mM sodium dithionite, 5 mM ATP, 10 mM MgCl₂, 0.2-0.4 mg BCR and 16 µl-80 µl of the saturated acetylene solution. The volume of the gas phase was approximately 1.5 ml, the reaction was started by addition of ATP. The samples were directly analyzed by HPLC using a MonoQ anion exchange column as described previously [7].

**Sample preparation for EPR- and Moessbauer spectroscopy**

All BCR samples for EPR- and Moessbauer spectroscopy were prepared in an anaerobic glove box under a 100 % nitrogen atmosphere (<1.0 ppm O₂). Prior to any sample preparation of BCR excess of dithionite and corresponding oxidation products were removed by passing the
concentrated enzyme sample (0.1-1 mM; 0.5-1 ml) over a Biogel P-6 (Biorad) desalting column (volume: 5 ml, diameter: 1 cm) which had been equilibrated with either a 100 mM Mops/KOH pH 7.3 or with 100 mM Tris/HCl buffer pH 8.0 both containing 10 mM MgCl₂. If not otherwise stated this dithionite free enzyme sample represented the starting material for all sample preparations.

*Reduction of the enzyme*

Reduction of BCR was performed according to standard anaerobic procedures by adding sodium dithionite from a freshly prepared stock solution (100 mM in 100 mM Mops/KOH pH 7.3) giving a final 10-fold excess of this reductant compared to the enzyme. The concentration of these dithionite solutions was checked from time to time by titration against ferricyanide, showing that they were between 80-90 % of the nominal concentration. The pH of the enzyme solutions was not affected by dithionite addition since the buffer concentration in the enzyme preparations was 10-100-fold higher than the final dithionite concentration (~ 1-10 mM). The reduced enzyme was quickly transferred into EPR tubes or Moessbauer sample holders and then frozen either inside the glove box on dry ice (Moessbauer-samples) or outside the glove box in an gas tight sealed EPR tube in liquid nitrogen. When BCR was reduced with deazaflavin/light the enzyme was brought to 100 mM Tris/HCl buffer pH 8.0 using a desalting P6-column desalting which had been equilibrated with this buffer. Deazaflavin was obtained as a kind gift from Prof. S. Ghisla (University at Konstanz, Germany). The final concentration of deazaflavin in the sample was 20-40 µM (for EPR samples) or 200 µM (for Moessbauer samples). The EPR samples were illuminated in airtight sealed glass tubes (diameter: 1 cm) for 15 min with white light (250 W halogen bulb) from both sides of the tube (distance: approximately 2-3 cm). Moessbauer samples were illuminated for 15 min by installing the sample holders inside and the light source outside a glove box consisting of transparent PVC (distance to the tube: 5-10 cm).

*Effect of MgATP, benzoyl-CoA and AdoPP[NH]P*

When the benzoyl-CoA-independent ATP hydrolyzing activity of dithionite-reduced BCR was studied, 10 mM ATP and 20 mM MgCl₂ for EPR spectroscopy and 25 mM MgATP and 40 mM
MgCl₂ for Moessbauer spectroscopy were used. Since ADP is a competitive inhibitor of BCR ($K_i$ for MgADP is 1.1 mM, $K_m$ for ATP is 0.6 mM, [8]) a MgATP regenerating system was added consisting of phosphoenolpyruvate (same concentration as ATP) and pyruvate kinase (Sigma, activity in the assay was 10 times higher than BCR activity). Prior to freezing of the EPR tubes or Moessbauer sample holders, BCR was incubated for less than 1 min to ensure that excess residual MgATP was present. To test the effect of the inactivating compound AdoPP[NH]P on the EPR and Moessbauer spectra, the enzyme was incubated for 10 min at room temperature with 0.05 mM (EPR-spectroscopy) or 0.2 mM (Moessbauer spectroscopy) of this compound in the presence of 20 mM MgCl₂.

Effect of acetylene

The effect of acetylene on the EPR spectrum of BCR was studied in the presence and absence of MgATP. After addition of 60 µl of a saturated acetylene solution prepared at 20°C as described above to 540 µl of a BCR solution (25 mg ml⁻¹ in 100 mM Mops/KOH and 20 mM MgCl₂ pH 7.3) the sample was incubated for 10 min at room temperature. Half of the solution was directly transferred into an EPR tube and frozen whereas the other half of the solution was incubated for 2 min with 10 mM ATP for 1 min at room temperature and then frozen in an EPR tube.

Redox titration experiments of BCR

Dye-mediated redox titration of BCR was performed in a Miller-Howe anaerobic glove box under a nitrogen atmosphere (<1 ppm O₂). The enzyme/mediator mixture (2.5 ml) was dissolved in 100 mM Mops/KOH pH 7. The concentration of BCR was 100-150 µM. The mediator consisted of methyl and benzyl viologens, neutral red, safranin O, phenosafranin, anthraquinone-2-sulfonate, 2-hydroxy-1,4-naphthoquinone, indigo disulfonate, resorufin, methylene blue, phenazine methosulfate and $N'N'N'N'$-tetramethyl-p-phenylenediamine at a final concentration of 40 µM each. The redox potential was adjusted with 10-100 mM sodium dithionite and potassium ferricyanide prepared freshly and anaerobically in the same buffer as BCR. Potentials reported in this paper are with reference to SHE and were obtained by using a potential of +243 mV vs SHE for the saturated calomel electrode at 22°C. Mediator/enzyme mixtures with a stable potential
could be obtained from -543 mV to +100 mV. Stabilization (drift <1 mV/min) of the potentials usually required 1-5 min under continuous stirring. Samples with defined redox potentials were immediately frozen anaerobically in EPR tubes and stored in liquid nitrogen. Redox titrations were normally performed in the oxidative direction but as a control for reversibility, some samples were prepared by re-reduction with dithionite.

**EPR spectroscopy**

X-band EPR spectra were recorded on an updated Bruker 200D-SRC spectrometer. Low temperature measurements were made using an Oxford Instruments ESR 900 cryostat modified to take sample tubes of up to 4-mm internal diameter. Recording conditions are described in the legends to the individual figures. Spin concentrations of ground-state transition EPR signals were determined by comparison with a 1.00 mM copper sulfate sample in 11 mM sodium EDTA.

**Moessbauer spectroscopy**

$^{57}$Fe Mössbauer spectra were recorded with a conventional constant acceleration spectrometer using a $^{57}$Co source in a Rh matrix (1 GBq using the bath cryostat; 1.3 GBq using the magnet cryostat). Measurements at 4.2 K and 77 K were performed with a bath cryostat (Oxford Instruments) and a permanent magnet mounted outside the cryostat producing a field of $20 \text{ mT} \perp \gamma$. High-field measurements were performed with a cryostat equipped with a superconducting magnet (Oxford Instruments). The spectra were analyzed assuming Lorentzian line shape; the isomer shift is quoted relative to $\alpha$-Fe at room temperature.

**Other methods**

Benzoyl-CoA was synthesized according to Schachter and Taggart [11]. Protein concentration was determined by the Bradford method using bovine serum albumin as standard [12]. SDS PAGE were performed as described by Laemmli [13]. Protein was visualized by Coomassie blue staining [14].
RESULTS

1. Nature of the iron-sulfur centers

The nature of the iron-sulfur centers of BCR was studied by Moessbauer and EPR spectroscopy. Metal analysis of BCR revealed the presence of 10-12 mol iron/mol enzyme, and substoichiometric amounts of zinc. Earlier EPR studies [7] had indicated the presence of two [4Fe-4S] and additional Fe-S clusters which were assigned to [2Fe-2S] clusters or to a novel metal cluster with an unknown structure. In addition, as part of this work, similar EPR signals typical of [3Fe-4S] clusters were detected in partially inactivated BCR preparations (data not shown).

Moessbauer spectroscopy: oxidized BCR

For Moessbauer spectroscopy studies, BCR was purified from extracts of T. aromatica grown anaerobically with $^{57}$Fe as sole source of iron. High $^{57}$Fe enrichment was achieved as estimated from the resonance absorption effect of about 9 % for each subspectrum of the oxidized sample, which corresponds to about 11 mM $^{57}$Fe (sample concentration was about 1 mM and isolated benzoyl-CoA reductase samples were shown to contain 10-12 mol Fe/mol enzyme). The Moessbauer spectrum of thionine-oxidized BCR taken at 4.2 K in a small external field of 20 mT perpendicular to the $\gamma$-beam shows a slightly asymmetric quadrupole doublet (Fig. 2A). For reasons which will be discussed later the spectrum was analyzed using three subspectra of equal area. The isomer shifts and quadrupole splittings so obtained ($\delta$~0.45 mm s$^{-1}$ and $\Delta E_Q$~1 mm s$^{-1}$, see Table 1) are typical of delocalized mixed-valence iron sites in tetrahedral sulfur coordination and are characteristic of [4Fe-4S]$^{2+}$ clusters [15]. The slightly different quadrupole splittings of the subspectra indicate structural differences between the three [4Fe-4S]$^{2+}$ clusters which each contain two delocalized mixed-valence iron-pairs with spins $S_{12}$=9/2 and $S_{34}$=9/2 with antiparallel coupling yielding a total cluster spin of zero. Indeed, the spectrum recorded in a high external field of 7T parallel to the $\gamma$ beam (Fig. 2B) shows magnetic splitting only due to the external field and thus reveals the diamagnetic nature of the clusters in the oxidized BCR sample.
These results allowed us to exclude the presence of [2Fe-2S] and [3Fe-4S] clusters since both types of oxidized clusters ([2Fe-2S]^{2+} and [3Fe-4S]^{+}) show subspectra with parameters typical for Fe^{3+}S_{4} (δ~0.25 mm s^{-1}; ΔE_{Q}~0.7 mm s^{-1}), which are clearly absent in the Mössbauer spectra of thionine oxidized BCR. Additionally [3Fe-4S]^{+} clusters would reveal paramagnetic features in a high external field of 7T, whereas the oxidized sample contained clearly only diamagnetic species.

**Moessbauer spectroscopy: reduced BCR**

The Mössbauer spectrum of deazaflavin reduced BCR recorded at 120 K (Fig 3A) and 4.2 K (Fig 3B) in a small external field of 20 mT ⊥ γ is shown in Fig. 3A and 3B. The 4.2 K spectrum shows the presence of a paramagnetic species and a quadrupole doublet, the latter with parameters (δ ~0.45 mm s^{-1} and ΔE_{Q}~1 mm s^{-1}, see Table 1) similar to those observed in the oxidized sample. EPR investigations (see EPR section of this work) showed the presence of S=1/2 species and therefore the paramagnetic species were analyzed by using the spin-Hamiltonian approximation for a total cluster spin S=1/2 [16]. As starting parameters for the simulation parameters for [4Fe-4S]^{1+} published in [15] were used. With this procedure 33% of the total area were attributed to a [4Fe-4S]^{1+} cluster with parameters given in Table 1. The remaining 66% of the total area could be simulated with two doublets whose parameters are similar to those (see Table 1) found for components 1 and 2 in the thionine oxidized sample. The spectrum taken at 4.2 K in an high external field of 7T || γ (Fig. 3C) was simulated with the same parameter set as the low-field data. Subspectra 1 and 2 represent diamagnetic [4Fe-4S]^{2+} clusters as in thionine oxidized BCR. By elevating the temperature to 120 K (Fig. 3a) the diamagnetic subspectra exhibit the isomer shifts δ_{1}=0.41 mm s^{-1}, δ_{2}=0.4 mm s^{-1} and the quadrupole splittings ΔQ_{1}=1.04 mm s^{-1}, ΔE_{Q2}=0.76 mm s^{-1}. The spectrum of [4Fe-4S]^{1+} can be simulated with one quadrupole doublet with the parameters δ =0.55 mm s^{-1}, ΔE_{Q2}=0.95 mm s^{-1}, which are similar to those reported for the reduced ferredoxin from *C. pasteurianum* [15].

**EPR spectroscopy**

Based on the results obtained from Moessbauer spectroscopy we reinvestigated the [4Fe-4S] clusters of reduced BCR using temperature dependent and power saturation EPR studies. To
achieve a high degree of reduction, the dithionite used in previous studies was replaced by
deaflavin/light as reductant. Our aim was a clear assignment of individual EPR-subspectra to
the three [4Fe-4S] clusters of BCR as identified by Moessbauer spectroscopy. For this purpose
we first compared the EPR spectra of unlabeled with $^{57}$Fe-labeled enzyme. All EPR features
clearly broadened in the $^{57}$Fe spectrum as a result of unresolved hyperfine interaction of unpaired
electrons with the nucleus of $^{57}$Fe (I=1/2), (Fig. 4). This confirmed that high $^{57}$Fe enrichment was
achieved and that no other paramagnetic metal than iron dominated the EPR spectra of our
sample.

Further EPR studies were performed with unlabeled BCR. Unusually for [4Fe-4S] clusters, which
normally relax rapidly [17], an EPR signal from a [4Fe-4S]$^{+1}$ cluster was observed up to 77 K. At
60 K a rhombic EPR spectrum characteristic of a single [4Fe-4S]$^{+1}$ cluster with g-values at 2.017,
1.938, 1.908 was observed (Fig. 5A); this cluster is referred to cluster I. A minor EPR signal at
g=2.004 with less than 0.05 spin/mol protein was superimposed on this spectrum and indicated
the presence of a radical of unknown nature. At temperatures below 40 K additional features at
g=2.053 and around g=1.93 intensified indicating the rise of an EPR signal from an additional
[4Fe-4S]$^{+1}$ cluster relaxing significantly faster than cluster I. At temperatures below 25 K all
features started to saturate simultaneously at 2 mW (Fig. 5A). A difference spectrum between the
spectra recorded at 20 K/ 0.2 mW and the spectrum taken at 60 K and 2 mW normalized for
temperature and microwave power is presented in Fig. 5A. The difference spectrum was rather
broad and axial and is assigned to cluster II with g-values at 2.053, 1.93, and 1.93. The
dependencies of the EPR signals of both clusters on temperature and microwave power were
highly similar resulting in only a weak difference spectrum. The total spin concentration in the
dithionite reduced sample was not higher than 0.82 mol/mol enzyme, indicating only a partial
reduction of the clusters. This was in agreement with the results obtained from Moessbauer
spectroscopy where the highest degree of reduction was 33 %. EPR signals from the putative
third [4Fe-4S] cluster of BCR, as expected from Moessbauer spectroscopy, could not be detected
in the deaflavin-reduced sample indicating that it was not reducible by this system. The
temperature dependence of the total spin concentration between 20 K to 77 K fitted well to the
observation that cluster II relaxes faster than cluster I (Fig. 5B). After normalization for
temperature, the spin concentration approximately doubled from 0.42 (60 K) to 0.82 (20 K)
spins/enzyme. This increase is considered to be mainly a result of the increased intensity of the
cluster II EPR signal which is not detectable at temperatures above 40 K.

At temperatures below 15 K, broad unresolved features covering more than 200 mT started to
develop. These broad features also have been detected in a previous study and were ascribed to
two interacting 2[4Fe-4S] clusters [8]. Even at 4 K and 20 mW microwave power these features
could not be saturated [8]. It was concluded that in the some protein molecules the clusters
interacted magnetically, whereas others showed EPR features of single isolated [4Fe-4S]+1
species, indicating a conformational heterogeneity. Since spin integration of the broad signal
extending over 200 mT could not be carried out accurately it was impossible to give an exact
ratio of coupling/non-coupling clusters. An additional problem was that all clusters were at best
partially reduced, so it was not clear which of the [4Fe-4S] clusters contributed to this broad
signal. A rough estimate indicated that the spin concentration of the broad signals at 4 K was in
the same range as the total spin concentration detected at 25 K. Therefore, we estimate that
photoreduction with deazaflavin resulted in a 50 % reduction of all clusters with half of the
paramagnetic clusters interacting. This value is slightly higher than that in the samples used for
Moessbauer spectroscopy (33 %).

The results obtained from Moessbauer spectroscopy and the content of iron and acid-labile sulfur
(10-12 mol/mol enzyme) strongly indicated the presence of three [4Fe-4S] clusters but only two
clusters could be identified clearly by EPR spectroscopy in the deazaflavin reduced state, although
the broad signal of interacting clusters could not be assigned. In order to identify a third [4Fe-4S]
cluster by EPR spectroscopy, we studied BCR under steady state conditions of ATP-dependent
benzoyl-CoA reduction. In Fig. 6B a representative EPR-spectrum of BCR recorded at 40 K is
presented showing that clusters I and II are both more oxidized than in the dithionite-reduced
sample, as indicated by a 80 % decrease of signal intensity at g=2.05 and g=1.93,1.91. In parallel,
novel EPR features were observed in the g=2 region. After subtraction of the EPR spectrum of
dithionite-reduced BCR (× 0.2) from the one obtained in the steady state of substrate reduction, an EPR signal was left with features at \( g = 2.037, 1.995, 1.97 \) and \( 1.96 \) (Fig. 6 BΔC). These features could not be ascribed to an isolated \( S = 1/2 \) species, but instead are typical of an interaction with a second magnetic site. With this assumption, the EPR-signal indicated the presence of a [4Fe-4S] cluster with \( g \)-values \(-2.03, 1.995 \) and \( 1.965 \) with splitting constants in the range 2-3 mT. A decrease of the modulation amplitude to 0.3 mT did not resolve the signal further (not shown). Its spin concentration amounted to less than 0.35 spins/enzyme indicating that cluster III was reduced to approximately the same extent as the other two clusters in the photoreduced sample. Optimal EPR conditions were 30 K and 2 mW. The nature of the second magnet involved in the interaction with cluster III is not known. It is rather unlikely that this is either cluster I or II since the 40 K difference spectrum in Fig. 6D was obtained easily, which would not have been the case had one of these clusters been coupled magnetically with cluster III. A hyperfine interaction with a nuclear spin, such as a proton, is an unlikely explanation since this type of interaction usually results in splitting constants significantly below 2 mT, although this cannot be ruled out absolutely. It is likely that the complex EPR spectrum of cluster III is due to a dipole-dipole interaction with an additional unknown paramagnet, such as a radical species. At temperatures higher than 77 K an isotropic radical signal remains (Fig. 6A). It is not clear whether the decrease of spin concentration of the radical at lower temperatures was only due to saturation or also to an interaction with cluster III.

2. Redox properties of the clusters

The natural electron donor for BCR is a reduced ferredoxin with two [4Fe-4S] clusters with reduction potentials of -431 mV and -587 mV vs SHE [4,19]. The reduction potentials of the iron-sulfur clusters of BCR therefore are expected to be below -400 mV. As discussed above, Moessbauer studies indicated that only 1/3 of all clusters could be reduced by dithionite or deazaflavin/light, indicating that the reduction potential of at least two clusters must be far below -500 mV.
We have studied the redox potential dependence of the EPR spectra recorded at different temperatures. Our goal was to investigate the redox potential-dependent rise of both the signals of the interacting and the non-interacting clusters. At potentials more positive than -400 mV, only weak EPR-signals were detected, originating from adventitious iron at g = 4.3, or in some batches minor signals (spin concentration less than 0.3 mol/mol enzyme) of an oxidized [3Fe-4S]^{+1} cluster (not shown). This EPR signal is assigned to a partial degradation of [4Fe-4S] to [3Fe-4S] clusters and was similar to the spectra obtained when BCR was exposed to air, leading to an irreversible inactivation of the enzyme (see also Fig. 10). In Fig. 7B samples recorded at 25 K at potentials from -467 mV to -535 mV are shown. Usually the redox titration was performed in oxidative direction by oxidation of dithionite-reduced enzyme with ferricyanide. At -467 mV the 25 K spectrum displays features of cluster I (see 60 K spectrum in Fig. 5A) as indicated by the ratio of signal amplitudes at g=1.93 to 1.908 and the typical rhombicity. When the potential was lowered further, additional features appeared at g=2.053. In parallel, the shape in the g<2 region changed as a result of an increase of intensity at g=1.91. These two features were typical for an increase of spin concentration of cluster II which increasingly dominated the spectrum as the potential was poised lower. Due to the strong radical signal of the mediators the spin concentration at -543 mV and 25 K could only be estimated to be in the range of 0.4-0.6 mol/mol enzyme; the spin concentration was lower than in photoreduced sample (0.82 mol/mol).

We also recorded EPR-spectra of the same samples at 4 K in order to investigate the redox potential-dependent features of the EPR signals of the magnetically interacting clusters (Fig.7A). At -467 mV, when the 25 K spectrum mainly displayed features of cluster I, a very poorly resolved and broad signal around g=2 started to develop. In parallel to reduction of cluster II, the EPR signal of the interacting [4Fe-4S]^{+} clusters also increased, indicating that these signals derive from the magnetic interaction of cluster II with at least one of the other two clusters. However, our results show that this interaction did not involve all reduced cluster II, since at higher temperatures EPR signals typical of non-interacting clusters could be ascribed to isolated clusters I and II. No sign of even a partial reduction of the putative cluster III was observed at potentials down to -543 mV. However one has to take into account that the EPR signals ascribed to this
cluster (g=2.037, 1.97, 1.96) are quite narrow and close to those of the strong radical of the redox dyes, so that they could be obscured by the latter. Additionally, cluster III might couple with cluster II resulting in the broad signal observed at low temperatures. It was not possible to determine accurately the spin concentration from the low temperature spectra.

Since none of the clusters was even half-reduced a fit to a Nernst curve was impossible. The spectra shown in Fig.7B clearly imply that the reduction potential of cluster I and cluster II must be lower than -500 mV. However due to only a partial reduction a fit to Nernst curve was impossible.

3. Effect of MgATP and AdoPP[NH]P on the clusters

Reduced BCR catalyzes both a stoichiometric ATP hydrolysis coupled to benzoyl-CoA reduction, and benzoyl-CoA-independent ATP hydrolysis [8]. In a previous EPR study three major effects were observed under steady state conditions of substrate-independent ATPase activity. First, new EPR signals were induced: an isotropic signal at g=5.15 accompanied by a minor one at g=12 [8]. These two signals were assigned to the transitions of the first excited state and ground state an S=7/2 high spin system. Second, the broad signal at g=2 assigned to two interacting [4Fe-4S] clusters sharpened up, indicating that the interaction between the clusters was diminished after prolonged incubation with MgATP. Third, a concomitant loss of S=1/2 spins was observed in the course of the experiment. In these studies the nature of the paramagnet with the S=7/2 high-spin system was not identified. Moessbauer studies should indicate whether the switch from the S=1/2 to a S=7/2 high spin system occurred in one isolated cluster, or in two interacting [4Fe-4S] clusters. Additionally, we wanted to clarify whether the two effects of MgATP described above were the result of binding or hydrolysis of MgATP. For this purpose the non-hydrolyzable ATP analog AdoPP[NH]P was used; this compound does not act as a competitive inhibitor but inactivated BCR irreversibly in a time-dependent reaction [8].

The Moessbauer spectra of BCR under steady state conditions of benzoyl-CoA-independent ATPase activity are shown in Fig. 8. The spectra recorded at 4.2K at 20 mT ⊥ γ (Fig. 8B) and
7T || γ (Fig. 8C) exhibit an additional magnetic hyperfine pattern when compared to Fig. 3. EPR studies on an equivalent sample revealed the presence of a S=7/2 species [8]. We therefore simulated this magnetic pattern using the spin-Hamiltonian approximation with S=7/2 and with D = 4.3 cm⁻¹ and E/D = 0.12 derived from EPR spectra [8]. In the spectrum taken at 120 K (Fig. 8A) there is a slight shoulder at about 1.5 mm s⁻¹ which can be assigned to the S=7/2 species as it did not appear in the spectrum of deazaflavin/reduced reduced BCR (Fig. 3A). This line originates from Fe²⁺ sites similar to those in the S=7/2 species of the selenium-substituted 2[4Fe-4Se]⁺ clusters in the ferredoxin from Clostridium pasteurianum [20]. Therefore the same pattern of electron localization as described in [20] was assumed, i.e. a S=7/2 species with two subspectra in the ratio Fe²⁺:Fe³⁺ = 3:1. For the Fe³⁺ site values δ=0.39 mm s⁻¹ and ΔE_Q=1.17 mm s⁻¹ taken from [20] were used. For the Fe²⁺ sites slightly different values (δ=0.58 mm s⁻¹ and ΔE_Q=1.55 mm s⁻¹) were used in order to be consistent with the data taken at 120 K. The low- and high-field spectra of Fig. 8B,C were fitted taking η, Aₓ, Aᵧ, Az and the total area of the two subspectra of the S=7/2 as free parameters, and using the same parameters as for the deazaflavin/light-reduced sample for the [4Fe-4S]⁺ (S=1/2) and [4Fe-4S]²⁺ clusters. The mean values of the resulting A-Tensors of the S=7/2 species (Tab.1) are comparable with the theoretical and the experimental mean values given in [20]. Again 66% of the spectral area represent diamagnetic [4Fe-4S]²⁺ clusters, 25 % belong to paramagnetic (S=1/2) [4Fe-4S]¹⁺ and 8% can be assigned to [4Fe-4S]¹⁺ clusters with spin S=7/2. The fact that the ratio of diamagnetic clusters to paramagnetic clusters is 2:1 for two different preparations strongly indicates that BCR contains three discrete [4Fe-4S] clusters.

In order to distinguish between the effects of binding and hydrolysis, EPR-spectra of BCR were recorded at 4 K and 0.2 mW in the presence of MgATP and AdoPP[NH]P (Fig. 9). Under these conditions it was certain that the fast relaxing signals of the interacting [4Fe-4S] clusters were not saturated. In the steady state of benzoyl-CoA-independent ATPase activity, the broad EPR signal of the interacting [4Fe-4S] clusters sharpened up (indicated by arrow 2, Fig. 9A,B). In parallel the typical isotropic signal at g=5.15 of the first excited state transition of an S=7/2 system was observed (arrow 1 in Fig. 9B). The accompanying signal at g=12, which results from a ground
state transition of the S=7/2 system [8], represents only a minor contribution to the total spin and therefore was only detectable at very high enzyme concentrations (not shown). When ATP (10 mM) was replaced by AdoPP[NH]P virtually no signal of an S=7/2 spin system was observed (Fig. 9C). However, in the same way as with MgATP, the broad EPR-signal of the interacting [4Fe-4S] clusters changed to sharper signals of non-interacting clusters. We therefore conclude that binding of the nucleotide eliminates the interaction between two [4Fe-4S] clusters, whereas hydrolysis of ATP induces the switch from S=1/2 to S=7/2 of a [4Fe-4S] cluster. The latter could not be observed in the presence of the non-hydrolyzable ATP analog AdoPP[NH]P. No significant changes were induced by ATP or by AdoPP[NH]P in the EPR signals of the non-interacting [4Fe-4S] clusters recorded at higher temperatures.

4. Effect of oxygen

BCR is highly sensitive to oxygen, with a half life in the absence of reducing agents of less than 30 s [3]. We investigated this irreversible inactivation by recording EPR spectra of BCR after exposure to air and after re-reduction of the enzyme with dithionite. In Figure 10A the full scale 4.8 K spectrum of air-oxidized BCR displays two main features at g=4.3 and g=2.01. The former signal was assigned to adventitiously bound tetrahedrally coordinated Fe³⁺. It was clearly increased compared to an intact mildly thionine-oxidized sample (not shown), indicating the partial release of iron atoms from the [4Fe-4S] clusters as a result of degradation. Note, that the optimal temperature for this signal was between 20-30 K and therefore it was highly saturated under the conditions of Fig. 10. The second axial EPR signal was typical of an oxidized [3Fe-4S]⁺⁺⁺ cluster with g~2.013 and was not saturated at 4.8 K and 2 mW microwave power. An EPR spectrum displaying the g=2 region of the axial spectrum of the [3Fe-4S]⁺⁺⁺ cluster is shown in more detail in Fig.10D. In addition to the sharper features at g=2.033 and g=2.013, a weak and broad signal was observed at g=1.938. Note that a radical signal at g=2.00 also contributed to the spectrum shown. The reduction potential of the [3Fe-4S]⁺⁺⁺ cluster was between −100 mV and −150 mV as estimated from EPR spectra recorded at potentials between +100 mV to −300 mV (not shown). The rise of [3Fe-4S]⁺⁺⁺ EPR signals after incubation on air was an indication of the degradation of one or more [4Fe-4S] clusters to [3Fe-4S] clusters. However, the spin
concentration of this signal was less than 1 mol/mol enzyme indicating that 5 min incubation resulted only in a partial cluster degradation. A full scan 4 K EPR-spectrum of the enzyme sample which had been anaerobically re-reduced by excess of dithionite after exposure to air is presented in Fig. 10B. The EPR-signal of the [3Fe-4S]$^{+1}$ signal completely disappeared as a result of its reduction to a [3Fe-4S]$^0$ cluster. Additionally, a broad signal around $g=12$ was detected characteristic of an S=1 state of a [3Fe-4S]$^0$ cluster. This signal sharpened up when the EPR spectrum was recorded with a parallel mode cavity confirming the presence of an integer spin system. (Fig. 10C). Anaerobic incubation of air-treated BCR in the presence of excess dithionite and Fe$^{2+}$ for 8 h at 21°C did not affect the EPR signals of the [3Fe-4S] clusters. Although [3Fe-4S] clusters could be identified in oxygen treated BCR, EPR signals in the $g=2$ region from intact [4Fe-4S] clusters were still observed after re-reduction of the enzyme (Fig. 10B). A 25 K spectrum of re-reduced BCR is presented in Fig.10E showing a signal very similar to that of cluster II, with $g$-values at 2.05, 1.93 and 1.93 (see difference spectrum in Fig. 5A) and a radical signal at $g=2.004$. The spin concentration of the former signal was three times higher than that of the [3Fe-4S]$^{+1}$ cluster of the air-oxidized sample shown in Fig.10D. Therefore, we can rule out that cluster II is degraded to a [3Fe-4S] form to a significant extent. It is more likely the [3Fe-4S] clusters are degradation products of cluster I or III. If the total loss of activity of the air-treated sample resulted from cluster degradation only, a higher [3Fe-4S] content would have been expected. Therefore we assume that either the [4Fe-4S] clusters are further degraded, to [2Fe-2S] clusters or to free Fe-atoms, or another unknown oxygen-sensitive group, such as a radical-containing moiety, is irreversibly inactivated.

5. Effect of acetylene

In order to explore alternative substrates for BCR we tested acetylene which is used in routine assays for testing nitrogenase activity. As reported, nitrogenase and BCR share some analogous features as both couple stoichiometric ATP-hydrolysis to the reduction of a chemically inert molecule [3,8].
In the presence of BCR and 10 mM MgATP, no electron flux from reduced methyl viologen to reduction of acetylene (0.8-4 mM) was observed. However, the benzoyl-CoA-independent ATPase activity of dithionite-reduced enzyme was increased by 50%. This increase was independent of acetylene concentration in a range from 0.8-4 mM (not shown). The rate of ATP-dependent benzoyl-CoA reduction was not affected by acetylene (0.8-4 mM). We also tested the effect of acetylene (4 mM) on the EPR spectrum of the dithionite reduced enzyme in the presence and absence of the MgATP (Fig.11). In the absence of MgATP acetylene did not induce major changes in the EPR spectrum of dithionite reduced BCR as shown in Fig. 11A and 11 B. However, in the presence of both acetylene and MgATP new EPR signals were induced (Fig. 11C). A difference EPR spectrum between acetylene-treated BCR in the presence and absence of MgATP is presented in Fig.11CΔB. This spectrum was dominated by an axial signal with all g-values higher than 2 and with features at g=2.14, 2.12, 2.08, 2.06 and 2.017. Additionally, a broad feature appeared in the g=1.92 region. These signals were not induced by MgATP in the absence of acetylene. Optimal EPR conditions for the novel signals were 20 mW and 30 K, where cluster I and II were slightly saturated. The spin concentration amounted to 0.15 mol spin/mol enzyme. Neither the S=1/2 EPR signals of cluster I and II, nor the rise of the S=7/2 high spin signals observed in the steady-state of benzoyl-CoA independent ATP-hydrolysis, were affected by addition of acetylene plus MgATP.
DISCUSSION

1. Nature of the metal centers in BCR

The enzymatic reduction of the aromatic ring requires electron transport at very low potentials. Therefore, the redox centers of BCR were expected to have a very special nature to catalyze such a demanding process at pH 7 and room temperature. The most prominent example of a similar ATP-driven redox reaction is nitrogenase, which contains unique redox centers (P-cluster, Moco-factor) [21]. Surprisingly EPR and Moessbauer spectroscopy revealed that BCR contains three ‘normal’ [4Fe-4S] centers as its sole metal redox modules. The molecular composition and the amino acid sequence is clearly different to nitrogenase, but is very similar to another enzyme system as discussed below.

The identification of the three [4Fe-4S] clusters in BCR was based on data from Moessbauer spectroscopy, EPR spectroscopy and metal analysis. Although the data obtained with both spectroscopic techniques were consistent with our major conclusions, some minor differences need to be discussed. In all Moessbauer samples the degree of overall reduction was 33 % which would be consistent with one reduced and two oxidized [4Fe-4S] clusters. However, the results obtained by EPR spectroscopy pointed to a different interpretation: two partially reduced isolated clusters were identified with different EPR spectroscopic properties, and EPR signals of magnetically interacting clusters were detected.

Since the Moessbauer spectroscopic properties were quite similar for all clusters, it was difficult to distinguish between the presence of one reduced cluster and two half-reduced clusters. Different conditions used for the both techniques could have influenced the spectroscopic properties of BCR. Because of the different requirements of each technique, the EPR samples were ten times less concentrated than the Moessbauer samples (200 mg/ml), in which the concentration of the [4Fe-4S] clusters reached 3 mM which may have resulted in a strong redox buffering effect that diminished the extent of their reduction. The degree of reduction was usually lower in the Moessbauer samples (33 %) than in the EPR samples (up to 50 %). At the high
concentrations used for Moessbauer spectroscopy the viscosity was increased which also could have influenced the protein conformation. The presence of both interacting and non-interacting clusters observed by EPR indicated the presence of a microheterogeneity in the BCR samples. The ‘interacting’ and ‘non-interacting’ forms of BCR are considered as two different conformational states; note that binding of ATP eliminates the interaction [8]. In our enzyme samples we usually found varying amounts of FAD (0.1-0.3 mol/mol enzyme) [4]. No correlation between FAD content and enzyme activity has been observed, indicating that it binds nonspecifically to the enzyme, most probably to the ATP binding sites; substoichiometric binding of FAD may result in the observed microheterogeneity. In addition minor variations of external factors, e.g., protein and salt concentration or viscosity, especially in combination with different freezing procedures (EPR samples were frozen in liquid nitrogen, Moessbauer samples on dry ice) could have resulted in microheterogeneity as has been reported for other proteins [22]. Interprotein magnetic interactions may also play a role at very high concentrations. Major enzyme concentration effects have been demonstrated, e.g., for glutamine phosphoribosylpyrophosphate amidotransferase of *E. coli* where the spin state of a [4Fe-4S] cluster switched from 3/2 to 5/2 when the concentration was increased to 2 mM [23].

The EPR signals observed under various conditions are summarized in Table 2. Clusters I and II exhibit usual EPR spectroscopic properties for a [4Fe-4S]^{2+/1} cluster, with the expected g-values, although cluster I shows a rather atypical slow spin relaxation. These clusters were reducible to some extent by both dithionite or deazaflavin/light in the absence of the substrate. However, the spectroscopic and electrochemical properties of cluster III were more unusual. First, its reduction was only observed under steady state conditions of substrate reduction, possibly due to a very negative reduction potential. Second, cluster III displayed an unusual narrow spectrum expanding over only approximately 18 mT (Fig. 6). In contrast to cluster III, the EPR spectra of clusters I and II covered a range of more than 30 mT each (Fig. 5). Third, and most remarkably, the spectrum displayed a split line-shape that was most likely due to the interaction with a second paramagnetic site. Since a radical mechanism is proposed for BCR [5,6] a free radical would be an attractive candidate as coupling partner of cluster III. Although radical signals have been
detected in different BCR preparations (see, e.g., spectrum A in Fig. 6) these signals could not be assigned to a protein or substrate radical, and artifacts cannot be ruled out. If a radical anion or a free radical were formed on the substrate, its lifetime is expected to be very short since the subsequent protonation is considered to be a faster process [24]. Therefore, pre-steady-state EPR and ENDOR spectroscopy experiments may allow the detection of such a radical using $^{13}$C-labeled substrates.

The oxygen-sensitivity of BCR fits with the observed irreversible degradation of [4Fe-4S] to [3Fe-4S] clusters upon exposure to air; such cluster conversions are well described and are reversible in some cases [25]. Two observations need to be reconciled: first the high sensitivity of BCR towards oxygen is consistent with the low reduction potentials of the [4Fe-4S] clusters ($E^\circ < -550$ mV for cluster I and II as estimated from redox titration experiments); secondly benzoyl-CoA reductase is able to accept electrons from reduced methyl viologen (MV) with $E^\circ = -448$ mV [26]. The reaction rate is linear up to an MV$_{ox}$:MV$_{red}$ ratio of at least 4:1, indicating that potentials of -400 mV are sufficiently low for an optimal electron transfer rate to the primary electron accepting site of the enzyme. Furthermore, at –400 mV the spin concentration of paramagnetic [4Fe-4S] clusters was less than 1 %. This may indicate that electron transfer to the enzyme is not the rate-limiting step. Cluster I or II are suitable candidates for the primary electron accepting site, whereas cluster III is probably involved in electron transfer from cluster I or II to the substrate.

Acetylene stimulated benzoyl-CoA-independent ATPase activity and induced novel axial EPR signal with all g-values higher than 2.00 (Fig. 11, Table 2). Such signals are typical for [4Fe-4S]$^{3+}$ clusters of several high-potential iron-sulfur (HiPIP) proteins [27]. It is rather unlikely that acetylene induced the oxidation of a [4Fe-4S]$^{1+/-2}$ cluster to the +3 state, although the presence of all three redox states (+3,+2,+1) has been demonstrated for the _Rhodophila globiformis_ HiPIP under non-denaturing conditions [27]. Although electron transfer from reduced methyl viologen to acetylene was not observed, we cannot rule out the possibility that the enzyme reduced acetylene to ethylene at concentrations similar to that of BCR. Both acetylene and ethylene are
potential ligands for metal complexes. Note, however, that addition of ethylene to BCR did not cause such effects (not shown) but rather partially inactivated the enzyme [3]. Surprisingly, EPR signals similar to acetylene plus MgATP-treated BCR have also been found in transient complexes between ethylene and the nitrogenase-Mo-Fe-protein from Klebsiella pneumoniae [28]. Although the metal clusters of the Mo-Fe protein of nitrogenase are structurally unrelated to the [4Fe-4S] clusters of BCR it is likely that acetylene (or the putative reduced product ethylene) forms complexes with a [4Fe-4S] cluster of BCR, altering its EPR spectroscopic properties. Since the EPR signals of clusters I and II are not affected by acetylene treatment, cluster III is the most probable candidate for such a complex formation.

2. Comparison with similar enzymes

Amino acid sequence comparisons of the four subunits of BCR from T. aromatica revealed that they show highest similarities the four putative subunits of benzoyl-CoA reductase from Rhodopseudomonas palustris [29,30] and to the 2-hydroxy-glutaryl-CoA dehydratase/activase systems from amino acid fermenting bacteria [29,31-33]. The biochemistry of the former enzyme has not been studied yet, whereas 2-hydroxyglutaryl-CoA dehydratases from several bacteria have been purified and characterized [31,32]. These enzymes catalyze the mechanistically difficult elimination of water from the α-position of (R)-2-hydroxyglutaryl-CoA affording (E)-glutaconyl-CoA. A similar catalytic mechanism, involving a ketyl radical, has been proposed for both BCR and 2-hydroxyglutaryl-CoA dehydratase [5,34]. In order to initiate enzymatic dehydration these enzymes require a second protein component, termed activase [35]. Activase catalyzes the ATP hydrolysis-dependent activation of electrons accepted from an external source, such as the artificial electron donor Ti(III) citrate.

The 2-hydroxyglutaryl-CoA dehydratases from Acidaminococcus fermentans and Clostridium symbiosum have been studied in detail [33,36]. Both comprise two different subunits (Hgd A and Hgd B) with 38-41% amino acid similarities to the β- and γ-subunits of BCR (Bcr B and Bcr C) [29]. They contain two (C. symbiosus) or one (A. fermentans) [4Fe-4S] clusters and various amounts of FMN and/or riboflavin. The biochemistry of the corresponding activase from A.
*fermentans* has been studied. It is a homodimeric protein (Hgd C) containing a single [4Fe-4S] cluster [35]. The amino acid sequences of the α- and δ-subunit of BCR (Bcr A and Bcr D) show high similarities to each other (45% if the extra N-terminal loop of BCR A is disregarded) and to Hgd C (52%) [29]. Bcr A, Bcr D and Hgd C all contain identical phosphate and adenosine-binding motifs similar to many sugar kinases [29,35]. Note that enzymatic ring reduction requires stoichiometrical ATP-dependent electron activation whereas ATP hydrolysis is only required in catalytic amounts in the dehydration process: once an activated electron is transferred from activase to dehydratase it will stay in the activated form for several catalytic cycles [35]. BCR can be considered as a fusion product of ATP hydrolyzing/electron activating (Bcr A and D) and ketyl radical forming (Bcr B and C) moieties which are located on two different proteins in the dehydratase/activase enzyme system. The total amount of three [4Fe-4S] clusters is the same in BcrBCAD from *T. aromatica* and in the 2-OH-glutaryl-CoA dehydratase/activase system, HgdABC2 from *C. symbiosum* [33].

Due to the similarities of the amino acid sequences of the BCR and the 2-hydroxyglutaryl-CoA dehydratase/activase subunits, similar spectroscopic and electrochemical properties of the [4Fe-4S] clusters were expected. Indeed, neither of the one or two [4Fe-4S] clusters of the 2-hydroxyglutaryl-CoA dehydratases from *A. fermentans* or *C. symbiosum* was reducible with commonly used reducing agents [33,37]. The single cluster of activase, however, was reducible to a paramagnetic S=3/2 high spin state [37]. This incomplete reduction of the whole dehydratase/activase systems resembles the situation with BCR where only 33-50% of the three [4Fe-4S] clusters were reducible.

Only two cysteines are conserved in Hgd C, Bcr A and Bcr D; these are separated by a 38 amino acid spacer. Therefore, one of the [4Fe-4S] clusters of BCR is probably ligated by two cysteines from Bcr A and Bcr D, respectively, as has been suggested for the homodimeric activase (HgdC2) [33]. This would be similar to the [4Fe-4S] cluster ligation of nitrogenase Fe-protein which is buried at the interface of two identical subunits [21]. The S=3/2 high spin state of the [4Fe-4S] cluster in both nitrogenase Fe-protein and activase, HgdC, support this assumption, although no
amino acid similarities between the two proteins have been found. No S=3/2 EPR signals were detected in BCR samples, which may be a result of the differences between the composition of the one-protein component BCR and the two-protein component systems of nitrogenase and dehydratase/activase. However one important common feature is shared by all three enzymes: all contain a moiety with low potential redox modules of which the electron transferring operation is strictly coupled to ATP-hydrolysis. Since in BCR, reduction of cluster III is only observed in the presence of MgATP and benzoyl-CoA, it is likely that ATP hydrolysis is required for electron transport from cluster I or II to cluster III.

3. Effect of adenosine nucleotides

The ability to study electron transfer and ATPase activity separately is important because it allows us to study the effect of ATP hydrolysis alone. Two spectroscopic effects have been reported, the rise of S=7/2 EPR signals and the elimination of the magnetic interaction of two clusters [7]. The Moessbauer spectroscopy described in this work has confirmed the presence of the S=7/2 species assigned it to a [4Fe-4S] cluster which changed from the S=1/2 to the S=7/2 state. This latter state has only been observed previously for biological iron-chalconide clusters where a bridging sulfur of a [4Fe-4S] has artificially been replaced by selenium [38,39] and for P-clusters in the thionine oxidized MoFe protein of nitrogenase [40]. In BCR the switch from the S=1/2 to the S=7/2 state parallels enzymatic activity and is probably the result of structural changes in the vicinity of a [4Fe-4S] cluster induced by ATP hydrolysis. In a [4Fe-4S]$^{+1}$ cluster, all individual Fe-atoms are in the high-spin state and are coupled antiferromagnetically to give an overall S=1/2 spin due to double- and super-exchange. Structural changes in the protein are likely to induce changes in the double- and super-exchange interactions, and in vibronic coupling, which could generate a S=7/2 ground state in [4Fe-4S] clusters [41]. Clearly such changes could have large effects on the redox properties of a [4Fe-4S] cluster, although it is not possible to define a simple relationship between spin state and reduction potential.

It is possible to distinguish between the effects of binding and hydrolysis of MgATP using the non-hydrolyzable ATP analogue AdoPP[NH]P. Such experiments have shown that binding of the
nucleotide eliminates the interaction of two [4Fe-4S] clusters, whereas hydrolysis is responsible for the switch from the $S=1/2$ to the $S=7/2$ state. We propose that the competitive inhibitor ADP accumulates as a result of ATPase activity and binds to the enzyme which therefore remains in the ‘non-interacting’ state. This would indicate that the hydrolysis of ATP, but not its binding, is responsible for the re-coupling of non-interacting [4Fe-4S] clusters.

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REFERENCES


37. Buckel, W., University of Marburg, Germany; personal communication.


LEGENDS TO FIGURES

Fig. 1. Reaction catalyzed by benzoyl-CoA reductase.

Fig. 2. Mössbauer spectra of $^{57}$Fe-enriched benzoyl-CoA reductase oxidized by thionine. A. Spectrum taken at 4.2 K in an applied field of 20 mT $\parallel \gamma$. B. spectrum taken at 4.2 K in an applied field of 7 T $\parallel \gamma$. The solid lines are the sum of subspectra 1,2,3 (see Table 1).

Fig. 3. Mössbauer spectra of $^{57}$Fe-enriched benzoyl-CoA reductase photoreduced by deazaflavin. A. Spectrum taken at 120 K in an applied field of 20 mT $\perp \gamma$. B. spectrum taken at 4.2 K in an applied field of 20 mT $\perp \gamma$. C. spectrum taken at 4.2 K in an applied field of 7 T $\parallel \gamma$. The solid lines are the sum of subspectra 1,2,3,4 (see Table 1).

Fig. 4. EPR spectra of deazaflavin/light reduced benzoyl-CoA reductase. (----) unlabeled enzyme, (-----) $^{57}$Fe-labeled enzyme. EPR parameters: microwave frequency: 9.414 GHz, modulation amplitude, 1.17 mT, microwave power 2 mW; temperature, 25 K.

Fig. 5. Temperature-dependent EPR spectra of deazaflavin/light-reduced benzoyl-CoA reductase. The enzyme (120 µM) was reduced anaerobically with 20 µM deazaflavin in 100 mM Tris/HCl, pH 7.5 for 10 min in white light. A. Representative spectra recorded from 60 K to 15 K. EPR parameters: microwave frequency, 9.415 GHz; modulation amplitude, 1.17 mT; microwave power, 2 mW for 60 K to 25 K spectra and 0.2 mW for 20 K and 15 K spectra. Note that the dominant features of the EPR spectrum recorded at 60 K are assigned to cluster I in this work. 20 K-60 K is the difference spectrum of the 20 K spectrum minus the 60 K spectrum and is assigned to cluster II. The spectra were normalized for temperature and microwave power. The numbers in the figure refer to g-values. B. Total S=1/2 spin concentration of the spectra recorded at 15 K to 77 K. All spin concentrations were corrected for temperature-dependence according to Curie’s Law.
Fig. 6. EPR spectra of dithionite-reduced benzoyl-CoA reductase in the presence of MgATP (20 mM) and benzoyl-CoA (3 mM). A. Spectrum recorded at 77 K, B. Spectrum recorded at 40 K, C. 40 K spectrum of dithionite-reduced enzyme in the absence of MgATP and benzoyl-CoA. B+C. Difference spectrum B minus 0.2 times spectrum C. EPR parameters: microwave frequency, 9.415 GHz; microwave power, 2 mW; modulation amplitude: 1.27 mT.

Fig. 7. EPR spectra of benzoyl-CoA reductase at different redox potentials. The potentials were poised by adding ferricyanide to a dithionite-reduced benzoyl-CoA reductase preparation in the presence of a redox mediator mixture. A. Spectra recorded at 4 K, B. Spectra recorded at 25 K. All spectra were recorded under non-saturating conditions. The numbers in B are g-values. EPR-parameters: microwave frequency, 9.415-9.416 GHz; microwave power: 0.2 mW in A. and 2 mW in B; modulation amplitude 1.17 mT.

Fig. 8. Mössbauer spectra of $^{57}$Fe-enriched BCR reduced by dithionite in the presence of MgATP. a: spectrum taken at 120 K in an applied field of 20 mT $\perp \gamma$. b: spectrum taken at 4.2 K in an applied field of 20 mT $\perp \gamma$. c: spectrum taken at 4.2 K in an applied field of 7T $\parallel \gamma$. The solid lines are the sum of subspectra 1,2,3,4,5,6 (see Table 1).

Fig. 9. EPR spectra of dithionite-reduced benzoyl-CoA reductase (180 µM) in the presence of MgATP and MgAdoPP[NH]P. 4 K EPR spectra of A. reduced enzyme as isolated, B. reduced enzyme after 1 min incubation with 20 mM MgATP, C. reduced enzyme after 1 min incubation with 0.5 mM MgAdoPP[NH]P. Arrow 1 points to the ATP-induced isotropic EPR-signal at g=5.1; arrow 2 to the broad shoulder in the spectrum of dithionite reduced enzyme in the absence of ATP or AdoPP[NH]P. EPR parameters: microwave frequency, 9.414 GHz; microwave power 0.2 mW; modulation amplitude, 1.17 mT.

Fig. 10. EPR spectra of air-inactivated benzoyl-CoA reductase (250 µM). A. Full scan spectrum recorded at 4 K after 5 min incubation of BCR at air, B. Re-reduction of the enzyme in A. with excess of dithionite (10 mM), C. EPR spectrum of B. using a parallel mode cavity. D.
g=2 region of spectrum A. E. g=2 region of spectrum B. EPR parameters: microwave frequency, 9.409 GHz for spectrum A,B,D,E and 9.250 GHz for spectrum C.; microwave power and temperature, 2 mW and 4 K in A,C, and D, 0.2 mW and 4 K in B, 2 mW and 26 K in E; modulation amplitude, 1.17 mT.

Fig. 11. Effect of acetylene on the EPR spectra of dithionite-reduced benzoyl-CoA reductase. Dithionite reduced benzoyl-CoA reductase was anaerobically incubated with acetylene (~4 mM) in the presence and absence of MgATP (20 mM) for 10 min at 20°C. Note that the EPR conditions are optimized for the acetylene-induced signal, under these conditions the usual S=1/2 EPR signals of the [4Fe-4S] clusters are saturated. A. Dithionite reduced enzyme as isolated. B. Reduced enzyme in the presence of acetylene. C. Reduced enzyme in the presence of acetylene and MgATP. EPR parameters: microwave frequency, 9.354 GHz; microwave power, 20 mW; modulation amplitude, 1.17 mT; temperature, 30 K.
Table 1: Parameters for the simulations of the samples A, B, C of Fig. 2,3 and 8 at 4.2K in external fields of 20 mT $\perp \gamma$ and 7 T $\parallel \gamma$. $\eta$ is the asymmetry parameter, $\Gamma$ is the full linewidth at half maximum, $\hat{A}/gN\mu_N$ is the effective magnetic hyperfine coupling tensor.

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<td>0.98</td>
<td>0.98</td>
</tr>
<tr>
<td>$\eta$</td>
<td>0.7</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>$\Gamma$ [mms$^{-1}$]</td>
<td>0.27</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>$S_{tot}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$\hat{A}/gN\mu_N$ [T]</td>
<td>0</td>
<td>1/2</td>
<td>1/2</td>
</tr>
<tr>
<td>Rel. area [%]</td>
<td>33.3</td>
<td>33.3</td>
<td>33.3</td>
</tr>
</tbody>
</table>

|            | 2           | 2           | 2                   |
| $\delta$ [mms$^{-1}$] | 0.45        | 0.45        | 0.45                |
| $\Delta E_Q$ [mms$^{-1}$] | 1.32        | 1.29        | 1.29                |
| $\eta$    | 0.7         | 0.7         | 0.7                 |
| $\Gamma$ [mms$^{-1}$] | 0.27        | 0.30        | 0.30                |
| $S_{tot}$ | 0           | 1/2         | 1/2                 |
| $\hat{A}/gN\mu_N$ [T] | 0           | (-24; -24; -20) | (+21; +5; +1) |
| Rel. area [%] | 33.3        | 33.3        | 33.3                |

|            | 3           | 3           | 3                   |
| $\delta$ [mms$^{-1}$] | 0.45        | 0.50        | 0.39                |
| $\Delta E_Q$ [mms$^{-1}$] | 1.14        | 1.30        | 1.17                |
| $\eta$    | 0.4         | 0.8         | 0.2                 |
| $\Gamma$ [mms$^{-1}$] | 0.27        | 0.30        | 0.3                 |
| $S_{tot}$ | 0           | 1/2         | 7/2                 |
| $\hat{A}/gN\mu_N$ [T] | 0           | (-24; -24; -20) | (+7.7; +8.5; -16.3) |
| Rel. area [%] | 33.3        | 12.5        | 16.6                |

|            | 4           | 4           | 5                   |
| $\delta$ [mms$^{-1}$] | 0.45        | 0.58        | 0.58                |
| $\Delta E_Q$ [mms$^{-1}$] | 1.29        | 1.60        | 1.17                |
| $\eta$    | 0.7         | 0.4         | 0.2                 |
| $\Gamma$ [mms$^{-1}$] | 0.30        | 0.30        | 0.3                 |
| $S_{tot}$ | 0           | 1/2         | 7/2                 |
| $\hat{A}/gN\mu_N$ [T] | 0           | (+21; +5; +1) | (+7.7; +8.5; -16.3) |
| Rel. area [%] | 33.3        | 12.5        | 16.6                |

|            | 5           | 6           |                    |
| $\delta$ [mms$^{-1}$] | 0.39        | 0.58        |                    |
| $\Delta E_Q$ [mms$^{-1}$] | 1.17        | 1.55        |                    |
| $\eta$    | 0.2         | 0.3         |                    |
| $\Gamma$ [mms$^{-1}$] | 0.3         | 0.3         |                    |
| $S_{tot}$ | 0           | 7/2         |                    |
| $\hat{A}/gN\mu_N$ [T] | 0           | (-2.4; -8.8; -16.3) |                    |
| Rel. area [%] | 33.3        | 12.5        | 16.6                |
### Tab. 2: EPR-signals of benzoyl-CoA reductase.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>g-values</th>
<th>Condition</th>
<th>Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>[4Fe-4S]$^{+1}$ cluster, S=1/2</td>
<td>2.017, 1.938, 1.908</td>
<td>Dithionite-reduced</td>
<td>Rhombic, observable up to 60 K</td>
</tr>
<tr>
<td>[4Fe-4S]$^{+1}$ cluster, S=1/2</td>
<td>2.053, 1.93, 1.93</td>
<td>Dithionite-reduced</td>
<td>Axial, observable up to 40 K</td>
</tr>
<tr>
<td>[4Fe-4S]$^{+1}$ cluster, S=1/2</td>
<td>~2.03,~1.995,~1.965</td>
<td>Dithionite reduced, steady state of benzoyl-CoA reduction*</td>
<td>Rhombic, observable up to 60 K</td>
</tr>
<tr>
<td>2 interacting [4Fe-4S]$^{+1}$ cluster, S=1/2</td>
<td>Around 2</td>
<td>Dithionite reduced</td>
<td>Very broad, observable up to 10 K</td>
</tr>
<tr>
<td>[4Fe-4S]$^{+1}$ cluster, S=7/2</td>
<td>5.15 (excited state)</td>
<td>Dithionite reduced</td>
<td>Isotropic (g=5.15), optimal T = 30 K</td>
</tr>
<tr>
<td></td>
<td>11.5 (ground state)</td>
<td>Steady state of ATP hydrolysis**</td>
<td>Poor ‘absorption’ signal (g=12)</td>
</tr>
<tr>
<td>[3Fe-4S] cluster, S=1/2</td>
<td>2.013,~2.01,~2.01</td>
<td>Oxygen oxidized</td>
<td>Axial signal, observable below 20 K</td>
</tr>
<tr>
<td>[3Fe-4S] cluster, S=1</td>
<td>12</td>
<td>Oxygen oxidized, re-reduced with dithionite</td>
<td>Broad signal, observable below 10 K, sharpens up in parallel mode EPR</td>
</tr>
<tr>
<td>?</td>
<td>2.14,2.12,2.08,2.00</td>
<td>Dithionite reduced, in the presence of acetylene and MgATP</td>
<td>Rhombic with $g_{av} &gt; 2.0$, optimal at 30 K</td>
</tr>
</tbody>
</table>

*In the presence of MgATP and benzoyl-CoA

**In the presence of MgATP and the absence of benzoyl-CoA
COSCoA + ATP + H₂O → ADP + Pi + COSCoA

2 Fd(red) → 2 Fd(ox)
A

Intensity

2.017
1.938
1.908
2.053
1.93
20 K - 60 K

g-value

2.14 2.1 2.05 2 1.95 1.9 1.85 1.8 1.75

B

Spin Concentration (mol/mol protein)

0.9
0.8
0.7
0.6
0.5
0.4
0.3

Temperature (K)

20 30 40 50 60 70 80