Protein–Protein Interactions Studied by EPR Relaxation Measurements: Cytochrome c and Cytochrome c Oxidase

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The complex formed between cytochrome c oxidase from Paracoccus denitrificans and its electron-transfer partner cytochrome c has been studied by multi-frequency pulse electron paramagnetic resonance spectroscopy. The dipolar relaxation of a fast-relaxing paramagnetic center induced on a more slowly relaxing center can be used to measure their distance in the range of 1–4 nm. This method has been used here for the first time to study transient protein–protein complex formation, employing soluble fragments for both interacting species. We observed significantly enhanced transversal relaxation of the Cu A center in cytochrome c oxidase due to the fast-relaxing iron of cytochrome c upon complex formation. The possibility to measure cytochrome c oxidase in the presence and absence of cytochrome c permitted us to separate the dipolar relaxation from other relaxation contributions. This allowed a quantitative simulation and interpretation of the relaxation data. The specific temperature dependence of the dipolar relaxation together with the high orientational selectivity achieved at high magnetic field values may provide detailed information on distance and relative orientation of the two proteins with respect to each other in the complex. Our experimental results cannot be explained by any single well-defined structure of the complex of cytochrome c oxidase with cytochrome c, but rather suggest that a broad distribution in distances and relative orientations between the two proteins exist within this complex.

Introduction

Protein–protein interactions are important in a large variety of biological processes such as photosynthesis, respiration, and signal transduction. The formation of protein–protein complexes is essential for redox processes in the mitochondrial and bacterial respiratory chain, where the transfer of electrons is coupled to translocation of protons across the membrane generating an electrochemical proton gradient used for ATP synthesis.1–3 In electron transfer interactions, the binding of the electron carriers has to be both specific and transient in order to ensure catalytic efficiency and high turnover. Because of their transient nature, redox complexes are difficult to study and only a small number of structures has been determined so far by X-ray and NMR spectroscopy (e.g., refs 4–7). Computational docking studies have been employed to obtain more information about the structure of protein–protein complexes (e.g., refs 8–10).

Cytochrome c oxidase (CrO, complex IV) and cytochrome c552 (from here on referred to as c552) are two membrane proteins involved in the respiratory electron transport chain of Paracoccus denitrificans. CrO is the terminal enzyme of the respiratory chain and catalyzes the four-electron reduction of oxygen to water.11 The membrane-anchored protein c552 is the electron donor for this reaction in this bacterium as it shuttles electrons between complex III (cytochrome bc1 complex) and complex IV.12 The first electron acceptor in CrO is the binuclear copper center CuA, located in subunit II.11 Although the structures of CrO and c552 from P. denitrificans have been solved,13–16 no structure of their complex has been obtained up to now.

The complex of c552 with CrO is of transient nature, based mainly on electrostatic interactions. Extensive mutagenesis studies have been carried out in order to identify the docking site on CrO.17 A set of surface exposed acidic residues around the binuclear CuA center has been found to play a crucial role in protein binding. Mutagenesis studies, kinetic studies, as well as NMR experiments on the redox partner c552 have shown that positively charged amino acid residues around the heme cleft constitute the complementary binding site.17–19 Computational docking studies for the electron-transfer complexes between CrO and c552 or horse heart cytochrome c (from here on referred to as c63) have been performed, and binding models have been proposed on the basis of these results.8–10

Pulse EPR methods, like pulsed electron–electron double resonance (PELDOR),20 double quantum coherence (DQC),21 and relaxation measurements,22 are well-established techniques to measure distances between two paramagnetic centers in the range of 10–70 Å. All of them use the magnetic dipole–dipole coupling between the paramagnetic centers to determine the distance between them. Whereas PELDOR and DQC experiments have been used mainly on nitroxide spin labels and other slowly relaxing paramagnetic centers, relaxation experiments have been applied to obtain distances between a fast relaxing spin (e.g., a metal ion) and a more slowly relaxing spin, as for example nitroxide spin labels or transition metal ions.22–26

Both paramagnetic centers involved in this study have been characterized thoroughly by EPR spectroscopy in the past. The binuclear CuA center of different bacterial and mitochondrial...
CrO and of the CuA-containing soluble fragment of subunit II (from here on referred to as CuCo) exhibits an EPR spectrum typical for a mixed-valence $[\text{Cu}^{1.5+}, \text{Cu}^{1.5+}]^2$ $S = 1/2$ binuclear copper with g-tensor values $g_{\alpha} = 1.99$, $g_{\eta} = 2.02$, and $g_{\zeta} = 2.18$.\textsuperscript{27,28} Mitochondrial cytochrome $c$ shows a rhombic g-tensor with values $g_{\alpha} = 1.25$, $g_{\eta} = 2.26$, and $g_{\zeta} = 3.06$, typical for biological class I low spin $S = 1/2$ ferricytochrome.\textsuperscript{29} The electron spin–lattice relaxation times $T_1$ of CuA in CrO and of mitochondrial cytochrome $c$ in the temperature range of 1.5–15 K have been measured.\textsuperscript{30}

Here, we apply X-band (9 GHz) and G-band (180 GHz) pulse EPR spectroscopy to investigate the magnetic dipole–dipole interaction between the cytochrome low-spin Fe$^{3+}$ and the binuclear CuA center from CrO in the CrO$_{2}$:cytochrome $c$ protein–protein complex. Stoichiometric 1:1 complex formation is observed in mixtures of the two proteins as a significant enhancement of the transversal relaxation rate of the slowly relaxing CuA spin by the fast-relaxing Fe$^{3+}$ spin in the complex. The specific temperature dependence of the dipolar relaxation and high orientational resolution obtained at G-band frequency provide information about the distribution of distances and relative orientations of the two proteins in the complex.

**Theory**

**Dipolar Interaction.** Two spins in a magnetic field sense each other through a magnetic dipole–dipole interaction.\textsuperscript{31} The strength of this interaction depends on the distance $R$ between the spins and on the orientation of the vector $\mathbf{R}$ connecting them with respect to the two magnetic moments:

$$H_{\text{dd}} = \frac{\mathbf{\mu}_A \cdot \mathbf{\mu}_B}{R^3} - \frac{3(\mathbf{R} \cdot \mathbf{\mu}_A)(\mathbf{R} \cdot \mathbf{\mu}_B)}{R^5} \tag{1}$$

In magnetic resonance with an external magnetic field in the $z$-direction, this Hamilton operator can be expressed as

$$H_{\text{dd}} = \frac{g_A g_B R^2}{R^3} (A + B + C + D + E + F) \tag{2}$$

where $g_A$ and $g_B$ are the orientation dependent effective g-values of spins A and B, respectively. The secular term A of this Hamiltonian is given by

$$A = (1 - 3 \cos^2(\theta_D)) g_A^2 g_B^2 \tag{3}$$

$\theta_D$ is the angle between the external magnetic field and the dipolar vector; $S_A^z$ and $S_B^z$ are the respective spin operators. Therefore, the dipolar splitting $(2A)$ of the resonance lines of spins A and B is orientation dependent, as will be explained in more detail later on.

**Dipolar Relaxation.** For two unlike coupled spins, of which one relaxes much faster than the other, dipolar coupling may manifest itself as a change in the relaxation behavior of the slower relaxing spin. From time-dependent perturbation theory, it can be shown that a local minimum in the longitudinal relaxation time $T_1$ of the slow-relaxing spin (spin A) occurs when the relaxation rate $K = 1/T_{1}\text{B}$ of the fast-relaxing spin B equals the Larmor frequency of spin A.\textsuperscript{32} Similarly, a minimum in the transversal relaxation time of spin A can be found when the relaxation rate $K$ is equal to the dipolar coupling strength $\Delta$ in angular frequency units. For the two paramagnetic centers investigated here, only the second process will be effective in the accessible temperature range (5–30 K).

**Relaxation Measurements.** Relaxation measurements were performed with a two pulse Hahn echo sequence ($\pi/2$-pulse, delay time $\tau$, $\pi$-pulse, delay time $\tau$, echo signal). The relaxation behavior caused by dipolar coupling has been calculated to be\textsuperscript{33}

$$\Phi_{\text{dd}}(2\tau) = C^{-2} \left[ \frac{K}{2} ((K + C)e^{-(K+C)2\tau} + (K - C)e^{-(K+C)2\tau}) - \Delta^2 e^{-K^2\tau} \right] \tag{4}$$

with $C^2 = K^2 - \Delta^2$.

In the slow-relaxing ($K \ll \Delta$) and the fast-relaxing limit ($K \gg \Delta$), eq 3 reduces to simple monoexponential decay curves:

$$\Phi_{\text{dd}}(2\tau) = \exp \left( -\frac{2\tau}{T_1\text{B}} \right) \tag{5}$$

and

$$\Phi_{\text{dd}}(2\tau) = \exp \left( -\Delta^2 T_1\text{B}\tau \right) \tag{6}$$

respectively.

As $T_1\text{B}$ depends strongly on temperature,\textsuperscript{30} the dipolar relaxation traces are also dependent on temperature.

**Orientation and Temperature Dependence.** The dipolar coupling $\Delta$ depends on the angle $\theta_D$ of the dipolar vector with respect to the external magnetic field. In an experiment on disordered frozen solution samples, where molecules with many different orientations of the dipolar vector are excited (as in our case at X-band frequencies), the resulting dipolar echo decay is a sum of the decays caused by all excited orientations. This manifests itself in a non-exponential echo decay curve at higher temperatures where the dipolar relaxation is sensitive to $\Delta$ (explained in more detail in part A of Supporting Information).

This effect is taken into account in our numerical simulations by explicitly including the copper hyperfine coupling and by averaging over all molecular orientations that are in resonance with the chosen microwave frequency within an inhomogeneous line width, which is determined by other unresolved hyperfine interactions.

For spin systems with such large $g$-anisotropies as cytochrome $c$, the dipolar splitting depends not only on the orientation of the dipolar axis with respect to the external magnetic field but also strongly on the orientation of the cytochrome $c$. Some of the other terms, in particular C and D, also contribute to the dipolar splitting (up to 10%). These effects change the width and shape of the dipolar Pake pattern and have been included in the numerical simulations of our data.

At G-band frequency (180 GHz), the anisotropic $g$-tensor dominates all other interactions of the CuA paramagnetic species by far and leads to a well-resolved powder pattern (see Figure 1b). In this case, depending on spectral position, spins with a much smaller distribution of orientations can be excited, which makes this experiment very sensitive to the orientation of the dipolar vector with respect to the CuA $g$-tensor frame. Different spectral positions within the powder spectrum relax according to their effective dipolar coupling $\Delta(\theta_D)$, resulting in anisotropic relaxation.

**Extraction of Dipolar Relaxation Traces.** The total echo signal decay is given as the product of an intrinsic signal decay of spin A (which includes its own intrinsic relaxation and electron spin echo envelope modulation (ESEEM) effects) and the dipolar relaxation from spin B:

$$\Phi_{\text{tot}}(2\tau) = \Phi_{\text{Adecay}}(2\tau) \Phi_{\text{Ahr}}(2\tau) \Phi_{\text{dd}}(2\tau) \tag{7}$$
The samples used for EPR measurements typically contained E. coli, containing 220 amino acid residues expressed in Escherichia coli and purified as previously reported. The CcO II concentration was determined by taking absorption coefficients at 552 nm, and the cytochrome c concentration was measured by recording redox difference spectra with an extinction coefficient of 21,800 M⁻¹ cm⁻¹. This division can be easily accomplished experimentally in the case of protein–protein complexes, because \( \Phi_{\text{tot}} \) and \( \Phi_A \) can be measured independently. The extraction of the pure dipolar relaxation function \( \Phi_{\text{dd}} \) is a prerequisite for a quantitative simulation and interpretation of such relaxation measurements.

Materials and Methods

Sample Preparation. The CuA-containing soluble fragment of CcO (CcOII) and the cytochrome c552 soluble fragment from P. denitrificans have been expressed in a heterologous system in Escherichia coli and purified as previously reported. The soluble fragment of cytochrome c1 was derived from the bc1 complex, containing 220 amino acid residues expressed in E. coli. It carries negative surface charges like CcOII and therefore does not interact with CcOII and was used as a negative control in the EPR experiments. Cytochrome c from horse heart (Sigma-Aldrich) was dissolved in 25 mM HEPES-KOH buffer at pH 7.0.

To fully oxidize CcO and CcII for EPR experiments, these fragments were incubated with catalytic amounts of cytochrome c oxidase from P. denitrificans for 30 min and then purified by gel filtration using a 5 mM HEPES-KOH buffer and 10% glycerol at pH 7.0. Cytochrome CcO, CcII, and CcI concentrations were determined by recording redox difference spectra with extinction coefficients \( \Delta \varepsilon_{550-535} = 21.0 \), \( \Delta \varepsilon_{557-540} = 19.4 \), \( \Delta \varepsilon_{553-540} = 19.4 \) M⁻¹ cm⁻¹ for \( \varepsilon_{\text{abc}} \), CcII, and CcI, respectively. The CcOII concentration was determined by taking absorption spectra with an extinction coefficient of 3.0 M⁻¹ cm⁻¹. The samples used for EPR measurements typically contained 100 \( \mu \)M fully oxidized CcOII and 100 \( \mu \)M fully oxidized cytochrome in 5 mM HEPES-KOH buffer and 10% glycerol at pH 7.0. The samples were transferred into standard quartz EPR tubes and subsequently frozen in liquid nitrogen.

X-Band Pulse EPR Spectroscopy. Electron spin echo decay measurements were performed using a Bruker Elexsys-800 X-band spectrometer equipped with a Bruker MD5-W1 cavity and an Oxford CF935 helium flow cryostat with ITC-5025 temperature controller. A two-pulse \( \pi/2 - \tau - \pi \) Hahn echo sequence was used to measure both the field-swept EPR spectra (Figure 1a) and the echo decay traces. The CuA center in CcOII exhibits a spectrum as observed in literature before, and the bacterial cytochromes have g-tensor values and relaxation rates in the temperature range 5–15 K that are very similar to those observed for mitochondrial cytochrome c.

The two-pulse echo decay experiments were performed in the temperature range 10–25 K and taken at a field position corresponding to the maximum of the CuA signal (corresponding to the \( g_{\perp} = g_{\perp} = g_{\perp} \) position). The lengths of the microwave pulsing sequences were 20 and 40 ns, respectively, and the shortest value for \( \tau \) was 120 ns, because of the dead time of the spectrometer. The echo decay traces of the protein mixtures and CcOII alone were taken with exactly the same experimental settings, and both traces were corrected for baseline artifacts by subtraction of off-resonance traces. The echo decay traces were reproducible to a very high accuracy for the same protein concentrations and did not depend on the freezing procedure. The signal amplitude, however, was not so reproducible, and therefore, the echo decay traces were normalized to 1 for the shortest \( \tau \) value.

G-Band Pulse EPR Spectroscopy. Echo decay measurements were performed on a home-built 6.4 T, 180 GHz pulse EPR spectrometer. A two-pulse \( \pi/2 - \tau - \pi \) Hahn echo sequence was as described above was used for all measurements, with typical \( \pi/2 \)-pulse lengths of 35–40 ns and a minimum \( \tau \) value of 200 ns. The relaxation measurements were mainly performed between the signal maximum and the high-field edge (Figure 1b), corresponding to the \( g_{\perp} \) and \( g_{\perp} \) positions, respectively, in a temperature range of 5–15 K. The temperature was measured by a sensor at the sample position with an estimated error of less than 1 K. The superconducting magnet contains a sweep coil with a span of 0.15 T, so in order to obtain the full field-swept spectra of CuA (approximately 0.7 T wide) the main coil needed to be swept. This method does not provide us with accurate absolute values of the magnetic field, but calibration with an internal Mn²⁺ standard and simulations indicate that the field sweep is linear within the needed accuracy. Such field-swept spectra were taken with different \( \tau \) values to look for anisotropy of the dipolar relaxation at high fields. Due to the strongly increased spectral width of the CuA spectra at G-band
frequency, the typical sample concentration was 200 µM for these measurements.

Analysis of Experimental Data. A home-written MatLab simulation program based on the theory described above has been used to simulate the dipolar relaxation time traces at different temperatures for the protein–protein complex between CrOII and cytochromes. The experimental dipolar relaxation traces at different temperatures were simultaneously fitted via either a SIMPLEX or a sequential quadratic programming algorithm. Fit parameters for a single binding geometry were obtained for a single binding geometry were as follows: the distance between the two paramagnetic centers R; the polar angles of the dipolar vector with respect to the CrOII g-tensor with respect to the CuA g-tensor frame (α, β, γ) of the cytochrome; the exchange coupling J; and an offset to account for the amount of unbound CrOII. Additionally, the literature values of the cytochrome T1 relaxation times as a function of temperature were allowed to vary within a factor of 2 to account for experimental errors. The consistency and significance of the obtained fit parameters were tested by repeated fit minimization procedures with arbitrary starting values of the fit parameters. In all cases, the obtained minima were reproduced for many different starting values and are therefore assumed to be global minima.

Results

Extraction of Dipolar Relaxation Traces. In the protein–protein complexes under study, two S = 1/2 spins are coupled to each other: the binuclear mixed-valence CuA in CrOII as the slowly relaxing observer spin and Fe3+ in its low-spin state in cytochrome c as the rapidly relaxing spin. Electron spin echo decay measurements of CuA were performed in order to examine the distance and orientation between the redox partners CuA, Cu, and Fe3+ in cytochrome c, bound in a protein–protein complex. In this experiment, the intensity of the Hahn echo was recorded as a function of the separation time τ between the two pulses. Figure 2 shows the two-pulse echo decay traces of CrOII alone in comparison with the decay of the CrOII and c552 mixture measured under the same experimental conditions. The presence of Fe3+ caused a significantly faster decay of the echo of CuA due to dipole–dipole interactions between the two paramagnetic centers. As described in the theoretical section and shown in Figure 2, a division of these two time traces removes all intrinsic relaxation and hyperfine modulation of the CuA paramagnetic center and allows extraction of the pure dipolar relaxation traces. The division method was applied to all experimental echo decay traces shown further on.

Protein–Protein Complex Formation. The dipole–dipole interaction of CuA with the Fe3+ of three different cytochromes has been investigated: a soluble fragment of c552, which in the bacterium serves as a membrane-anchored electron donor to CrOII;12 CrOII, which is often used as a substrate in enzymatic assays for the bacterial oxidase, providing high turnover activity;19 and c1, a soluble fragment derived from the P. denitrificans cytochrome b1 complex, which due to its highly negative surface potential cannot form a complex with CrOII and is used as a negative control.35 The dipolar relaxation traces of CuA in CrOII with these three different cytochromes are shown in Figure 3. The dipolar relaxation traces of the mixtures of CrOII with both binding cytochromes c1 and c552 are very similar and decay much faster than the trace with the control protein c1. This is because the distance between the two paramagnetic centers for the specifically bound protein–protein complexes is much shorter than the average intermolecular distance between randomly distributed paramagnetic centers. The paramagnetic centers in the complexes involving cytochromes c552 and c1 must have an interspin distance on the order of 2 nm for electron-transfer reactions to occur,7–10 whereas non-binding c1 has a significantly larger average intermolecular distance (approximately 25 nm for a cytochrome concentrations of 100 µM). The concentration dependences of the binding and non-binding cytochromes are also very different. A linear concentration dependence of the echo decay function was observed for the non-binding cytochrome c1, whereas the other two cytochromes showed a very different behavior, depending on the stoichiometric ratio of CrOII and cytochrome c (see Supporting Information part B).

The relaxation rate calculated for the mixture of the nonbinding c1 with CrOII is only slightly smaller than the experimentally observed value.35 Altogether, these results are clear evidence for the formation of specific protein–protein complexes between CrOII and either c552 or c1, whereas in the case of c1 only dipolar interactions of randomly distributed cytochromes were detected.
Temperature Dependence of Dipolar Relaxation Traces. Due to the strong temperature dependence of the $T_1$ relaxation time of cytochrome $c$, the dipolar relaxation traces depend strongly on temperature with a minimum in dipolar relaxation time for the condition $1/T_1 = \Delta$. The dipolar relaxation traces in the temperature range 12–23 K of the complex of CrO$_{11}$ with $c_{552}$ are shown in Figure 4 and with $c_{hh}$ in Figure 5. Both complexes exhibit a pronounced minimum in dipolar relaxation (Figures 4 and 5). In accordance with theory, the low-temperature decay trace (12 K) is monoexponential, whereas the higher temperature decay traces show strongly non-exponential behavior due to orientation dependence of the dipolar coupling (see Supporting Information part A). The temperature dependences of the echo decay traces of the complexes with $c_{552}$ and $c_{hh}$ are qualitatively similar but not identical. This probably reflects differences in complex structure as will be discussed later on.

The temperature dependence of the echo decay traces for the $c_1$ mixture is again very different (data shown in Supporting Information part C). The dipolar relaxation enhancement is much weaker in this case; the decay curves are monoexponential at all temperatures and do not show any pronounced minimum in dipolar relaxation. Again, this is in full agreement with our notion that $c_1$ does not form a specific protein–protein complex with CrO$_{11}$.

High-Field Pulsed EPR Measurements. Despite the higher concentration of the protein samples used at G-band (200 $\mu$M at G-band, 100 $\mu$M at X-band), again a strong temperature dependence was measured for the dipolar relaxation of Cu$_A$ upon mixture with $c_{hh}$ (Figure 6). However, the high-field data could not be fitted with the cytochrome $T_1$ values from literature, which implies that the $T_1$ at G-band frequency differs from that at X-band frequency.

Discussion

Specific Protein–Protein Complex Formed. The significant relaxation enhancement of the Cu$_A$ center in CrO$_{11}$ in the presence of $c_{552}$ or $c_{hh}$ observed by our pulse EPR experiments confirms a previous observation by NMR spectroscopy$^{18}$ that complex formation between the two fully oxidized proteins takes place despite the fact that no electron is transferred to the oxidized Cu$_A$ center. For both the natural electron donor $c_{552}$ and the non-homologous $c_{hh}$, a 1:1 complex was formed with approximately 90% yield at low ionic strength, as was seen by comparison of dipolar relaxation traces for different concentrations of cytochrome $c$ (see Supporting Information part B). In contrast to the other two cytochromes, $c_1$ does not form a complex with CrO$_{11}$, which was proven by the very different concentration and temperature dependences of the dipolar relaxation for the mixture of CrO$_{11}$ and $c_1$.

Extraction of Dipolar Relaxation Traces. The pure dipolar relaxation traces were obtained by measuring CrO$_{11}$ indepen-
dently from the CrO₂⁺:cytochrome c complex, followed by a division of the two experimental traces. This enabled a more precise analysis and quantitative numerical simulation of the experimental traces. The fact that the ESEEM modulation vanished by the division confirmed that the electronic structure of Cu₄̄ does not change upon cytochrome c binding. This was already indicated by the observation of identical g- and hyperfine tensors of Cu₄ in the presence and absence of cytochrome c.

Temperature Dependence of the Dipolar Relaxation—Low-Temperature Behavior. The monoexponential X-band relaxation traces taken at 12 K of the complexes with c₁₈ and c₁₃ could be both simulated very well by the known values of the cytochrome c T₁ relaxation times as theoretically predicted by eq 5 and not by a correlation time given by the expression \( \sqrt{T_1 T_2} \), which was suggested for relaxation studies performed on a spin-labeled myoglobin and a porphyrin–nitroxide model system. This is evidence that the intrinsic relaxation of Cu₄ is the same in the CrO₂⁺ protein whether it is bound to cytochrome c or not. The small non-exponential contribution of these experimental decay traces at short \( \tau \) values arises from overlap of the fast-decaying cytochrome signal still visible at low temperatures.

Temperature Dependence of the Dipolar Relaxation—High-Temperature Behavior. The 23 K data of both complexes show a strongly non-exponential decay with a surprisingly large offset (the dipolar relaxation does not go to zero) of about 50%. Origins of this offset could be that (1) unbound CrO₂⁺ is present and, hence, Cu₄ spins that are unaffected by dipolar relaxation show up as a constant contribution, having no dipolar decay, or (2) there is a contribution of spin pairs whose dipolar angle (\( \theta_D \)) is approximately the magic angle; see also Supporting Information part A. Both origins could be excluded here because (1) no such contribution from unbound CrO₂⁺ was observed at lower temperature (12 K), and (2) in a powder sample, it is impossible for 50% of the spin pairs to have \( \theta_D \) near to the magic angle for any geometry of the protein complex.

Therefore, simultaneous fits of the X-band dipolar decay traces at all temperatures were performed to investigate the protein complex structure. Assuming a single binding geometry of the complex, a number of structures was found with a similar quality of the fit as the one shown in Figure 5. The \( R \) values of these fit structures range from 1.9 to 2.6 nm and have very specific values of the exchange coupling J and dipolar and Euler angles. The fit algorithm had optimized the dipolar coupling distribution in such a way that the low-frequency dipolar coupling distribution of the disordered sample average was almost identical for all these structures and had a sharp and narrow maximum around zero frequency. This feature was necessary to account for the large offset at long \( \tau \) values by causing very fast dipolar relaxation that had almost fully decayed during the spectrometer dead time. At X-band frequency, the simulated echo decays corresponding to these structures are indistinguishable due to experimental dead time, which allows no values shorter than 120 ns for the pulse separation time \( \tau \) (see Supporting Information part A).

Orientation Selectivity at G-Band Frequencies. To test if any of these structures describes the protein complex in reality, we performed high-field G-band EPR relaxation measurements. As described before, the possibility to selectively observe molecules with a specific orientation with respect to the magnetic field provides us with more detailed information on the geometry of the complex in disordered frozen solution samples. The different temperature dependence observed at G-band (Figure 6) can be explained by assuming a significant increase of the relaxation rate of the fast-relaxing cytochrome. The spectrum of cytochrome c is, however, too broad to be measured at G-band. An estimate of the cytochrome T₁ at G-band can be made by determining the dipolar relaxation of Cu₄ in the presence of c₁₈ at low temperatures where the dipolar relaxation equals the longitudinal relaxation of the fast-relaxing spin (eq 5). From the 5 K measurements, we estimated 2 orders of magnitude faster relaxation rates at G-band frequency for cytochrome c (1.6 ms at X-band versus 6 \( \mu \)s at G-band). Such enhanced relaxation rates at higher frequencies are in agreement with theoretical predictions for the direct and the Raman process, which are the dominant relaxation mechanisms at these temperatures for cytochrome c. As the T₁ of cytochrome c is an important parameter for our quantitative simulations, it is very difficult to extract a reliable value of the dipolar coupling strength by fitting the temperature-dependent relaxation traces at G-band. Nevertheless, it is possible to obtain valuable angular information of the protein complex from the G-band measurements performed at different spectral positions at a temperature where the relaxation enhancement is sensitive to the dipolar coupling \( \Delta \) (see eq 6). Simulations of all structures from fits of the X-band temperature-dependent relaxation traces showed a pronounced anisotropy of the dipolar relaxation traces at \( g_x \) and \( g_y \) spectral positions at G-band. However, no differences in dipolar relaxation were observed experimentally in the spectral range between \( g_x \) and \( g_y \) within the given experimental signal-to-noise ratio. Field-swept echo-detected spectra for different \( \tau \) values also did not show any indication of strong relaxation anisotropy. On the basis of these experimental results, we discarded all the solutions found by X-band fits assuming a single complex structure.

Two-Site Model for the Protein—Protein Complex. The lack of relaxation anisotropy at G-band can be explained by the existence of several structures with different cytochrome orientations, dipolar angles, and/or distances. To test this hypothesis, a simple model with two distinct binding sites was incorporated in the fitting procedure. This led to more free parameters, but nevertheless, a pronounced minimum was repeatedly found for fits with random starting values. One Fe³⁺ to Cu₄ distance was always in the range of 1.8–2.3 nm, and a second long distance was found with approximately 4 nm. The populations of the two sites varied between 1:1 and 2:1. The Euler angles, describing the relative orientations of the molecules in the complex, are not well-defined within this model.

The complex with the short distance causes a strong decay within the dead time of the experiment and is therefore only present in the time traces with a reduced relative intensity (see Supporting Information part A). This component accounts mostly for the large offset of the dipolar relaxation traces at high temperatures. The long distance complex accounts for most of the details of the non-exponential decay curves. Simulations based on this model are in much better agreement with the high-field EPR data. Even for this oversimplified model of just two binding geometries, some of the solutions found by fit-minimization of the X-band data predict only small relaxation anisotropy at G-band frequency, which is in agreement with our experimental G-band results. Such a fit with the model assuming two complex geometries is overlaid with the experimental traces in Figure 5, and the parameters are given in the figure caption.

The fact that a single complex geometry does not agree with the high-field data may be explained on the one hand by the two-step model for complex formation between electron-transfer proteins proposed previously. This model, based on kinetic
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studies, NMR data, and MD simulations, suggests that in a first step a rather unspecific encounter complex is formed, which then, in a second step, rearranges to a more specific complex that is optimized for electron transfer. Whereas the first step is guided by electrostatic interactions to achieve high affinity, the formation of the latter is driven by specific van der Waals forces. Hence, cytochromes bound in electron-transfer complexes with a short CuA to Fe3+ distance and one well-defined geometry, as well as cytochromes bound in encounter complexes (at larger distances) may be present in frozen solution samples.

On the other hand, the protein complex may consist of a dynamic ensemble of conformations with distances in the range of 2—4 nm where an electron is transferred whenever the two redox centers are close enough.6,10 Both models are in agreement of 2 dynamic ensemble of conformations with distances in the range distances) may be present in frozen solution samples. Significant experimental differences between the relaxation method to protein complexes where more than two paramagnetic centers in complexes with CrOII and c552 protein mixtures, and temperature dependence of dipolar relaxation for cytochrome c1. This material is available free of charge via the Internet at http://pubs.acs.org.

Conclusions

For the first time, pulse EPR techniques have been used successfully to study the interaction between two electron-transfer proteins. Electron spin echo decay measurements of the paramagnetic CuA center in the soluble fragment of subunit II of CrO in complexes with different cytochromes were performed. Binding and non-binding cytochromes could be clearly distinguished. The division method provides pure dipolar relaxation traces that can be quantitatively analyzed to obtain details of the structure of a protein—protein complex, without the necessity of taking into account the intrinsic relaxation properties of the observed paramagnetic species. In the investigated system, the temperature dependence of the dipolar relaxation traces measured at X-band and the orientation-selective measurements at high frequency together suggest that there is a broad distribution of complex structures with interspin distances of 2—4 nm, rather than there being one single well-defined conformation of the protein—protein complex.

Complementary to the PELDOR method, which works preferentially for nitroxide spin labels with long relaxation times and narrow line widths, the relaxation method can be applied to spectrally broad paramagnetic centers in coupled spin pairs, where one of the spins relaxes extremely fast. Therefore, dipolar interactions between endogenous metal centers in enzymes can be directly measured. In addition, it is possible to apply this method to protein complexes where more than two paramagnetic centers are involved. Our preliminary echo decay experiments of full-size CrOII, which contains a total of four paramagnetic centers, in complex with cytochrome c showed that the division method also in this case removes all contributions from other internal paramagnetic centers in CrOII to the CuA signal and retains only the dipolar relaxation due to external cytochrome c. Extensions to membrane-embedded larger protein complexes and supercomplexes can be envisaged and have been initiated in our laboratory.

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Supporting Information Available: Detailed description of temperature and orientation dependence of dipolar relaxation, concentration dependence of dipolar relaxation for CrOII and c552 protein mixtures, and temperature dependence of dipolar relaxation for cytochrome c1. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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