Redox Centers of 4-Hydroxybenzoyl-CoA Reductase, a Member of the Xanthine Oxidase Family of Molybdenum-containing Enzymes*

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4-Hydroxybenzoyl-CoA reductase (4-HBCR) is a key enzyme in the anaerobic metabolism of phenolic compounds. It catalyzes the reductive removal of the hydroxyl group from the aromatic ring yielding benzoyl-CoA and water. The subunit architecture, amino acid sequence, and the cofactor/metal content indicate that it belongs to the xanthine oxidase (XO) family of molybdenum cofactor-containing enzymes. 4-HBCR is an unusual XO family member as it catalyzes the irreversible reduction of a CoA-thioester substrate. A radical mechanism has been proposed for the enzymatic removal of phenolic hydroxyl groups. In this work we studied the spectroscopic and electrochemical properties of 4-HBCR by EPR and Mössbauer spectroscopy and identified the pterin cofactor as molybdopterin mononucleotide. In addition to two different [2Fe-2S] clusters, one FAD and one molybdenum species per monomer, we also identified a [4Fe-4S] cluster/monomer, which is unique among members of the XO family. The reduced [4Fe-4S] cluster interacted magnetically with the Mo(V) species, suggesting that the centers are in close proximity, (<15 Å apart). Additionally, reduction of the [4Fe-4S] cluster resulted in a loss of the EPR signals of the [2Fe-2S] clusters probably because of magnetic interactions between the Fe-S clusters as evidenced in power saturation studies. The Mo(V) EPR signals of 4-HBCR were typical for XO family members. Under steady-state conditions of substrate reduction, in the presence of excess dithionite, the [4Fe-4S] clusters were in the fully oxidized state while the [2Fe-2S] clusters remained reduced. The redox potentials of the redox cofactors were determined to be: [2Fe-2S]1+/2 = -205 mV, [2Fe-2S]3+/2 I, -255 mV; [2Fe-2S]3+/2 II, -255 mV; FAD/FADH: FADH2, -230 mV/470 mV; [4Fe-4S]1+/2 = -465 mV and Mo(VI)/(V)/(VI), -380 mV/500 mV. A catalytic cycle is proposed that takes into account the common properties of molybdenum cofactor enzymes and the special one-electron chemistry of dehydroxylation of phenolic compounds.

Aromatic compounds comprise a large group of natural products many of which contain hydroxyl or methoxyl functionalities. Methoxyl groups are usually converted to hydroxyl groups and C1 units before further metabolism takes place. In recent years a growing number of anaerobic bacteria have been identified that use low molecular mass aromatic compounds as their sole sources of cell carbon and energy. In the presence of molecular oxygen, hydroxylation is catalyzed by mono- or dioxygenases, and oxidative aromatic ring cleavage is catalyzed by dioxygenases. However, in the absence of the highly reactive co-substrate oxygen, alternative strategies have developed in anaerobic bacteria (1, 2). Many compounds are converted to the central intermediate benzoyl-CoA, which becomes deaeromized by benzoyl-CoA reductase (BCR)1 (1). This enzyme couples the difficult ring reduction to a stoichiometric ATP hydrolysis (3). It is assumed that BCR also catalyzes the direct ring reduction of ortho- or meta-substituted hydroxy-, amino- or methyl-derivatives of benzoyl-CoA.2 Examples are 3-hydroxy- or 3-methyl-benzoyl-CoA (5). However, the aromatic ring of para-substituted derivatives, for example 4-hydroxybenzoyl-CoA (4-HBCoA) cannot be reduced by BCR or similar enzymes for mechanistic reasons.2 Therefore, an additional group of enzymes should exist that reductively removes hydroxy or amino groups from the ring prior to deaeromatization. The only enzyme of this group of which the biochemistry has been studied so far is 4-HBCR from the denitrifying bacterium Thauera aromatica (6). This enzyme plays a key role in the anaerobic metabolism of phenolic compounds (1). 4-HBCR catalyzes the reductive dehydroxylation of 4-HBCoA to benzoyl-CoA as two electrons are transferred to the substrate (6) (Fig. 1). The natural electron donor is a reduced 2[4Fe-4S] ferredoxin (7), which also serves as the in vivo electron donor for the next enzyme in aromatic metabolism, benzoyl-CoA reductase (8). 4-HBCR has a molecular mass of 260 kDa and consists of three subunits of 75 (α), 35 (β), and 17 kDa (γ), suggesting an (αβγ)3 composition (6). Purified 4-HBCR contained 15 mol of iron, 12.5 acid-labile sulfur, 1.3 mol of FAD and 1.9 mol of molybdenum per mol of dimeric enzyme (7). The enzyme was inactivated by cyanide giving initial evidence that it may belong to the xanthine oxidase (XO) family of molybdenum cofactor-containing enzymes (6).

The genes coding for the three subunits of 4-HBCR have been cloned and sequenced in T. aromatica (7) and in the

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1 The abbreviations used are: BCR, benzoyl-CoA reductase; 4-HBCR, 4-OH-benzoyl-CoA reductase; XO, xanthine oxidase; 4-HBCoA, 4-hydroxybenzoyl-CoA; CODH, carbon monoxide dehydrogenase; XDH, xanthine dehydrogenase; Mops, 4-morpholinopropanesulfonic acid; mW, milliwatt.

2 H. Möbitz and M. Boll, submitted for publication.
Rhodopseudomonas palustris (9). They are highly conserved between these two organisms and also show high similarity to several enzymes of the XO family. This was highest with carbon monoxide dehydrogenases (CODH) from Oligotropha carboxydovorans and Pseudomonas thermo-carboxydovorans, with nicotine dehydrogenase from Arthrobacter nicotinovorans, and with eukaryotic xanthine dehydrogenases (XDH) (7).

The XO family of molybdenum-cofactor-containing enzymes comprises a large group of enzymes that usually catalyze hydroxy- or oxo-transfer to the substrate. They usually contain a molybdopterin-cofactor and [2Fe-2S] clusters; many also harbor hydroxy- or oxo-transfer to the substrate. They usually contain a throbacter nicotinovorans, and with eukaryotic xanthine dehydrogenases and 4-HBCR was highest with carbon monoxide dehydrogenases (CODH) are highly conserved between these two organisms and also molybdenum, plus an additional [4Fe-4S] cluster that is unique to 4-HBCR contains the expected [2Fe-2S] clusters, FAD and

The Mo(V) EPR signals are typical of the XO family enzymes. Based on these results, a catalytic mechanism is proposed.

**EXPERIMENTAL PROCEDURES**

**Growth of Bacterial Cells**—T. aromatica (DSM 6984) was isolated in our Freiburg laboratory and has been deposited in the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany) (18). It was grown anoxically at 28 °C in a mineral salt medium in a 200-liter fermenter with 4-OH-benzoate and nitrate in a molar ratio of 1.35 as sole sources of energy and cell carbon. Continuous feeding of the substrates, cell harvesting, and storage of cell extracts were carried out as described previously (10). 57Fe-enrichment of 4-HBCR was achieved by adding 57Fe as sole source of iron to the medium. For this purpose 175 mg of metallic 57Fe (95.7% enriched, Advanced Materials and Technology Consulting, New York, NY) was dissolved in 2.2 ml of 12 M HCl overnight at 80 °C. After the metal was completely dissolved, the solution was added drop-wise to 100 ml of 0.6 M nitritriacetic acid, pH 5.2, under continuous stirring. During this procedure the pH was held constant between 3 and 5 by adding 2 M NaOH. With this amount of 57Fe T. aromatica was grown in a 100-liter batch culture with continuous feeding of 4-OH-benzoate and nitrate to 4-HBCR was estimated to >90% enriched in 57Fe (20).

**Protein Purification, Enzyme Activity Assay, Purity Control, and Sample Storage**—Purification of 4-HBCR from extracts of T. aromatica was performed under strictly anaerobic conditions in a glove box under a N2/H2 atmosphere (95.5, by vol.) as described earlier (6). The procedure included three chromatographic steps using anion exchange chromatography on DEAEP-Sepharose (Amersham Biosciences, Inc.), chromatography on Hi Load Q-Sepharose (Amersham Biosciences, Inc.), and affinity chromatography on Cibacron Blue-Agarose (Sigma). Concentration of the protein samples was achieved by centrifugation (8000 × g) in Microsep Microconcentrators (exclusion limit 50 kDa). The concentration of the enzyme was ~150 mg ml–1 for Mössbauer studies or 15–30 mg ml–1 for EPR spectroscopy. The purity of these enzyme preparations was >90% as estimated by Coomassie staining of SDS gels. Enzyme activity was determined in a continuous spectrophotometric assay recording the 4-HBCoA-dependent oxidation of reduced methyl viologen at 730 nm at 37 °C (εmax = 2.4 mm−1 cm−1), (6). 25–35 mg of purified 4-HBCR was obtained from 200 g of cells (wet mass) with specific activities of 8–12 pmol 4-HBCoA reduced min–1 mg–1. Concentrated protein samples were stored anaerobically in tubes sealed with gas-tight stoppers at ~80 °C for several months without loss of activity.

**Sample Preparation for EPR and Mössbauer Spectroscopy**—All 4-HBCR samples for EPR and Mössbauer spectroscopy were prepared in an anaerobic glove box under a 100% nitrogen atmosphere (~1.0 ppm O2). Prior to sample preparation, excess 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 (Bio-Rad) desalting column (volume: 5 ml, diameter: 0.7 cm) equilibrated with either 100 mM Mops/KOH, pH 7.5, or 100 mM Hepes/HCl, pH 8.0, both containing 100 mM NaCl. Unless stated otherwise, this dithionite free enzyme was the starting material for all sample preparations.

**Reduction and Oxidation of the Enzyme**—Reduction of 4-HBCR was performed by adding sodium dithionite from a freshly prepared stock solution (100 mM in 100 mM Mops/KOH, pH 7.5) giving a final 10-fold excess of this reductant compared with the enzyme. The enzyme was quickly transferred into EPR tubes or Mössbauer sample holders and then frozen either inside the glove box on dry ice (Mössbauer-samples) or outside the glove box in a gas-tight sealed EPR tube in liquid nitrogen. Anaerobic oxidation of 4-HBCR was achieved by titrating the enzyme with an anaerobically prepared filtered thionine stock solution (~10 ml in the buffer described above). 4-HBCR was considered fully oxidized when the color of the enzyme solution remained blue for at least 5 min.

**Redox Titration of 4-HBCR**—Dye-mediated redox titration of 4-HBCR was performed in an anaerobic glove box under a nitrogen atmosphere (~1 ppm O2). The enzyme/mediator mixture (2.5 ml) was in 100 mM Mops/KOH, pH 7. The concentration of 4-HBCR was 80–100 μM. The mediator consisted of methyl and benzyl viologen, neutral red, safranin O, phenosafranin, anthraquinone-2-sulfonate, 2-hydroxy-4-naphthoquinone, indigo disulfonate, resorufin, methylene blue, phenazine methosulfate and N′N′N′-tetramethyl-p-phenylenediamine at a final concentration of 30 μM each. The redox potential was adjusted anaerobically with freshly prepared 10–100 mM sodium dithionite and potassium ferricyanide solutions in the same buffer as the 4-HBCR. Potentials are reported with reference to standard hydrogen electrode and were obtained by using a potential for the saturated calomel elec-

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**FIG. 1. Reaction catalyzed by 4-OH-benzoyl-CoA reductase.**

Two electrons are transferred from reduced ferredoxin to the substrate.
trode at 22 °C of +243 mV versus standard hydrogen electrode. Mediator/enzyme mixtures with a stable potential could be obtained from −573 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 in anaerobic 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 1.0-mmt copper sulfate sample in 11 mM sodium EDTA.

Mössbauer Spectroscopy—57Fe Mössbauer spectra were recorded with a conventional constant acceleration spectrometer using a 57Co source in a Rh matrix (1 GBq using the bath cryostat; 1.3 GBq using the magnet cryostat). Measurements at 80 and 77 K were performed with the magnet cryostat (Oxford Instruments) and a permanent magnet mounted outside the cryostat producing a field of 20 mT. High-field measurements were performed with a cryostat equipped with a superconducting magnet (Oxford Instruments). The spectra were analyzed assuming Lorentzian line shape. Isomer shifts are quoted relative to α-Fe at room temperature.

Molybdenum Cofactor Analysis—Molybdenum cofactor analysis was carried out based on the method of Johnson et al. (21). This procedure converts the molybdenum cofactor to its oxidized fluorescent degradation product (form A). The starting solution was 80 μg of purified 4-HBCR in 4 ml of 100 mM Tris/HCl, pH 7.2. The sample was incubated with the oxidizing solution (1% I2, 2% KI in 1 M HCl) at 25 °C overnight under shaking. The sample was subsequently treated with 20 mM pyrophosphatase and left at room temperature for 2 h. The sample was then clarified by centrifugation and applied to a QAE-Sephadex (Amer sham Biosciences, Inc. FPLC column (volume: 35 ml) that had been equilibrated with 1 M ammonium acetate. Once the extract was loaded onto the column, it was washed with four column volumes of water. To elute the dephospho-form A, 10 mM acetic acid was added to the column, and fractions collected. To elute any form A-nucleotide variant, 50 mM HCl was applied to the column. The pooled fractions were neutralized with NaOH and then concentrated by freeze-drying overnight.

The samples were taken up in 2 ml of water and separated by reverse phase high-performance liquid chromatography with 3% methanol/50 mM ammonium acetate as the mobile phase. Peaks were collected and a 500-μl aliquot was added to 20 ml of 20 mM Tris/HCl, pH 7.6, MgCl2 to a final concentration of 2 mM and 1 unit of pyrophosphatase. The sample was left at room temperature for 1 h, and the fluorescence before and after pyrophosphatase treatment was analyzed as described (21). As standards, oxidized 4-OH-benzoyl-CoA was synthesized via the N-hydroxysuccinimide ester of the carboxylic acid according to Gross and Zenk (22). Protein concentrations were determined by the Bradford method using bovine serum albumin as standard (23). SDS-PAGE was performed as described by Laemmli (24). Protein was visualized by Coomasie Blue staining (25).

RESULTS
Nature of the Iron-Sulfur Clusters

The metal analysis of 4-HBCR revealed the presence of 15–16 mol of iron/mol of dimeric enzyme (α2β2γ2) indicating that the iron content was double of that of typical members of the XO family that have 8 mol of iron/mol of dimeric enzyme. Amino acid sequence analysis suggested the presence of an additional [4Fe-4S] cluster in the β-subunit of 4-HBCR in addition to the two [2Fe-2S] clusters that are usually found in the small γ-subunit of XO-type enzymes. To elucidate the nature of the iron-sulfur clusters Mössbauer and EPR spectroscopic studies were performed.

Mössbauer Spectroscopy of Thionine-oxidized 4-HBCR—For Mössbauer spectroscopy studies, BCR was purified from extracts of T. aromatica grown anaerobically with 57Fe as the sole source of iron. High 57Fe enrichment was achieved (>90%) as estimated from the resonance absorption effect of each subspectrum of the oxidized sample. In Fig. 2, the Mössbauer spectra of the thionine-oxidized 57Fe-labeled 4-HBCR taken at 4.2 K in external fields of 20 mT γ (A) and 7 T γ (B) are shown. Spectrum A consists of two doublets with an area ratio of 1:1. One of the doublets exhibits parameters typical of delocalized mixed-valence high-spin iron sites (Fe2.5+) with tetrahedral sulfur-coordination (δ = 0.44 mm s−1; ΔEq = 1.12 mm s−1; Γ = 0.33 mm s−1; dotted line in Fig. 2A) as in [4Fe-4S]1+ and in [3Fe-4S]0 clusters (26). The second doublet is characteristic of high-spin Fe3+ sites tetrahedrally coordinated with sulfur ligands (δ = 0.29 mm s−1; ΔEq = 0.61 mm s−1; Γ = 0.27 mm s−1; dashed line in Fig. 2A). Such Fe3+ sites are present in [2Fe-2S]1+ clusters and [3Fe-4S]0 clusters (26).

Applying a high external field (Fig. 2B) demonstrates the presence of two diamagnetic components with area ratio 1:1. This observation rules out the existence of paramagnetic [2Fe-2S]1+ and [3Fe-4S]0 clusters. Therefore, thionine-oxidized HBCR contains [4Fe-4S]1+ clusters and [2Fe-2S]1+ clusters in a ratio of 1:2. Together with the measured Fe content of 15–16 iron per protein molecule our results indicate that a dimer of 4-HBCR contains two [4Fe-4S]1+ clusters and four [2Fe-2S] clusters. This result agrees completely with the predictions made by amino acid sequence analysis (7).

Mössbauer Spectroscopy of Dithionite-reduced 4-HBCR—Fig. 3 shows the Mössbauer spectra of dithionite-reduced 4-HBCR taken at 120 K (A) and at 4.2 K in external fields of 20 mT γ (B) and 7 T γ (C). The central region of spectrum A (measured at 120 K) shows a strongly asymmetric doublet, the right part of which (in the interval 0.5–1.0 mm s−1) is not resolved as it is in Fig. 2A. This behavior indicates the superposition of several subspectra. Since spectrum B, measured at 4.2 K in an applied magnetic field of 20 mT, contains diamagnetic as well as paramagnetic components, we have included subspectra characteristic of diamagnetic [4Fe-4S]2+ and [2Fe-2S]2+ clusters, as well as subspectra characteristic of paramagnetic [4Fe-4S]1+ and

FIG. 2. Mössbauer spectra of 57Fe enriched 4-0H-benzoyl-CoA reductase (650 μg) oxidized by thionine. A, spectrum taken at 4.2 K in an applied field of 20 mT γ. B, spectrum taken at 4.2 K in an applied field of 7 T γ. The solid lines are the sum of two subspectra.
centers, samples of 4-HBCR were taken at potentials between −100 mV and −570 mV at −30 mV steps. In these studies, we attempted to assign EPR signals to individual clusters and to elucidate their redox properties.

At potentials below −190 mV EPR signals appeared between 330 and 350 mT, which were almost fully developed at −350 mV. EPR signals of at least two different paramagnetic species were observed with characteristic features at $g_z = 2.04$ and $g_x = 2.02$ (using the convention $g_x < g_y < g_z$). Representative EPR spectra recorded at −190 mV and −284 mV are presented in Fig. 4, A and C. The −190 mV spectrum was greatly obscured by the radical EPR signal of the redox dyes. After subtraction of this signal, a rhombic $S = \frac{1}{2}$ EPR signal with $g$ values at 2.04, 1.995, and 1.964 ($g_{av} = 1.999$) was obtained which we assign to an Fe-S cluster (Fig. 4B). This spectrum also included additional minor sharp features between $g = 1.96$ and 1.98, which can probably be assigned to a resting Mo(V) signal (for a detailed characterization of the Mo(V) EPR signals, see below). The difference spectrum between the EPR spectra recorded at −284 mV and −190 mV (after normalized subtraction of the redox dye radical signal) is presented in Fig. 4D. It displays a slightly rhombic EPR signal, which we assign to a second Fe-S cluster with typical $g$ values at 2.02, 1.977, and 1.961 ($g_{av} = 1.986$). The redox potential-dependent rise of both signals is shown in Fig. 5A. The g = 2.04 EPR signal-fitted to a Nernst curve with $E'_0 = −205$ mV and the g = 2.02 signal to one with $E'_0 = −255$ mV. To characterize these signals in more detail, a redox potential-dependent microwave power saturation study was performed. The signals differed considerably in their relaxation properties at 40 K (Fig. 5B and C). The saturation of the g = 2.02 EPR signal occurred only at microwave powers greater than 10 mW and was independent of the poised potential (Fig. 5C). In contrast, the g = 2.04 EPR signal exhibited a strong dependence on the redox potential: at −190 mV (when the g = 2.02 feature was only weakly present) power saturation was already observable at 0.2 mW, whereas at −284 mV (when the g = 2.02 signal was strongly developed) the signal required microwave powers >2 mW for saturation. Obviously, the reduction of the “g = 2.02-cluster” strongly influences the relaxation properties of the “g = 2.04-cluster” indicating that these two clusters interact magnetically. Since [4Fe-4S]$^{1+}$ clusters usually relax much faster than this, we refer to the signals as [2Fe-2S]$^{1+}$ I ($g_{av} = 1.999$) and [2Fe-2S]$^{1+}$ II ($g_{av} = 1.986$). The maximum spin concentration was 1.5–1.8 spins/monomer of 4-HBCR. It is interesting to note that [2Fe-2S]$^{1+}$ I has an unusually slow relaxation rate (Fig. 5B) and was observable at temperatures up to 120 K.

At potentials below −400 mV the EPR spectra of 4-HBCR became highly complex, with representative spectra shown in Fig. 6A; new features at $g = 2.06$ and below $g = 1.95$ developed. Both features showed substantially faster relaxation than [2Fe-2S]$^{1+}$ I and II (no saturation occurred at 20 K and 2 mW). Thus, we assign these EPR signal features to the [4Fe-4S]$^{1+}$ cluster of 4-HBCR. A difference spectrum taken from reduced 4-HBCR at −573 mV at 20 mW and 20 K minus one taken at 2 mW and 40 K is shown in Fig. 6B. This spectrum could be simulated as a rhombic $S = 1/2$ species with $g$ values at 2.063, 1.954, and 1.934, which can be interpreted as the EPR signal of a single non-interacting [4Fe-4S]$^{1+}$ cluster. However, due to the complexity of the spectra, presumably because of multiple magnetic interactions, this difference spectrum has to be taken cautiously and may only represent the part of the [4Fe-4S]$^{1+}$ clusters in a non-interacting form. As described above, this [4Fe-4S]$^{1+}$ cluster was also identified by Mössbauer spectroscopy; with this technique its redox potential has already been estimated to be substantially more negative than those of the...
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Subspectra 3–6 were simulated using a spin-Hamiltonian yielding spin expectation values that are used in the nuclear Hamiltonian to describe the magnetic hyperfine field, i.e. $H_{\text{hf}} = \langle S \rangle \cdot A$, where $I$ is the nuclear spin and $A$ is the effective magnetic hyperfine coupling tensor, the main components of which are given in the table. δ, isomer shift; ΔEgg, quadrupole splitting; Γ, line width; η, asymmetry parameter.

| Cluster | [4Fe-4S] | [2Fe-2S] | [2Fe-2S] | [4Fe-4S] |
|---------|----------|----------|----------|----------|
| Subspectrum | [Fe(2S)5-] | [Fe(2S)5-] | [Fe(2S)5-] | [Fe(2S)5-] |
| Total spin | 0 | 0 | 0 | 0 |
| δ (mm$^{-1}$) | 0.44 | 0.29 | 0.30 | 0.64 |
| ΔEgg (mm) | 1.12 | 0.61 | 0.90 | -3.20 |
| Γ (mm s$^{-1}$) | 0.33 | 0.27 | 0.36 | 0.36 |
| η | 0.3 | 0.5 | 0.6 | 0 |
| Axx (T) | | | -29 | 8 |
| Axy (T) | | | -34 | 12 |
| Azz (T) | | | -36 | 25 |
| Rel. area (%) | 25 | 12.5 | 18.75 | 12.5 |
| Reference | | | | |

|   | [2Fe-2S] | [2Fe-2S] | [2Fe-2S] | [2Fe-2S] |
|---|----------|----------|----------|----------|
|    | Power | Power | Power | Power |
|    | 0.2 mW | 2.6 mT | 284 mV | 190 mV |
|    |       |       |       |       |

Fig. 4. EPR spectra of 4-HBCR (75 μM) recorded at potentials at $-190$ mV and $-284$ mV. A, spectrum recorded at $-190$ mV. B, as A after subtraction of the isotropic radical signal of the redox dyes. C, spectrum recorded at $-284$ mV. D, spectrum recorded at $-284$ mW. EPR parameters: microwave frequency, 9.410–9.412 GHz; modulation amplitude, 2.6 mT; temperature, 40 K.

[2Fe-2S] clusters. The gradual rise of the [4Fe-4S]$^{-1}$ signal features was accompanied by a loss of intensity of all features assigned to [2Fe-2S]$^{-1}$ clusters, e.g. those at $g = 2.04, 2.02, and 1.97$ (Figs. 4 and 6A). To demonstrate this more clearly, features at $g = 2.04$ (assigned to [2Fe-2S] I), $g = 1.97$ (assigned to [2Fe-2S]$^{-1}$ I and II), $g = 2.06$, and $g = 1.935$ (assigned to [4Fe-4S]$^{-1}$) were plotted versus the redox potential. Both the rise of the EPR signal of the [4Fe-4S]$^{-1}$ cluster and the disappearance of the features assigned to the [2Fe-2S] clusters followed a Nernst-like curve with $E_0 = -465$ mV (Fig. 7) giving further evidence that reduction of the [4Fe-4S] cluster was associated with the disappearance of the EPR signals of the [2Fe-2S]$^{-1}$ clusters. The results obtained from Mössbauer spectroscopy of dithionite-reduced 4-HBCR neither indicated the reduction of the [2Fe-2S]$^{-1}$ clusters to the fully reduced [2Fe-2S] state nor the formation of a high-spin system. Thus, we suggest that the complete loss of the low-spin [2Fe-2S]$^{-1}$ EPR signals results from strong magnetic interactions with each other and/or other paramagnets. Such interactions might result in a broadening and/or a splitting of EPR signals making a clear assignment highly complex. Moreover, the maximal total spin concentration determined at 40 K and 2 mW was 2–2.5 spins/monomeric 4-HBCR at $-470$ mV. At this potential the [4Fe-4S] cluster was only $\approx 50\%$ reduced. When the potential was lowered to $-573$ mV, the spin concentration/monomer decreased again to 1.7 ± 0.3 spins at $-573$ mV. This suggests that spins were escaping detection due to broadening effects resulting from magnetic interactions among clusters.

Fig. 5. Effect of redox potential and microwave power on the EPR spectra of the [2Fe-2S] clusters of 4-HBCR. The redox potentials were poised by adding ferricyanide to a dithionite-reduced 4-HBCR preparation in the presence of a redox mediator mixture. A, relative signal intensity of (○) [2Fe-2S] I and (●) [2Fe-2S] II as determined by the signal amplitudes of their g-x tensor (at $g = 2.04$ and $g = 2.02$, respectively). Nernst curves for $E_0 = -205$ mV (dashed line) and $E_0 = -255$ mV (solid line). B, effect of the microwave power on the signal intensity of [2Fe-2S] I at 40 K at (○) $-190$ mV, (◇) $-226$ mV, (▼) $-256$ mV, (◇) $-284$ mV, and (●) $-284$ mV. C, effect of the microwave power on the signal intensity of [2Fe-2S] II at (○) $-226$ mV and (◇) $-284$ mV.

EPR Spectroscopy of the Mo(V) State and the Flavin Semiquinone

Mo(V) EPR Signal of Reduced 4-HBCR As Isolated—Depending on the amount of dithionite added and the incubation time of enzyme with excess dithionite different types of Mo(V) signals are usually obtained in the XO family; according to Bray’s nomenclature these are termed “slow” and “rapid” (30). Isolated, dithionite-reduced 4-HBCR exhibited an axial Mo(V)
EPR signal with $g = 1.990$, 1.965, and 1.965, similar to rapid-type signals observed in other members of the XO family (Fig. 8). At temperatures above 120 K this EPR signal was not obscured by those of paramagnetic Fe-S clusters. However, we did not observe a time and/or dithionite concentration-dependent change in the shape and intensity of the Mo(V) EPR signal. Thus, we exclude the possibility that a significant amount of a slow-type EPR signal can be obtained from the dithionite-reduced enzyme. The split features of the Mo(V) EPR spectrum of 4-HBCR are typical for a hyperfine interaction of a Mo(V) species with one or more protons, such as that bound to the terminal sulfur ligand of molybdenum, as reported for several other members of the XO family (10–12).

Mo(V) EPR Signals at Different Redox Potentials—Many members of the XO family in the as-prepared, resting, oxidized state display a Mo(V) EPR signal that usually accounts for 1–10% of the total molybdenum and is assigned to a “Resting signal” (30). Traces of such a signal were found with 4-HBCR at positive potentials ($> -200$ mV) in a redox titration study (see Fig. 4, spectrum A). This signal was obscured significantly by the redox dye radical, and its spin concentration was estimated to be less than 0.1% of total molybdenum. We did not investigate this EPR signal further. In a redox titration experiment Mo(V) EPR signals occurred at potentials below $-300$ mV, which were identical to the one observed in the as-isolated, dithionite-reduced state (Fig. 8). Such Mo(V) signals were observed over an unusually broad potential range from $-300$ mV to $-570$ mV. In Fig. 9D the relative signal intensities of the Mo(V) species are plotted versus the poised potential. A fit with Nernst curves gave the following apparent redox transitions of the molybdenum site in 4-HBCR: Mo(VI)/Mo(V) $= -380$ mV and Mo(V)/Mo(IV) $= -500$ mV (Fig. 9D). The maximum total spin concentration of the Mo(V) species was 1.6/native dimeric enzyme, which matches the molybdenum content of 1.9 mol/mol dimeric enzyme.

To detect magnetic interactions with other paramagnets a power saturation/temperature study of the Mo(V) signal was performed at different potentials. At $-354$ mV the Mo(V) EPR signal did not change significantly in shape and/or line width when the temperature was lowered stepwise from 200 K to 60 K (Fig. 9A). In contrast, at $-482$ mV, the shape of the Mo(V) EPR signal showed a strong dependence on the temperature (Fig. 9B). When the temperature was lowered stepwise from 200 K to 80 K, the hyperfine pattern of the bound protons was gradually lost, probably by broadening of the overall spectrum (Fig. 9B), and at temperatures below 60 K it appeared again. As shown above, at $-354$ mV both [2Fe-2S] clusters were in the paramagnetic state, but the [4Fe-4S] cluster was not. This indicates that the reduction of the [4Fe-4S] cluster, which is mainly in the paramagnetic state at $-482$ mV, induced the temperature-dependent changes in the Mo(V) signal so that an interaction exists between the [4Fe-4S]$^{-1}$ cluster and the Mo(V). To test this further we also determined the power saturation behavior of the Mo(V) signal at potentials where the
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FIG. 9. Effect of redox potential and temperature on the Mo(V) EPR signals of 4-HBCR (60–80 μM). A, Mo(V) signals at −354 mV at the temperatures as indicated. B, Mo(V) signals at −482 mV at the temperatures as indicated. All spectra were normalized for temperature and were recorded under non-saturating conditions. C, relative signal intensities of the Mo(V) signal at different redox potentials. The Nernst curves were simulated with the same parameters as those used for the dithionite-reduced sample (Fig. 3, Table I). The main difference, however, is that in the

[4Fe-4S] cluster was diamagnetic and paramagnetic. At −354 mV, the Mo(V) EPR signal became saturated at 80 K between 2 and 20 mW, and at 200 mW the signal intensity fell to 50% of the non-saturated value (Fig. 9C). However, at −482 mV the Mo(V) EPR signal did not even saturate at 80 K and 200 mW. This can be explained by a magnetic interaction of the Mo(V) site with the faster relaxing paramagnetic [4Fe-4S]−1 cluster.

Flavin Semiquinone EPR Signals—FAD has been previously determined to be the flavin cofactor in 4-HBCR (7). We followed the rise of the isotropic flavin semiquinone radical signal in a redox titration study. At high temperatures (>100 K) an isotropic EPR signal at g = 2.008 was observed (not shown). Its line width (1.9 mT at 200 K and 2 mW) clearly differs from that of the radical EPR signal of the mediator mixture (1.3–1.4 mT). It is typical for an EPR signal of a blue neutral semiquinone FADH+ as has also been suggested for the radical species found in the closely related quinoline 2-oxidoreductase (32). An accurate quantitative redox titration study to determine the mid-point potentials of the redox transitions of the flavin was complicated by the high ratio of redox dyes (30 μM) to that of the enzyme (80 μM). Thus, in the redox titration we followed both the change in line width as well as the total spin concentration of the isotropic EPR signal. Taking both parameters into account we estimate E′₀ = −380 mV and E″₀ = −500 mV. (●) normalized signal intensities of the Mo(V) signal at different redox transitions.

Effect of 4-OH benzoyl-CoA (5 mM) on the Mo(V) EPR signals of 4-HBCR (650 μM).

The enzyme was incubated for 5 s at 4 °C and subsequently frozen on dry ice. A, spectrum taken at 120 K. B, spectrum taken at 4.2 K in an applied field of 20 mT. C, spectrum taken at 4.2 K in an applied field of 7 T. The envelope lines through the data points are the sum of four subspectra (see Table I) corresponding in the present case to cluster stoichiometry [4Fe-4S]3+:[2Fe-2S]1+:[2Fe-2S]2= 2:1:3.

FIG. 10. Effect of 4-OH benzoyl-CoA (5 mM) on the Mössbauer spectra of 57Fe-enriched, dithionite-reduced 4-HBCR (650 μM).

The enzyme was incubated for 5 s at 4 °C and subsequently frozen on dry ice. A, spectrum taken at 120 K. B, spectrum taken at 4.2 K in an applied field of 20 mT. C, spectrum taken at 4.2 K in an applied field of 7 T. The envelope lines through the data points are the sum of four subspectra (see Table I) corresponding in the present case to cluster stoichiometry [4Fe-4S]3+:[2Fe-2S]1+:[2Fe-2S]2= 2:1:3.

Effect of 4-HBCoA on the Fe-S Clusters

Mössbauer spectra of dithionite-reduced 4-HBCR (650 μM) taken after rapid mixing with 5 mM 4-HBCoA at 4 °C and subsequent freezing on dry ice are shown in Fig. 10. Spectra were recorded at 120 K (A) and at 4.2 K in external fields of 20 mT (B) and 7 T (C). From the specific activity of the enzyme under the conditions used, 4-HBCoA was not depleted. In principle, the subspectra could be simulated with the same parameters as those used for the dithionite-reduced sample (Fig. 3, Table I). The main difference, however, is that in the steady state of substrate reduction no [4Fe-4S]3+ clusters were observed. The cluster stoichiometry obtained for [4Fe-4S]3+: [2Fe-2S]1+: [2Fe-2S]2+ = 2:1:3 (Fig. 10) showing that all [4Fe-4S] clusters were oxidized, whereas 75% of the [2Fe-2S] clusters were reduced. This indicates that under steady-state
conditions of 4-HBCoA reduction, either the [2Fe-2S] clusters are not oxidized at all or that reduction of these clusters (by excess of dithionite present in the sample) is much faster than their substrate-dependent oxidation. A similar experiment was also performed using EPR spectroscopy. Spectra taken at 40 K and 0.2 mW were very similar to those obtained at −350 mV in the redox titration (Fig. 6) indicating that in the steady-state of substrate reduction most but not all of the [2Fe-2S] clusters were in the reduced state, whereas all [4Fe-4S] clusters were in the oxidized state. This also confirms that the complex changes of the Fe-S EPR spectra observed between −400 mV and −500 mV are mainly due to the reduction of the [4Fe-4S] cluster.

**Nature of the Molybdenum Cofactor of 4-HBCR**

Once released from molybdoenzymes, the molybdenum cofactor is extremely labile and in the presence of oxygen is oxidized to a number of stable variants including form A, form B, and urothione (21). However, controlled oxidation in the presence of iodine converts molybdopterin to form A and the molybdopterin mononucleotide and not a molybdenum to the Mo(IV) state (22). The function of this cluster can be assumed to be to mediate low potential electrons from the natural electron donor ferredoxin to the molybdenum site. As the redox potential of the two [4Fe-4S]1½ clusters of this ferredoxin are −435 mV and −585 mV, respectively (31), a direct electron transfer from each of the ferredoxin clusters to the [4Fe-4S] cluster of 4-HBCR would be possible. The reduction of the [4Fe-4S] cluster marked by the EPR properties of both the [2Fe-2S] clusters and the Mo(V) species, indicating magnetic interactions between these redox cofactors. The [4Fe-4S] cluster is located in the β-subunit of 4-HBCR that contains an extra loop with 35–40 amino acids including five cysteine residues of which four have been considered to be involved in cluster ligation (7). Ligation of this cluster by four cysteines agrees with our Mössbauer spectroscopy data. A similar loop, with four conserved cysteines, has also been deduced from the gene coding for the β-subunit of 4-HBCR in the anaerobic phototrophic bacterium *R. palustris* (9) but has not yet been described for any other member of the XO family. Notably, this amino acid insertion is located directly adjacent to FAD binding motifs, suggesting that the [4Fe-4S] cluster is close to the flavin (7). These motifs include two boxes that are involved in pyrophosphate binding and are typical for the FAD-binding domain of the vanillyl-alcohol oxidase family (33). Notably, the molybdenum containing CODH to which 4-HBCR shows high amino acid similarities also contains such a FAD-binding domain (34). The redox properties of FAD in 4-HBCR are different from those of other XO family members (see Table III). The gap between the two redox transitions from FAD to FADH is unusually high (200 mV) and the FAD/FADH(−470 mV) couple is much more negative when compared with other XO family members (Table III). A possible function of the FAD could be the mediation of electron transfer between the “low potential” (4Fe-4S) cluster, Mo(VI/V) transition) and “high-potential” electron carriers (both [2Fe-2S] clusters).

The three-dimensional structures of molybdenum and flavin containing enzymes available so far, e.g. those of CODH (34), XDH, or XO (35), show a common arrangement of the redox cofactors with the flavin being spatially separated from the molybdenum cofactor. The distance in all cases >20 Å making direct electron transfer between them unlikely. In contrast, the architecture of these enzymes suggests an electron transfer chain from the flavin via both [2Fe-2S] centers to the molybdenum. Our EPR spectroscopy data provide evidence for a magnetic interaction between the [4Fe-4S] cluster and the molybdenum, suggesting that an alternative electron transfer chain exists in 4-HBCR. This would also fit with the redox potentials determined for the cofactors. Full reduction of molybdenum to the Mo(V) state (E’o = −500 mV) by the [2Fe-2S]+ clusters (E’o = −205 mV and −255 mV) is thermodynamically unlikely (Table II), and it is more likely that the fully reduced FADH (FADH/FADH −470 mV) or the [4Fe-4S]+ clusters...
cluster \((E'_0 = -465 \text{ mV})\) could donate single electrons to the molybdenum. If indeed a different electron transfer chain exists in 4-HBCR it is necessary to assume a different tertiary and/or quaternary structure from those of the solved structures of CODH or XO. There are two possibilities for alternative arrangement of the cofactors in the light of the spectroscopic data obtained in this work: (i) a different arrangement of the three subunits shifting the \([4\text{Fe}-4\text{S}]\) cluster/FAD closer to the Mo-site of the other one. toward each other enabling an electron transfer from the \([4\text{Fe}-4\text{S}]\) cluster of one trimer to the Mo-site of the other one. Both possibilities are conceivable since the presence of the additional domain containing the \([4\text{Fe}-4\text{S}]\) cluster could result in such structural alternatives.

\(\text{[2Fe-2S]}\) Clusters—Usually two different types of \([2\text{Fe-2S}]\) clusters are found in enzymes of the XO family (11, 12). It has generally been accepted that they can be distinguished and assigned by their typical EPR spectroscopic properties. Usually, the cluster referred to as \([2\text{Fe-2S}]\) I is of the plant type ferredoxins with \(g_{av} \approx 1.96\) being observable up to 100 K, whereas \([2\text{Fe-2S}]\) II exhibits a wider range of g values and relaxes substantially faster (optimal temperature is generally below 20 K). Typical differences between the anisotropies of both clusters in several members of the XO family have been compared elsewhere (36). Although some variations and overlapping existed, the results suggested that in all members of the XO family the differences and main EPR characteristics of both \([2\text{Fe-2S}]\) clusters are conserved. However, the EPR properties of the two \([2\text{Fe-2S}]\) centers of 4-HBCR do not follow this classification. None of the clusters exhibits either an unusual fast relaxation behavior nor a wide spread of g values. In contrast to other XO family enzymes the cluster with a wider spread of g values, referred to as \([2\text{Fe-2S}]\) I, relaxes much slower than cluster II. Both clusters interact magnetically in 4-HBCR presumably because of their close proximity as demonstrated in other enzymes of the family, e.g., \(Desulfovibrio\) \(gigas\) aldehyde oxidoreductase (closest distance: 13.5 A) (37) or CODH of \(O.\ carboxydotovans\) (12.6 A) (34). More unusually, the reduction of the \([4\text{Fe}-4\text{S}]\) cluster strongly affected the EPR properties indicating a strong interaction between these clusters and produced the curious effect that the EPR signals of the \([2\text{Fe-2S}]\) clusters gradually disappeared, presumably because of broadening and/or splitting effects; shifts to the fully reduced or high-spin states of the \([2\text{Fe-2S}]\) clusters can be excluded. So far, such a situation has not been described for \([2\text{Fe-2S}]\) clusters of XO family enzymes.

Molybdenum Cofactor—Molybdopterin mononucleotide is the common cofactor of eukaryotic molybdoenzymes but has only rarely been reported as the cofactor among prokaryotes, such as in XDH from \(R.\ capsulatus\) (39). The impact of the presence or absence of a second nucleotide moiety at the pterin cofactor on the catalytic properties of an enzyme is not clear. It is generally accepted that catalysis takes place at the molybdenum cofactor. Thus, the lack of the second nucleotide in the pterin mononucleotide cofactor might enable the spatial positioning of the CoA-ester substrate of 4-HBCR adjacent to the molybdenum cofactor.

The EPR properties of the Rapid Mo(V) species are very similar to those of other XO family members; at least one proton is coupled to the Mo(V) species, possibly derived from an \(-\text{SH}\) or \(-\text{OH}/\text{H}_2\text{O}\) group (10–12). No evidence was obtained for a Slow Mo(V) signal. The big difference of \(-120 \text{ mV}\) between the two redox transitions Mo(V)/Mo(IV) \((E'_0 = -380 \text{ mV})\) and Mo(V)/Mo(VI) \((E'_0 = -500 \text{ mV})\) and the extremely low redox potential of the latter redox couple are more unusual. Similar potentials have only been reported for aldehyde oxidoreductase but for the slow signal rather than the rapid as we have found in 4-HBCR (38). Most other reported Mo(VI)/Mo(V)/Mo(IV) couples are in a narrow range around \(-350 \text{ mV}\) (Table III). Our proposed catalytic mechanism for 4-HBCR, as discussed below, requires low potential electrons, which fits well with the unusually negative redox potentials of the Mo-cofactor.

### Mechanistic Aspects of 4-OH-Benzoyl-CoA Reductase

It is agreed that in general an essential role of enzymes containing a molybdenum-pterin cofactor is the catalysis of controlled two-electron transitions, or oxygen transfers, between a substrate and spatially separated one-electron carriers such as ferredoxins, flavodoxins, or cytochromes. In the mechanism of known XO family enzymes one can normally distinguish two half-cycles. First, the two-electron oxidation of the substrate occurs as Mo(VI) is reduced to Mo(IV). Secondly, Mo(VI) is oxidatively regenerated in two single electron transfer steps (10–12). As discussed above, in 4-HBCoA reduction two-electron transfer chemistry is most unlikely, and a model of alternate one-electron and one-proton transfer steps to the aromatic ring has been suggested to be the most plausible mechanism. This is by analogy with the Birch reduction of organic chemistry for the dehydroxylation of an aromatic ring. Due to the electron-withdrawing character of the \(-\text{hydroxy}\) group, a single electron transfer to 4-HBCoA is facilitated compared with electron transfer to non-substituted benzoyl-CoA.\(^2\) A single electron transfer would yield a radical anion \((E'_0 = -465 \text{ mV})\) and the extremely low redox potential of the latter redox couple are more unusual. Similar potentials have only been reported for aldehyde oxidoreductase but for the slow signal rather than the rapid as we have found in 4-HBCoA (38). Most other reported Mo(VI)/Mo(V)/Mo(IV) couples are in a narrow range around \(-350 \text{ mV}\) (Table III). Our proposed catalytic mechanism for 4-HBCR, as discussed below, requires low potential electrons, which fits well with the unusually negative redox potentials of the Mo-cofactor.

### Table III

Redox potentials of the cofactors from different enzymes of the xanthine oxidase family

| Enzyme | [2Fe-2S] I | [2Fe-2S] II | [4Fe-4S] | FAD/FADH(H) | FAD/FADH | Mo(V)/Mo(VI) + | FAD/FADH | Mo(V)/Mo(VI) + | Reference |
|--------|------------|-------------|----------|-------------|-----------|---------------|-----------|---------------|-----------|
| 4-HBCR | -205       | -255        | -465     | -250        | -470      | -380          | -500      | this work     |           |
| XO (milk) | -280       | -245        | N.P.     | -310        | -220      | -355          | -335      | [4]           |           |
| XDH (chicken liver) | -280       | -275        | N.P.     | -345        | -377      | -357          | -337      | [4]           |           |
| AOR (Desulfovibrio gigas) | -280       | -285        | N.P.     | -258        | -212      | -351          | -351      | [4]           |           |
| AOR (rabbit liver) | -297       | -310        | N.P.     | -258        | -212      | -351          | -351      | [4]           |           |
| QuiOR (Pseudomonas putida) | -155       | -195        | N.P.     | N.D.        | -390      | -390          | N.D.      | [32]          |           |
| IsoOR (Pseudomonas diminuta) | +65        | +10         | N.P.     | N.D.        | N.D.      | N.D.          | N.D.      | [32]          |           |
| QualOR (Arthrobacter sp.) | -250       | -70         | N.D.     | N.D.        | N.D.      | N.D.          | N.D.      | [32]          |           |

\(^a\) Redox titration of Mo(V) rapid type.

\(^b\) Mo(V) slow type EPR signal.
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half-cycle (with respect to the oxidation of the molybdenum) proceeds in two single electron transitions to the substrate, whereas the reductive half includes a two-electron reduction from Mo(VI) to Mo(IV). Due to the striking similarity of 4-HBCR and other members of the XO family with respect to the catalytic cycle (with respect to the oxidation of the molybdenum) yields benzoyl-CoA. Alternatives to this homolytic cleavage, such as formation of a phenyl cation or a H₂O⁺ species are extremely unfavorable thermodynamically. Interestingly, a rate-limiting electron transfer step has been predicted at ~360 mV in earlier kinetic studies (17) fitting to the Mo(VI)/Mo(V) transition (~380 mV).

Step V: Reductive Two-electron Regeneration of the Mo(IV) —Two electrons and two protons are transferred to the molybdenum center in order to close the cycle. The mechanism presented is consistent with known chemistry and the properties of this enzyme.

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